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I. Description

Brevibacillus (Bacillus brevis) Expression System provides an excellent high-yield protein production featuring an efficient secretory expression. *Bacillus brevis*, a gram-positive bacterium, is characterized by its ability to secretory/produce large amount of proteins[1]. This characteristic has been successfully used in the production of a large number of heterologous proteins. This system has the following features and is capable, in particular, of producing secretory proteins.

- Secretory/produces proteins extracellularly in large quantity
- Exhibits very little protease activity
- Produces active form of proteins
- Easy to culture and sterilize
- Amenable to genetic manipulations
- A safe host

Table 1 shows some proteins that have been successfully produced using this system, including enzymes, antigens, and cytokines, among other proteins, all of which were expressed in high level and confirmed to be in the active form. This system also has a solid track record of producing bacteria-, archaebacteria-, and eukaryote-derived proteins, demonstrating its capability regardless of the origin of a gene. Especially, the productions of eukaryote-derived secretory proteins, which generally have an S-S bond in their structures, are known to be difficult in most prokaryotic expression systems. *Brevibacillus*, however, features a secretory production, which has been shown to produce even S-S-bonded proteins efficiently.

Table 1. Examples of Successful Production of Heterologous Proteins Using *B. choshinensis* Host-Vector System

Protein	Origin	Production (g/L)	Reference
Enzymes			
α -Amylase	*B. licheniformis*	3.7	
Sphingomyelinase	*B. cereus*	3.0	
Xylanase	*B. halodurans*	0.2	
CGTase	*B. macerans*	1.5	2)
Chitosanase	*B. circulans*	1.4	
Hyperthermostable protease	*A. pernix*	0.1	
Hyperthermostable nuclease	*P. horikoshii*	0.7	
PDI	Human	1.0	3)
Antigen			
Surface antigen	*E. rhusiopathiae*	0.9	
Surface antigen	*T. pallidum*	0.8	
Cytokine			
EGF	Human	1.5	4)
IL-2	Human	0.6	5)
NGF	Mouse	0.2	
IFN- γ	Chicken	0.5	6)
TNF- α	Bovine	0.4	
GM-CSF	Bovine	0.2	
GH	Flounder	0.2	

By a high transformation efficiency by electroporation, this host is amenable to genetic manipulation. Using a shuttle vector between *B. choshinensis* and *E. coli,* expression vectors can be constructed in *E. coli.*
Two types of culture medium (refer to V-9. Medium components) are used to produce a target protein. The production process is simple by culturing in culture tubes or flasks on a shaker and harvesting the culture supernatant by centrifugation. With no need to rupture bacterial cells, centrifugation of the cell culture will remove cells and yield a clear supernatant containing the target protein, which offers an advantage for the subsequent purification procedure.

II. Components

● His-Tag Fusion Expression Vectors

pNC-HisT DNA	10 µg (Cat. #HB121)
pNC-HisF DNA	10 µg (Cat. #HB122)
pNC-HisE DNA	10 µg (Cat. #HB123)

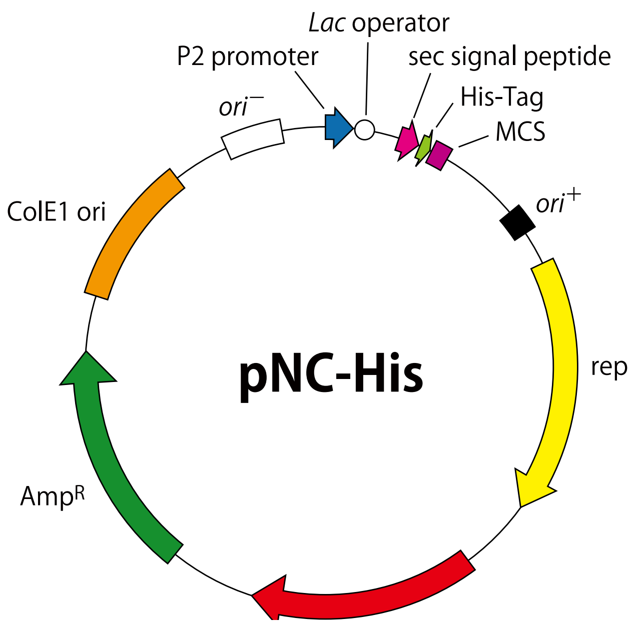
III. Storage   −20℃

IV. Overview of *Brevibacillus* Expression System His-Tag Fusion Expression Vector

The flow of experiments to secretory/produce the target protein using this product is described as below.

IV-1. Selection of Expression Vectors

pNC-HisT DNA, pNC-HisF DNA, and pNC-HisE DNA are shuttle vectors between *Brevibacillus* and *E. coli* derived from partially altered pNCMO2 DNA (Cat. #HB112), a secretory expression vector. These vectors are constructed with insertions of a His-Tag sequence (6 x His sequence) located downstream of the secretion signal and of a protease recognition sequence for tag removal. The same as with pNCMO2, expression plasmids are constructed in *E. coli* and then transformed into *Brevibacillus* to conduct of expression experiments.
The His-Tag sequence is followed by a thrombin sequence in pNC-HisT DNA, a factor Xa sequence in pNC-HisF DNA, or an enterokinase sequence in pNC-HisE DNA, offering the option to select a vector that is optimal for the target protein.
Like pNCMO2, these vectors use a P2 promoter derived from the host cell wall protein as its expression promoter. This P2 promoter is useful for cloning target genes because it is a very powerful promoter in *Brevibacillus* while it does not work in *E. coli*, thereby allowing for an efficient protein production in *Brevibacillus.*
This potent promoter activity, nevertheless, may interfere with the growth of transformants. In such a case, it is recommended to use pNY326 DNA (Cat. #HB111), which has a weaker promoter activity and can be stably maintained in the host cell. (However, His-Tag cannot be inserted into the N-terminus.)



pNC-HisT DNA : 5,260 bp
pNC-HisF DNA : 5,260 bp
pNC-HisE DNA : 5,263 bp

Figure 1 Vector Map of pNC-His DNA

<Features of pNC-His-Series Vectors>

P2 promoter	Uses a part of 5′ sequence of cell wall protein (HWP) gene, which exhibits very little activity in *E. coli* but is a potent promoter in *Brevibacillus*
Sec signal peptide	Secretion signal sequence of HWP is modified to increase the secretion efficiency
MCS (multicloning site)	Protease recognition sequence: 9 restriction enzyme cleavage sites
His-Tag + protease recognition sequence	Enterokinase (pNC-HisE) Factor Xa (pNC-HisF) Thrombin (pNC-HisT)
Terminator	A 46 bp nucleotide sequence functioning as a terminator is introduced at the downstream of the multi-cloning site
Rep	Gene involved in plasmid replications (pUB110 derived)
Ori	Replication origin allowing replication and maintenance of the plasmid in *Brevibacillus* (pUB110 derived)
Nm^R	Neomycin resistance gene, working as a selection marker in *Brevibacillus*
ColE1 *ori*	Replication origin allowing replication and maintenance of the plasmid in *E. coli* (pUC derived).
Amp^R	Ampicillin resistance gene Selection marker in *E. coli*

<Cloning sites of pNC-HisT DNA>

```
AAGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTAATTGTGAGCGGATAA  120
        P2-35                  P2-10          lac operator
CAATTATTCTGCATGGCTTTCCTGCGAAAGGAGGTGACACGCGCTTGCAGGATTCGGGCT  180
                               SD1
TTAAAAAGAAAGATAGATTAACAACAAATATTCCCCAAGAACAATTTGTTTATACTAGAG  240
                                                         SD2
                                          Hpa I
GAGGAGAACACAAGGTCATGAAAAAAAGAAGGGTCGTTAACAGTGTATTGCTTCTGCTAC  300
                 M  K  K  R  R  V  V  N  S  V  L  L  L  L
                                      Nco I
TGCTAGCTAGTGCACTCGCACTTACTGTTGCTCCCATGGCTTTCGCTGCAGGTAGCCACC  360
L  L  A  S  A  L  A  L  T  V  A  P  M  A  F  A ▲A  G  S  H
      Sec Signal peptide                      Signal cleavage
                                   Cloning site
                        BamH I     Xba I  Xho I  EcoR I  Kpn I
ATCACCATCACCATCTGGTTCCACGTGGATCCGTCGATCTAGACTCGAGGAATTCGGTAC  420
H  H  H  H  H  L  V  P  R  G  S
   His-Tag        Thrombin

Sma I  BstB I  Cla I  Hind III
CCCGGGTTCGAAATCGATAAGCTTCGGCATTATAG
```

<Cloning sites of pNC-HisF DNA>

```
AAGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTAATTGTGAGCGGATAA  120
        P2-35                  P2-10          lac operator
CAATTATTCTGCATGGCTTTCCTGCGAAAGGAGGTGACACGCGCTTGCAGGATTCGGGCT  180
                               SD1
TTAAAAAGAAAGATAGATTAACAACAAATATTCCCCAAGAACAATTTGTTTATACTAGAG  240
                                                         SD2
                                          Hpa I
GAGGAGAACACAAGGTCATGAAAAAAAGAAGGGTCGTTAACAGTGTATTGCTTCTGCTAC  300
                 M  K  K  R  R  V  V  N  S  V  L  L  L  L
                                      Nco I
TGCTAGCTAGTGCACTCGCACTTACTGTTGCTCCCATGGCTTTCGCTGCAGGTAGCCACC  360
L  L  A  S  A  L  A  L  T  V  A  P  M  A  F  A ▲A  G  S  H
      Sec Signal peptide                      Signal cleavage
                                   Cloning site
                        BamH I     Xba I  Xho I  EcoR I  Kpn I
ATCACCATCACCATATCGAAGGTCGTGGATCCGTCGATCTAGACTCGAGGAATTCGGTAC  420
H  H  H  H  H  I  E  G  R
   His-Tag     Factor Xa

Sma I  BstB I  Cla I  Hind III
CCCGGGTTCGAAATCGATAAGCTTCGGCATTATAG
```

<Cloning sites of pNC-HisE DNA>

AAGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTAATTGTGAGCGGATAA 120
P2-35 P2-10 *lac* operator

CAATTATTCTGCATGGCTTTCCTGCGAAAGGAGGTGACACGCGCTTGCAGGATTCGGGCT 180
SD1

TTAAAAAGAAAGATAGATTAACAACAAATATTCCCCAAGAACAATTTGTTTATACTAGAG 240
SD2

Hpa I
GAGGAGAACACAAGGTCATGAAAAAAAGAAGGGTCGTTAACAGTGTATTGCTTCTGCTAC 300
M K K R R V V N S V L L L L

Nco I
TGCTAGCTAGTGCACTCGCACTTACTGTTGCTCCCATGGCTTTCGCTGCAGGTAGCCACC 360
L L A S A L A L T V A P M A F A ▲ A G S H
Sec Signal peptide Signal cleavage

Cloning site
*Bam*H I *Xba* I *Xho* I *Eco*R I
ATCACCATCACCATGATGACGATGACAAAGGATCCGTCGATCTAGACTCGAGGAATTCGG 420
H H H H H D D D D K
His-Tag **Enterokinase**

Kpn I *Sma* I *Bst*B I *Cla* I *Hin*d III
TACCCCGGGTTCGAAATCGATAAGCTTCGGCATTATAG

IV-2. Cloning into expression vectors

The expression vectors employ a secretion signal derived from cell wall protein. These vectors' design incorporates a multicloning site for insertion of the target gene at the downstream of a secretion signal, a His-Tag sequence, and a protease recognition site. Using two different restriction enzyme sites on the multicloning site, the insert DNA is cloned in the intended direction.

IV-3. Transformation of *Brevibacillus*

The transformation of *Brevibacillus* is achieved by electroporation. Selection is based on neomycin resistance. When a shuttle vector is used for subcloning in *E. coli,* ampicillin resistance can be used as the selection marker.

If no electroporation device is available, the Tris-PEG method may be used for gene transformation at *Brevibacillus*. In such case, due to the low transformation efficiency, it is recommended to use a completed expression plasmid constructed using *E. coli* as a host.

IV-4. Detection of protein production and scale-up

Use a negative control to confirm expression of the target protein. Pick up the transformant introduced with the expression plasmid for the target protein and culture in the specified liquid medium on a shaker for 48 - 64 hours to obtain the target protein. Apply the culture supernatant to SDS-PAGE analysis or to a similar test in order to confirm the presence or absence of expression. Scale up the production to raise yield. Large scale culturing of *Brevibacillus* is relatively easy.

V. Protocol

V-1. *Brevibacillus* strain

Standard genetic engineering technique is applicable.

V-1-1. Genotype

An essential gene for spore formation cascade is disrupted in *B. choshinesis* SP3 so that sterilization of the transformants is easily done. Furthermore, the trace activity due to intracellular protease gene (*imp*) and extracellular protease gene (*emp*) is nullified by gene disruption to secure the intactness of protein products.

V-1-2. Storage of recombinant *Brevibacillus*

Short-term storage (about 1 week)

Pick a single colony and spread it on MTNm plate. Place it overnight in the incubator kept at 30℃. Seal the plate and store it at room temperature (about 20℃).

[Note] Do not store the plates in a refrigerator!

Long-term storage (1 month or longer)

Pick a single colony innoculate it into 2SYNm medium. Culture it overnight on a shaker. Transfer the broth to a freezing vial and add equal amount of LB medium containing 40% glycerol.

Store it at -80℃.

V-2. *E. coli* host

A *lac* operator is inserted in the pNC-His series to weaken the promoter activity in *E. coli*. Hence, host strain inserted with an F factor (*lac* I^q), such as JM109, has to be used. The genotype of JM109 is shown below for reference.

JM109 : *recA*1, *endA*1, *gyrA*96 *thi*-1, *hsdR*17 (r_K^- m_K^+), *e*14$^-$ (*mcrA*$^-$), *supE*44, *relA*1, △ (*lac-proAB*) /F' [*traD*36, *proAB*$^+$, *lac* I^q, *lacZ* △ M15]

V-3. Construction of expression vectors using pNC-His series

Procedures and precautions for the construction of expression vectors using pNC-His series

E. coli strains having *lac* I^q and *recA*, such as JM109, are recommended as the host for plasmid constructions.

An insert DNA should be cloned in the vector in-frame at the downstream of the secretion signal and His-Tag.

Stop codon must be introduced at the 3' end of the gene.

When expressing a bacterial secretory protein, using the protein's own secretion signal may in some cases provide a better result. In such case, the use of pNY326 vector is required (His-Tag cannot be inserted into the N-terminus.)

V-3-1. Cloning inserts into pNC-His series

<Gene amplification by PCR>

Design primers to allow the insertion of the target gene downstream of the secretion signal. Introduce into both ends of the PCR product two different restriction enzyme sites, appropriate for the expression vector, to provide direction and to amplify the target gene by PCR. Select PCR conditions appropriate for each gene and PCR enzyme. It is recommended to use high-fidelity PCR enzymes for higher accurate PCR (e.g., PrimeSTAR HS DNA Polymerase (Cat. #R010A)).

<Construction of expression plasmids via ligation>

Treat the insert and the vector (0.5 - 1.0 µg) with two kinds of restriction enzymes. Recover and purify the target fragments from each by electrophoresis on agarose gel.
Use 100 ng of each purified DNA for ligation reaction with a ligation reagent (e.g., DNA Ligation Kit <Mighty Mix> (Cat. #6023)). Use 1/5 of the reaction mixture for the transformation of *E. coli*.

<Cloning using in-fusion cloning system>

Clontech's In-Fusion® HD PCR Cloning Kit offers the convenience of simple and fast directional cloning even in the absence of an appropriate restriction enzyme site. Please follow the protocol for the In-Fusion cloning system.

<Transformation of *E. coli*>

Use *E. coli* hosts for cloning that are of high transformation efficiency, for instance, *E. coli* JM109 Competent Cells (Cat. #9052) and *E. coli* JM109 Electro-Cells (Cat. #9022).

V-3-2. Analysis of *E. coli* recombinants

Seed 100 - 200 µl of the transformed mixture onto LB plates containing 50 - 100 µg/ml of ampicillin. Incubate at 37℃ for 15 - 18 hours.
Select 10 - 20 ampicillin-resistant colonies and inoculate in 2 ml of LB medium (containing 50 - 100 µg/ml of ampicillin)
Harvest transformants after incubation at 37℃ for 15 - 18 hours. Extract plasmid using a commercial kit. In general, 1.5 - 3 µg of plasmid DNA can be recovered.
Cut an appropriate amount of DNA with restriction enzyme cleavage. Generally the same restriction enzymes that was employed for cloning are used for this digestion. Confirm the presence/absence of the insert DNA by agarose gel electrophoresis.
Along with confirming the insertion of the target gene by restriction digestion, it is still necessary to perform sequencing to make sure the target gene is inserted and that no error is introduced by PCR.

V-3-3. Sequencing

For sequence verification, the following sequences may be used as the forward and the reverse primers. (pNCMO2 and pNY326 share the same primer sequences.)

Forward Sequencing Primer : 5'-CGCTTGCAGGATTCGG-3'
Reverse Sequencing Primer : 5'-CAATGTAATTGTTCCCTACCTGC-3'

V-3-4. Purification of expression vector

Incubate the transformant inserted with the target gene, and purify the expression vector using a commercial kit.

V-4. Transformation of *Brevibacillus*

Use a host with high transformation efficiency (the use of *Brevibacillus* Competent Cells, Cat. #HB116, is recommended).

V-4-1. Preparation

Prepare the following reagents and materials.

Brevibacillus Competent Cells (Cat. #HB116)
Brevibacillus Competent Cells
- MT Medium**
- Solution A
- Solution B

Plasmid for target gene expression
MTNm Plates*
Culture Tubes
Sterilized Microtubes

＊: Medium Composition
MTNm Plate
Suspend 7.5 g of agar in 500 ml of MT liquid medium** and sterilize using an autoclave. Let stand at room temperature until it has cooled to approximately 50℃ and then add neomycin solution (10 mg/ml stock solution) to a final concentration of 10 μg/ml. Mix gently then dispense into plates.

＊＊: MT liquid medium

Glucose*1	10.0 g/L
Polypeptone	10.0 g/L
Meat Extract	5.0 g/L
Yeast Extract	2.0 g/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
$MnSO_4 \cdot 4H_2O$	10 mg/L
$ZnSO_4 \cdot 7H_2O$	1 mg/L
$MgCl_2 \cdot 6H_2O$	4.1 g/L

Adjust to pH7.0

*1 : Sterilize glucose and glucose-free media separately. Mix after sterilization.

2) NTP Transformation Method

(1) Thaw Solution A, Solution B and MT medium.

(2) Remove from storage only the number of tubes of *Brevibacillus* Competent Cells that you need for transformation, and transport in dry ice/ethanol.

(3) Thaw the *Brevibacillus* Competent Cells quickly (approximately 30 seconds) in a 37℃ water bath.

(4) Centrifuge the cells (12,000 rpm, 30 seconds to 1 minute) to form a cell pellet and remove the supernatant with a micropipette.

Perform the following procedures at room temperature.

(5) Mix the plasmid DNA solution (prepared to a volume of 5 µl or less)[*1] with 50 µl of Solution A.

(6) Add all of the mixed DNA solution to the bacterial cell pellet from step (4) and vortex to completely suspend the bacterial pellet.

(7) Allow to stand for 5 minutes.

(8) Add 150 µl of Solution B (PEG solution)[*2] and vortex until the solution is uniform (5 - 10 seconds).

(9) Microcentrifuge the cells (5,000 rpm, 5 minutes) and completely remove the supernatant.

(10) Microcentrifuge briefly (5,000 rpm, 30 seconds) and completely remove the supernatant.

(11) Add 1 ml of MT medium and suspend completely with a micropipette.

(12) Incubate at 37℃ in an orbital shaker (120 rpm, 2 hours).

(13) Use a sterile inoculating loop to remove a small sample from the culture. Streak on an MTNm plate and culture overnight at 37℃.

(14) Select isolated colonies for plasmid analysis or protein expression.

*1: Use 10 - 100 ng of the purified plasmid.

*2: Solution B (PEG solution) is highly viscous - use a micropipette for 1,000 µl and suction slowly.

V-5. Expression of target proteins by *Brevibacillus* recombinants

After completing the expression clone, conduct a small-scale protein expression. A general protocol for verifying expression is stated below.

V-5-1. Overview

Once the expression transformant has been identified, conduct an expression check with negative control including a vector alone.

The production of the target protein may vary across transformants depending on the nature of the target protein. The size of colonies may also vary. Thus, six to ten colonies should be selected at random (including both large and small colonies) for incubating in culture tubes. If the plates are left for several days after transformation, the protein productivity may decrease. In such case, perform another transformation to obtain fresh transformants.

V-5-2. Culture medium

Use TM medium and 2SY medium as the basic medium for expression test. The protein productivity may vary depending on the medium. The balance between the production potential and growth is important. Therefore, verify the productivity using both media.

V-5-3. Culture for production (secretory production)

Incubate a negative control along with the expression clone to confirm protein production by comparative analysis.
The protocol for expression test is as follows.

(1) Select single colony and inoculate it into both 3-ml of 2SYNm and TMNm liquid media in culture tubes (φ 16 mm). Incubate at 30 - 33℃ with shaking at 120 rpm for 48 - 64 hours. During the incubation, aliquots of the culture should be taken every 24 hours to confirm target protein production.
(2) At the end of incubation, isolate the supernatant fraction by centrifugation (5,000X *g*, 5 minutes). Suspend the cell precipitate in an equal volume of PBS*.
(3) Perform SDS-PAGE (CBB staining or Western blotting) or activity assay on the supernatant and the precipitate fraction.

* : For ease of preparation, use PBS Tablets (Cat. #T900).

V-6. SDS-PAGE analysis

Perform electrophoresis using a SDS-PAGE gel suitable for analyzing the target protein.

V-6-1. Sample preparation

Add 10 µl of 5X SDS-PAGE loading buffer to 40 µl of culture supernatant or cell precipitate.
Mix and heat at 100℃ for 10 minutes to prepare samples for electrophoresis.

V-6-2. Control

Use following samples as the control.

a. Molecular size marker of protein
b. The target protein standard
c. Sample from the culture of *B. choshinensis* SP3 introduced with an expression vector without the insert (negative control)

V-6-3. Analysis of protein expression

The presence/absence of protein production can be confirmed by comparing the target protein standard and the culture supernatant on a SDS-PAGE gel. In cases when detection is difficult due to low expression, low solubility, or masking by the background proteins, the protein production should be verified by Western blot analysis with an antibody specific to a target protein, functional evaluation (e.g., specific activity), or protein purification by a special technique. When pNC-His series is used as an expression vector, the Universal His Western Blot Kit 2.0 (Cat. #635642) or a western blotting by an anti-His-tag antibody can be used to detect the target protein. Site-specific Proteases are purchased from Merck (Novagen).

V-7. Optimization of protein expression

A number of experiments resulted in high-level expression that exceeded 1 mg/ml, and most proteins can be produced in producibility of 100 µg/ml or more. In the case of a lower or no production of a protein, please refer to the guideline provided below.

V-7-1. Low expression

a. To change the promoter activity, please try both vectors (pNC-His series or pNY326 sold separately). The same as with the pNC-His series, in some cases the higher the promoter activity is, the greater the output may be. The output may also be raised by improving the growth of bacteria through the use of a vector having a weak promoter activity such as pNY326.
b. Use a different type of medium. The production output may vary depending on the type of medium.
c. Please compare the number of plasmid copies with the control. If there was a large drop in the number of copies, try using the pNY326 vector, switching to a different medium, or increasing the antibiotic concentration (400 µg/ml), among other things.
d. Some proteins may not be suitable for secretory production. In that case, try intracellular expression by using an intracellular expression vector of the pNI series. In case of a minute production, try ammonium sulfate precipitation or concentration by ultrafiltration membrane.

V-7-2. No expression

Proceed with the same experiments as V.7.1. "Low expression." If those remedies result in no improvement, consider the following points:

a. Check the secondary structure of mRNA. The presence of a high-energy palindrome structure may cause translation abnormalities. In such case, it is necessary to insert mutation to the repeat sequence to remove stacking.
b. Inappropriate sequence neighboring the signal cleavage site may affect secretory production. If inserting an additional sequence to the N-terminus does not create any problem to the target protein's activity, the following approaches may improve: inserting a purification tag and/or a detection tag, inserting a random sequence by PCR, and screening for a high-level expression sequence.

V-8. Protein purification

Purification methods vary according to the type of target protein. Once the target protein is successfully secreted, a bacteria removal procedure will yield a clear supernatant, which offers an advantage for the subsequent purification procedure.
If pNC-His series is used, the target protein can be easily purified using TALON® Metal Affinity Resin (Cat. #635501) or another histidine tagged protein purification resin. Moreover, the added His-Tag can be easily removed after purification by treatment with the respective protease (Enterokinase, Factor Xa, or Thrombin) corresponding to each recognition sequence. For conditions about purification with histidine-tagged protein purification resin and about protease treatment, please refer to the inserted manual for each product.

V-9. Medium components

2SY liquid medium

Components	
Glucose*	20.0 g/L
Bacto Soytone (Becton Dickinson)	40.0 g/L
BactoYeast Extract (Becton Dickinson)	5.0 g/L
$CaCl_2 \cdot 2H_2O$	0.15 g/L

*: Sterilize the mixture of glucose and $CaCl_2$ separately from the other components. Mix after sterilization.

2SY Nm liquid medium

Add neomycin solution (stock solution: 50 mg/ml) to 2SY liquid medium to a concentration of 50 µg/ml.

TM liquid medium

Components

Glucose*	10.0 g/L
Polypeptone	10.0 g/L
Meat extract	5.0 g/L
Yeast extract	2.0 g/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
$MnSO_4 \cdot 4H_2O$	10 mg/L
$ZnSO_4 \cdot 7H_2O$	1 mg/L

Adjust to pH 7.0.

*: Sterilize glucose and glucose-free medium. Mix separately after sterilization.

TMNm medium

Add neomycin solution (stock solution 50 mg/ml) to TM liquid medium to a concentration of 10 µg/ml.

For the following used in TM medium and TMNm medium, please refer to the products stated below.

Glucose	(Wako Pure Chemical; Cat. #041-00595)
Polypeptone	(Nihon Pharmaceutical Co.; Cat. #394-00115)
Meat extract	(Wako Pure Chemical (Ehrlich); Cat. #054-03705)
Yeast extract	(Nihon Pharmaceutical Co.; Cat. #393-00521)
Neomycin	(Sigma; Cat. #N-1876)

MT liquid medium (TM liquid medium containing 20 mM $MgCl_2$)

Components

Glucose*	10.0 g/L
Polypeptone	10.0 g/L
Meat extract	5.0 g/L
Yeast extract	2.0 g/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
$MnSO_4 \cdot 4H_2O$	10 mg/L
$ZnSO_4 \cdot 7H_2O$	1 mg /L
$MgCl_2$	4.1 g/L

Adjust to pH 7.0.

*: Sterilize glucose and glucose-free media separately. Mix after sterilization.

MTNm plate

Suspend 7.5 g of agar in 500 ml of MT liquid medium and sterilize using an autoclave. Let stand at room temperature until it has cooled to approximately 50℃ and then add neomycin solution (10 mg/ml stock solution) to a final concentration of 10 µg/ml. Mix gently then dispense into plates.

VI. Example Experiment: Expression and Purification of *Bacillus licheniformis* α-amylase (BLA) using pNC-HisT

In expression experiment using the plasmid inserted with the BLA gene into pNC-HisT in TMNm medium (30℃, 48 hours), a SDS-PAGE analysis showed a high production of approximately 0.2 mg/ml in the culture supernatant (Figure 2). Next, from the supernatant recovered by centrifugation, the histidine tagged BLA was purified by using TALON® Metal Affinity Resin. A SDS-PAGE analysis performed thereafter showed (Figure 3A) a high degree of purification nearly at a 100% recovery rate. Then the purified protein was digested by Thrombin. The change in mobility on SDS-PAGE confirmed the removal of His-Tag (Figure 3B). This was further verified by a Western blotting in which the protease-digested proteins showed no reaction with the His-Tag antibody (data not shown).

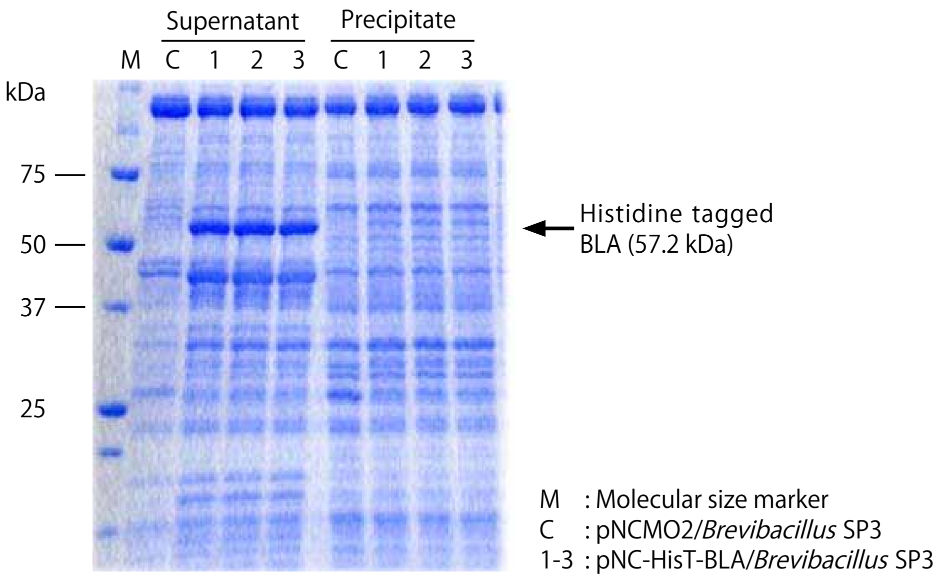
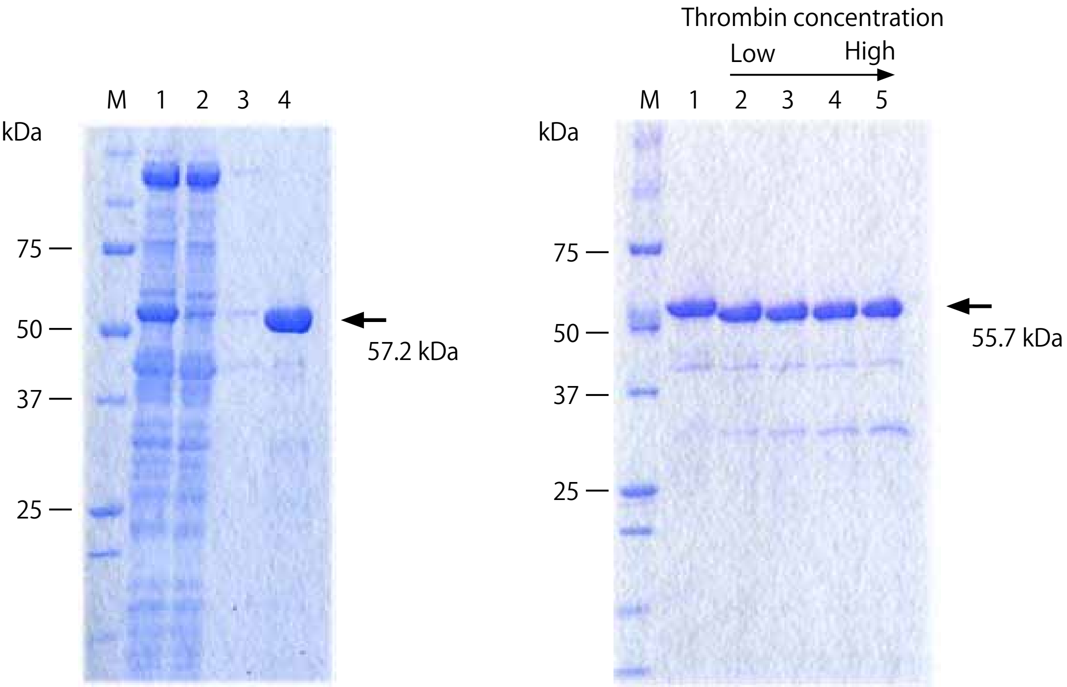


Figure 2. Secretory Expression of α -amylase (BLA) using pNC-HisT

A. TALON® resin purification

B. His-Tag removal



M : Molecular size marker
1 : Culture supernatant
2 : Path-through
3 : Washing
4 : Elution fraction

M : Molecular size marker
1 : Purified histidine-tagged BLA
2-5 : Thrombin treated (16 hr)

Figure 3. Purification of α -amylase (BLA) expressed using pNC-HisT

The expression and purification of BLA using pNC-HisF or pNC-HisE followed by Factor Xa-or Entero-kinase-digestion, yielded as excellent results as in Figures 2 and 3 (data not shown).

VII. Related Products

[*Brevibacillus* Secretory Expression System]
Brevibacillus Expression System II (Cat. #HB200)
Brevibacillus Competent Cells (Cat. #HB116)
pNY326 DNA (Cat. #HB111)
pNCMO2 DNA (Cat. #HB112)
pNY326-BLA DNA (Cat. #HB114)

[Intracellular Expression Vector]
pNI DNA (Cat. #HB131)
pNI-His DNA (Cat. #HB132)

[Histidine-tagged Protein Purification]
HisTALON™ Cartridge Purification Kit (Cat. #635649)
HisTALON™ Cartridge (Cat. #635650)
HisTALON™ Buffer Set (Cat. #635651)
TALON® Metal Affinity Resin (Cat. #635501/635502/635503/635504)

[Histidine-tagged Protein Detection]
Universal His Western Blot Kit 2.0 (Cat. #635642)

[Others]
In-Fusion® HD PCR Cloning Kit (Cat. #639619 etc.)
E. coli JM109 Competent Cells (Cat. #9052)
E. coli JM109 Electro-Cells (Cat. #9022)
DNA Ligation Kit <Mighty Mix> (Cat. #6023)
PrimeSTAR™ HS DNA Polymerase (Cat. #R010A)
PBS Tablets (Cat. #T900)

VIII. References

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