Cat. # HB200

For Research Use

TaKaRa

Brevibacillus Expression System II

Product Manual

v202104

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I. Description

The *Brevibacillus* (*Bacillus brevis*) Expression System is an efficient system for producing high yields of secreted protein. Using this system, it is possible to produce an unprecedented number of heterologous proteins. The *Brevibacillus* Expression System offers the following advantages, and is particularly effective in the production of secretory proteins.

- Extracellular secretion of large amounts of protein
- Almost no detectable protease activity
- Produces active proteins
- Simple procedures for genetic manipulation and culturing
- Safe host bacteria

Examples of protein production using this system are shown in Table 1. High expression levels were obtained for a variety of proteins (enzymes, antigens, and cytokines), regardless of gene origin (bacteria, archaea and eukaryotes). In particular, secretory proteins of eukaryotic origin typically contain S-S bonds for biological activity, and it is generally difficult to produce these proteins using other prokaryotic expression systems. The characteristic secretory production of *Brevibacillus* allows high-efficiency production, even for proteins with S-S bonds.

Table 1: Examples of Heterologous Protein Expression with the *B. choshinensis* Host-Vector System

Protein	Origin	Quantity of Expression (g/L)	References
Enzymes			
Alpha-Amylase	*B. licheniformis*	3.7	
Sphingomyelinase	*B. cereus*	3.0	
Xylanase	*B. halodurans*	0.2	
CGTase	*B. macerans*	5.0	
Chitosanase	*B. circulans*	1.4	
Hyperthermophilic protease	*A. pernix*	0.1	
Hyperthermophilic nuclease	*P. horikoshii*	0.7	
PDI	Human	1.0	3)
Antigens			
Surface antigen	*E. rhusiopathiae*	0.9	
Surface antigen	*T. pallidum*	0.8	
Antibodies			
VHH of anti-NDOM antibody	Llama	3.0	
scFv of anti-fluorescein antibody	Mouse	0.2	
Fab of anti-erbB antibody	Mouse	0.4	
Cytokines			
EGF	Human	7.0	
NGF	Mouse	0.2	
IFN- γ	Chicken	0.5	5)
TNF- α	Cow	0.4	
GM-CSF	Cow	0.2	
GH	Flounder	0.2	

It is possible to construct the expression vector using a shuttle vector in *E. coli* or by directly introducing the DNA ligation solution into the expression host. Target protein may be obtained by culturing transformed host bacteria in test tubes or flasks (two types of media can be used; see Section VI. 9 for medium composition), and collecting protein from the culture supernatant. Homogenization of bacteria is unnecessary; the target protein is obtained from the clear supernatant fraction following centrifugation (to pellet the bacterial cells) and may be purified using standard techniques.

II. Components

Brevibacillus Expression System II (Cat. #HB200)

- Expression Vectors
  - pNY326 DNA — 10 µg
  - pNCMO2 DNA — 10 µg (Cat. #HB112)
- Control Vector
  - pNY326-BLA DNA — 1 µg
- Competent Cells
  - *Brevibacillus* Competent Cells (Cat. #HB116)
    - *Brevibacillus* Competent Cells — 100 µl x 10
    - MT medium — 1 ml x 10
    - Solution A — 1 ml
    - Solution B — 1 ml x 2

III. Materials Required but not Provided

1. Reagents

- DNA ligation kit
- LB (+Amp) plates
- MTNm plates
- TMNm plates
- TM liquid medium
- 2SYNm medium
- MT liquid medium
- Neomycin

Note: See Section VI. 9: Medium Composition

2. Materials

- Sterilized culture tubes
- Sterilized microtubes
- Microcentrifuge
- Vortex
- Incubating orbital shaker

IV. Storage

Expression Vectors	pNY326 DNA, pNCMO2 DNA	-20℃
Control Vector	pNY326-BLA DNA	-20℃
Competent Cells	*Brevibacillus* Competent Cells	-80℃
MT medium, Solution A, Solution B		-80℃

V. Overview of the *Brevibacillus* Expression System

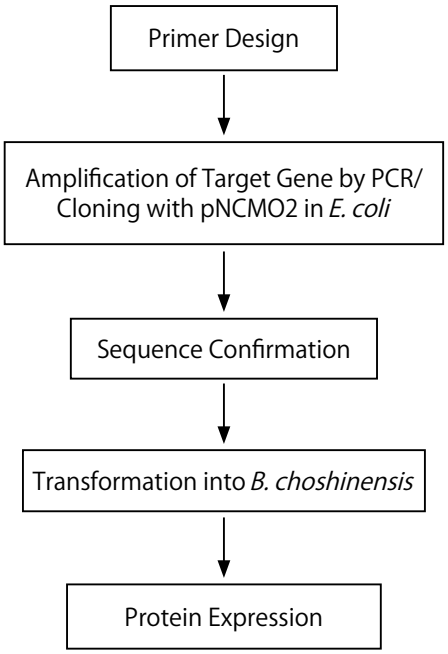
The experimental process is shown below, up to secretory production of the target protein.

1. Selection of Expression Vector

A. pNCMO2

pNCMO2 is a *Brevibacillus* and *E. coli* shuttle vector. Accordingly, the expression plasmid is constructed in *E. coli* and then is introduced into *Brevibacillus* for protein expression.

The P2 promoter, derived from a cell-wall protein of the host bacterium, is used as the expression promoter for pNCMO2. Because the P2 promoter is not functional in *E. coli*, it is useful for cloning the target gene. However, it is an exceptionally strong promoter in *Brevibacillus* and results in efficient protein production. This strong promoter activity may inhibit the growth of transformants in some cases. If this occurs, consider using the pNY326 vector.



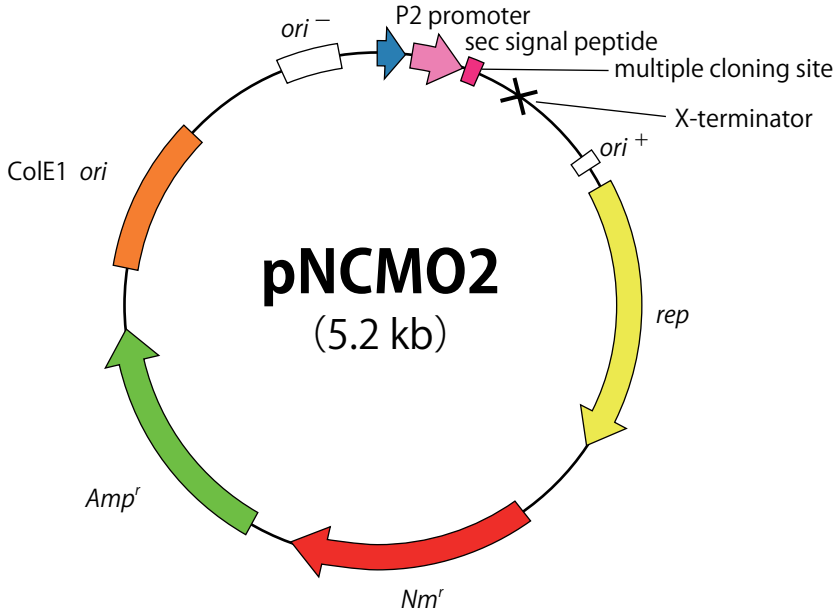


Figure 1. pNCMO2 DNA Vector Map

<Vector Information for pNCMO2>

P2 Promoter	Uses part of the 5' sequence of the cell-wall protein gene. Has almost no activity in *E. coli*. Very strong expression in *Brevibacillus*.
Sec Signal	A modified secretion signal to increase versatility
Multiple Cloning Site	11 restriction enzyme cutting sites
X-Terminator	A 46-bp terminator structure downstream from the multiple cloning site
Rep	Gene relating to plasmid replication (from pUB110)
Ori	Replication origin for replication of the plasmid in *Brevibacillus* (from pUB110)
Nm^r	Neomycin resistance gene Selection marker in *Brevibacillus*
ColE1 *Ori*	Origin of replication for the plasmid in *E. coli* (pUC derived)
Amp^r	Ampicillin resistance gene Selection marker in *E. coli*

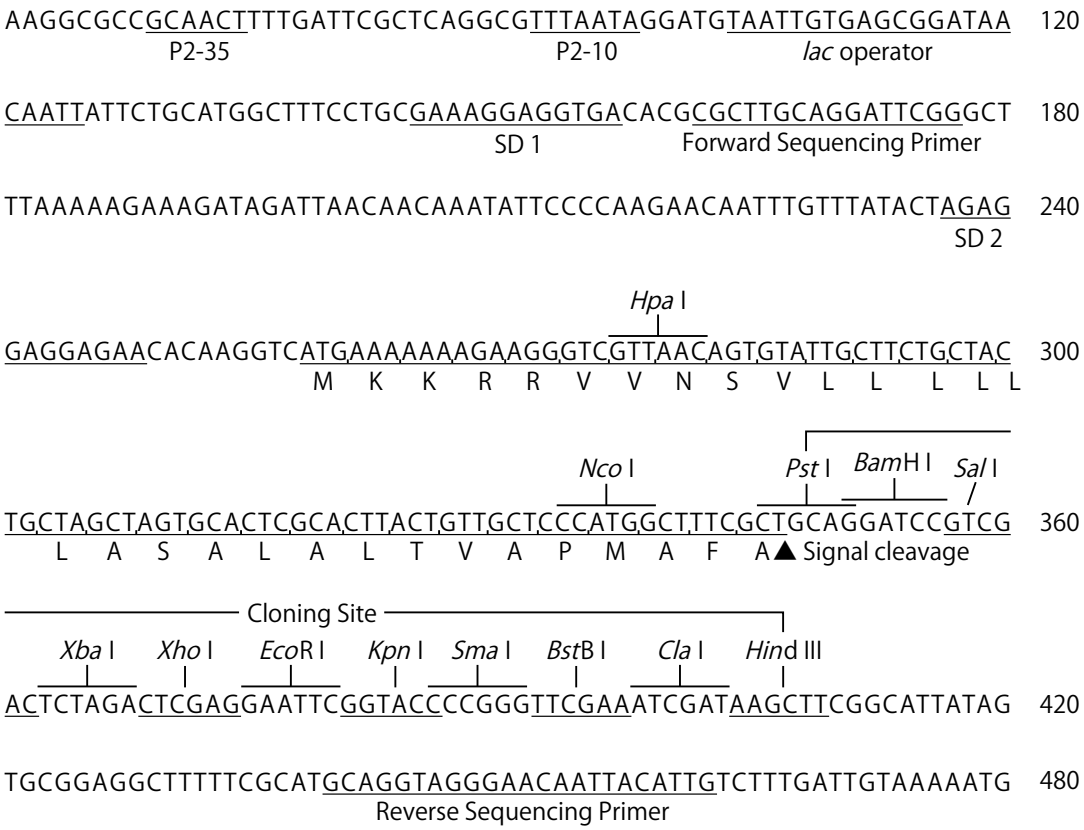


Figure 2. Sequence of the Region Between the Multiple Cloning Site and the P2 Promoter of pNCMO2.

The P2 promoter is the main promoter in cell wall protein synthesis. A *lac* operator sequence introduced immediately upstream of this promoter controls promoter activity inside of *E. coli*. SD sequences (ribosome binding site) are present at two sites (SD1, SD2) and are translated using the same frame.

B. pNY326

pNY326 can replicate in *B. choshinensis* cells and its size (3.4 kb) is much smaller than that of pNCMO2 (5.2 kb). In addition, the promoter activity of the pNY326 vector is substantially weaker than that of pNCMO2. Because of these features, it can be maintained stably even if it expresses proteins that are toxic to the host cells, and, in some cases, higher protein production can be achieved using this vector because of the improved growth of the transformants.

pNY326 exhibits stable productivity and is especially well-suited to large scale culture. The expression plasmid must be constructed in one step using *B. choshinensis* as the host (the vector can be maintained only in *B. choshinensis*). Because the transformation efficiency must be sufficiently high, the use of *Brevibacillus* Competent Cells (10^5 transformants / μg DNA; Cat. #HB116) is recommended.

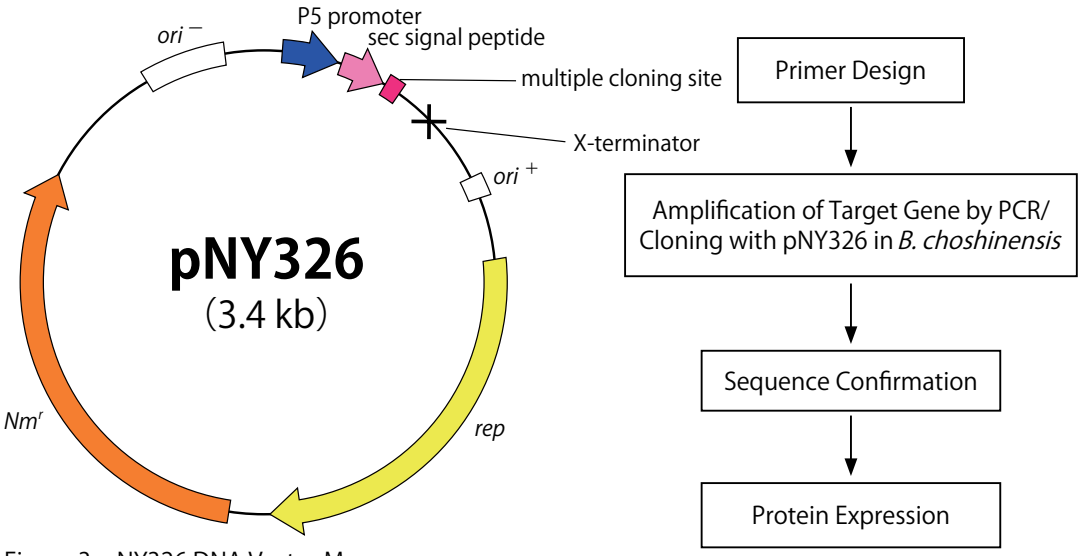


Figure 3. pNY326 DNA Vector Map

<Vector Information for pNY326>

P5 Promoter	Uses part of the 5' sequence of the cell wall protein gene. Strong expression in *Brevibacillus*.
Sec Signal	A modified secretion signal to increase versatility
Multiple Cloning Site	11 restriction enzyme cutting sites
X-Terminator	A 26-bp terminator structure downstream from the multiple cloning site
Rep	Gene relating to plasmid replication (from pUB110)
Ori	Replication origin allowing replication of the plasmid in *Brevibacillus* (from pUB110)
*Nm^r^*	Neomycin resistance gene Selection marker in *Brevibacillus*

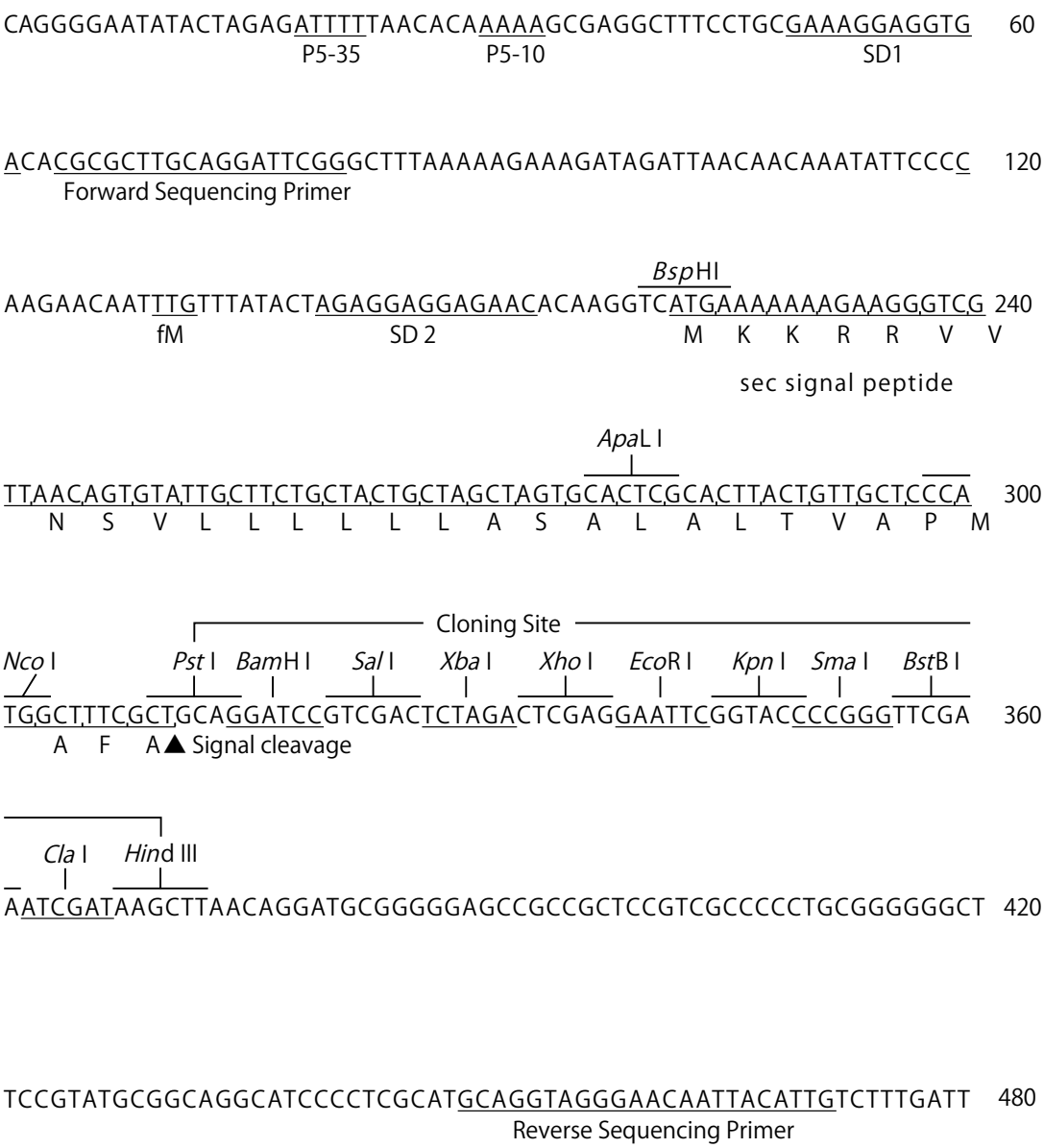


Figure 4. Sequence of the Region Between the Multiple Cloning Site and the Promoter Region of pNY326.

The P5 promoter exhibits comparatively weak promoter activity in *Brevibacillus*. SD sequences (ribosome binding site) are present in 2 locations (SD1, SD2) and are translated using the same frame.

2. Cloning into the Expression Vector

A secretion signal that is derived from a cell wall protein is used in the expression vector. Design primers so that the target gene is inserted downstream of the secretion signal cleavage site. The use of two types of restriction enzyme sites in the multiple cloning site allows directional cloning of the target gene.

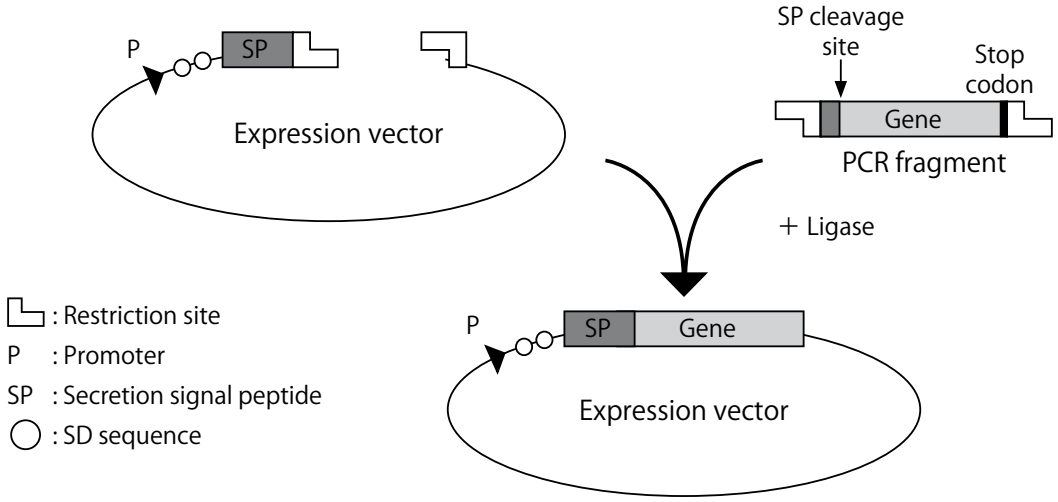


Figure 5. Construction of *B. choshinensis* expression plasmids

3. Transformation of *Brevibacillus*

Transformation of *Brevibacillus* is carried out using the New Tris-PEG (NTP) method (VI-4-C). Transformants are selected by resistance to neomycin. When subcloning using the shuttle vector pNCMO2 is carried out in *E. coli*, transformants are selected using ampicillin resistance.

4. Confirmation of Protein Production and Scale-Up

Target protein expression is confirmed using a negative control (empty shuttle vector) and a positive control (pNY326-BLA: Alpha-amylase from *Bacillus licheniformis*). Pick transformants harboring the expression plasmid and culture in liquid medium. After growing the culture in a 37°C orbital incubator (120 rpm) for 48 - 64 hours, isolate the culture supernatant by centrifuging the culture. An aliquot of the supernatant should be used for SDS-PAGE analysis to confirm production of the target protein. If production is not sufficient, try scaling-up.

VI. Protocol

1. *Brevibacillus* Strain

B. choshinensis is a highly safe host bacterium and can be easily genetically engineered.

A. Genotype

Because genes relating to sporulation have been disrupted, it is easy to perform sterilization of the strain. Additionally, disruption of the intracellular protease gene (imp) and the extracellular protease gene (emp) minimizes degradation of expressed recombinant proteins.

B. Control DNA

pNY326-BLA DNA is a positive control plasmid for *Brevibacillus* secretory production which contains the gene encoding *Bacillus licheniformis* α-amylase. The size of the protein is about 55 kDa and the secretory production level is 0.1 g/L or more.

C. Storage of Recombinant *Brevibacillus*

1) Short-Term Storage (approximately 1 week)
   1. Pick a single colony with a sterile inoculating loop and spread on a MTNm plate.
   2. Culture overnight at 30℃.
   3. Seal the plate and store at room temperature (approximately 20℃).

   Note: Never store plates in a refrigerator.

2) Long-Term Storage (1 month or longer)
   1. Pick a single colony with a sterile inoculating loop, inoculate in 2SYNm medium, then incubate at 37℃ on an orbital shaker (120 rpm) overnight.
   2. Transfer to a vial for cryopreservation and add an equal volume of LB medium containing 40% glycerol.
   3. Store frozen at -80℃.
   4. Thaw individual vials immediately before use and inoculate at 0.1 - 1.0% volume of liquid medium.

   Note: Do not subject cell stocks to repeated freeze-thaw cycles.

2. *E. coli* Host Strain

A *lac* operator is inserted into pNCMO2 to weaken promoter activity within *E. coli*. This makes it necessary to use an *E. coli* host that contains F factor (*lacI*^{q}), such as JM109. The JM109 genotype is shown below as a reference.

JM109: *recA1*, *endA1*, *gyra96*, *thi-1*, *hsdR17* (r_K^- m_K^+), *e14* $^-$ (*mcrA* $^-$), *supE44*, *relA1*, △ (*lac-proAB*) /F' [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*△M15]

3. Construction of Expression Vectors Using pNCMO2

A. Precautions for the Construction of Expression Vectors using pNCMO2

1) Use an *E. coli* host strain such as JM109 that has *lac I*^{q} and is *recA*$^-$.
2) Insert DNA should be cloned in-frame downstream from the secretion signal peptide.
3) Introduce a stop codon at the 3'-end of the target gene to be cloned.
4) When expressing a secreted protein of bacterial origin, improved results may be obtained by using the protein's own secretion signal. When using the target protein's secretory signal, the pNY326 vector is a useful expression vector because it contains a *Bsp*H I site at the start codon of the signal peptide sequence.

B. Cloning the Target DNA into pNCMO2

1) Select one of the following methods for inserting target DNA into pNCMO2.
   a. Gene Amplification using PCR

      Design PCR primers so that it is possible to insert the target gene downstream from the secretion signal. Introduce two types of restriction enzyme sites at the ends of the PCR product to establish directionality. Amplify the target gene using PCR. Select PCR conditions based on the primers and PCR enzyme used in the reaction. The use of a high-fidelity PCR enzyme, such as PrimeSTAR® Max DNA Polymerase (Cat. #R045A), is recommended.

   b. Construction of the Expression Vector through Ligation

      Select two restriction enzyme sites in the expression vector multiple cloning site that match both ends of the target DNA insert and provide directionality. Treat 0.5 - 1.0 μg of the insert and vector DNA with the restriction enzymes. Perform agarose gel electrophoresis on both the vector and insert DNA restriction enzyme reactions, then collect and purify the target fragments.

      Ligate ~ 100 ng of each purified DNA fragment using a ligation reagent such as DNA Ligation Kit <Mighty Mix> (Cat. #6023). Use a portion of the reaction solution for transformation of *E. coli*. Use the remainder for direct transformation to *Brevibacillus* (Section VI. 4. C.)

   c. Cloning with the In-Fusion® Cloning System

      The In-Fusion HD Cloning Kit can be used to simply and quickly carry out directional cloning even when there are no appropriate restriction enzyme sites. Refer to the In-Fusion Cloning System protocol.

2) Transformation of *E. coli*

   Use an *E. coli* host with high transformation efficiency. *E. coli* JM109 Competent Cells (Cat. #9052) or *E. coli* JM109 Electro-Cells (Cat. #9022) are recommended.

C. Analysis of Recombinant pNCMO2/*E. coli*

1) Inoculate an LB plate supplemented with 50 - 100 μg/ml of ampicillin with 100 - 200 μl of the transformation mixture. Incubate at 37°C for 15 - 18 hours.
2) Select 10 - 20 ampicillin-resistant colonies and add each to separate culture tubes containing 2 ml LB medium supplemented with 50 - 100 μg/ml of ampicillin.
3) Incubate at 37°C for 15 - 18 hours in an orbital incubator (250 rpm), then collect the cells using centrifugation. Extract plasmids using a commercially-available kit. Usually, 1.5 - 3 μg of plasmid DNA can be collected.
4) Perform restriction enzyme analysis using 0.5 - 1 μg DNA. Design the digest so that you can easily determine if your plasmid contains an insert. Confirm the presence of the insert DNA with agarose electrophoresis; run uncut plasmid on the same gel for comparison.
5) After the presence of the insert is detected, perform sequencing to confirm that the insert does not contain PCR-introduced errors and is in-frame with the signal sequence.

D. Sequencing

The following forward and reverse primers can be used to confirm the insert DNA sequence. (Primer sequences are shared by pNCMO2 and pNY326.)

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

4. Construction of Expression Vectors Using pNY326

A. Precautions for the Construction of Expression Vectors using pNY326

1) Use *Brevibacillus* Competent Cells to construct plasmids.
2) Insert DNA should be cloned in-frame downstream from the sec signal on the pNY326 vector.
3) Introduce a stop codon at the end of the target gene that is to be cloned.
4) When expressing a secretory protein of bacterial origin, use the endogenous secretion signal in addition to the sec signal on the pNY326 vector. When doing so, clone the insert in-frame from the start codon at the *Bsp* HI site.
5) pNY326 should be selected when transformants cannot be obtained using pNCMO2 or when higher stability is required.

B. Cloning of the Insert into pNY326

1) Select one of the following methods for inserting target DNA into pNY326.

a.Gene Amplification using PCR

Design PCR primers so that it is possible to insert the target gene downstream from the secretion signal. Introduce two different restriction enzyme sites that correspond to the expression vector at the ends of the PCR primers, to establish directionality and amplify the target gene using PCR. Select PCR conditions based on the primers and PCR enzyme used in the reaction. The use of a high-fidelity (high accuracy) PCR enzyme, such as PrimeSTAR Max DNA Polymerase (Cat. #R045A), is recommended.

b. Construction of the Expression Vector through Ligation

Select two restriction enzyme sites in the expression vector multiple cloning site that match both ends of the target DNA insert and provide directionality. Treat 0.5 - 1.0 μg of the insert and vector DNA with the restriction enzymes. Perform agarose gel electrophoresis on both the vector and insert DNA restriction enzyme reactions, then collect and purify the target fragments.

Ligate ~100 ng of each purified DNA using a ligation reagent such as DNA Ligation Kit <Mighty Mix> (Cat. #6023). The ligation reaction is used for transformation into *Brevibacillus.*

C. Transformation of *Brevibacillus*

Use a host with high transformation efficiency [*Brevibacillus* Competent Cells (Cat. #HB116) is recommended].

1) Preparation

Prepare the following reagents and materials.

Plasmid for target gene expression
MTNm plates＊1
MT liquid medium＊1
Culture tubes＊2
Sterilized microtubes

＊1 Refer to VI. 9. Medium Composition
＊2 e.g., 14-ml round-bottom sterile tube (falcon tube).

2) NTP Transformation Method

(1) Thaw Solution A, Solution B, and MT medium.
(2) Remove only the number of tubes of *Brevibacillus* Competent Cells needed for transformation from storage, and keep them on 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 for 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 (in a volume of 5 μl or less)＊1 with 50 μl of Solution A.
(6) Add all of the DNA solution to the bacterial cell pellet (from Step 4) and vortex to completely resuspend the bacterial pellet.＊2
(7) Allow to stand for 5 minutes at room temperature.
(8) Add 150 μl of Solution B (PEG solution)＊3 and vortex until the solution is uniform (5 - 10 seconds).
(9) Centrifuge the cells (5,000 rpm for 5 minutes) and remove the supernatant.
(10) Centrifuge briefly (5,000 rpm for 30 seconds) and remove the supernatant completely.
(11) Add 1 ml of MT medium and resuspend completely with a micropipette.
(12) Transfer the medium containing the cells into a culture tube, then incubate for 2 hours at 37℃ in an orbital shaker (120 rpm).
(13) Use a sterile inoculating loop to remove a small sample from the culture. Streak on an MTNm plates and culture overnight at 37℃.
(14) Select isolated colonies for plasmid analysis or protein expression.

＊1 When DNA ligation solution is used, mix 5 μl of the reaction solution with Solution A. When using purified plasmids, use 10 - 100 ng.
＊2 Be sure to resuspend well, as incomplete dispersion of cells will decrease the efficiency of transformation.
＊3 Solution B (PEG solution) is highly viscous - use a 1,000 μl micropipette and pipette slowly.

D. Analysis of Recombinant *Brevibacillus*

1) Randomly select 10 - 20 of the colonies from the MTNm plate and inoculate in 2 ml of TMNm medium.
   Incubate the cultures with vigorous shaking under sufficient ventilation.
2) Incubate the cultures at 37°C in an orbital shaker (120 rpm) for 15 - 18 hours. Centrifuge at 5,000 rpm for 5 minutes to collect the bacteria. Extract plasmid DNA using a commercial plasmid extraction kit. In general, 1 - 2 μg of plasmid DNA can be recovered.
3) Analyze the plasmid by restriction enzyme digestion. Run uncut plasmid on the same gel for comparison.
4) After confirming the presence of the insert DNA, perform sequencing to confirm that the gene has no errors introduced by PCR and is in-frame with the signal sequence. (See Section VI. 3. D for sequencing primers.)

5. Target Protein Expression with Recombinant *Brevibacillus*

Perform small-scale protein expression with the transformed *Brevibacillus* cells. A standard method for confirming expression is given here.

A. Overview

After transformants are prepared, perform expression testing. Use the following transformants as the controls:

Positive Control:	*B. choshinensis* SP3/pNY326-BLA transformant
Negative Control:	*B. choshinensis* SP3/pNCMO2 or pNY326 (vector only) transformant

Protein expression level and colony size of transformants may vary depending on the target protein. Therefore, 6 - 10 colonies (including both large and small colonies) should be randomly selected for expression testing in culture tube. If plates stand for multiple days after transformation, protein production may decrease. If this occurs, perform transformation again.

B. Culture Medium

Use TM and 2SY culture media for expression testing. Protein productivity may vary depending on the medium used. Examine protein production using both types of media because a different medium may cause differences in protein expression level.

C. Secretory Protein Production

Determine whether or not culture conditions are appropriate by confirming production of *Bacillus licheniformis* alpha-amylase (approximately 55 kDa) using *B. choshinensis* SP3/pNY326-BLA as the positive control. At the same time, prepare cultures of the transformants that express the target protein and a negative control, then confirm production by comparing their protein expression levels.

The protocol for expression testing is shown below.

1) Select individual colonies and inoculate in test tubes (16 mm diameter) containing 3 ml 2SYNm and TMNm liquid medium. Incubate the cultures at 30 - 33℃ with shaking (120 rpm) under sufficient ventilation for 48 - 64 hours. During this time, remove an aliquot every 24 hours and measure production of the target protein.
2) After culture is complete, isolate the supernatant by centrifuging at 5,000*g* for 5 minutes. Suspend the cell pellet in an equal amount of PBS.*
3) Evaluate protein expression in the culture supernatant and pellet fraction using SDS-PAGE (CBB staining or Western blotting) or measure protein activity.

* PBS tablets (Cat. #T900) are recommended.

6. SDS-PAGE Analysis

A. Preparation of Samples

Add 10 μl of 5X SDS-PAGE loading buffer to 40 μl of each supernatant and pellet suspension.

After mixing, heat at 100℃ for 10 minutes to obtain denatured samples for electrophoresis.

B. Controls

Use the following samples as controls

a. Standard target protein
b. Culture supernatant of *B. choshinensis* SP3 with an expression vector that does not contain insert DNA (negative control)
c. Culture supernatant of *B. choshinensis* SP3/pNY326-BLA (positive control)
d. Molecular weight marker

C. Analysis of Protein Expression

It is possible to determine if protein was produced by comparing the standard target protein and the culture supernatant by SDS-PAGE. Detection may be difficult when protein production is low, when solubility is poor, or when there is high background. In these cases, confirm protein production using a method such as Western blotting using a specific antibody, evaluation of function (such as measurement of enzyme activity), or purification.

7. Optimization of Protein Expression

The probability of protein expression using this system is about 30 - 40%. Protein production level is 100 mg/L or more for most experiments, and the production may exceed 1 g/L in some experiments. If target protein productivity is quite low or no product can be detected, refer to the guidelines described below.

A. Low Protein Expression

1) Try using BIC System (Cat. #HB300). The success rate should improve by 20 - 30%.
2) Try using both vectors, pNCMO2 and pNY326. These vectors have different promoter activities. In some cases, as with pNCMO2, higher promoter activity improves productivity. Alternately, the use of a vector with weaker activity such as pNY326 may increase productivity by improving bacterial growth.
3) Try different types of media (TM and 2SY), as this may lead to changes in productivity.
4) Some target proteins are not well-suited for secretory production; try to produce the protein by intracellular expression and measure protein production in the cell fractions.

   If production is very low, concentrate the protein using ammonium sulfate precipitation or ultrafiltration.

B. No Protein Expression

First, try the suggestions in Section VI. 7. A. If there is no improvement, consider the following possibilities.

1) Confirm the conformation of the target mRNA. A high-energy palindromic structure may result in abnormal translation. In this case, it is necessary to introduce mutations in repetitive sequence to eliminate stacking.
2) If the sequences in the vicinity of the signal cleavage site are unsuitable, there may be effects on secretory production. If the addition of extra sequence at the N-terminal end does not affect the activity of the target protein, consider introducing purification tags or detection tags, or random sequences.

8. Protein Purification

Select a purification method suitable for the type of target protein used. When the target protein is produced through secretion, it is localized to the culture medium. Centrifugation will pellet the bacteria, allowing separation of the culture medium and target protein from the cells. Target protein can then be purified from the culture medium using standard methods such as ion exchange, hydrophobic, or affinity chromatography.

It is possible to obtain purified proteins more easily by adding his tags or other purification tags to the target gene. His tags can be added to either the 5′ or 3′ end of the target protein gene. Beforehand, check that the tag is compatible with the activity of protein.

The following his tag vectors have a his tag sequence and a protease recognition sequence for tag region removal inserted downstream of the pNCMO2 DNA secretion signal:

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

Note: Directly applying 2SY medium to a Ni-chelate column may cause Ni to leach from the column resin. If 2SY medium is used for culturing the recombinant cells, purification can be successfully performed after dialysis of the culture sample. Dialysis is unnecessary when using Ni Sepharose Fast Flow (GE Healthcare).

9. Medium Composition

・2SY Liquid Medium

Components

Glucose＊1	20.0 g/L
Bacto Soytone	40.0 g/L
Bacto Yeast Extract	5.0 g/L
$CaCl_2$ $2H_2O$	0.15 g/L

Adjust to pH7.2 with NaOH

＊1 Mix glucose and $CaCl_2$ and sterilize separately from the other components. Mix after sterilization.

・2SYNm Liquid Medium

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

・TM Liquid Medium

Components

Glucose＊2	10.0 g/L
Phytone peptone	10.0 g/L
35%Ehrlich Bonito Extract	5.75 g/L
Yeast extract Blue label	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 pH7.0 with NaOH

＊2 Sterilize glucose and the other components separately. Mix after sterilization.

・TMNm liquid medium

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

- MT Liquid Medium
  Add $MgCl_2$ to the TM liquid medium to a concentration of 20 mM.
- MTNm Plate
  Suspend 7.5 g of agar in 500 ml of MT liquid medium and autoclave. After cooling to approximately 50℃, add neomycin (50 mg/ml stock solution) to a concentration of 50 μg/ml, mix gently, and dispense into plates.

Note: For the components of the 2SY medium and the TM medium, the following manufacturers are recommended:

Bacto Soytone	(Becton Dickinson, Code. 243620)
Bacto Yeast Extract	(Becton Dickinson, Code. 212750)
Phytone Peptone	(Becton Dickinson, Code. 211906)
35%Ehrlich Bonito Extract	(Kyokuto Pharmaceutical, Code. 551-01212-5)
Yeast extract Bluel label	(Oriental Yeast Co., Ltd.)

VII. References

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VIII. Related Products

BIC System (Cat. #HB300)
pBIC DNA Set (Cat. #HB310)
pNC-HisT DNA (Cat. #HB121)
pNC-HisF DNA (Cat. #HB122)
pNC-HisE DNA (Cat. #HB123)
pNI DNA (Cat. #HB131)
pNI-His DNA (Cat. #HB132)
In-Fusion® HD Cloning Plus (Cat. #638909-638920)
DNA Ligation Kit <Mighty Mix> (Cat. #6023)
PrimeSTAR® Max DNA Polymerase (Cat. #R045A)
PBS (Phosphate Buffered Salts) Tablets (Cat. #T900)
E. coli JM109 Competent Cells (Cat. #9052)
E. coli JM109 Electro-Cells (Cat. #9022)

IX. Notice: Living Modified Organisms

Brevibacillus Competent Cells (Cat. #HB116) and *Brevibacillus* Expression System II (Cat. #HB200) include a genetically "Living Modified Organism (LMO)" defined in "The Cartagena Protocol on Biosafety". The supplied *Brevibacillus* Competent Cells in these kits contain partial sequences of 2 μm plasmid DNA derived from *Saccharomyces cerevisiae*.

Please follow the guidelines or the laws and regulations that are specific to your country and pay attention to safe handling, storage, transport and disposal.

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