Cat. # HB300
HB310

For Research Use

TaKaRa

Brevibacillus Expression System
BIC System (Cat. #HB300)
pBIC DNA Set (Cat. #HB310)

Product Manual

v202104

Table of Contents

I. Description

The *Brevibacillus* Expression System is an efficient system for producing secreted proteins and is ideal for eukaryotic recombinant protein expression. *Brevibacillus choshinensis* is a gram-positive bacterium well-suited for heterologous protein expression and is capable of producing high yields of active protein. This system offers the following advantages:

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

Examples of protein production using this system are shown in Table 1. High expression of biologically active protein (enzymes, antigens, and cytokines) was obtained regardless of gene origin (bacterial, archaea, or eukaryotic). In particular, eukaryotic secretory proteins often contain disulfide (S-S) bonds and are generally difficult to produce in prokaryotic expression systems. The *B. choshinensis* Host-Vector System can produce high yields of secretory proteins, including those with disulfide 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	4)
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	6)
TNF- α	Cow	0.4	
GM-CSF	Cow	0.2	
GH	Flounder	0.2	

The high transformation efficiency of *Brevibacillus* makes cloning target genes simple. The BIC (*Brevibacillus in vivo* Cloning) method used in this system allows expression plasmids to be constructed by simply by mixing PCR fragments with the vector DNA and transforming the mixture into the host bacteria.

In addition, culturing methods for protein production are also simple. Culturing for production of the target protein uses one of two types of media (see V-8. Composition of Medium). For secretory protein expression, medium is inoculated with transformed host bacteria, and the target protein accumulates in the culture supernatant. After culturing, the protein-containing solution can be obtained by removing the cells by centrifugation, and protein can be further purified as needed.

II. Components

Brevibacillus Expression System - BIC System (Cat. #HB300)

- pBIC DNA Set (Cat. #HB310)
  - Linear Expression Vector DNA
    - pBIC1 DNA 5 μg (100 ng/μl)
    - pBIC2 DNA 5 μg (100 ng/μl)
    - pBIC3 DNA 5 μg (100 ng/μl)
    - pBIC4 DNA 5 μg (100 ng/μl)
  - Control (Insert DNA)
    - BLA 1 μg (80 ng/μl)
- 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. Storage

- pBIC DNA Set
  - pBIC1 - pBIC4 DNA -20℃
  - BLA -20℃
- Competent Cells
  - *Brevibacillus* Competent Cells -80℃
  - Others -80℃

IV. Overview of the BIC System

IV-1. Features

BIC (*Brevibacillus in vivo* Cloning) is an extremely simple method for obtaining recombinant expression plasmids. With this method, the plasmid is constructed by mixing a linearized vector and an insert DNA amplified by PCR, and transforming this mixture into the host bacteria. Because the procedure does not include conventional restriction enzyme digestion and ligation, the BIC system can save time, work, and expense. It is often difficult to select restriction enzyme sites when cloning large-sized genes with traditional methods; the BIC method overcomes this problem. In addition, multiple gene expression strains can be created simultaneously through the use of multiple target genes.

IV-2. Principles

DNA that codes for the target protein is generated by PCR with the addition of 15 base pairs of sequence that is homologous to each end of the linear vector. The PCR product is mixed with the linear vector and is introduced into competent cells. Recombination occurs between the homologous sequences in the cell and the expression plasmid is formed (Figure 1).

Because the recombination reaction can also occur at sites near the ends of the linear vector, it is possible to construct plasmids for intracellular expression that lack the secretion signal or plasmids for direct secretory expression that lack the his tag sequence. To do this, mix the vector with PCR fragments that contains 15 base pair overhangs that are homologous to the sequence upstream of the secretion signal or the his tag sequence, respectively. This system includes pBIC1-pBIC4, expression vectors that include 4 types of secretion signal sequences derived from proteins that have extremely high secretion in *B. choshinensis*.

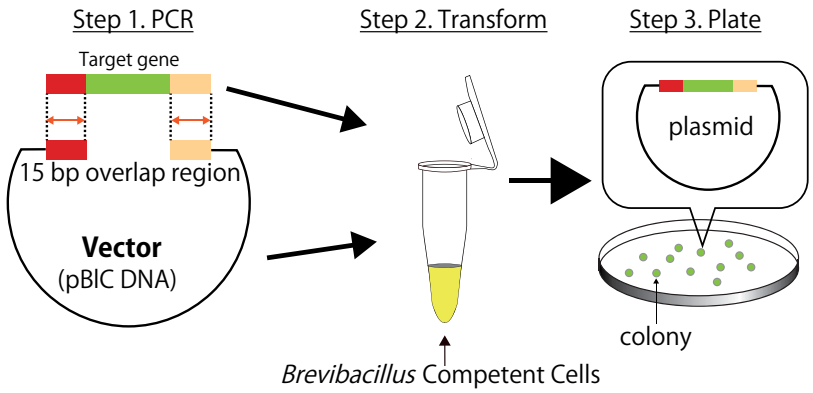
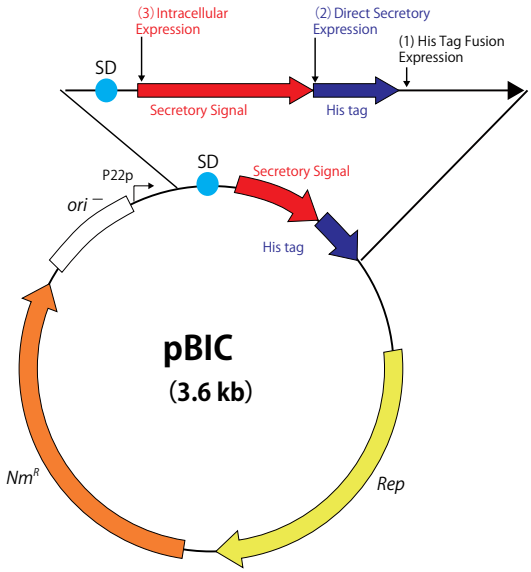


Figure 1. Principles of BIC

IV-3. Expression Vector

<Selection of Expression Type>

For target protein expression using *Brevibacillus*, it is possible to select secreted expression or intracellular expression based on the characteristics of the protein. This system is particularly effective for expression of secretory proteins. For secretory expression, the target gene is inserted downstream of the secretion signal sequence or his tag. Intracellular expression is recommended to efficiently produce proteins that are located and function in intracellular locations. It is possible to use *Brevibacillus* to obtain high-level soluble production of proteins that are insoluble with *E. coli*.



The pBIC DNA can be used to construct the following 3 types of plasmids for protein expression.

(1) Secretory Expression (His Tag Fusion Expression)
Secreted with two amino acids (AD) + his tag +DDDDK (enterokinase site) attached to the N terminus, allowing purification of the target protein using a Ni-chelate column.*1 When introducing genes, homologous recombination is performed using sequences within the enterokinase recognition sequence.

(2) Secretory Expression (Direct Secretory Expression)
A target gene is introduced downstream of the secretion signal. When introducing the gene, homologous recombination is performed using the sequence upstream of the secretion signal cutting site.

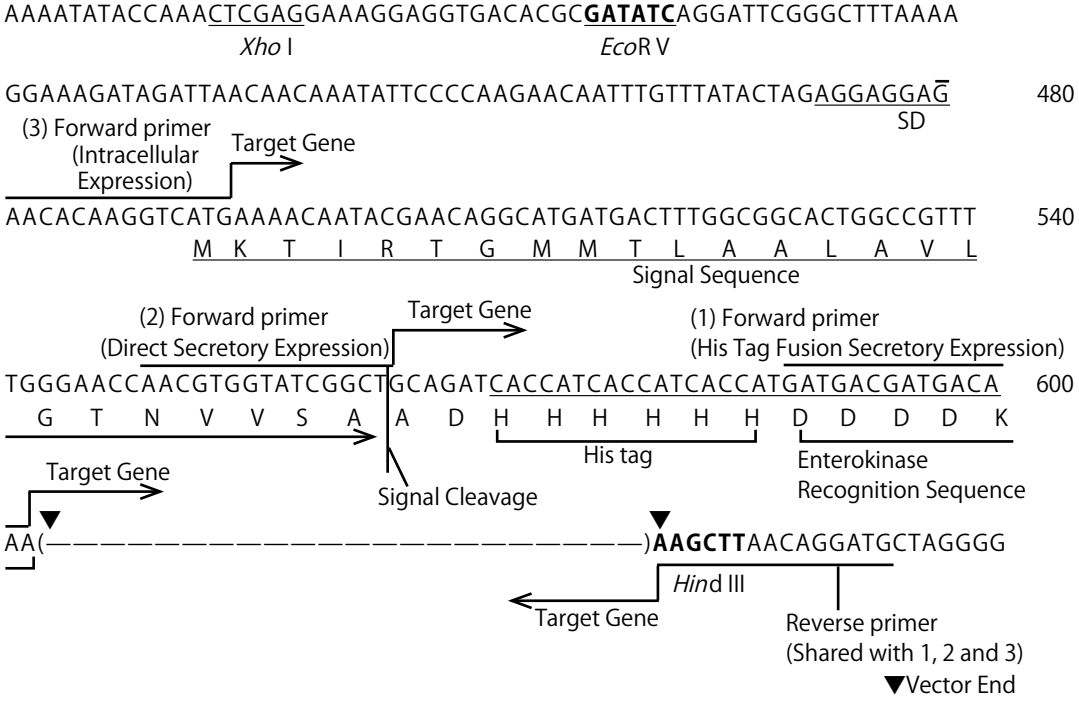
(3) Intracellular Expression
A target gene is introduced downstream of ATG, the translation initiation site. Homologous recombination is performed using the sequence upstream of the ATG.

*1 Be aware that directly applying cultures in 2SY medium to a Ni-chelate column may cause Ni to leach from the column resin. In this case, perform dialysis prior to column purification. Dialysis is not necessary when using Ni Sepharose Fast Flow (GE Healthcare).

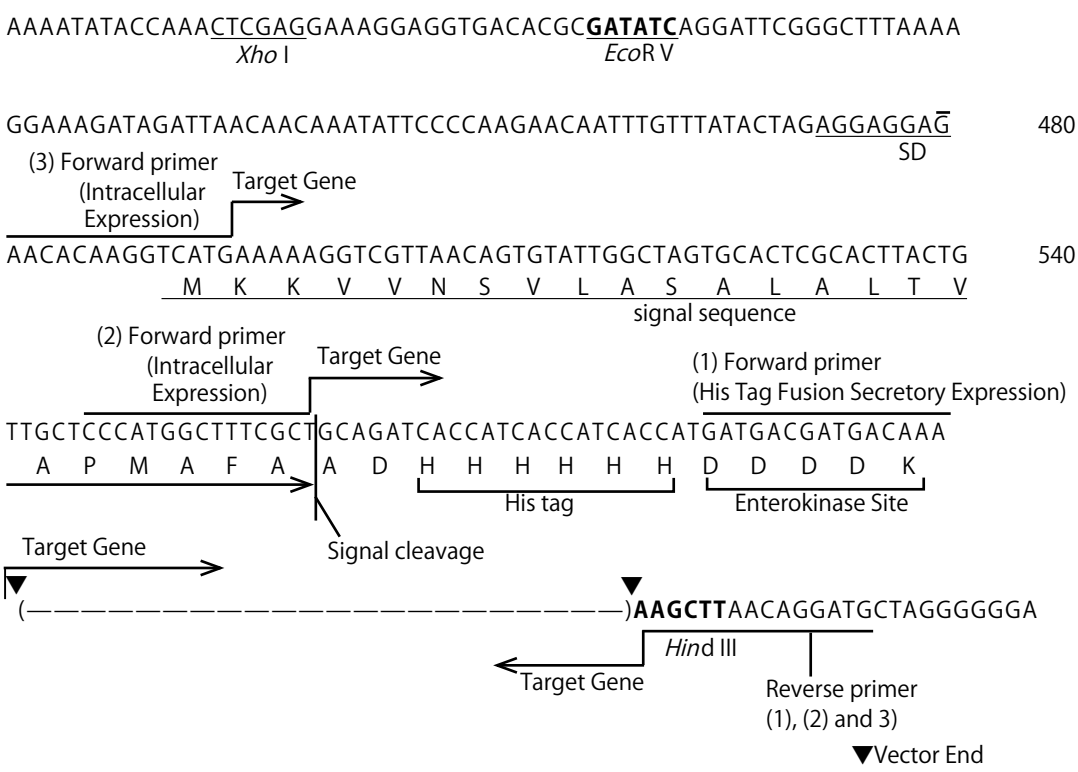
<Secretion Signals>

The secretion signal is a factor that greatly affects the secretory efficiency of the protein. Because there is no universal secretion signal that allows secretion of any protein with maximum efficiency, it is necessary to select a secretion signal that is suitable for the target protein. This system includes pBIC1-pBIC4 expression vectors that contain 4 types of secretion signal sequences derived from proteins that have extremely high secretion in *B. choshinensis*. By introducing the target gene into these vectors and comparing protein production, it is possible to screen for the expression plasmid that allows optimal secretory expression.

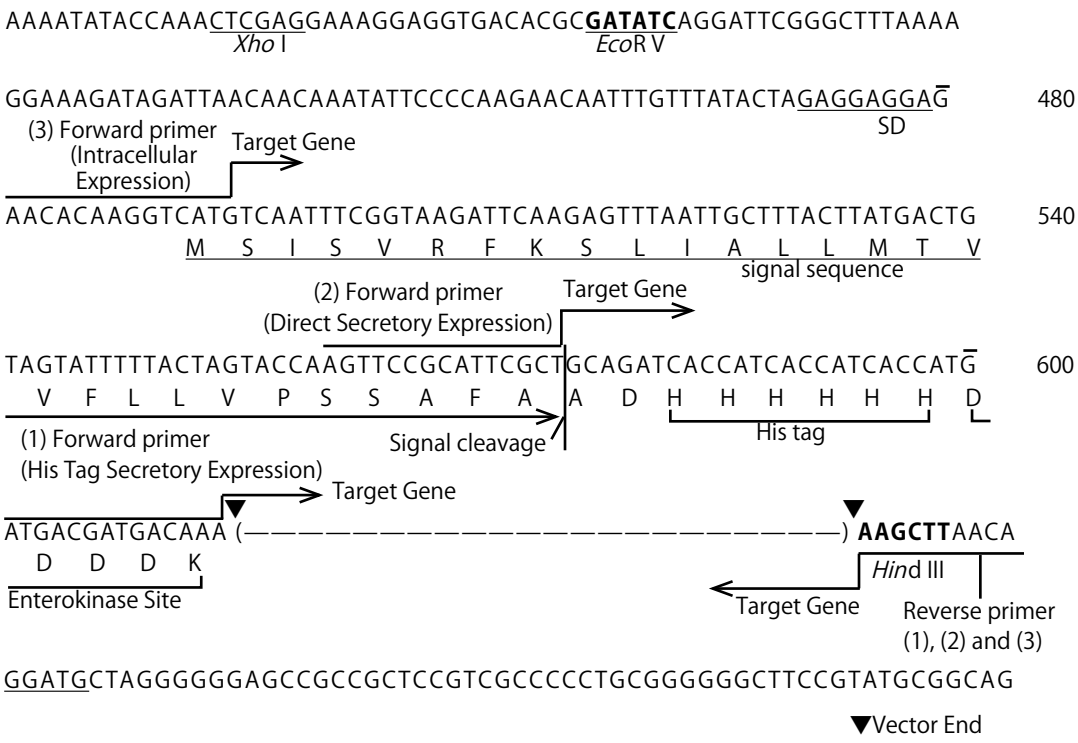
pBIC1 DNA



pBIC 2 DNA



pBIC3 DNA



pBIC4 DNA

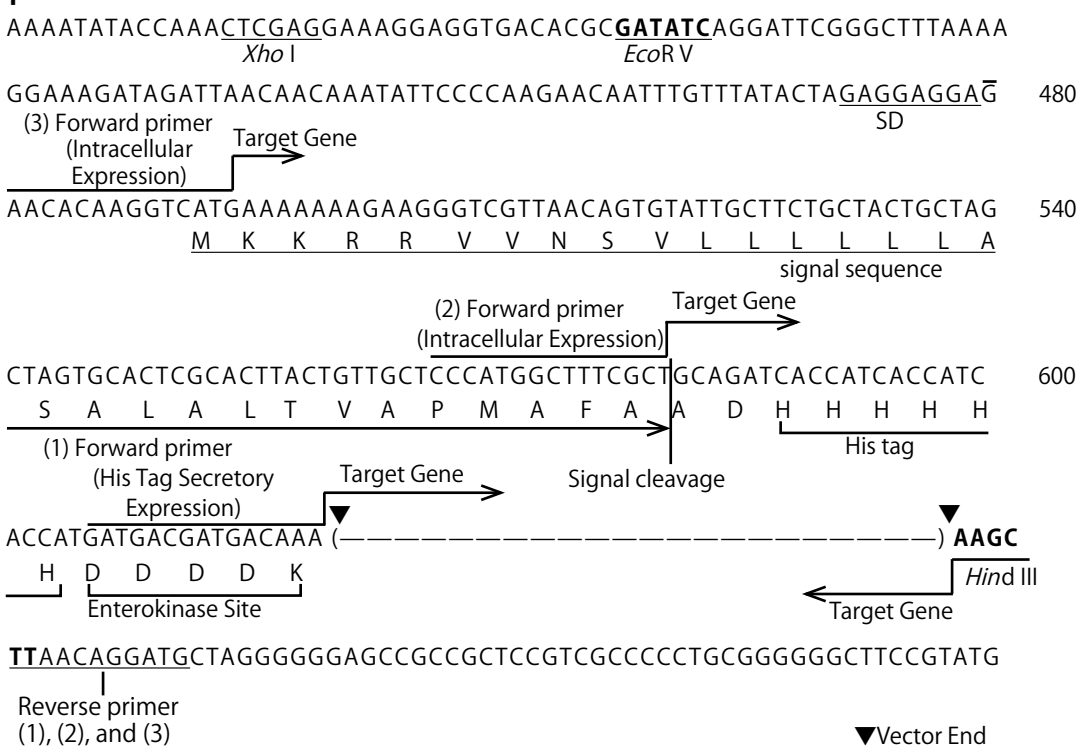


Figure 3. Nucleotide Sequence of the Secretion Signal in pBIC vectors

V. Protocol

V-1. About *Brevibacillus*

B. choshinensis SP3 is a highly safe host bacterium that is included on the GILSP list (Japan) and that can be easily genetically engineered.

V-1-1. Genotype

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

V-1-2. Control DNA

BLA: This insert DNA encodes alpha-amylase from *Bacillus licheniformis and* can be used as a positive control for expression of a his-tagged, secreted protein; the control DNA is 1,480 bp.
The 55 kDa alpha-amylase protein expression of >50 mg/L in 2SY medium can be expected.

V-1-3. Storage of recombinant *Brevibacillus*

Short-Term Storage (approximately 1 week)

1. Pick a single colony 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 the plate in a refrigerator (4℃).

Long-Term Storage (1 month or more)

1. Pick a single colony, inoculate 2SYNm medium (See V-8. Composition of Medium), and culture overnight with shaking.
2. Add an equal volume of LB medium containing 40% glycerol and transfer to a vial for cryopreservation.
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.

V-2. Construction of pBIC Expression Vectors

V-2-1. pBIC DNA Structure

<Vector Information for pBIC>

Promoter	A portion of the 5' sequence upstream of the *B. choshinensis* P22 gene, which is expressed strongly in *Brevibacillus*
Secretion Signal	pBIC1: Secretion signal for BbrPI from *B. choshinensis* pBIC2: Secretion signal for a cell wall protein from *B. brevis* pBIC3: Secretion signal for the P22 gene from *B. choshinensis* pBIC4: Modified secretion signal for pBIC2
Terminator	A 46-bp termination signal is inserted downstream from the cloning site
Rep	Encodes a protein related to plasmid replication (derived from pUB110)
Ori	Plasmid replication origin (derived from pUB110)
Nm^R	Neomycin resistance gene (selection marker)

Refer to Figure 3 in Section IV-3. for additional details.

V-2-2. Cloning into pBIC

<PCR Amplification of Target Genes>

Design primers for the target sequence based on (1) - (3) below.
Design primers with 15-base 5′ overhangs that are homologous to the vector, and with sequences (18 - 20 bases) for amplifying the target gene at the 3' ends.

(1) For Secretory Expression (His tag Fusion Expression)
Forward: 5′ -GATGACGATGACAAA- 5' sequence of the target gene
Reverse*1: 5′ - CATCCTGTTAAGCTT- 3' sequence of the target gene

(2) For Direct Secretory Expression

- Primer design for pBIC1:
  Forward: 5′ -AACGTGGTATCGGCT- 5' sequence of the target gene
  Reverse*1: 5′ -CATCCTGTTAAGCTT- 3' sequence of the target gene
- Primer design for pBIC2:
  Forward: 5′ -CCCATGGCTTTCGCT- 5' sequence of the target gene
  Reverse*1: 5′ -CATCCTGTTAAGCTT- 3' sequence of the target gene
- Primer design for pBIC3:
  Forward: 5′ -AGTTCCGCATTCGCT- 5' sequence of the target gene
  Reverse*1: 5′ - CATCCTGTTAAGCTT- 3' sequence of the target gene
- Primer design for pBIC4:
  Forward: 5′ - CCCATGGCTTTCGCT- 5' sequence of the target gene (same as pBIC2)
  Reverse*1: 5′ - CATCCTGTTAAGCTT- 3' sequence of the target gene

(3) For Intracellular Expression
Forward: 5′ -GAACACAAGGTCATG- 5' sequence of the target gene (without the start codon)
Reverse*1: 5′ - CATCCTGTTAAGCTT- 3' sequence of the target gene

*1 The Reverse primer is identical in (1), (2), and (3).

Amplify the target gene by PCR, using the primers designed above. Determine the appropriate PCR conditions based on the product length and the PCR enzyme. A high-fidelity PCR enzyme such as PrimeSTAR® Max DNA Polymerase (Cat. #R045A) is recommended for PCR.

<Purification of the PCR Product>

1. Perform agarose gel electrophoresis using an aliquot of the PCR reaction. If only specific amplification products are observed, purify the PCR product using a commercial DNA purification column (such as NucleoSpin Gel and PCR Clean-up).*2
2. If non-specific amplification is observed by agarose gel electrophoresis, purify the DNA from the agarose gel by using a kit such as NucleoSpin Gel and PCR Clean-up.

*2 You may omit purification of the PCR product if there is only a small amount of non-specific products.

<Transformation>

Mix 1 μl (100 ng) of pBIC DNA with the insert DNA at a molar ratio of approximately 1 : 2, and add sterilized water to a total volume of 5 μl. Transform *Brevibacillus* Competent Cells (Cat. #HB116)*3 with the mixture. Refer to Section V-3. *Brevibacillus* Transformation for a protocol.

*3 In general, transformation efficiency is approximately 80%, but the efficiency may decrease if the expressed protein is toxic to the bacteria. For a control experiment, this kit includes the BLA (alpha-amylase derived from *B. licheniformis*) gene as a positive control. Mix 1 μl (100 ng) of the vector with 1 μl (80 ng) of BLA and transform the competent cells for a control. When the expression plasmid is constructed correctly using BLA, it is possible to identify positive clones by the formation of a halo surrounding colonies on an agar plate that contains 2% starch. However, because the secretory expression of BLA is slightly toxic to the host cells, the number of colonies formed and the transformation efficiency with pBIC4 (which has higher expression) will be lower than with pBIC1-pBIC3.

V-2-3. Analysis of Recombinant Plasmids

1) Randomly select 6 - 10 various sizes of colonies on a MTNm plate and inoculate each colony in 2 ml of TMNm medium.
Incubate the cultures with vigorous shaking under sufficient ventilation.

2) After incubating the cultures for 15 - 18 hours at 37℃, centrifuge at 5,000 rpm for 5 minutes. Purify the plasmids from the cell pellet using a commercial plasmid extraction kit. In general, 1 - 2 μg of DNA can be recovered.

3) Analyze the plasmid by restriction enzyme digestion.

4) After confirming insertion of the target gene, validate the target gene sequence by sequencing analysis. The following forward and reverse primers can be used for sequencing.

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

* The primers can be used for sequencing inserts in both intracellular expression and secretory expression plasmids.

V-3. *Brevibacillus* Transformation

V-3-1. Preparation

Brevibacillus Competent Cells (Cat. #HB116)
(Components) *Brevibacillus* Competent Cells
MT medium
Solution A
Solution B

MTNm plates
Tubes for culture*
Sterile microtubes

* e.g., 14-ml round-bottom sterile tube (falcon tube).

V-3-2. Transformation

(1) Bring Solution A, Solution B, and MT medium to room temperature.

(2) Thaw the *Brevibacillus* Competent Cells rapidly (approximately 30 seconds) in a 37℃ waterbath.

(3) Centrifuge at 12,000 rpm for 1 - 2 minutes, and remove the supernatant completely using a micropipette.

Perform the following steps at room temperature

(4) Mix the DNA solution (5 μl*1) with 50 μl of Solution A.

(5) Add all of the DNA mixture to the tubes in (3) and vortex to completely resuspend the cell pellets.*2

(6) Allow to stand at room temperature for 5 minutes.

(7) Add 150 μl of Solution B (PEG solution)*3 and vortex until a uniform mixture is obtained (5 - 10 seconds).

(8) Pellet the cells using a microcentrifuge (5,000 rpm for 5 minutes) and remove the supernatant.

(9) Centrifuge briefly again (5,000 rpm for approximately 30 seconds) and completely remove the supernatant.

(10) Add 1 ml of MT medium and resuspend the pellet completely using a micropipette.

(11) Transfer the medium containing the cells into a culture tube, then incubate for 2 hours at 37℃ in an orbital shaker (120 rpm).

(12) Spread cells onto the MTNm plate and incubate overnight at 37℃.

(13) Use the colonies for plasmid analysis or protein expression.

*1 Mix pBIC DNA (100 ng) and the insert DNA at a molar ratio of 1 : 2, add sterilized water to obtain a total of 5 μl, and then mix with Solution A. For purified plasmids, use 10 - 100 ng of DNA.

*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; pipette slowly using a 1,000 μl pipette tip.

V-4. Protein expression with Recombinant *Brevibacillus*

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

V-4-1. Precaution

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 test tube culture.
If plates stand for multiple days after transformation, protein production may decrease. If this occurs, perform transformation again.

Perform protein expression testing using the positive control which expresses the BLA gene in pBIC DNA. If there are large and small colonies on the selective plates, use the small colonies for the BLA protein expression.

V-4-2. Culture Medium

TM medium and 2SY medium are used as the media for expression testing. Examine the protein production using both types of media because different medium may cause differences in protein expression level.

V-4-3. Secretory Protein Production

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 the 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 centrifugation at 5,000*g* for 5 minutes. Suspend the cell pellet in an equal amount of PBS.*

(3) Evaluate protein expression in the supernatant and pellet fraction using SDS-PAGE (CBB staining or Western blotting) and measure protein activity.

V-4-4. Intracellular Protein Production

(1) Select each colony and inoculate test tubes (16 mm diameter) containing 3 ml of 2SYNm and TMNm liquid medium. Culture at 30 - 33℃ with shaking at 120 rpm for 48 - 64 hours. Remove an aliquot every 24 hours and examine production of the target protein.

(2) After culture is complete, isolate the supernatant by centrifugation at 5,000*g* for 5 minutes. Suspend the pellet using an equal amount of PBS*, then sonicate. Alternatively, protein can be easily and gently extracted using the xTractor Buffer Kit (Cat. #635623).

(3) After cell disruption using sonication or xTractor Buffer, centrifuge at 12,000*g* for 10 minutes to recover the supernatant (soluble fraction). Then, evaluate protein expression using SDS-PAGE and measure protein activity.

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

V-5. SDS-PAGE Analysis

Perform target protein analysis using SDS-PAGE.

V-5-1. Sample Preparation

Add 10 μl of 5X SDS-PAGE loading buffer to 40 μl of the culture supernatant and the pellet suspension. After mixing, heat at 100℃ for 5 minutes to obtain the denatured sample for electrophoresis.

V-5-2. Controls

Use the following samples as controls.

a. Molecular weight markers
b. Target protein standards
c. The culture supernatant of *B. choshinensis* SP3/pBIC-BLA that expresses alpha-amylase (approximately 55 kDa) (positive control).

V-5-3. Analysis of Protein Expression

It is possible to determine whether or not protein was produced by comparing the target protein standards 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 with a specific antibody, evaluation of function (such as measurement of enzyme activity), or purification.

V-6. Optimization of Protein Expression

With the BIC System, the probability of protein expression is ～ 60%. Protein production of 100 μg/ml can be achieved for most experiments, and expression exceeding 1 mg/ml is possible for some experiments.

V-6-1. Low productivity

a. Try another medium. A change in medium may increase protein production.

b. Some target proteins are not well-suited for secretory production. Try to produce the protein by intracellular expression and examine protein expression in the cell fractions.
If very little protein is produced, concentrate the protein using ammonium sulfate precipitation or an ultrafiltration membrane.

V-6-2. No protein production

First, try the suggestions in Section V-6-1. Low productivity. If there is no improvement, consider the following possibilities:

a. 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 sequences to eliminate stacking.

b. 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 sequence.

V-7. Purification of the Target Protein

When the target protein is produced through secretion, it is present in the culture medium. Centrifugation will pellet the bacteria, allowing separation of the target protein in the culture medium from the cells. Then, the target protein may be purified from the culture medium using conventional methods such as ion exchange, hydrophobic, or affinity chromatography.

A his tag has been introduced downstream of the cloning site of pBIC DNA. It is possible to obtain his-tagged proteins that can be purified using a Ni-chelate column.
Please note that 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).

V-8. Composition of Medium

・2SY Liquid Medium

Composition

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

Adjust to pH 7.2 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 2 SY liquid medium to reach 50 μg/ml.

・TM Liquid Medium

Composition

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 pH 7.0 with NaOH

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

・TMNm Liquid Medium

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

・MT Liquid Medium

Add $MgCl_2$ to the TM liquid medium to a final concentration of 20 mM.

・MTNm Plates

Suspend 7.5 g of agar in 500 ml of the MT liquid medium and autoclave. After cooling to approximately 50℃, add neomycin (50 mg/ml stock solution) to obtain a final concentration of 50 μg/ml, mix gently, and dispense into plates.

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 Blue label	(Oriental Yeast Co., Ltd.)

VI. Related Products

Brevibacillus Expression System II (Cat. #HB200)
pNC-HisT DNA (Cat. #HB121)
pNC-HisF DNA (Cat. #HB122)
pNC-HisE DNA (Cat. #HB123)
pNI DNA (Cat. #HB131)
pNI-His DNA (Cat. #HB132)
PrimeSTAR® Max DNA Polymerase (Cat. #R045A/B)
PrimeSTAR® GXL DNA Polymerase (Cat. #R050A/B)
xTractor Buffer Kit (Cat. #635623)
NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250)

VII. References

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VIII. Notice: Living Modified Organisms

Brevibacillus Competent Cells (Cat. #HB116) and *Brevibacillus* Expression System BIC System (Cat. #HB300) 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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