Cat. # 3380

For Research Use

TaKaRa

B. subtilis Secretory Protein Expression System

Product Manual

v201801Da

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I. Description

Recombinant protein production using *Bacillus subtilis* as host facilitates soluble expression and secretory expression. The *B. subtilis* expression host is particularly effective for proteins that have complex structures, such as S-S bonds. Unlike the *E. coli* expression systems, recombinant proteins produced by *B. subtilis* are free of endotoxin.

T Eggert, *et al*. have reported that the level of secretion of recombinant proteins is significantly influenced by the type of signal peptide used.[1)] Takara Bio has developed a system to screen for efficient expression of secreted proteins using *B. subtilis*. This system makes it possible to identify the signal peptides suitable for secretory expression of a target protein from among a library of 173 types of *B. subtilis*-derived secretory signal peptides. However, because the plasmids of *B. subtilis* have extremely low copy number, plasmid construction within *B. subtilis* is difficult. This system uses pBE-S DNA as a shuttle vector of *E. coli* and *B. subtilis*, which makes it possible to construct and propagate expression plasmids in *E. coli*.

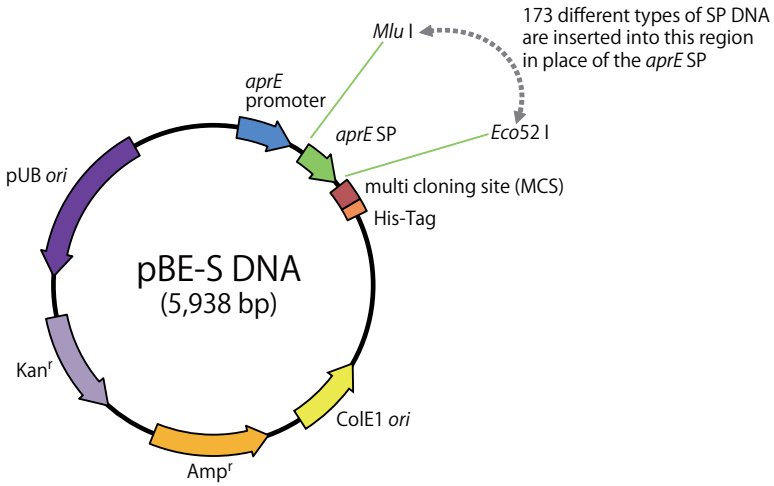
This system includes:

- SP DNA mixture - the DNA library of the secretory signal peptides of *B. subtilis*
- The *B. subtilis* / *E. coli* shuttle vector pBE-S DNA
- *B. subtilis* strain RIK1285

The SP DNA mixture contains DNA fragments (for In-Fusion® cloning) that encode 173 types of *B. subtilis* secretory signal peptides. The pBE-S DNA vector includes the pUB110-derived replication ori (pUB *ori*) and a kanamycin-resistant gene (Kan^r) which functions in *B. subtilis,* as well as the pUC-derived replication ori (ColE1 *ori*) along with an ampicillin-resistant gene (Amp^r) which functions in *E. coli*. Additionally, it includes a *B. subtilis*-derived subtilisin promoter (*aprE* promoter) and secretory signal peptide (*aprE* SP), which are located upstream from the multi-cloning site (MCS) and the His tag sequence. Once linearization of pBE-S DNA containing a target gene in the MCS is performed through the use of the restriction enzymes *Mlu* I and *Eco*52 I, the In-Fusion cloning method is used to introduce the DNA library of 173 types of secretory signal peptides in the SP DNA mixture in place of the *aprE* SP (see vector map below). This makes it possible to screen for an efficient secretory signal peptide suitable for the target protein.

Because the MCS of pBE-S DNA is compatible with the *E. coli* cold-shock expression system pCold™ vector (Cat. #3360 - 3365 and 3371)*, one can easily transfer the target gene between these vectors. The host strain *B. subtilis* RIK1285, provided with this kit, is deficient in two kinds of proteases,[2)] and therefore is suitable for the secretory expression of target proteins.

* Not available in all geographic locations. Check for availability in your area.



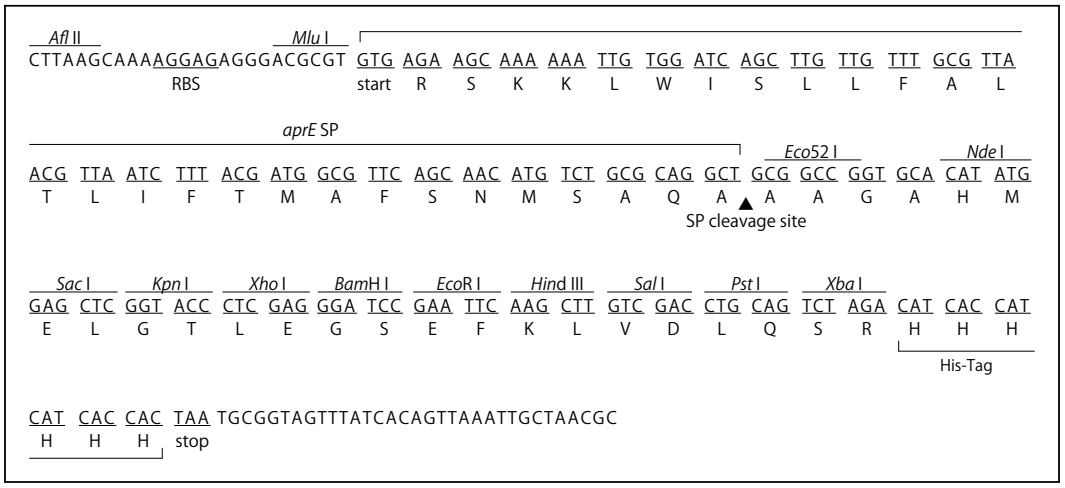


Figure 1. Sequence of the multi-cloning site of pBE-S DNA

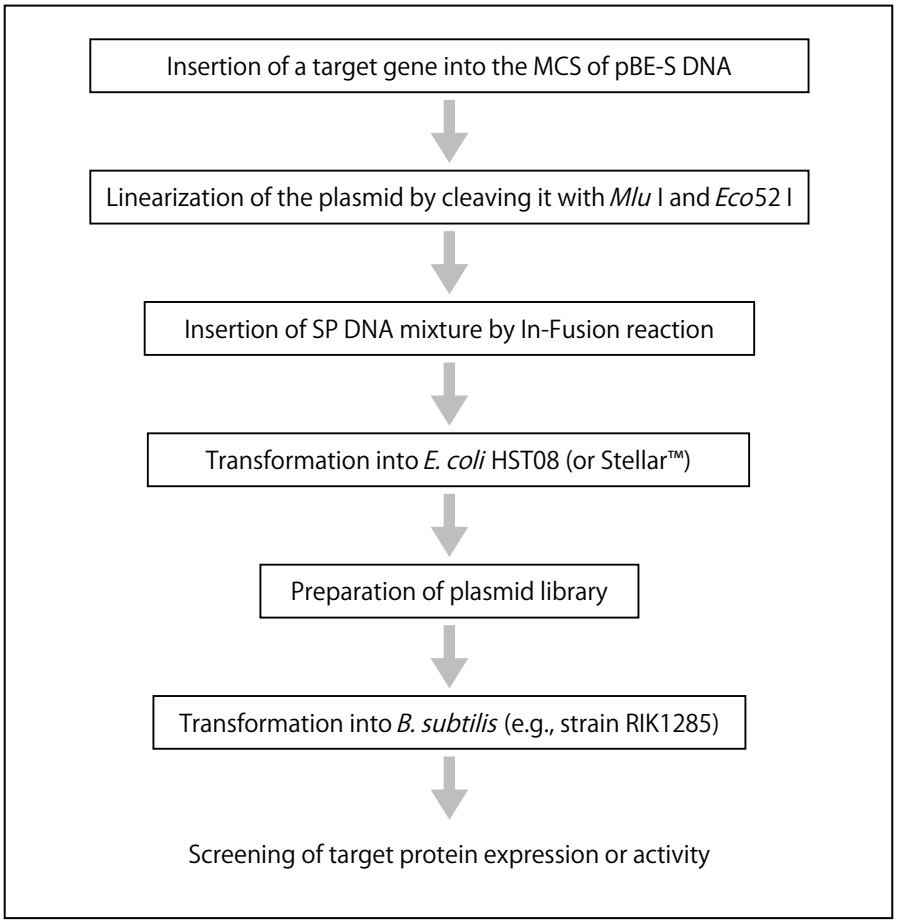


Figure 2. Flowchart of the experimental procedure for *B. subtilis* Secretory Protein Expression System

II. Components*1

SP DNA mixture (0.032 pmol/μl)*2	45 μl
pBE-S DNA (0.5 μg/μl)*3	20 μg
B. subtilis RIK1285*4 (glycerol stock)	100 μl x 2

*1 Library development (10 reactions)
*2 DNA mixture encoding secretory signal peptides from the 173 types of *B. subtilis* for use with In-Fusion cloning system (10 reactions)
For the sequence of secretory signal peptides, please see the product page for the *B. subtilis* Secretory Protein Expression System on the Takara Bio web site.
*3 in TE buffer (pH 8.0)
*4 Marburg 168 derivative: *trpC2*, *lys1*, *aprE* Δ3, *nprR2*, *nprE18*

III. Storage

- SP DNA mixture and pBE-S DNA: -20℃
- *B. subtilis* RIK1285 glycerol stock: -80℃

IV. Materials Required but not Provided

- In-Fusion HD Cloning Kit (Cat. #639633 through 639650)
- *E. coli* HST08 Premium Competent Cells (Cat. #9128) or Stellar Competent Cells (Cat. #636763/636766)
- *Mlu* I (Cat. #1071A)*1
- *Eco*52 I (*Xma* III) (Cat. #1039A)*1
- Other restriction enzymes as needed*2
- Commercially available plasmid purification kit
- Reagents for *B. subtilis* transformation (see Appendix, Section 1, Step 1, "Preparation of Reagents")

*1 Used to linearize pBE-S DNA containing target genes (for insertion of SP DNA mixture)
*2 Used in cloning of target genes to pBE-S DNA

V. Precautions for Use

- Please use this kit in combination with the In-Fusion HD Cloning System (Cat. #639645/639646/639647), supplied with competent cells. Alternatively, use the In-Fusion HD Cloning Kit (Cat. #639648/639649/639650), not supplied with competent cells, in combination with Stellar Comptent Cells (Cat. #636763/636766).
- The SP DNA mixture is prepared by combining PCR-amplified DNA fragments. There will be cases in which PCR-amplified byproducts such as primer dimers are cloned, although such occurrences will be infrequent.

VI. Protocol

VI-1. Construction of Expression Plasmid Library

For In-Fusion reaction procedures, please refer to the user manual of the In-Fusion HD Cloning System (supplied with competent cells) or In-Fusion HD Cloning Kit (Stellar Competent Cells sold separately). For the transformation of *E. coli* HST08 (Stellar) competent cells, please see the instructions that accompany the product.

1) Construct the expression plasmid by inserting a target gene into the multi-cloning site downstream from the secretory signal peptide of pBE-2 DNA, so that it matches the translation frame (see the vector map and Figure 1).

2) Use the restriction enzymes *Mlu* I and *Eco*52 I to completely digest the expression plasmid constructed in Step 1.*1

   *1 Simultaneous digestion with *Mlu* I and *Eco*52 I is not recommended. Perform the reactions sequentially. Isolate and recover the expression plasmid fragment using agarose gel electrophoresis.

   If a *Mlu* I or *Eco*52 I restriction site exists in the sequence of the target gene, linearize the expression plasmid by PCR amplification. See "VIII. Troubleshooting".

3) Prepare the In-Fusion reaction solution by mixing the SP DNA mixture and the expression plasmid digested by the restriction enzymes in Step 2 at a 2 : 1 molar ratio, as shown below.*2, 3

Linearized expression plasmid (*Mlu* I–*Eco*52 I cut)	100 ng	y mol
SP DNA mixture	x μl	2 x y mol
5X In-Fusion HD Enzyme Premix	2 μl	
Sterile purified water	up to 10 μl	

   *2 If 1 kb of the target gene is inserted into pBE-S DNA, the expression plasmid (cut with *Mlu* I–*Eco*52 I) will be approximately 6.8 kb, 100 ng of which will correspond to approximately 0.022 pmol. If the volume of the expression plasmid (cut with *Mlu* I–*Eco*52 I) and SP DNA mixture used in the reaction exceeds 7 μl, double the amount of 5X In-Fusion HD Enzyme Premix used and adjust the volume to 20 μl with sterile purified water.

   *3 To check background, simultaneously perform the same procedure with a reaction system that does not include the SP DNA mixture.

4) Mix the reaction solution by pipetting.

5) Allow the mixture to incubate at 50℃ for 15 minutes and then incubate on ice.

6) Thaw the *E. coli* HST08 (Stellar) Competent Cells on ice immediately prior to their use.

7) After thawing the cells, mix them gently to homogenize and transfer 100 μl of competent cells to a 14 ml rounded-bottom test tube. Do not vortex.

8) Add 2 μl of the In-Fusion reaction solution to the tube in Step 7.

9) Incubate on ice for 30 minutes.

10) Incubate at 42℃ for 45 seconds.

11) Incubate on ice for 1 - 2 minutes.
12) Add SOC culture medium pre-warmed to 37℃ to obtain a final volume of 1 ml.
13) Shake at 160 - 225 rpm for 1 hour at 37℃.
14) Spread a suitable volume on an LB plate containing ampicillin (100 μg/ml).
15) Incubate overnight at 37℃.*4, 5

*4 To confirm library size, we recommend counting the number of colonies. A colony count of 2,000 or more is necessary to prepare a sufficient plasmid library. (The insertion of 163 types of signal peptides has been confirmed empirically from 960 colonies.) The number of colonies obtained will vary according to the target genes inserted. If you do not obtain a sufficient number of colonies, increase the scale of transformation.

*5 If the background is high, repeat the process starting from Step 2.

16) After suspending the colonies from the plate in LB*6 and harvesting them, purify the plasmids to produce the plasmid library.*7

*6 When using a square plate (140 x 104 x 16 mm), collect colonies in 5 ml of LB culture medium and then wash twice in 3 to 5 ml of LB. Commercially available bacteria spreaders are convenient for use in harvesting.

*7 Ordinarily, 20 μg or more of plasmid DNA can be purified from the 2,000 colonies. Adjust the concentration to 0.1 to 1 μg/μl for use in *B. subtilis* transformation.

VI-2. *B. subtilis* Transformation

Using the plasmid library obtained in VI-1, transform *B. subtilis**8 and select transformants on the selection medium plate including kanamycin (10 μg/ml).

*8 For reference, an example of the procedure for *B. subtilis* transformation is shown in the Appendix, IX-1.

VI-3. Analysis of Recombinant *B. subtilis*

Incubate the colonies obtained in VI-2 in liquid selection medium containing kanamycin (10 μg/ml) [for example, in 5 ml of medium at 37℃ for 24 hours (or up to 48 hours)], and confirm the expression level of the target protein in culture supernatant by methods such as activity measurement or SDS-polyacrylamide gel electrophoresis.*9

*9 Refer to Appendix, IX-2 for a protocol to purify plasmids from *B. subtilis* (e.g., when plasmid purification is needed prior to determining the sequence of signal peptides for clones with high expression levels).

VII. Experimental Example: Screening of Secretory Signal Peptides Suitable for Ultra–Heat-Resistant Enzyme β-Glycosidase

[Methods]

DNA encoding β-glycosidase derived from *Pyrococcus furiosus* was amplified by PCR and inserted into the *Nde* I-*Xba* I site of pBE-S DNA to construct the expression plasmid. Subsequently, the expression plasmid library was constructed according to the protocol described in Section VI, and 1 μg, 2 μg and 4 μg of the plasmid library were added to 300 μl, 600 μl and 1,200 μl of competent cells of *B. subtilis* RIK1285, respectively, as prepared using the method described in the Appendix. Transformation was then performed, also using the method described in the Appendix. Each of 470 colonies was used to inoculate 1 ml LB medium with kanamycin (10 μg/ml). After cultures were incubated for 24 hours at 37℃, the β-glycosidase activity of each culture supernatant was measured with the use of a synthetic substrate. Plasmids from 24 clones with differing expression levels were purified, and the sequences of the inserted secretory signal peptides were determined.

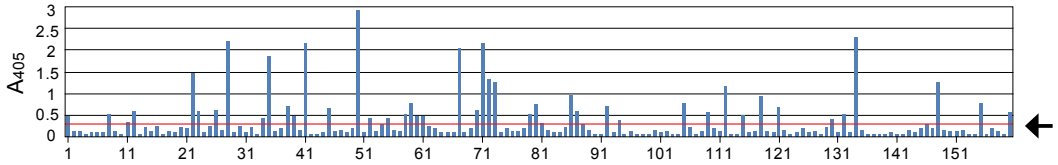
[Results]

Upon the preparation of the expression plasmid library according to the protocol, 28 μg of plasmids were obtained from 2,470 colonies. The following transformation efficiency was observed for *B. subtilis* RIK1285 with the use of these plasmids:

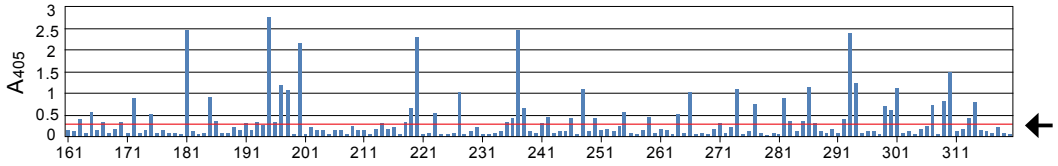
Volume of Competent Cells (μl)	Quantity of DNA (μg)	Colony Count	Transformation Efficiency (colonies/μg DNA)
300	1	104	1.0×10^2
600	2	244	1.2×10^2
1,200	4	497	1.2×10^2

When the β-glycosidase activity of 470 randomly selected clones was measured, it was confirmed that clones exhibiting activity of varying strengths had been obtained.

[No.1 - 160]



[No.161 - 320]



[No.321 - 470, A: pBE-S only, B:pBE-S + glycosidase gene]

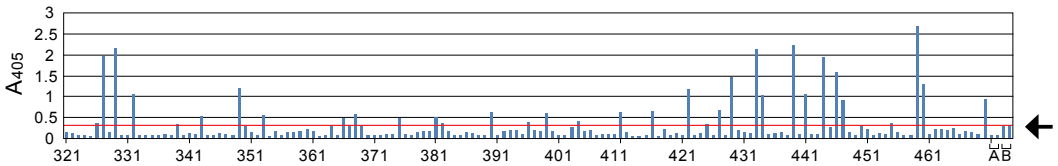


Figure 3. Results of measuring β-glycosidase activity of 470 clones

← indicates expression level in *aprE* signal peptide

Plasmid DNA was prepared from 24 of the clones having varying levels of activity, and the signal peptide sequences were determined. It was found that the level of secretary expression differed according to the secretary signal peptide (Table 1).

Table 1. Signal peptide DNA insertion

	signal peptide	Clone count	Clone No.
Strong activity	*ywsB*	7	28, 71, 134, 181, 200, 433, 439
	citH	4	41, 50, 195, 459
	phoB	1	444
	ybdG	1	329
	(*aspB*)*	1	293
Moderate activity	*ykwD*	2	45, 80
	ybbE	1	455
	ywaD	1	364
	abnA	1	307
No activity	*lipB*	1	3
	ybbR	1	461
	yopL	1	120
	ypbG	1	320
	ywmD	1	383

* Truncated version missing part of the *asp*B signal peptide, thought to be derived from a primer dimer.

This system makes it possible to select suitable secretory signal peptides for the secretory expression of target proteins. If secondary screening is performed, optimal signal peptides can be selected.

VIII. Troubleshooting

1. If few colonies are obtained when *E. coli* is transformed after the In-Fusion reaction:
   - Confirm complete digestion by *Mlu* I and *Eco*52 I in VI-1-2) (e.g., using electrophoresis). If the digestion is inadequate, an increase in background and a decrease in transformation efficiency will occur.
   - Use high-efficiency *E. coli* HST08 (or Stellar) Competent Cells.
   - Depending on the target genes inserted, it may be necessary to adjust the In-Fusion reaction conditions and optimize the experimental protocol. For details, please see the user manual of the In-Fusion HD Cloning Kit.

2. If few colonies are obtained when *B. subtilis* RIK1285 is transformed:
   - Confirm the transformation efficiency when 1 μg of the pBE-S DNA provided with the kit is used. If transformation is performed with the method described in the Appendix, IX-1, an efficiency of 3 to 8 x 10^2 colonies/μg will ordinarily be obtained. If the transformation efficiency is low, repeat the preparation of competent cells.
   - The transformation efficiency may vary with the target genes. Increase the reaction volume of the transformation as needed to ensure the necessary number of colonies.

3. If there is an *Mlu* I (or *Eco*52 I) site in the sequence of the target gene and it is not possible to use a restriction enzyme to linearize the expression plasmid:
   - Perform PCR amplification to prepare a linearized expression plasmid. Synthesize a primer that begins with a *Mlu* I or *Eco*52 I site, as shown below, and perform inverse PCR.

     [Examples of Primer Design]
     Perform inverse PCR with an M1-E1 primer pair.

     Primers starting with *Mlu* I:
     M1: 5′ - CGCGT CCCTC TCCTT TTGCT TAAGT TCAGA GTAG

     Primers starting with *Eco*52 I:
     E1: 5′ - GGCCG GTGCA CATAT GXXXX XXXXX XXXXX XXXX
     (Add additional nucleotides ("X") as needed to bring the length to approximately 34 nucleotides, depending upon the expression plasmid constructed.)

IX. Appendix

IX-1. Transformation Procedure Using *B. subtilis* RIK1285

IX-1-1. Preparation of Reagents

1) Prepare the following reagents and sterilize by autoclaving.
   - SP I salts (Per 1 L)

$(NH_4)_2SO_4$	2 g
K_2HPO_4	14 g
KH_2PO_4	6 g
Na-Citrate・$2H_2O$	1 g
$MgSO_4$・$7H_2O$	0.2 g

   - Casamino Acids/Yeast Extract (Per 100 ml)

Casamino Acids	2 g
Yeast Extract	10 g

   - 50% Glucose
   - 50 mM $CaCl_2$
   - 250 mM $MgCl_2$
   - 100 mM EGTA
     {ethylene glycol bis (β-amino ethyl ether)-N,N,N',N'-tetra-acetic acid}
     Adjust to pH 7.0 with NaOH.

2) Using sterile technique, mix the reagents prepared in Step 1 above to the composition shown below.
   - SP I medium

SP I salts	10 ml
50% glucose	100 μl
Casamino Acids/Yeast Extract	100 μl

   - SP II medium

SP I medium	5 ml
50 mM $CaCl_2$	50 μl
250 mM $MgCl_2$	50 μl

IX-1-2. Method for Preparing *B. subtilis* RIK1285 Competent Cells

1) Spread a suitable amount of glycerol stock of the host strain on an LB plate and incubate at 37℃ overnight (approximately 16 hours).
2) Inoculate 2 ml of LB medium with a loop full of the host strain incubated overnight on the LB plate. Grow the culture overnight (for approximately 16 hours) at 28℃, shaking at 150 - 180 rpm.
3) Add 50 μl of the culture broth to 5 ml of SP I medium, and incubate at 37℃ at 170 - 180 rpm.
4) Measure OD_{660} every 30 minutes beginning 1 hour after the start of culturing, and stop culturing once the culture has entered the plateau phase (Figure 4: near arrow).*1

   *1 Ordinarily, the plateau phase is reached within approximately 5 hours.

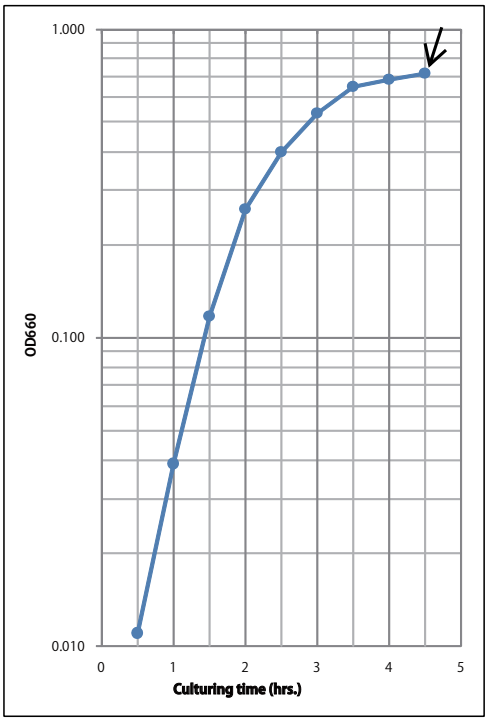


Figure 4. Growth curve of *B. subtilis* RIK1285

5) Add 0.5 ml of the culture broth to 4.5 ml of SP II medium, and incubate at 37℃, 90 - 100 rpm for 90 minutes.
6) Add 50 μl of 100 mM EGTA to the culture medium and shake at 37℃, 90 - 100 rpm for 5 - 10 minutes.
7) Divide the culture among 14 ml round-bottomed test tubes, dispensing 300 μl into each, and transform immediately.*2

*2 Once prepared, the competent cells cannot be stored. Use immediately after preparation.

IX-1-3. Transformation Method

1) Add 1 μg of DNA solution per 300 μl of the competent cells prepared in IX-1-2.
2) Incubate at 30℃, 90 - 100 rpm for 90 minutes.
3) Spread culture broth on LB plates containing kanamycin (10 μg/ml). Incubate overnight at 37℃.*3

*3 The transformation efficiency will differ according to the target genes that are inserted. If a sufficient number of colonies cannot be obtained by plating 300 μl of competent cells, perform transformation by plating with a volume equivalent to (1 μg of DNA solution per 300 μl of competent cells) x n so as to obtain a sufficient number of colonies for screening.

IX-2. Plasmid Preparation from *B. subtilis* Using NucleoSpin Plasmid

1) Inoculate 5 ml of LB culture medium containing kanamycin (10 μg/ml) with a single colony, and shake/incubate overnight (12 - 16 hours) at 37°C.
2) Centrifuge the culture for 30 seconds at 11,000*g* and harvest the cells.
3) Add to the pelleted cells 250 μl of buffer A1 with added lysosome*4 at a final concentration of 4 - 10 mg/ml. Vortex or pipette the sample to completely suspend the pellet, and transfer the suspension to a 1.5 ml microtube.
   *4 Lysozyme is not included in NucleoSpin Plasmid. Please purchase separately.
4) Incubate for 30 minutes at 37°C.
5) Following the instructions for NucleoSpin Plasmid, purify the plasmids, starting with the step at which buffer A2 is added.

X. References

1) Brockmeier U, *et al*. *J Mol Biol*. (2006) **362**: 393-402.
2) Murayama R, *et al*. *Biosci Biotechnol Biochem*. (2004) **68**(8): 1672-1680.

XI. Related Products

Mlu I (Cat. #1071A)
*Eco*52 I (*Xma* III) (Cat. #1039A)
In-Fusion® HD Cloning System (Cat. #639645/639646/639647)
In-Fusion® HD Cloning Kit (Cat. #639648/639649/639650)
Stellar™ Comptent Cells (Cat. #636763/636766)
E. coli HST08 Premium Competent Cells (Cat. #9128)
pCold™ Vector Series (Cat. #3360 through 3365 and 3371)*

* Not available in all geographic locations. Check for availability in your area.