Cat. # R160

For Research Use

TaKaRa

Bacterial 16S rDNA Clone Library Construction Kit

Product Manual

v201602Da

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I. Description

The Bacterial 16S ribosomal DNA (rDNA) Clone Library Construction Kit is a proprietary system developed for 16S rDNA clone library analysis, a technique used in microbial metagenomics. The kit can be used to construct clone libraries by PCR amplification and high-efficiency cloning of a specific region of 16S rDNA from genomic DNA of environmental bacteria. This system uses Tks Gflex™ DNA Polymerase, an enzyme that allows efficient PCR amplification even for crude samples, and pBackZero-alpha, a positive-selection vector that requires no screening. With these features, the system allows higher efficiency construction of 16S rDNA clone libraries than library generation with the conventional TA cloning strategy.

This product yields a cloning efficiency of >5 x 10^5 cfu/μg* and positive clones at a rate of >99%. Furthermore, despite blunt-end cloning, >99% of the clones produced are cloned in the same direction, allowing sequencing of the target DNA with primers that recognize the vector.

* : Cloning efficiency may decrease depending on experimental conditions, such as the transformation efficiency of the *E. coli* competent cells.

II. Components (For 20 reactions)

1.	Tks Gflex DNA Polymerase *1	(1.25 units/μl)	40 μl
2.	2X Gflex PCR Buffer (Mg^{2+}, dNTP plus) *1		1,000 μl
3.	pBackZero-alpha linear vector *2	(50 ng/μl)	20 μl
4.	16S-FA / 16S-R3 PCR primer Mix *3	(10 pmol /μl each)	40 μl
5.	DNA Ligation Kit <Mighty Mix> *4		150 μl
6.	sequencing primer BlaM1	(10 pmol/μl)	30 μl
7.	sequencing primer BlaRV	(10 pmol/μl)	30 μl
8.	DNA free H_2O		1 ml x 2
9.	Positive Control DNA *5	(1 ng/μl)	10 μl

*1 : These components are identical to those supplied with Tks Gflex DNA Polymerase (Cat. #R060A). The amount supplied is sufficient for 40 PCR reactions.

*2 : This vector is linearized and has blunt ends at the cloning site.

*3 : The 16S-FA/16S-R3 PCR primer mix is a set of 27f and 1492r universal primers for amplification of 16S bacterial ribosomal DNA[1)] with a sequence added to the 5′-terminus. This kit cannot be used with primers without the added sequence.

*4 : It is identical to DNA Ligation Kit <Mighty Mix> (Cat. #6023).

*5 : This is a plasmid DNA that contains genomic DNA (except for 16S rDNA) from *E. coli* JM109 and has sequences complementary to the 16S 27f/16S 1492r primers added to the end of the region. It is designed to allow the amplification of a 1,068 bp fragment with the 16S-FA/16S-R3 PCR primer Mix. The quantity supplied is sufficient for 10 positive control reactions.

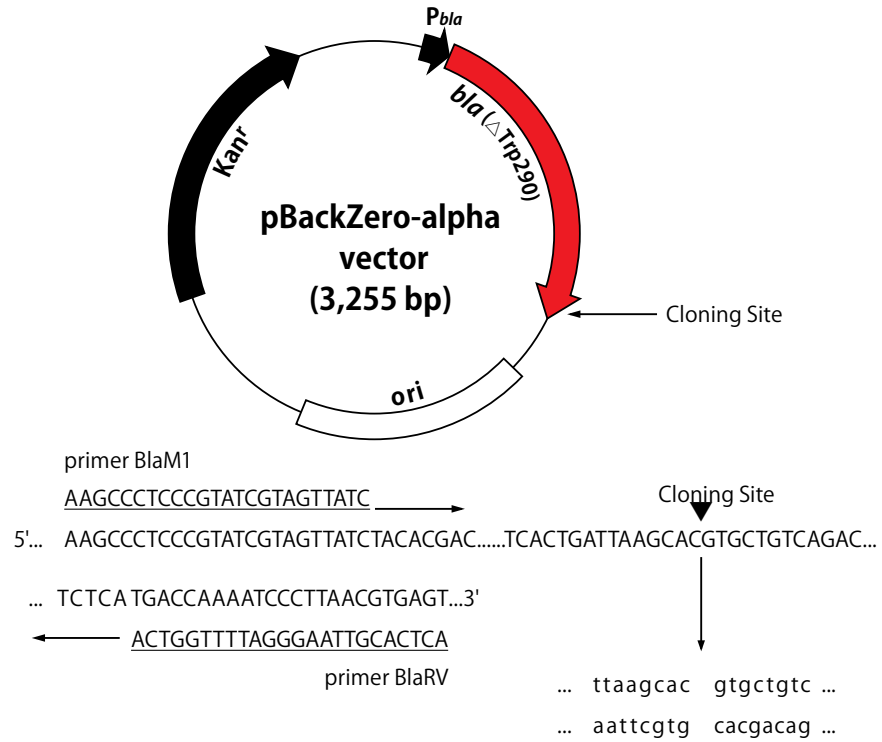


Figure 1. pBackZero-alpha vector map

[Primer sequences]

- 16S-FA PCR primer
  5'-TTTTAAAGAGTTTGATC (A/C) TGGCTCAG-3'
- 16S-R3 PCR primer
  5'-TTAATACGG (C/T) TACCTTGTTACGACTT-3'
- Sequencing primer BlaM1
  5'-AAGCCCTCCCGTATCGTAGTTATC-3'
- Sequencing primer BlaRV
  5'-ACTCACGTTAAGGGATTTTGGTCA-3'

III. Materials Required but not Provided

- Reagents for preparing bacterial genomic DNA from environmental samples
  NucleoSpin Soil (Cat. #740780.10/.50/.250), etc.
- Reagents for purifying PCR products
  NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250), etc.
- Competent cells
  E. coli HST08 Premium Competent Cells (Cat. #9128), etc.
- SOC culture medium
- LB plates containing 100 μg ampicillin/ml

- PCR reagents for colony screening
  EmeraldAmp® MAX PCR Master Mix (Cat. #RR320A)
  EmeraldAmp PCR Master Mix (Cat. #RR300A/B)*, etc.
- Plasmid DNA purification reagents
  NucleoSpin Plasmid (Cat. 740588.10/.50/.250), etc.

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

IV. Storage

− 20℃

V. Principle

The pBackZero-alpha vector is designed for efficient cloning of blunt-end PCR products amplified with α-type PCR enzymes, such as Tks Gflex DNA Polymerase. The plasmid contains a cloning site with a modified β-lactamase (*bla*) gene (an ampicillin-resistance gene) that lacks a TGG codon (ΔTrp290) at the 3′ end. Given that *bla*(ΔTrp290) encodes a non-functional β-lactamase, the vector does not confer ampicillin resistance. However, PCR products amplified with a primer containing a sequence (such as TTTTAA) which codes an aromatic amino acid and a stop codon at the 5′ terminus can be cloned into this site, thus allowing expression of active β-lactamase protein and thereby conferring ampicillin resistance. With this strategy, there is no need for blue/white selection to identify clones, and >99% of the viable clones on LB-Amp plates contain an insert. When cloning is performed with PCR products amplified from the supplied Positive Control DNA using this kit, each reaction can produce up to 5 x 10^5 cfu/μg PCR product.

Despite using blunt-end cloning, this kit allows directional cloning. With the TTTTAA sequence added to the 5′-terminus of only one of the PCR primers (16S-FA), only clones with 16S rDNA inserted in the same direction as the *bla* gene will exhibit ampicillin resistance; clones with the 16S rDNA inserted in the reverse direction and clones without the insert will be sensitive to ampicillin. Thus, >99% of the clones obtained from LB-Amp plates have the 16S rDNA inserted in the same direction as that of the *bla* gene. This is extremely advantageous for sequencing the cloned 16S rDNA fragments. If sequencing is performed using the BlaM1 primer, sequence information from the 27f-side of 16S rDNA can be reliably obtained.

Tks Gflex DNA Polymerase is an improved PCR enzyme derived from *Thermococcus sp*. This enzyme possesses high fidelity characteristic of α-type DNA polymerases and excellent extension activity through suppression of non-specific binding activity. Furthermore, the enzyme is paired with Takara Bio's proprietary elongation factor and a compound that increases priming specificity (patent pending) to provide amplifications with exceptionally high specificity from samples with a wide range of nucleic acid concentrations.

Tks Gflex DNA Polymerase improves PCR amplification of GC- or AT-rich sequences and long targets that are difficult to amplify with conventional PCR enzymes. Moreover, since the reaction buffer includes components that absorb PCR-inhibitors and that enhance amplification, high efficiency amplification is possible even from crude samples.
The polymerase is used for hot start PCR and includes a monoclonal antibody that suppresses DNA polymerase activity and 3′-5′ exonuclease activity at room temperature.

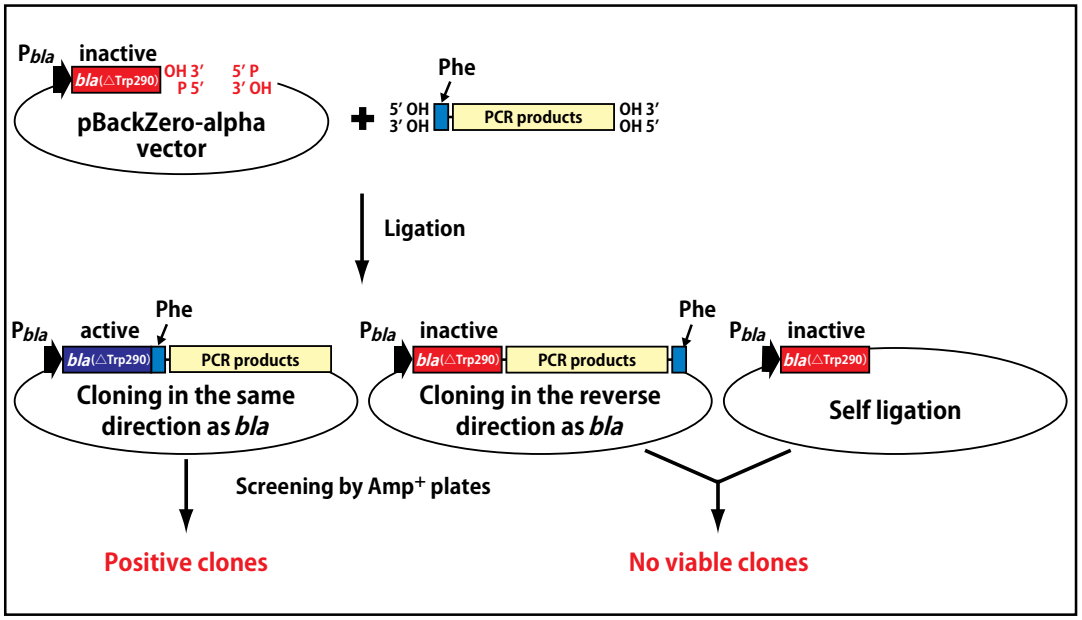


Figure 2 The cloning strategy of the Bacterial 16S rDNA Clone Library Construction Kit

VI. Precautions

1. Environmental samples, particularly soil, activated sludge, and compost, contain high levels of PCR inhibitors. Please use appropriate reagents and kits to obtain high-purity DNA when extracting bacterial genomic DNA from environmental samples.
   NucleoSpin Soil (Cat. #740780.10/.50/.250) is recommended for extracting DNA from environmental samples.
2. Always use fresh ampicillin-containing LB plates. Prepare plates with ampicillin at a concentration of 100 μg/ml. Because selection is based on β-lactamase activity, the concentration of ampicillin is very important for clone selection.

VII. Protocol

A. PCR Amplification of 16S rDNA

A-1. Prepare the following reaction mixture in PCR tubes.

Reagent	Sample	Positive Control	Negative Control
Template DNA	100 - 500 ng*1	1 µl	—
2X Gflex PCR Buffer	25 µl	25 µl	25 µl
16S-FA/16S-R3 PCR primer Mix (10 pmol/µl each)*2	1 µl	1 µl	1 µl
Tks Gflex DNA Polymerase (1.25 U/µl)	1 µl	1 µl	1 µl
DNA free dH_2O	to 50 µl	22 µl	23 µl
Total	50 µl	50 µl	50 µl

*1 : Typical PCR reactions with bacterial genomic DNA as template require no more than 0.1 to 1 ng of template DNA. When producing clone libraries, perform PCR with more template DNA and fewer PCR cycles to avoid the generation of chimeric clones. Excessive template DNA can decrease the efficiency of conventional PCR enzymes, but Tks Gflex DNA Polymerase provides excellent amplification results in this situation. Furthermore, because DNA prepared from an environmental samples may contain PCR inhibitors, the amount of template DNA added to the reaction mixture affects PCR efficiency. Tks Gflex polymerase contains a component that absorbs PCR inhibitors, allowing greater resistance to inhibitors than other conventional PCR enzymes. If PCR efficiency is low, reducing the amount of template DNA may improve the amplification efficiency.

*2 : The 16S-FA/16S-R3 PCR primer Mix contains 27f and 1492r universal primers for amplification of bacterial 16S rDNA. The primers are 5'-modified as described in section II. Components. The 16S-FA/16S-R3 PCR primer Mix amplify an approximately 1.5 kb fragment of 16S rDNA.

A-2. Perform PCR with the following conditions.

94℃ 1 min.
↓
98℃ 10 sec. ┐
55℃ 15 sec. ├ 15 - 20 cycles*3
68℃ *4 45 sec. ┘

*3: When producing clone libraries, perform PCR reactions with more template DNA and fewer cycles to avoid the generation of chimeric clones. Keep the number of cycles under 25, preferably between 15 and 20 cycles. Under this condition, high-efficiency Tks Gflex DNA Polymerase provides excellent amplification.

*4: Even for 3-step PCR, perform extension at 68℃.

A-3. Analyze the amplification products.

Perform agarose gel electrophoresis using 5 µl of the PCR reaction mixture to confirm the presence of an approximately 1.5 kb product. In addition, make sure there is no amplification in the Negative Control reaction. If the results show an approximately 1.5 kb band without non-specific amplification, proceed to B-1. PCR reaction mixture cleanup. If non-specific amplification products other than the 1.5 kb band are detected, perform purification as described in B-2. Purification of PCR products from agarose gels.

B. Purification of PCR Products

B-1. PCR reaction mixture cleanup

When a specific 1.5 kb 16S rDNA product is amplified, purify the PCR product using the NucleoSpin Gel and PCR Clean-up kit following the recommended protocol.

B-2. Purification of PCR products from agarose gels

If non-specific amplification products are present in addition to the 1.5 kb 16S rDNA amplification product, purify the 16S rDNA band (1.5 kb). Use the entire PCR mixture for agarose gel electrophoresis and purify DNA from the gel slices using the NucleoSpin Gel and PCR Clean-up kit following the recommended protocol. TAE buffer is recommended for electrophoresis.

C. Ligation and Transformation

C-1. Determine approximate DNA concentration by assaying the purified DNA solution using a spectrophotometer or by performing an agarose gel electrophoresis of part of the DNA solution (1 μl).

C-2. Prepare the following mixture for the ligation reaction.

pBackZero-alpha linear vector	(50 ng/μl)	1 μl
16S rDNA PCR products, purified		100 - 200 ng[*1]
DNA Ligation Kit <Mighty Mix>		5 - 7.5 μl[*2]
DNA free H_2O		x μl[*3]

*1 : The recommended ratio of vector to PCR product (by amount) is between 1:2 and 1:4. Avoid excessive PCR product, which can decrease reaction efficiency.
*2 : The amount of DNA Ligation Kit <Mighty Mix> is half of the total amount of the reaction mixture.
*3 : The total amount of the reaction mixture should be between 10 and 15 μl.

C-3. Perform the ligation reaction at 16℃ for 30 minutes.

C-4. Perform *E. coli* transformation using high-efficiency competent cells.

[For *E. coli* HST08 Premium Competent Cells]

(1) Thaw *E. coli* HST08 Premium Competent Cells on ice immediately before use.
(2) Once the cells are thawed, mix gently, and transfer 100 μl of the competent cells to a 14-ml round-bottom tube such as a Falcon tube on ice (do not vortex).
(3) Add 10 μl of the ligation reaction mixture.
(4) Let stand on ice for 30 minutes.
(5) Incubate at 42℃ for 45 seconds.
(6) Let stand on ice for 1 to 2 minutes.
(7) Add 890 μl of the SOC culture medium that has been pre-warmed to 37℃.
(8) Shake at 37℃ for 1 hour (160 - 225 rpm).
(9) Spread an appropriate amount on each LB-Amp plate.[*4]
(10) Incubate at 37℃ overnight.

*4 : When using competent cells with an efficiency >1.0 x 10^8 cfu/μg for pUC19 DNA, the efficiency generally is 5 x 10^5 to 1 x 10^6 cfu/μg DNA. A 1-ml aliquot of culture (equivalent to 50 ng vector DNA) that has undergone the transformation as described above is expected to yield 2.5 x 10^4 to 5 x 10^4 colonies. Therefore, when using a 9-cm plate, spread 1/100 to 1/500 of the culture volume.

D. Confirmation of Insert DNA

Using this system, >99% of clones have a correct insert DNA. To confirm the presence of an insert DNA, perform colony PCR or PCR with purified plasmid DNA as a template using the BlaM1/BlaRV sequencing primers or a 27f and 1492r universal primer pair (not included in this kit) for bacterial 16S rDNA amplification. The size of amplification products is approximately 1.6 kb when a PCR is performed using BlaM1/BlaRV, and approximately 1.5 kb when using 27f/1492r.

E. Sequence Analysis

Sequencing analysis of clone libraries generated with this kit can be performed using universal primers, such as 27f and 1492r primers, for 16S rDNA. In addition, as 99% or more of insert DNA is cloned in the same direction as the *bla* gene, the 27f-side of the 16S rDNA can be analyzed with the sequencing primer BlaM1, and the 1492r-side of the 16S rDNA can be analyzed using the sequencing primer BlaRV.

F. Analysis of Nucleotide Sequence

A homology search between the 16S rDNA sequence obtained and a nucleotide sequence database can be used to identify bacterial species.

Resources for data analysis:

- BLAST search
  NCBI : http://blast.ncbi.nlm.nih.gov/
  DDBJ : http://blast.ddbj.nig.ac.jp/top-j.html
- DNASIS Taxon V3.5 (Cat. #SK271)* can be used to deduce bacteria type
- Techno Suruga Lab microbial identification system (Cat. #14107142), etc.

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

VIII. Experimental Example

Constructing a soil-derived 16S rDNA clone library

<Methods>

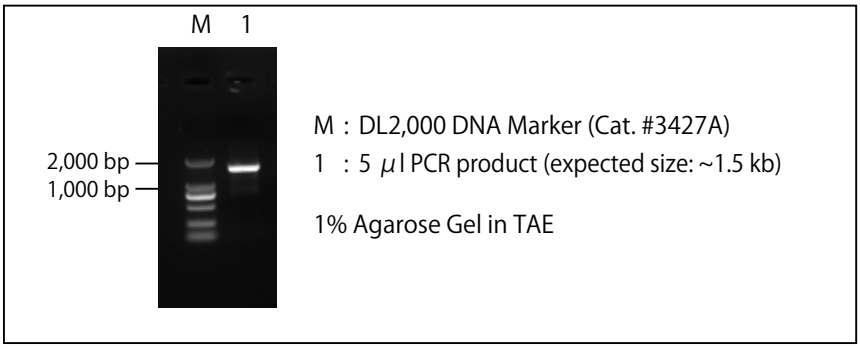
1. Genomic DNA from a 500-mg soil sample was purified using NucleoSpin Soil following the recommended protocol.
2. PCR was performed using 200 ng of the soil-derived genomic DNA as a template following the recommended protocol of this kit (20 cycles). The PCR products were purified with the NucleoSpin Gel and PCR Clean-up kit, and DNA concentration was determined by spectrophotometer (NanoDrop).
3. Ligation was performed with the reaction mixture below at 16℃ for 30 minutes.

pBackZero-alpha linear Vector	1 μl
Soil-derived 16S rDNA PCR products (100 ng/μl)	1 μl
DNA Ligation Kit <Mighty Mix>	5 μl
DNA free H_2O	3 μl
Total	10 μl

4. *E. coli* HST08 Premium Competent Cells were transformed with the entire reaction mixture (10 μl).
5. Fifty colonies from LB-Amp plates were picked and each cultured overnight in liquid LB medium containing 100 μg ampicillin/ml. Plasmid DNA was prepared with NucleoSpin Plasmid.
6. Sequence analysis of the plasmid DNA was performed with the sequencing primer BlaM1 and BlaRV.

<Results>

● PCR amplification bacterial 16S rDNA from soil-derived DNA



● Library analysis

Library titer (cfu/μg DNA)	1.0×10^6
Positive clones	100% (50/50)
Insert direction	100% identical

IX. Troubleshooting

Issue	Solution
No PCR products	Environmental sample-derived genomic DNA may contain impurities, including PCR inhibitors. Try to decrease the amount of genomic DNA template used in the PCR reaction. If no improvement is achieved, repurify the DNA.
Low transformation efficiency	Perform ligation with a vector to PCR product ratio (by amount) of between 1:2 and 1:4. Excessive DNA can reduce efficiency.
	Use high-efficiency competent cells such as *E. coli* HST08 Premium Competent Cells (Cat. #9128). Using competent cells with an efficiency of 1.0 x 10^8 cfu/ μg (pUC19) or better is recommended.
Low rate of positive clones (high level of backgrounds with no insert)	A high background may be attributable to degradation or low concentration of ampicillin. Always use fresh ampicillin-containing LB plates. Plates should contain 100 μg ampicillin/ml.

X. Related Products

TaKaRa PCR Thermal Cycler Dice™ Gradient	(Cat. #TP600)
TaKaRa PCR Thermal Cycler Dice™ *Touch*	(Cat. #TP350)*
0.2 ml Hi-Tube Dome Cap	(Cat. #NJ200)
NucleoSpin Soil	(Cat. #740780.10/.50/.250)
NucleoSpin Gel and PCR Clean-up	(Cat. #740609.10/.50/.250)
E. coli HST08 Premium Competent Cells	(Cat. #9128)
NucleoSpin Plasmid	(Cat. #740588.10/. 50/. 250)
EmeraldAmp® MAX PCR Master Mix	(Cat. #RR320A)
EmeraldAmp® PCR Master Mix	(Cat. #RR300A/B)*
DNASIS Taxon V3.5	(Cat. #SK271)*
Techno Suruga Lab microbial identification system	(Cat. #14107142), etc.

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

XI. Reference

Weisburg WG, Barns SM, Pelletier DA, and Lane DJ. (1991) *J Bacteriol*. **173**: 697-703.

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Thermal Cycler Dice is a trademark of TAKARA BIO INC.

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