# Rolling Circle Amplification-mediated *Bacillus* DNA Transformation

Cat#: BR100

# User Instruction

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Evomic Science LLC
725 San Aleso Ave, Suite 3,
Sunnyvale, CA 94085
USA

# Introduction

In contrast to Gram-negative bacteria, such as *E. coli*, introducing foreign DNA (DNA transformation) into Gram-positive cells, *such as Bacillus*, is notoriously difficult. It is presumed that at least two factors contribute this problem: 1). The extensive exterior network of peptidoglycan in Gram-positive cell walls physically restricts passage of exogenous DNA into the cell; 2). Cell wall-associated and intracellular nucleases in Gram-positive cell rapidly degrade the introduced DNA. Currently, phage transduction, electroporation, protoplast transformation and protoplast electroporation methods are recruited to introduce DNA into *Bacillus* strains. These methods are generally more laborious and less effective. Although some *Bacillus* are natural competence or induced competence by starvation media, the DNA transformation efficiency in those competent *Bacillus* is very low. Recently, an efficient approach through the regulatory expression of cluster transcription factors, e.g. *ComK*, in the non-naturally competent *Bacillus* strains, significantly benefit its DNA transformation. This well-tuned transient expression of *ComK* is becoming a robust and essential approach for *Bacillus* transformation.

In addition to the competence of *Bacillus*, the efficient DNA transformation in *Bacillus* is also dependent on the DNA format: DNA source, methylated status, and DNA concatemers, etc. Due to the host restriction and modification system, introducing a DNA fragment homolog to the recipient *Bacillus* strains into the foreign plasmid will increase the successful rate of transformation. The closer DNA homolog between the donor microorganisms and the recipient is, the higher the transformation rate will achieve. On the other hand, methylated DNA will decreased the transformation rate. Furthermore, *Bacillus* prefers to uptake DNA concatemers over short DNA fragments or circular DNA. Using our propriety SuperPhi™ enzyme (Patent Pending, product catalog# FP100), we thus developed a high efficient *Bacillus*-specific Rolling Circle Amplification (**bRCA**) kit to amplify the template DNA as concatemers and non-methylated DNA *in vitro*. The template DNA can be any circular DNA, such as the *in vitro* assembled or ligated circular DNA, plasmid, or the extended linear DNA (genomic DNA). The amplified DNA as concatemers can be directly applied to the *Bacillus* transformation with the highest transformation rate. The bRCA approach has been successfully applied to transform the nature competent or *ComK*-induced competent *Bacillus* strains, such as *Bacillus subtilis*, *Bacillus thuringiensis*, and *Bacillus licheniformis*. Compared to the traditional methods, the bRCA approach was 1,000-fold more efficient, which is of critical importance to *Bacillus* gene cloning, expression and library construction.

# Kit Components:

| Components | 100 Reaction | Storage |
|---|---|---|
| Sample Buffer | 800 µl | -20°C |
| Reaction Buffer | 500 µl | -20°C |
| SuperPhi™ Enzyme | 100 µl | -80°C |

# Detailed Protocol

The bRCA kit has the capacity to amplify as low as 1 picogram of the template DNA under optimal condition. The template DNA could be the extended linear DNA (genomic DNA), any circular DNA, such as *in vitro* assembly or ligated DNA mix, circular DNA from bacteria and yeast, including colony, or liquid culture. Typically, ten pictogram DNA, such as 0.2~0.5 μl of saturated overnight culture or 1/10 of the colony (approximately $10^2$ ~$10^4$ cells) would be enough for the *Bacillus*-specific DNA Rolling Circular Amplification reaction. To release plasmid or genomic DNA from gram-positive bacteria including *Bacillus*, lysozyme pre-treatment will be helpful. Please keep in mind that excess culture or colony will inhibit the amplification reaction! The protocol described below is a general protocol for amplifying the circular DNA from various sources. Yields and kinetics may vary if crude or un-quantified samples are used. We recommend to consider a starting point for adapting your specific reaction.

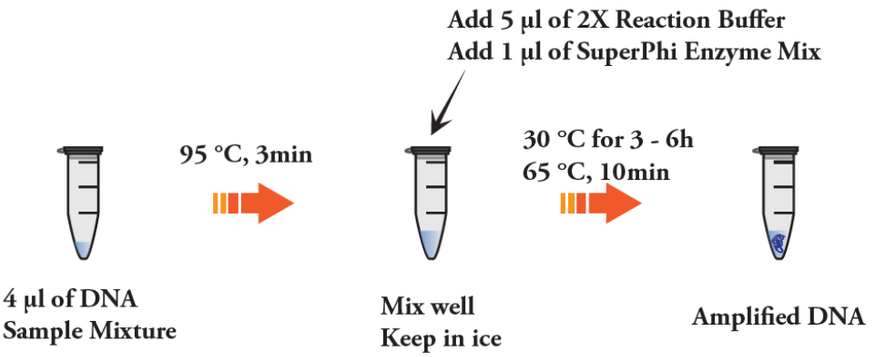



***Fig. 2. Schematic Representation of RCA-mediated DNA Amplification Protocol.***

## The protocol includes two parts:

1. Preparation of DNA samples for transformation;
2. *Bacillus* DNA Transformation.

Appendix I: Preparation of *ComK*-induction competent cells.

Appendix II: Preparation of natural Bacillus competent cells

## 1. Preparation of DNA Sample Mix for Transformation:

- DNA Sample Mix could be prepared, depending on material sources, as described below.
- Heat at 95 ˚C for 3 minutes and then quickly cool to 4˚C on the PCR machine.
- Keep the denatured DNA sample mix on ice until use.

***Note:*** *Heating at higher temperature or longer time may nick circular or host genomic DNA.*

### 1.1. Purified DNA or DNA ligation product or *in vitro* assembled product:

- ☐ Transfer 3 μl of Sample Buffer into a 0.2 ml PCR tube.
- ☐ Add 1 μl of the circular DNA (10 pg/μl to 10 ng/μl) to the above PCR tube.

### 1.2. Bacterial Colonies:

- ☐ Transfer 4 μl of Sample Buffer into a 0.2 ml PCR tube.

- Pick 1/100 of the colony (approximately $10^2$ ~$10^4$ cells) and add to the above PCR tube.
- If gram-positive bacteria are used, lysozyme pre-treatment will help to break down the cell wall to release the plasmid.

**1.3. Liquid Bacterial Culture:**

- Transfer 3.5 µl of Sample Buffer into a 0.2 ml PCR tube.
- Dilute the saturated overnight culture to 10-fold by sterile $dH_2O$, then add 0.5 µl to the above PCR tube.
- If gram-positive bacteria are used, lysozyme pre-treatment will help to break down cell wall to release the plasmid.

**1.4. Glycerol Stock:**

- Transfer 3.5 µl of Sample Buffer into a 0.2 ml PCR tube.
- Add 0.5 µl of glycerol stock to the above PCR tube.
- If gram-positive bacteria are used, lysozyme pre-treatment will help to break down the cell wall to release the plasmid.

**1.5. Bacterial Genomic DNA:**

- Genomic DNA can be isolated using the commercial genomic DNA isolation kit.
- Transfer 3 µl of Sample Buffer into a 0.2 ml PCR tube.
- Add 1 µl of 100 ng/µl genomic DNA to the above PCR tube.

## 2. DNA Amplification:

Add 5 µl of Reaction Buffer (**Vortex before use**) and 1 µl of SuperPhi™ Enzyme to the above PCR tube contains 4 µl of denatured DNA Sample Mix as shown below:

| Component | Volume per reaction |
|---|---|
| Sample Mix | 4 µl |
| Reaction Buffer | 5 µl |
| SuperPhi™ Enzyme | 1 µl |
| Final Volume | 10 µl |

One can prepare the master mix of the Reaction Buffer and the SuperPhi™ Enzyme for multiple reactions. The final volume is 10 µl. Mix well and incubate at 30 ˚C for 2~18 h.

***Note:*** *Due to the extremely high activity of our SuperPhi™ enzyme, the high concentration of concatemer DNA will become very sticky and even precipitate out after RCA reaction. If your template DNA is more than 100 ng in a 10 µl reaction, please DO NOT incubate longer than 4 hours at 30 ˚C.*

## 3. Inactivation: Incubating at 65 ˚C for 10 min, and then cool to 4 ˚C.

## 4. Transform bRCA product to *Bacillus* (ComK-induced or Natural Competence):

bRCA product with certain dilution can be directly transform either ComK-induced or natural *Bacillus* competent cells. If double crossover in chromosome was desired, bRCA product can be linearized with restriction enzyme and then transform *Bacillus*, but with lower transformation rate.

**4.1.** Take 2 µl of the above bRCA product to make 100x, 1,000x serial dilution with sterile $dH_2O$.

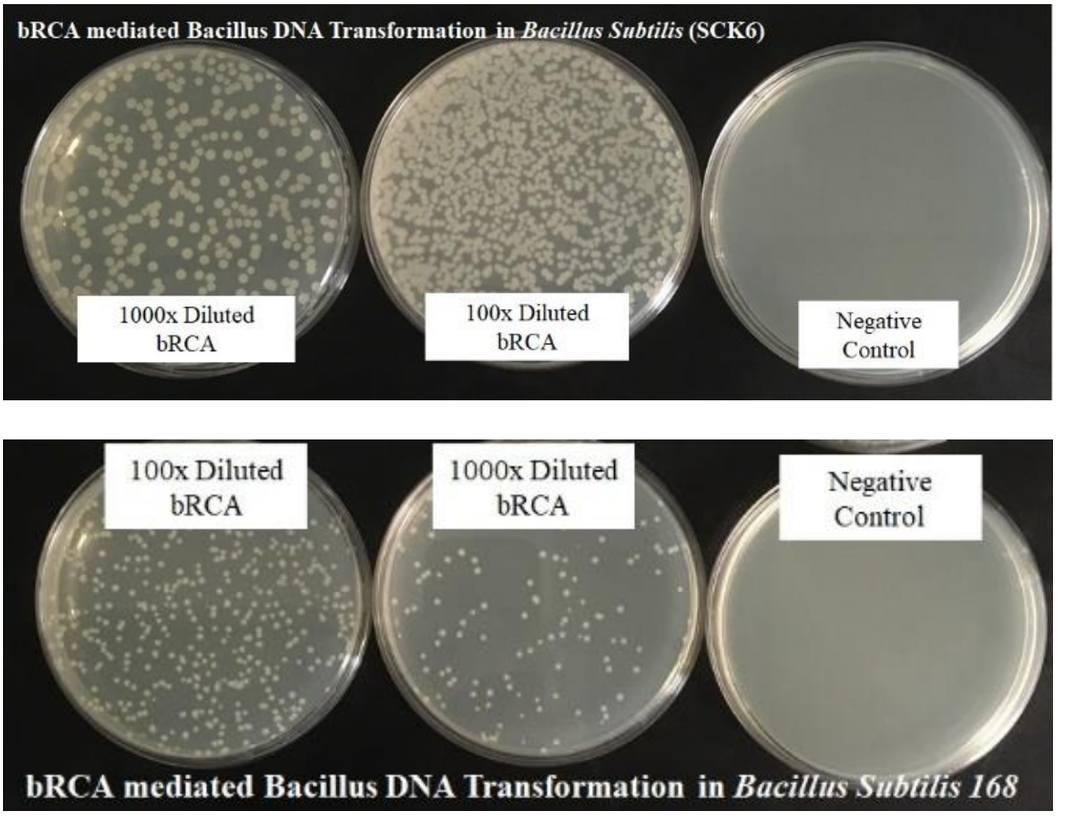
**4.2.** Prepare and label 3 of sterile 1.7 ml microcentrifuge tube or 2 ml Corning cryopreserve vials, then mix 2 µl of 100x, 1,000x diluted bRCA product with 100 µl of *ComK*-induced or natural competent *Bacillus* in each tube/vial.

**4.3.** Shake at 37 ˚C, 250 rpm for 1.5 h to recover transformants.

**4.4.** Plate all 100 µl of the transformants on antibiotic selective LB plates.

**4.5.** Incubate the plates at 37 ˚C overnight. Typically, you will have ~100 colonies of *Bacillus subtilis* with the 1,000x diluted bRCA product as shown below:

***Figure 1. Transformation Efficiency of bRCA Product in ComK-induced Competent Cells (SCK6) and natural competent cells (Bacillus subtilis 168)***. Six DNA fragments with average 1000bp, were assembled into vector with our U-Clone Master Kit (Evomic Science, Cat#:UC100). The resulting circular DNA was directly amplified with bRCA at 30ºC for 3 hrs. The concatemer DNA were diluted to the indicated concentration, and followed by Bacillus transformation directly. When bRCA products were diluted to 100 fold, there were too many colonies (upper panel, comK strain SCK6) on the antibiotic LB plate. There were still hundreds of colonies (upper panel, comK strain SCK6) on the antibiotic LB plate, when bRCA products were diluted to 1,000 fold. A similar result (lower panel, natural competent cells) was obtained when natural competent cells (*Bacillus subtilis* 168) was used.




## 5. FAQ and Troubleshooting: Please contact us at info@evomicscience.com.

## Appredix I: Preparation of ComK-induced Bacillus Competent Cells

**Starting materials:**

- Glycerol (or other) stock of *Bacillus* strain expressing ComK
- LB Broth and plates with antibiotic, if required
- 50% xylose stock (Filtered)

**Procedures:**

1. Day 1: Streak the stock of *Bacillus* strain on LB plate. Incubate plate overnight at 37 ˚C.
2. Day 2: Inoculate multiple colonies to obtain $OD_{600}$ = 0.2 to 0.3 in 20 ml of LB broth.
3. Shake at 37 ˚C, 250 rpm until $OD_{600}$ = 0.5~0.8 (~1.5 h). If overgrew, dilute with LB broth to obtain a 20ml culture with $OD_{600}$ = 0.8.
4. Add 120 µl of 50% xylose to the 20 ml culture (final xylose concentration=0.3%).
5. Induce cells for one hours at 37 ˚C (final $OD_{600}$~2.5).
6. **Frozen stock**: Add 8ml of 50% glycerol and 3.1 ml of DMSO to 20 ml of competent cells. Aliquot 100 µl to each 2 ml Corning cryogenic vial and flash freeze in liquid nitrogen or isopropanol/dry ice bath before transfer to -80 ˚C for storage.
7. **Test transformation efficiency:** transform with 50 ng of plasmid (0.5 µg/ml final concentration) to each 100 µl competent cell and select on LB+antibiotic plate. Usually the transformation efficiency is about 7-10%.
8. **Transform fresh/frozen cells**: Thaw 100 µl of cells in Corning cryogenic vial on ice and mix with appropriate amount of DNA (1µg/ml is regarded as saturated). Tighten cap and shake at 37 ˚C for 1-1.5 hr, 250 rpm. Plate cells onto the appropriate selective media at the appropriate dilutions and incubate at 37 ˚C overnight. Depending on the strain and antibiotic used for selection, colonies should appear within 14 to 20 h.

***Note: transformation is more efficient when incubating the cells and DNA mixture in glass tubes.***

## Appredix II: Preparation of Natural Bacillus Competent Cells

Natural competence development in *B. subtilis* is one of several stationary phase processes triggered by a nutritional downshift. This protocol assumes that you use a spectrophotometer that accepts 16×125 mm test tubes. If your spectrophotometer, like mine, works only with cuvettes, simply increase the culture volume to 10 or 20 ml in a 250-ml Erlenmeyer flask.

### Starting materials:

**Medium A (100 m)**

| | |
|---|---|
| Sterile water | 81 ml |
| 10× Medium A base | 10 ml |
| 10× Bacillus salts | 9 ml |

**Medium B**

| | |
|---|---|
| Medium A | 10 ml |
| 50 mM $CaCl_2 \cdot 2H_2O$ | 0.1 ml |
| 250 mM $MgCl_2 \cdot 6H_2O$ | 0.1 ml |

**10× Medium A base:**

| | |
|---|---|
| Yeast extract | 10 g |
| Casamino acids | 2 g |
| Distilled water | to 900 ml |
| Autoclave | |
| then add: | 50% glucose, filter-sterilized 100 ml |

**10× Bacillus salts**

| | |
|---|---|
| $(NH_4)_2SO_4$ | 20 g |
| $K_2HPO_4 \cdot 3H_2O$ | 183 g |
| $KH_2PO_4$ | 60 g |
| Na-citrate | 10 g |
| $MgSO_4 \cdot 7H_2O$ | 2 g |
| Water | to 1000 ml |

### Procedures:

1. Streak recipient strain on one-half of a Tryptose Blood Agar Base plate or LB plate. Incubate overnight (18 hr) at 37°C.
2. Inoculate a few colonies into 4.5 ml of Medium A in a 16×125 mm test tube that lacks visible scratches. Mix the contents of the tube thoroughly. Read its optical density at 650 nm in the spectrophotometer. Adjust the $OD_{650}$ to be 0.1-0.2, maintaining the volume at 4.5 ml.
3. Incubate at 37°C with vigorous aeration. Read the $OD_{650}$ every 20 min, plotting $OD_{650}$ against time on semi-log paper. After a brief lag, the OD should increase logarithmically---that is, they should fall on a straight line. Note the point at the culture leaves log growth—the graph points fall below the straight line. In *B. subtilis* genetics, this point is known as $t_0$. It should take 60-90 minutes of incubation and occur at $OD_{650}$=0.4-0.6.

4. Continue incubation for 90 minutes after the cessation of log growth ($t_{90}$). Transfer 0.05 ml of this culture into 0.45 ml of pre-warmed Medium B in a 16×125 mm test tube. Set up one tube for each transformation you intend to perform, plus an extra for a DNA-less control.
5. Incubate the diluted cultures at 37°C with vigorous aeration for 90 min. At this point, the cultures should be highly competent.
6. Add the appropriately diluted (100x diluted) bRCA product to the competent cells and incubate at 37°C with aeration for 30 minutes.
7. Plate aliquots of the transformed cells onto selective agar.

## References:

**Xiao-Zhou Zhang and Y.-H. Percival Zhang**. 2011. Simple, fast and high-efficiency transformation system for directed evolution of cellulase in *Bacillus subtilis.* Microb Biotechnol. 4(1): 98–105.

**Rahmer R, Morabbi Heravi K, Altenbuchner J.** 2015. Construction of a Super-Competent *Bacillus subtilis* 168 Using the P*mtlA*-com*KS* Inducible Cassette. **Front Microbiol**. 6:1431

**Yasbin, R. E., G. A. Wilson, and F. E. Young.** 1975. Transformation and transfection in lysogenic strains of *Bacillus subtilis:* Evidence for selective induction of prophage in competent cells. J. Bacteriol. **121:**296-304.