# OriCiro® Amp Kit

## OriCiro Amp Kit

The reaction consists of 26 purified enzymes involved in chromosome replication of *E. coli*. The chromosome replication cycle repeats autonomously at around 30˚C, enabling exponential amplification of circular DNA having *oriC* with extremely high fidelity ($10^{-8}$ error/base/cycle) (Figure 1). The kit yields up to 1 μg circular DNA per 10 μL reaction at 33˚C for 6 hr. The maximum amplification size is 50 kb in the current version of the kit.

**OriCiro Amp NEEDS *oriC* Cassette (0.4 kb) which can be inserted into circular DNA using OriCiro assembly kit.**

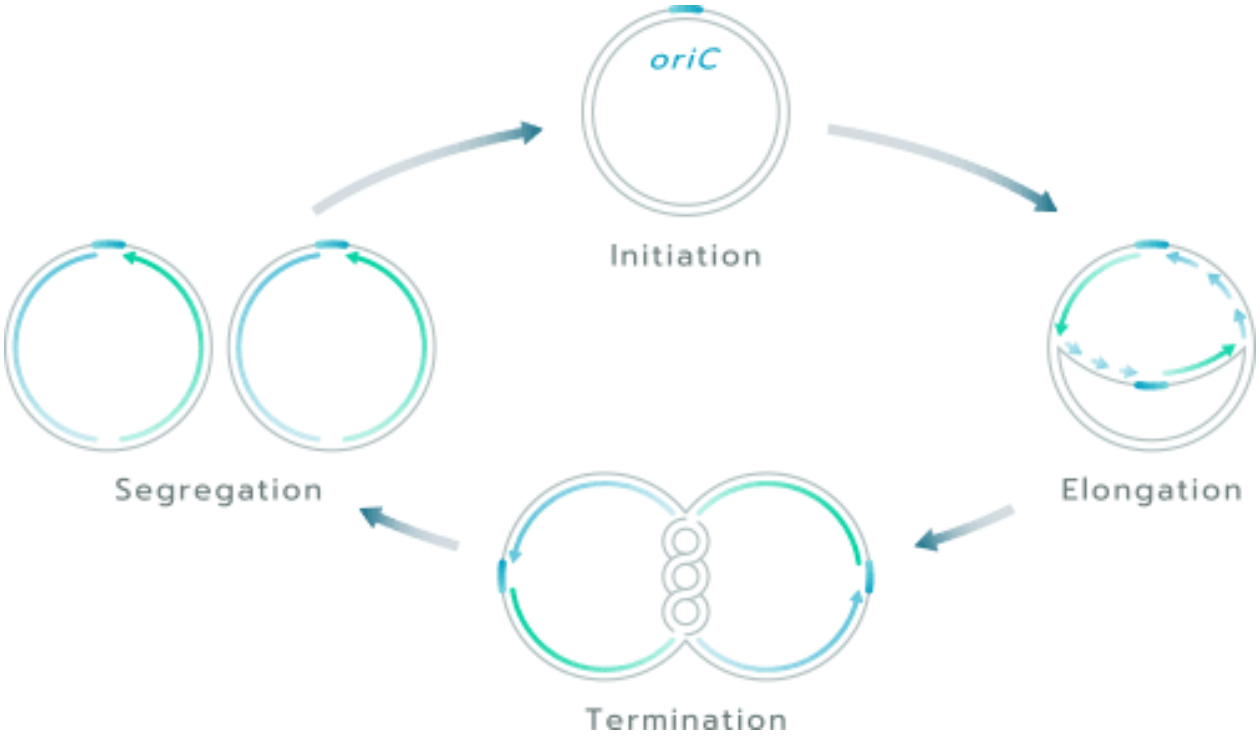


Figure 1. Amplification of circular DNA through chromosome replication cycle

# I. Components

| OriCiro Amp Kit | | (for 10 reactions) |
|---|---|---|
| (1) | 10X RE Mix | 10 µl |
| (2) | 5X Buffer I | 40 µl*1 |
| (3) | 5X Buffer II | 40 µl*1 |

*1: Extra volumes of 5X Buffer I and 5X Buffer II are added for optional Finalization reaction (see for Amplification Protocol).

# II. Equipment and materials required but not included

- Nuclease-Free Water
- Vortex mixer
- Microcentrifuge
- Thermal cycler or thermo block
- 0.2 ml microtubes (PCR tubes)
- Micropipettes (P-2, P-10) and tips

# III. Storage

OriCiro® Amp Kit is shipped on dry ice. Upon receipt, the kits must be immediately stored at **below -70°C.** 10X RE Mix contains enzymes, and repeated freeze-thaws must be avoided, although at least three times freeze-thaws are possible without loss of the function. 10X RE Mix must be frozen quickly by liquid nitrogen or dry ice ethanol before storing again at -70°C. Although 5X Buffer I and 5X Buffer II can be stored at below -20°C, all components including 10X RE Mix can be stored together at below -70°C for convenience.

# IV. Protocol

## AMPLIFICATION REACTION

(1) **Turn on a thermal cycler or an air incubator and preheat at 33°C.**
Avoid evaporation of the reaction during incubation. If the thermal cycler is used, its lid should be set at 40°C.

(2) **After 5X Buffer I and 5X Buffer II are thawed on ice, mix well with a vortex mixer and spin down with a microcentrifuge. After 10X RE Mix is thawed on ice, mix gently with the vortex mixer and spin down with the microcentrifuge.**

(3) **Prepare the following pre-mixture on ice. Mix before and after the addition of 10X RE Mix as indicated.** *1

| < Amp pre-mixture> | x1 reaction *2 |
|---|---|
| Nuclease-Free Water | 4 µl |
| 5X Buffer I | 2 µl |
| 5X Buffer II | 2 µl |
| → Vortex mixing | |
| 10X RE Mix | 1 µl |
| → Pipette mixing*3 | |
| Total | 9 µl |

*1: Use a 0.2 ml PCR tube to avoid evaporation.
*2: Amp premixture for multiple reactions can be prepared as a single "master mix" by multiplying the volume of each reagent by the number of reactions.
*3: For the pipette mixing, set pipette volume to the total mixture volume, and pipetting up and down four times with agitation.

(Option) Pre-incubation*4
Incubate the Amp pre-mixture at 33°C for 15 minutes.

*4: "Pre-incubation" option stimulates an initial stage of the amplification to allow stable amplification of the circular DNA particularly when the amplification is difficult due to a low amount of the template DNA molecules.

(4) **Add 1 µl of the assembly product (or *oriC* circular DNA), and mix with pipetting.*3**
**Incubate the mixture at 33°C for 6 hours*5 and hold at 12°C*6 or on ice before use.**

*5: • The incubation time can be shortened to 3 hours particularly when already supercoiled DNA is used as a template. The 6 hours incubation allows stable amplification particularly of the assembly product which requires gap-repair process.
• Higher temperature up to 40°C or longer incubation up to 16 hours is acceptable, but tends to produce other short DNA byproduct than your target.
*6: Thermal cycler program is useful to hold automatically at 12°C after the 33°C incubation.

(Option) Finalization[*7]

Dilute the reaction of step (4) two times with 1X Amp Buffer[*8] and further incubate at 33°C for 30 minutes.

*7: When replication intermediates (open circular or catenane DNA etc.) is abundant, "Finalization" option can convert them to supercoiled DNA.

*8: 1X Amp Buffer is prepared by mixing 5X Buffer I and 5X Buffer II to final 1X concentration with Nuclease-Free Water. Extra volumes of the 5X Buffers are provided for this option.

**(5) Check the amplified products using agarose gel electrophoresis.**[*9]

*9:
- Typical DNA concentration before Finalization option is 50-100 ng/µl
- The gel-loading buffer should contain SDS etc. to remove proteins from DNA.
- Because the product is supercoiled form, Supercoiled DNA Ladder (New England Biolabs) is recommended as a size maker. Alternatively, analyze it by restriction mapping.

The products can be stored at 4°C for several days. For long-term storage, add final 20 mM EDTA before storage at -20°C. Alternatively, purify the product with phenol/chloroform, followed by ethanol precipitation.

# V. Experimental examples

**Typical result of control reaction.**

- OriCiro assembly and amplification reaction was performed using *oriC* Cassette (0.4 kb) and Control Fragment (7.5 kb) in accordance with the protocol in this manual.
- The assembly reaction (total 5 µl) was performed at 42°C for 30 minutes.
- 1 µl of the assembly product was added into the amplification mixture (total 10 µl).
- The amplification reaction was performed at 33°C for 6 hours. An aliquot (5 µl) of the reaction was mixed with 5 µL of 1X Amp Buffer and further incubated at 33°C for 30 minutes (Finalization option).
- An aliquot (0.5 µl for the reaction without Finalization, or 1 µl for the reaction with Finalization) was diluted with 5 µl of Loading Buffer (final 25 mM Tris-HCl pH8.0, 25 mM EDTA, 0.1% SDS, 5% glycerol, 0.1% bromophenol blue). The DNA products without (lane 2) or with (lane 3) Finalization option were analyzed by 0.5% agarose gel electrophoresis in 0.5X TBE (60V for 60 minutes) and EtBr staining.
- RESULT: Supercoiled DNA (~8 kb) was observed as a major band in both reactions. The upper most band is concatemers generated due to rolling circles replication. Finalization option reduces the open circular replication intermediate (middle band). Note that the replication intermediates are not abundant in the case of control reaction and Finalization option is effective for the amplification of larger sized DNA.

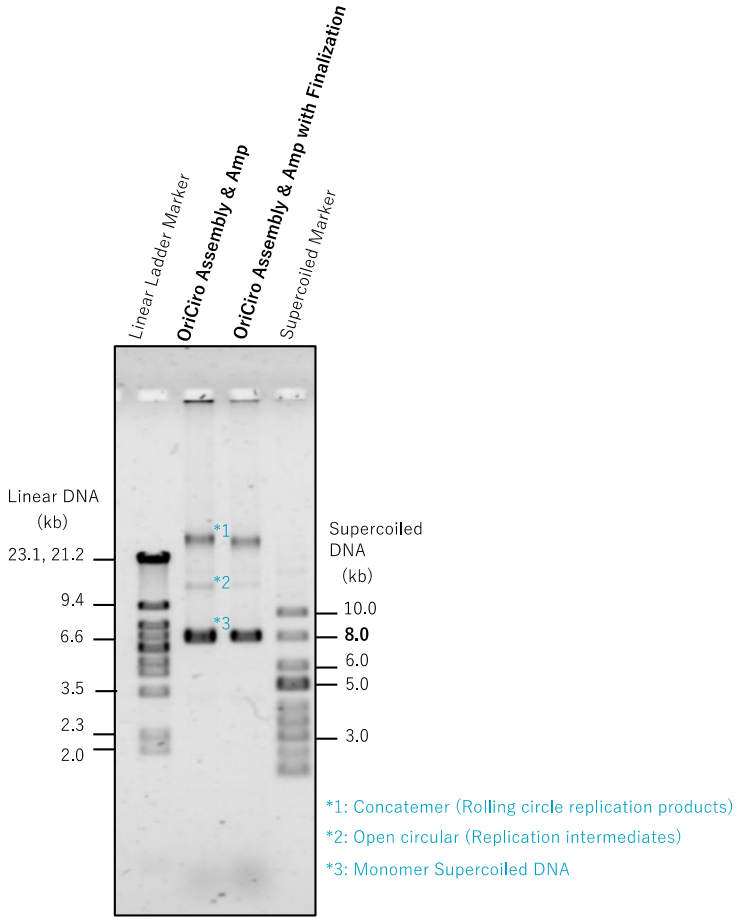


Figure 2. OriCiro Assembly and Amplification reaction of Control fragment (7.5 kb) and *oriC* cassette (0.4 kb)

# VI. FAQs

Q1 : How short or long DNA can be amplified?
A1 : Circular DNA from 2 kb to 50 kb is the optimal size for OriCiro amplification.

Q2 : How small amount of circular DNA can be used as the template?
A2 : Circular DNA at the single molecule level (atto-gram level) can be amplified in the case of DNA smaller than 10 kb.

Q3 : Can I re-amplify the amplified products with OriCiro Amp Kit?
A3 : Yes, it can amplify in a consecutive manner. Add the diluted products into the new amplification reaction mixture.

Q4 : Can I use a plasmid having a ColE1-type origin as a template?
A4 : The *oriC* sequence is essential for OriCiro Cell-Free Cloning System. If a plasmid has both ***oriC* and ColE1-type origins, the amplification reaction is not affected by the ColE1-type** origin.

Q5 : Can the amplified DNA be used for transformation of *E. coli* and transfection of cultured cells?
A5 : Yes.

Q6 : Could a circular plasmid contains both *oriC* and another *E. coli* replication origin?
A6 : Basically, *oriC* does not interfere with the function of the other plasmid origin like ColE1, p15A or R6K even if it were combined. However, when you combine with a high-copy pUC origin, then *oriC* will inhibit *E. coli* growth. Also, another plasmid origin does not interfere with the in vitro amplification.

Q7 : How does *oriC* cassette effect on plasmid copy number in *E. coli*?
A7 : *oriC* acts as low copy number (10-30 copies) plasmid origin.

# VII. Troubleshooting

**a. No amplification products are observed.**

1) Please check the DNA size is less than 50 kb. If the target DNA size exceeds 50 kb, the amplification requires a different protocol than described in this manual.
2) The assembly reaction is inhibited when salt and/or EDTA concentration of the DNA sample is too high.
3) Increase the temperature of the amplification reaction up to 37°C.
4) Increase the incubation time of the amplification reaction up to 18 hours.
5) Increase the template DNA amount added into the amplification reaction.

**b. Byproducts other than the target DNA are observed.**

1) DNA contamination
   Test negative control samples (the amplification reaction without DNA and with non-assembled DNA fragments) to ensure that the materials used are not contaminated. OriCiro Amp Kit amplifies DNA even from a single contamination molecule of circular DNA bearing

the oriC sequence.

2) Concatemer DNA
When the amplified DNA size is less than 4 kb, concatemer DNA may appear. It may be improved by shortening the incubation time of the amplification reaction. Also, contamination of E. coli genome causes concatemer DNA.

# VIII. References

1. T. Mukai, T. Yoneji, K. Yamada, H. Fujita, S. Nara, M. Su'etsugu, Overcoming the Challenges of Megabase-Sized Plasmid Construction in *Escherichia coli*, *ACS Synthetic Biology*, 2020, **9** (6), 1315-1327

2. T. Hasebe, K. Narita, S. Hidaka, M. Su'etsugu, Efficient Arrangement of the Replication Fork Trap for In Vitro Propagation of Monomeric Circular DNA in the Chromosome-Replication Cycle Reaction. *Life*, 2018, **8** (43)

3. M. Su'etsugu, H. Takada, T. Katayama, H. Tsujimoto, Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle, *Nucleic Acids Research*, 2017, **45** (20), 11525–11534

Note :

- This product is for research use only. It is not intended for use this product or its components for any purposes including but not limited to diagnostics, prophylactics, and/or therapeutics or otherwise clinical trials.
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