Ver. 4.0

Read the instruction manual thoroughly before you use the product.

For Research Use

OriCiro

OriCiro™ Cell-Free Cloning System

OriCiro™ Cell-Free Cloning System is an innovative tool enabling cell-free assembly and amplification of circular DNA molecules without *E. coli* transformation and culture. This product can not only streamline your workflow dramatically but also widen the scope of your genetic engineering techniques. This product is composed of OriCiro Assembly Kit and OriCiro Amp Kit and designed to produce the maximum effect when used in combination although each kit can be used independently.

OriCiro Assembly Kit assembles up to 50 DNA fragments simultaneously via their overlapping ends in a single isothermal enzymatic reaction. The assembly products are subjected directly to OriCiro Amp Kit for selective amplification of your target circular DNA. OriCiro Amp Kit enables to amplify exponentially circular DNA having *oriC* (*E. coli* chromosomal origin) in an isothermal condition even from a single template molecule. Approx. 200 ng – 1 µg circular DNA can be yielded as supercoiled form per 10 µl reaction.

OriCiro Cell-Free Cloning System allows an efficient construction and fast amplification of plasmid DNA without relying on *E. coli* cloning. The benefits include a simple and easy-to-handle procedure requiring less effort and shorter time compared to current methods and being suitable to automated processes. It is also possible to amplify any sequences including cell toxic and GC-rich sequences. The amplified products can be directly used for downstream applications.

I Components

a. OriCiro Assembly Kit (for 5 reactions)

(1)	2X RA Mix	12.5 µl
(2)	*oriC* Cassette (50 pg/µl)*1	10 µl
(3)	Control Fragment (1 ng/µl)	5 µl

b. OriCiro Amp Kit (for 10 reactions)

(4)	10X RE Mix	10 µl
(5)	5X Buffer I	40 µl
(6)	5X Buffer II	40 µl

Note

*1 : *oriC* Cassette is a 378 bp DNA fragment containing *oriC* (*E. coli* chromosomal origin) sequence. Both ends of the *oriC* Cassette have 40 bp overlapping sequences against Control Fragment (7.5 kb) (Fig. 1). See Appendix for sequence information.

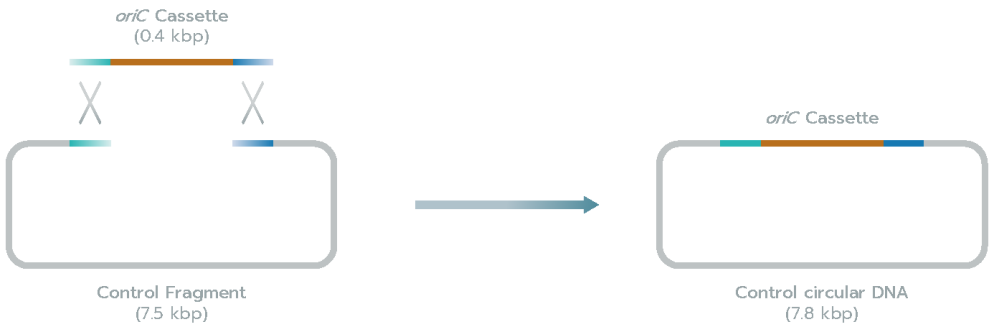


Figure 1. Schematic of *oriC* Cassette and Control Fragment

II Materials required but not included

a. Reagent

- Nuclease-Free Water
- Your intended *oriC* Cassette and DNA fragments

b. Material and Instruments

- Vortex mixer
- Mini centrifuge
- Heat block
- Thermal cycler (or Air incubator)
- 0.2 ml microtubes (PCR tubes)
- Micropipettes (P-2, P-10) and filter tips

III Storage

Below -70°C.

Before storing again at -80°C, 10X RE Mix should be frozen quickly by liquid nitrogen, etc.

IV Principle

a. Assembly Reaction

In OriCiro Assembly Kit, DNA fragments are assembled via overlapping ends having homologous sequences. First, each end of the fragments is digested by exonucleases to expose a single stranded homologous sequence. Then, corresponding fragments having the homologous sequence each other are annealed by enzymes (Fig. 2).

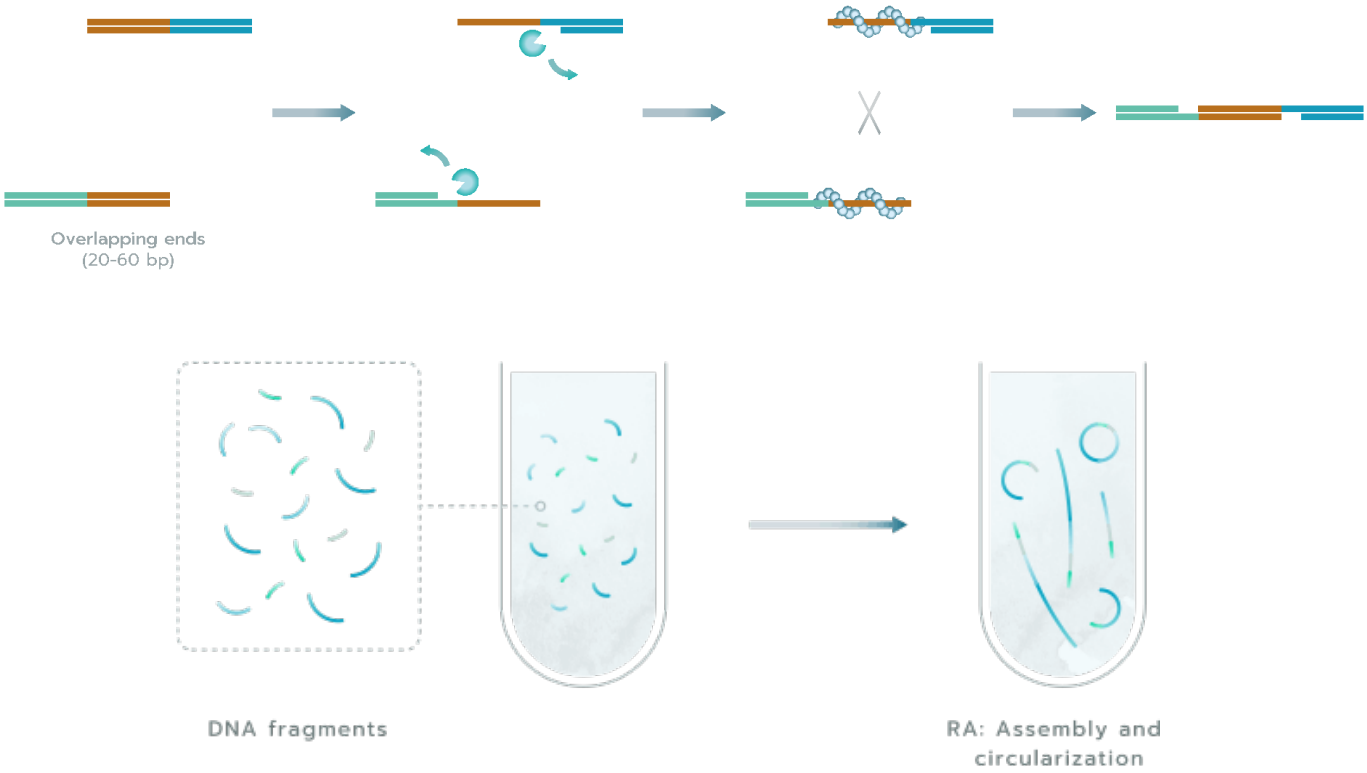


Figure 2. Assembly Reaction

b. Amplification Reaction

OriCiro Amp Kit contains 26 kinds of purified enzymes that are essential for the *E. coli* chromosome replication cycle. The replication cycle repeats autonomously, enabling exponential amplification of your circular DNA containing *oriC* (Fig. 3, 4, 5). For more detailed explanation, refer to the relevant publication (https://academic.oup.com/nar/article/45/20/11525/4209619).

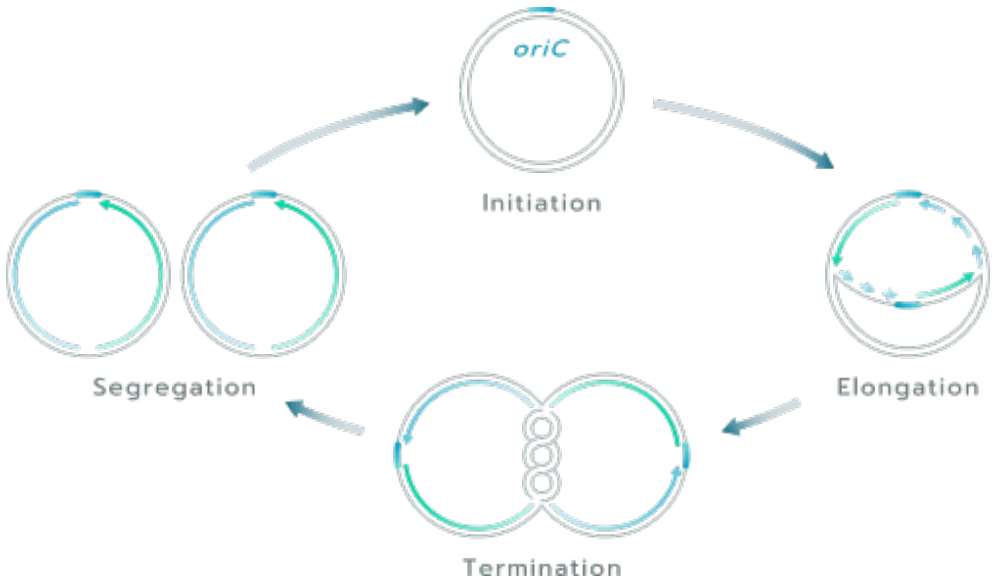


Figure 3. In-vitro reconstitution of chromosome replication cycle

Principle

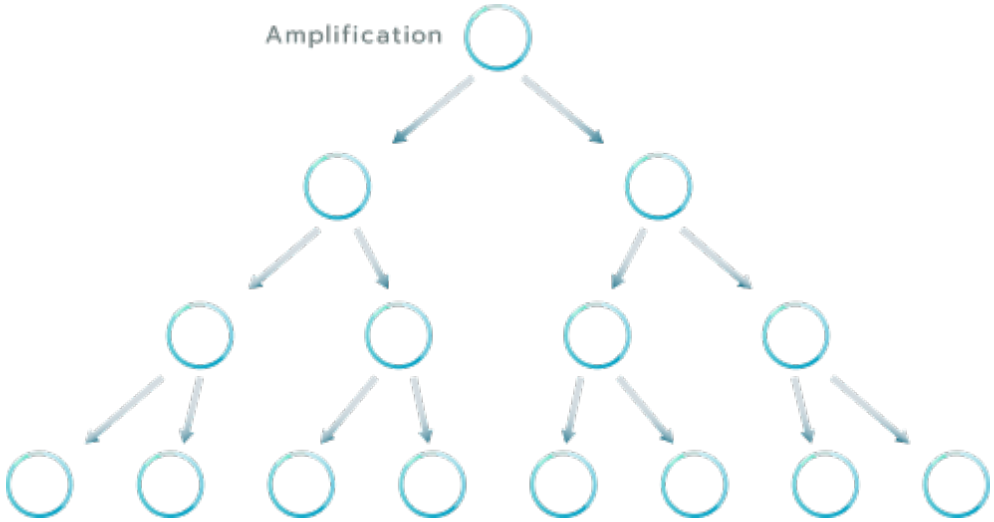


Figure 4. Cell-free hyper amplification

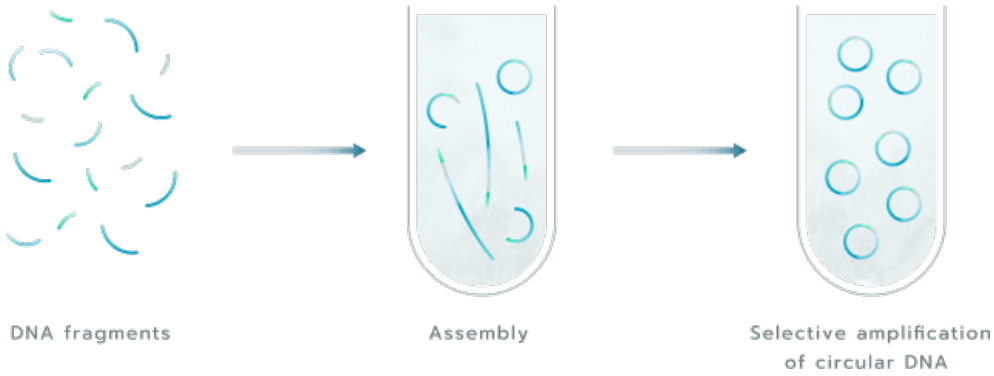


Figure 5. Efficient cell-free production of circular DNA from multiple DNA fragments

V Precautions for use

This section describes precautions for using this product. Please read carefully through the section before starting the protocol to achieve optimal results.

a. General Precaution

- Gently spin down components prior to use.
- Pipet enzymes slowly and carefully because of the high viscosity of these solutions.
- Use new disposable pipette tips to avoid contamination between samples when transferring reagents.
- To achieve better results, warm up the thermal cycler to the desired temperature before starting incubation. Lid temperature should be set at 7°C higher than the block temperature.

b. Assembly Reaction

- It is highly recommended to apply the amplification process followed by the assembly process.
- The size of DNA fragment that can be assembled by this product is from 200 bp to 50 kb.

c. Amplification Reaction

- Thaw the 10X RE Mix on ice.
- Template DNA must be in a circular form and must have the *oriC* sequences of *E. coli*.
- The minimum size of circular DNA is ~2 kb. If the target DNA size exceeds 50 kb, the amplification requires a different protocol than described in this manual. Please contact us at product@oriciro.com. (note: the maximum size that can be assembled and amplified by the protocol included in this Manual is 50 kb).
- OriCiro Amp products can be used as a template for the further round of the amplification reaction (passage amplification).

VI Protocol

a. Assembly Reaction

(1) Turn on heat block and preheat at 42°C.
(2) After 2X RA Mix is thawed on ice, mix well with vortex mixer for 30 seconds at maximum speed. Spin down with mini centrifuge.
(3) Prepare the following reaction mixture on ice[*1]. DNA fragments dissolved in TE buffer, Tris-HCl or Nuclease-Free Water can be used.

<per reaction>

Reagent	
Nuclease-Free Water	2.5 - X µl
DNA Fragments[*2]	X µl
2X RA Mix	2.5 µl
Total	5 µl

<per reaction, for Control Fragment>

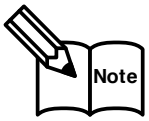
Reagent	
Nuclease-Free Water	0.5 µl
oriC Cassette	1 µl
Control Fragment	1 µl
2X RA Mix	2.5 µl
Total	5 µl

Mix the mixture well by pipetting to avoid foaming.

(4) Incubate the mixture at 42°C*3 for 30 minutes, then quench it on ice.

(Optional) Set the temperature of thermal cycler at 65°C and lid temperature at 72°C. Heat treatment by incubation at 65°C for 2 minutes, then quench it on ice*4. It is not recommended to heat gradually from 42°C to 65°C by thermal cycler.

(5) Storage condition of the assembled product is 4°C for a few days, and for further long-term storage, add final 20 mM EDTA before storage at -20°C. The following OriCiro Amp Kit reaction tolerates up to final 2 mM EDTA.



*1: Use 0.2 ml PCR tube to avoid evaporation.
*2: Includes your intended *oriC* cassette. Each DNA fragment should be added at equal molar ratio. The applicable quantity of DNA fragments is 1 pg – 20 ng. The optimal quantity for more than 10 fragments assembly is 20 ng as total DNA amount. See Appendix for how to calculate DNA fragment quantity to be added into the assemble reaction.
*3: 30–42°C is acceptable.
*4: Heat treatment represses mis-annealing products when you use a large number of fragments.

b. Amplification Reaction

(1) Turn on thermal cycler or air incubator and preheat at 33°C. set lid temperature at 40°C If a thermal cycler is used, the lid should be set at 40°C to avoid reaction inhibition due to evaporation of the reaction mixture.

(2) After 5X Buffer I and 5X Buffer II are thawed on ice, mix it well with vortex mixer and spin down with mini centrifuge. After 10X RE Mix is thawed on ice, mix it for 1 sec. x 3 times with vortex mixer and spin down with mini centrifuge.

(3) Prepare the following amplification reaction mixture on ice using 0.2 ml PCR tube.

<per reaction>

Reagent	
Nuclease-Free Water	4 µl
5X Buffer I	2 µl
5X Buffer II	2 µl
10X RE Mix	1 µl
Total	9 µl

(4) Incubate the amplification reaction mixture at 30°C for 15 minutes

(5) Add 1 µl of assembly product to the amplification reaction mixture and gently agitate the mixture by pipetting. Spin down with mini centrifuge.

(6) Incubate the mixture at 33°C for 6 hours – overnight.

(7) Prepare a new buffer (1X Buffer III) with 5X Buffer I, 5X Buffer II and Nuclease-Free Water to final 1X concentration of each component. Dilute immediately the reaction of step (5) two times with 1X Buffer III and incubate at 33°C for 30 minutes.

(8) Storage condition of amplified products is 4°C for a few days, and for further long-term storage, add final 20 mM EDTA before storage at -20°C. To avoid DNA shearing by freezing, if the length of amplified products is about 50 kb, please purify them with phenol/chloroform extraction and ethanol precipitation, and store the purified DNA at 4°C.

(9) Agarose gel electrophoresis is recommended to analyze amplified products. Since a band of circular supercoiled DNA of amplified products is not appeared at correct size, before agarose gel electrophoresis, digest amplified products with a proper restriction enzyme. Dilute the 0.5 µl of products with 5 µl of Loading Buffer (25 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.1% SDS, 5% glycerol, 0.1% bromophenol blue).

VII Appendix

a. Design and preparation of DNA fragments

Each end of DNA fragments must have overlapping sequences. For assembly of a smaller number of fragments, shorter overlap (~25 bp) is enough. Longer overlap (40–60 bp) is required to ensure the specificity for assembly of a larger number of fragments (>10 fragments). Tm value is not required to be considered.

The overlap does not have to be at the end of the DNA fragment, but must be no more than 100 bp from the end.

b. Design and preparation of your *oriC* Cassette

The *oriC* fragment having overlapping ends against your target fragments can be prepared by PCR using *oriC* Cassette (included in this kit) as a template, and primer pairs containing ~40 bp overlap sequences at their 5′ tails.

Typical sequence of primers to amplify your *oriC* Cassette will be as follows, that is designed to avoid annealing to *ter* sequences that exist at both ends of *oriC* sequence.

Forward primer:
5′ – (~40 nt overlap for your DNA fragment) + CTGCTCTGATGCCGCATAG –3′

Reverse primer:
5′ – (~40 nt overlap for your DNA fragment (Reverse)) + GTGTCGGGG CTGGCTTAAC –3′

c. DNA quantity calculation for OriCiro Assembly Kit.

Each DNA fragment should be added at equal molar ratio. The applicable quantity of DNA fragments is 1 pg – 20 ng. The optimal quantity for more than 10 fragments assembly is 20 ng as total DNA amount. DNA quantity added into the assembly reaction can be calculated by the following formula.

fragment size ÷ assembled size × DNA quantity (1 pg – 20 ng)

As an example of assembling 2 fragments, *oriC* Cassette (size: 0.4 kb) and Control Fragment (size: 7.5 kb), where the assembled size is 8 kb, the DNA quantity of each fragment should be as follows.

oriC Cassette: 0.4 kb ÷ 8 kb × 1 ng = 50 pg
Control Fragment: 7.5 kb ÷ 8 kb × 1 ng ≈ 1 ng

Fluorescence based quantification is recommended for assembly of more than 10 fragments.

d. Sequence of *oriC* Cassette

5′– ATGGTGCA CTCTCAGTAC AATCTGCTCT GATGCCGCAT
agtatgttgt aactaaagat ctactgtgga taactctgtc
aggaagcttg gatcaaccgg tagttatcca aagaacaact
gttgttcagt ttttgagttg tgtataaccc ctcattctga
tcccagctta tacggtccag gatcaccgat cattcacagt
taatgatcct ttccaggttg ttgatcttaa aagccggatc
cttgttatcc acagggcagt gcgatcctaa taagagatca
caatagaaca gatctctaaa taaatagatc ttctttttaa
tactttagtt acaacatact GTTAAGCCAG CCCCGACACC
CGCCAACACC CGCTGACGCG –3′

Small letters: *oriC* sequence
Capital letters: 40 bp overlapping sequences against Control Fragment
Enclosed: *ter* sequence to repress concatemer formation
Underlined: primer sequence for PCR amplification of OriC cassette

e. Sequence of Control Fragment (7.5 kb)

Control Fragment was constructed from pBeloBAC11 plasmid by PCR using the following primers;

5′ – CTATGCGGCATCAGAGCAG –3′ and

5′ – GTTAAGCCAGCCCCGACAC –3′ .

This fragment contains a chloramphenicol-resistant gene.

f. Quantification of circular DNA

Since the amplified product contains nucleotides, perform DNA quantification by absorbance method after purification by column or beads.

In the fluorescence method, the concentration of the amplified product, supercoiled DNA, is lower than that of linear DNA, so quantification should be performed after the amplified product is linearized.

Supercoiled DNA cannot be separated by capillary electrophoresis. If necessary, linearize the DNA and analyze it.

VIII Experimental examples

a. Typical result of control reaction.

- Assemble reaction and amplification reaction using *oriC* Cassette (0.4 kb) and Control Fragment (7.5 kb) was performed in accordance with the protocol for this product.
- Assembly reaction was performed at 42°C for 30 minutes.
- 1 µl of assembled product was added into amplification reaction.
- Amplification reaction was performed at 33°C for 6 hours.
- Applied 0.5 µl of amplified product to agarose gel electrophoresis.
- In the result of electrophoresis, well-amplified covalently closed supercoiled DNA was observed. The amplified DNA band appeared below 8 kb of general DNA marker because supercoiled DNA runs faster than the same size linear DNA in agarose gel.

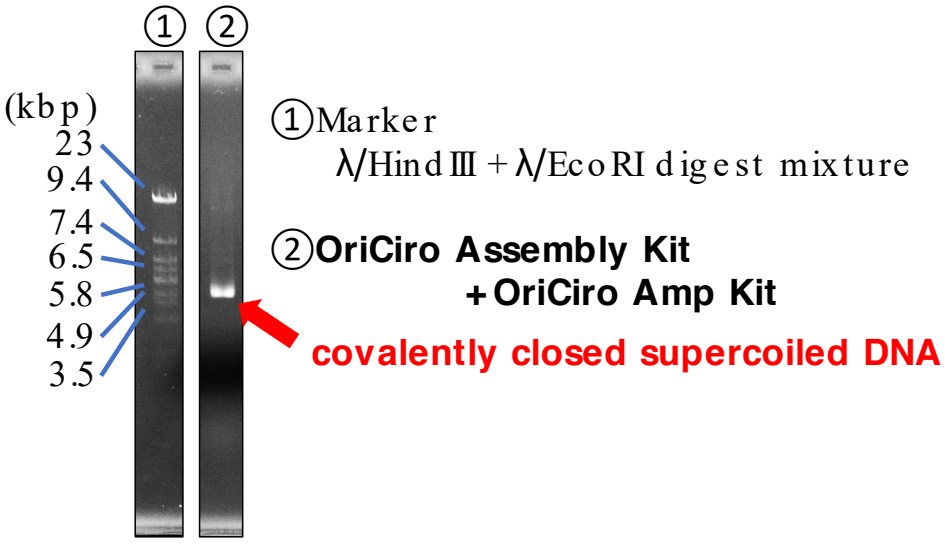


Figure 6. Result of agarose gel electrophoresis

IX Troubleshooting & FAQ

a. Assembly Reaction

Q1: Do I need purification of the assembly products before the OriCiro amplification?

A1: No. Add the assembly reaction (1 μl) directly into OriCiro Amp mixture (total 10 μl). OriCiro Amp selectively amplify circular DNA generated even slightly in the assembly reaction. The assembly intermediates (linear forms) are not amplified.

Q2: Can I use DNA fragments digested by restriction enzyme or CRISPR-Cas9?

A2: Yes.

Q3: No assembled products are observed.

A3: In certain conditions, the assembled product quantity may not be large enough to be observed after assembly reaction. It is highly recommended to perform following amplification reaction.

b. Amplification Reaction.

Q4: Can I reamplify the amplified products with OriCiro Amp Kit?

A4: Yes, it is possible to amplify in a consecutive manner (passage amplification). Dilute the amplified products (~10^{-6}), prepare new amplification reaction mixture and incubate again. It has been checked that the amplified products can be reamplified consecutively up to 10 times.

Q5: Can I use a plasmid having a ColE1-type origin as a template.

A5: It cannot be used a plasmid only having a ColE1-type origin, the optimized *oriC* sequence is essential for OriCiro Cell-Free Cloning System. If a plasmid having both *oriC* and ColE1-type origins, the amplification reaction is not affected by the ColE1 type origin.

Q6: Extra bands are observed in agarose gel electrophoresis?

A6-1: DNA contamination.

Put negative control sample (reaction without DNA) for the amplification reaction. OriCiro Amp Kit amplifies DNA even from a single contamination molecule. To identify and remove DNA contamination, monoclonal amplification from a single DNA molecule following limiting dilution is useful.

A6-2: Concatemer DNA

When the amplified DNA size is less than 4 kb, extra bands by concatemer DNA may appear. The situation may improve by shortening the reaction time.

Q7: No amplified products are observed.

A7-1: 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. Please contact us at product@oriciro.com.

A7-2: The assembly reaction will be inhibited when salt and/or EDTA concentration of the DNA sample is too high.

A7-3: Increase the amplification reaction temperature up to 37°C.

A7-4: Increase the amplification reaction time up to 18 hours.

A7-5: Increase the template DNA amount added into the amplification reaction.

Q8: Can the amplified DNA be used for transformation of *E. coli* and transfection of cells in culture?

A8: It can be used. Purification of the amplified product is not necessary for heat shock transformation.If electroporation is used, purify the product by ethanol precipitation or dialysis to remove the salt.

X Reference

1. 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)

2. 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

3. 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

Note :

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