OriCiro® Cell-Free Cloning System

OriCiro® Cell-Free Cloning System is a rapid and powerful tool replacing cumbersome DNA cloning (plasmid construction) process relying on *E. coli*. The system consists of two kits. **OriCiro Assembly Kit** allows seamless assembly of multiple overlapping DNA fragments. The assembly product can be added directly to **OriCiro Amp Kit** to get selective amplification of your target circular DNA (Figure 1). The amplified product is supercoiled DNA topologically identical to plasmid DNA isolated from *E. coli*.

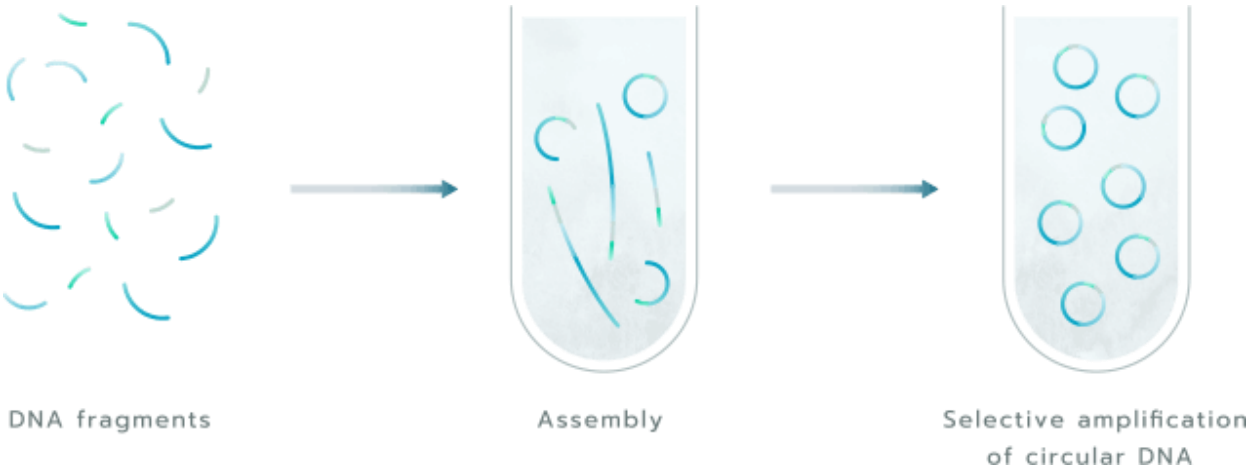

Figure 1. Assembly & Amplification to produce circular DNA

OriCiro Assembly Kit

Multiple DNA fragments are assembled seamlessly at 42˚C for 30 minutes via ~40 bp overlapping ends (Figure 2). DNA fragments generated by PCR or restriction enzyme digestion are available. Our unique enzyme-based annealing mechanism allows powerful assembly up to 50 fragments simultaneously.

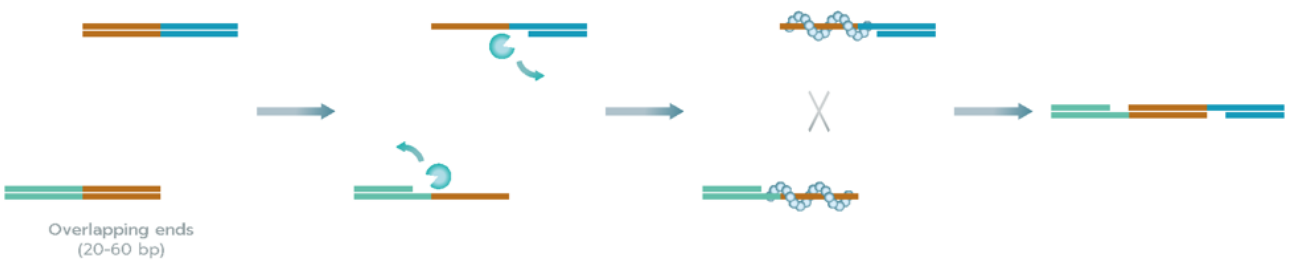

Figure 2. OriCiro Assembly

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 3). 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.

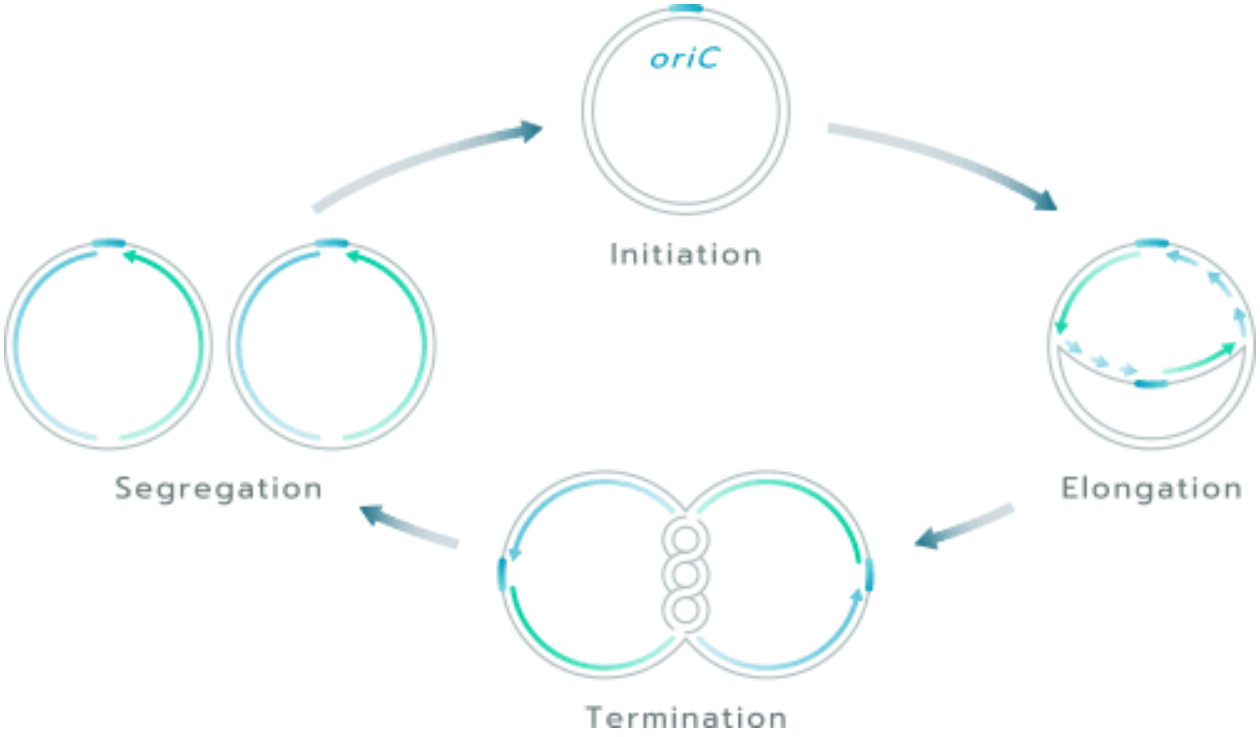

Figure 3. Amplification of circular DNA through chromosome replication cycle

I. Components

OriCiro Assembly Kit	(for 5 reactions)		**OriCiro Amp Kit**	(for 10 reactions)	
(1)	2X RA Mix	12.5 µl	(4)	10X RE Mix	10 µl
(2)	*oriC* Cassette*1 (50 pg/µl)	10 µl	(5)	5X Buffer I	40 µl*2
(3)	Control Fragment*1 (1 ng/µl)	5 µl	(6)	5X Buffer II	40 µl*2

*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) (Figure. 4). See Appendix for sequence information.

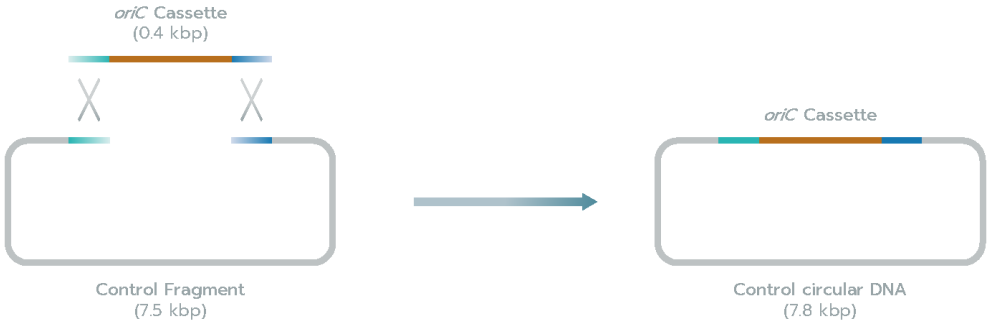

Figure 4. *oriC* Cassette and Control Fragment

*2: 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® Cell-Free Cloning System is shipped on dry ice. Upon receipt, the kits must be immediately stored at **below -70°C**. 2X RA Mix and 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 2X RA Mix, *oriC* Cassette, Control Fragment, 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

ASSEMBLY REACTION

(1) **Thaw 2X RA Mix on ice, mix it well with a vortex mixer at a maximum speed and spin down with a microcentrifuge.**

(2) **Prepare the following mixture on ice, and mix well by pipetting.**[*1]

< Sample >	
Nuclease-Free Water	2.5 – X µl
DNA Fragments[*2] (up to 20 ng as total fragments)	X µl
2X RA Mix	2.5 µl
Total	5 µl

< Positive control >	
Nuclease-Free Water	0.5 µl
oriC Cassette (50 pg/µl)	1 µl
Control Fragment (1 ng/µl)	1 µl
2X RA Mix	2.5 µl
Total	5 µl

*1: • Use a 0.2 ml PCR tube to avoid evaporation.
• Vortex mixing is not recommended because of small volume of viscous sample.
• For the pipette mixing, set pipette volume to the total mixture volume, and pipetting up and down four times with agitation.

*2: • Include your intended *oriC* Cassette for the subsequent amplification reaction.
• 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 fragments. (see Appendix c. for the quantity calculation).
• DNA fragment dissolved in TE buffer is acceptable.

(3) **Incubate the mixture at 42°C**[*3] **for 30 minutes and hold on ice before use.**[*4]

*3: 30–42°C is acceptable.

*4: Although the assembly product can be stored at 4°C for a few days before the subsequent amplification step, immediate use is recommended for best results.

(Option) Heat treatment[*5]
Immediately after the 42°C incubation in step (3), transfer the mixture to 65°C block and heat it for 2 minutes, followed by quick cooling on ice.

*5: "Heat treatment" option can eliminate mis-assembly by products, and is recommended for the best results particularly when you intend to assembly a large number of fragments (over five fragments).

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. Appendix

a. Design and preparation of DNA fragments (Figure 5)

Each end of DNA fragments must have overlapping sequences which is typically introduced by PCR using tailed primers. For assembly of a smaller number of fragments, shorter overlap (~25 bp) is enough. Longer overlap (40-60 bp) can improve the assembly specificity and is particularly required for assembly of a larger number of fragments (>10 fragments) or larger sized DNA (>10 kb). Tm value does not have to be considered. The overlap ~10 bp away from the fragment end is acceptable. Restriction digested fragments with 5' or 3' sticky end are also available.

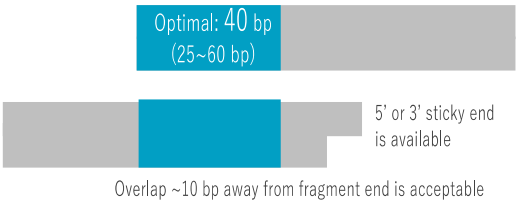

Figure 5. Design of overlapping

b. Design and preparation of your oriC Cassette (Figure 6)

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 primer sequence to amplify your oriC Cassette will be as follows:
Forward primer:5'-[~40 nt overlap for tail of your DNA fragment] +CTGCTCTGATGCCGCATAG-3'
Reverse primer: 5'-[~40 nt overlap for head of your DNA fragment (Reverse)] +GTGTCGGGGCTGGCTTAAC-3'

c. DNA quantity calculation for OriCiro Assembly Kit

Each DNA fragment should be added at equal molar ratio. The applicable amount of DNA fragments in the 5 µl assembly reaction is 1 pg - 20 ng as total fragments. Total 20 ng DNA is optimal for more than 10 fragments assembly. The amount of each DNA fragment is calculated by the following formula.

[Fragment size] / [Total assembly size] × [Total DNA amount (1 pg – 20 ng)]

As an example of assembly for two fragments; "*oriC* Cassette (0.4 kb)" and "Control Fragment (7.5 kb)", where the "total assembly size" is 8 kb, the amount 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

The equal molar ratio is important for the assembly of a larger number of fragments (>10 fragments). In this case, we recommend use of DNA fragments with approximately equal length, whose concentrations are quantified precisely using fluorescence-based method (e.g., Q-bit, Thermo Fisher Scientific) or Agilent 2100 Bioanalyzer (Agilent Technologies).

d. *oriC* Cassette (378 bp)

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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'
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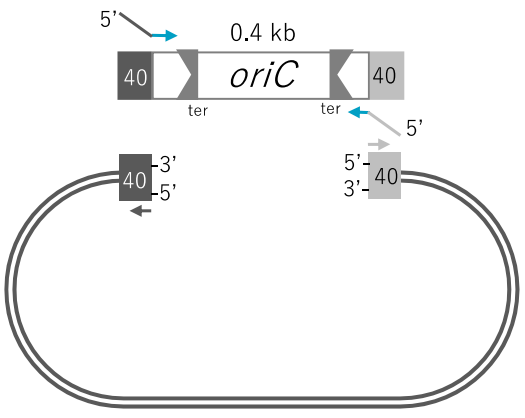

Figure 6. Design of *oriC*

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. Control Fragment (7.5 kb)

Control Fragment was constructed by PCR amplification of the whole **pBeloBAC11 plasmid using the following primers: 5'- CTATGCGGCATCAGAGCAG -3' and 5'- GTTAAGCCAGCCCCGACAC -3'**. The ***oriC*** Cassette will be inserted into a site downstream of the lacZa gene of pBeloBAC11. The resultant plasmid has ***oriC***, BAC origin and **a chloramphenicol-resistant gene**, and is able to maintain the plasmid in *E. coli* after transformation.

VI. 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.

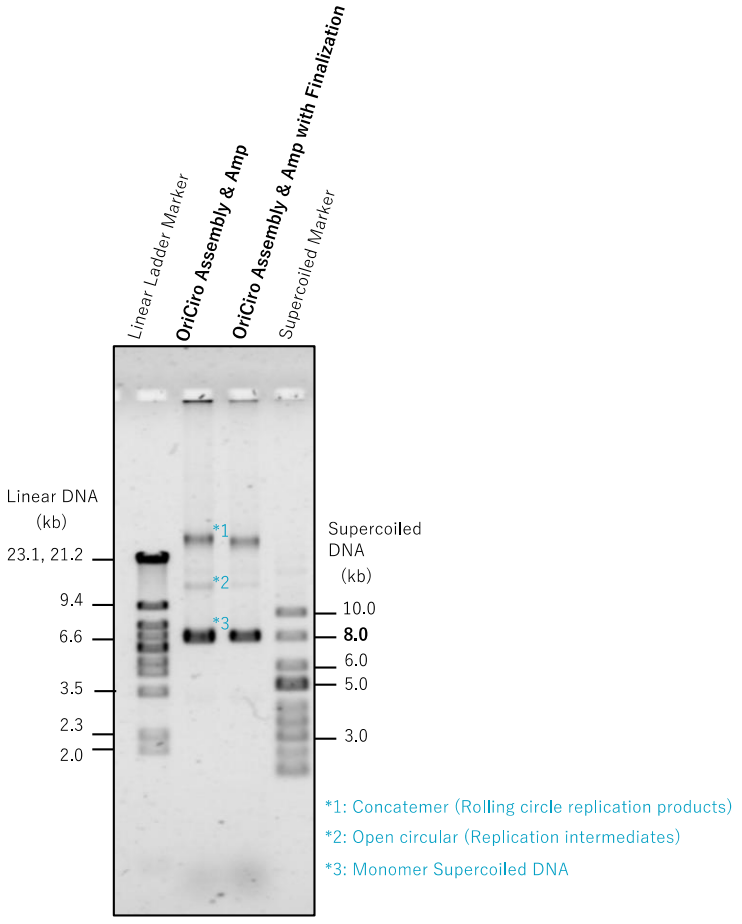

Figure 7. OriCiro Assembly and Amplification reaction of Control fragment (7.5 kb) and *oriC* cassette (0.4 kb)

VII. FAQs

a. Assembly Reaction

Q1 : How short or long DNA can be assembled?
A1 : DNA fragment from 0.2 kb to 50 kb can be assembled in OriCiro assembly kit.

Q2 : Can I use DNA fragments digested by a restriction enzyme or CRISPR-Cas9?
A2 : Yes. A digested fragment with blunt, 5' or 3' sticky end is available for the assembly reaction. Overlap sequences located several bp away from the digestion site is also acceptable. In most cases, the digestion reaction after heat-inactivation of endonucleases can be used directly for the assembly reaction without any purification step.

Q3 : How many DNA fragments can be assembled?
A3 : Up to 50 fragments can be assembled simultaneously using a set of similar sized fragments whose concentrations are accurately quantified.

Q4 : What size is optimal for the overlapping end sequences?
A4 : 40 bp is optimal. 15-300 bp is acceptable by customization of the assembly condition.

Q5 : Do I need care about Tm value of the overlapping end sequences?
A5 : No. Just add 40 bp sequences overlapping with the neighboring fragments.

Q6 : Do I need purification of the assembly products before the OriCiro amplification?
A6 : No. Add the assembly reaction (1 µl) directly into the amplification mixture (total 10 µl).

Q7 : How does the total amount of the fragments affect the assembly?
A7 : The assembly reaction is optimized for 20 ng of the total DNA fragments. Excess amount will reduce the assembly efficiency. On the other hand, very low amount of the fragments (1 pg) is acceptable for assembly of 2-3 fragments to produce circular DNA up to 10 kb by the following amplification step.

Q8 : Have you tested OriCiro system for cloning direct repeat containing sequences?
A8 : Yes. We have amplified 60kb plasmid which contains 171 bp with 352 repeats, and also a 5kb plasmid containing ~100 triplet repeats.

b. Amplification Reaction

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.

VIII. 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) Failure of the assembly reaction
The undesirable circular DNAs may be produced in the assembly reaction. Please check the purity of DNA fragments and the overlapping sequence specificity of DNA ends for the assembly reaction.

3) 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.

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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