OriCiro® Cell-Free Cloning System/PASS V4.1.2

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[1]OriCiro Genomics

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OriCiro Genomics

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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.

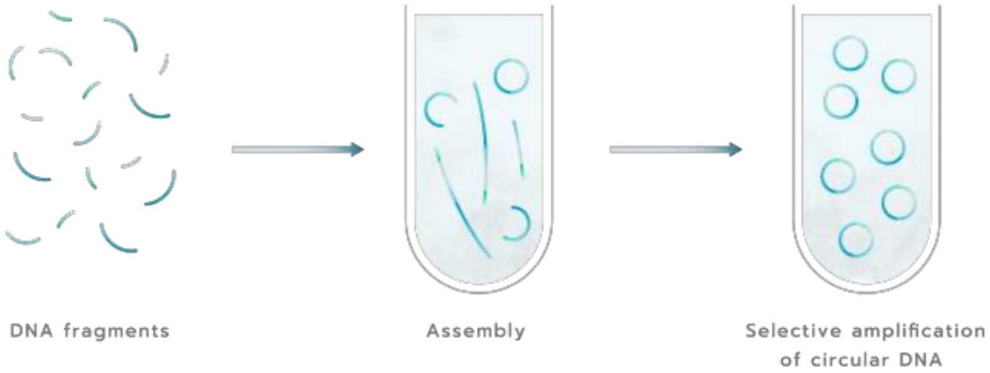

Figure 1. Assembly & Amplification to produce circular DNA

1. 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.

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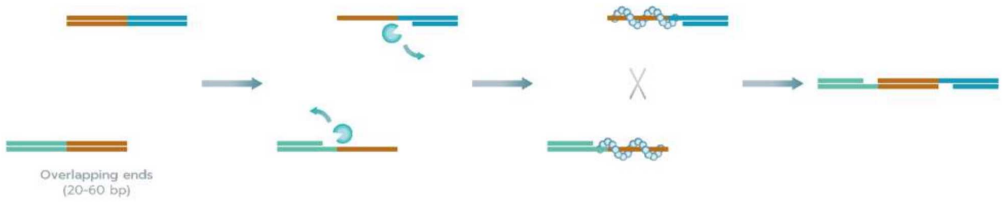

Figure 2. OriCiro Assembly

2. 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 37°C for 6 hr. The maximum amplification size is 50 kb in the current version of the kit.
n.b. OriCiro Amp NEEDS *oriC* Cassette (0.4 kb) which can be inserted into circular DNA using OriCiro assembly kit.

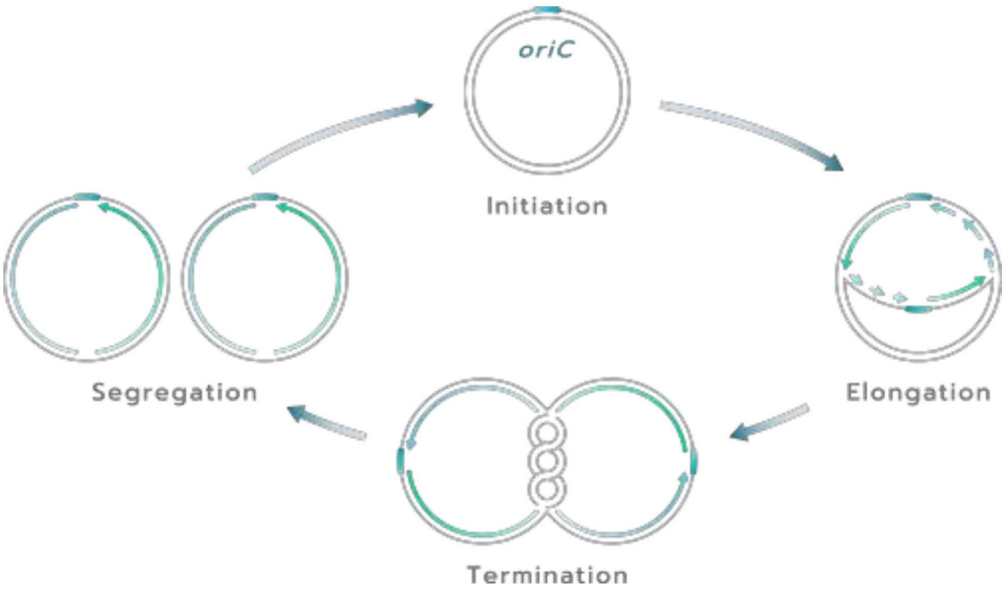

Figure 3. Amplification of circular DNA through chromosome replication cycle

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a. Design and preparation of DNA fragments

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.

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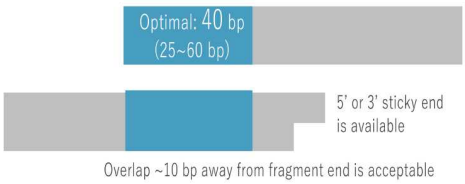
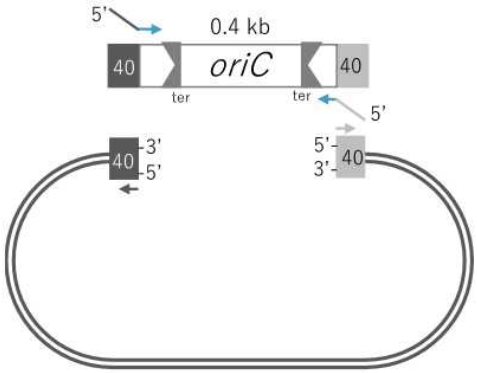


b. Design and Preparation of your oriC cassette

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 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
ControlFragment:7.5kb/8kb×1ng ≈ 1ng

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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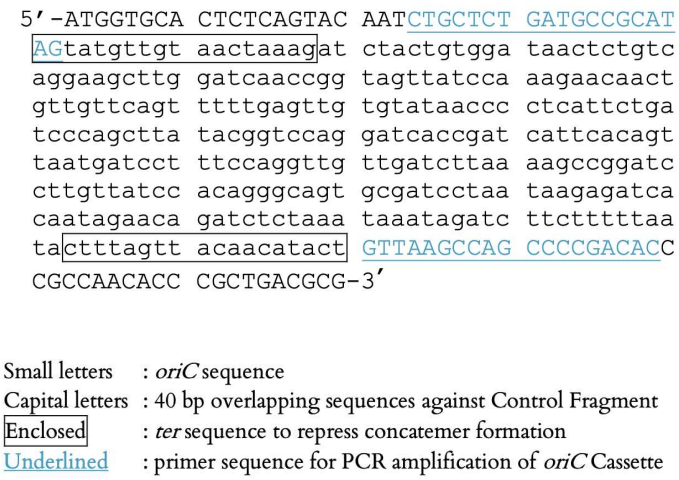

e. Control Fragment (7.5 kb)

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.

I. Kit Components

OriCiro® Assembly Kit (for 5 reactions):

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

OriCiro® Amp Kit (for 10 reactions)**:**

A	B
Component	**Volume (µl)**
10X RE Mix	10
5X Buffer I	40 µl *2
5X Buffer II	40 µl *2

*1: oriC Cassette is a 378 bp DNA fragment containing oriC (E. coli chromosomal origin)

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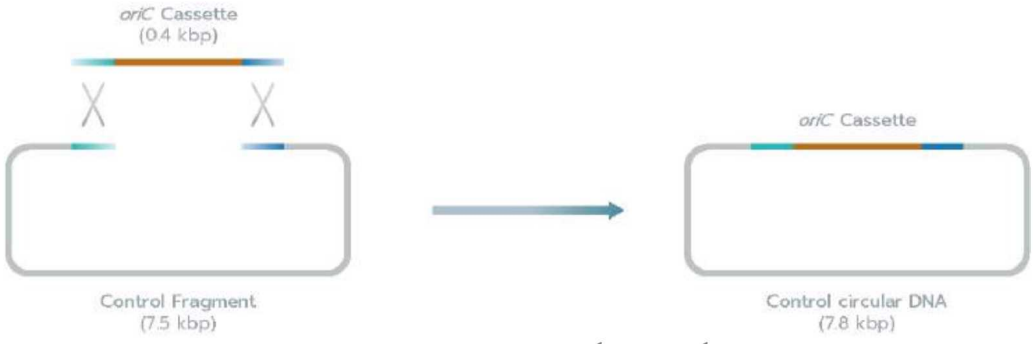
sequence. Both ends of the oriC Cassette have 40 bp overlapping sequences against Control Fragment (7.5 kb). See Guidelines tab for sequence information.



*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.

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 micro-centrifuge.

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

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Sample

A	B
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

Control

A	B
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): Needs 30-42°C
(*4): Although the assembly product can be stored at -4°C for a few days before the subsequent amplification step, i mmediate use is recommended for best results.

4 **Optional Step (*5): Heat Treatment**

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” 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).

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Amplification Reaction

5 **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.

6 **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.**

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

A	B
< Amp pre-mixture>	x1 reaction (*2)
Nuclease-Free Water	4µl
5X Buffer I	2µl
5X Buffer II –> Vortex Mixing	2µl
10X RE Mix –> Pipette mixing (*3)	1µl
Total	9µl

(*1): Use a 0.2ml 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.

8 (Optional) Pre-incubation (*4):

Incubate the Amp pre-mixture at 33°C for 15 minutes.

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(*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.

9 **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):

- A thermal cycler program is useful to hold automatically at 12°C after the 33°C incubation.

10 (Optional) Finalization (*7):

Dilute the reaction of step (9) 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.

11 **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.

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• 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.

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