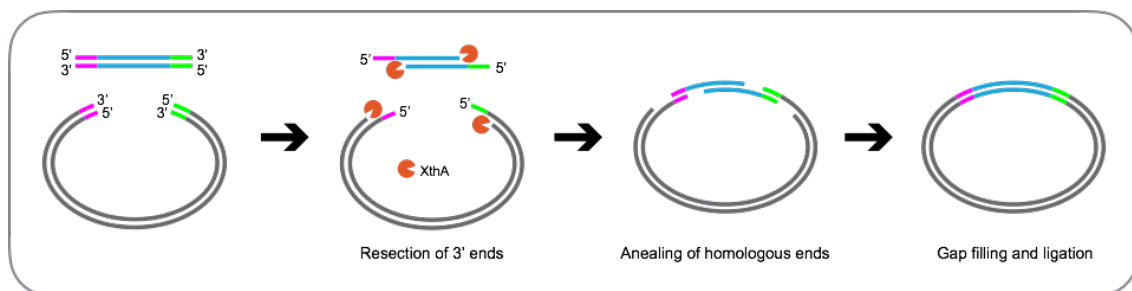


Instructions for iVEC3

iVEC is a simple method to carry out seamless cloning just by introducing DNA fragments into *E. coli*. NBRP *E. coli* has released iVEC3 in which multimerization of plasmids doesn't occur. In previous iVEC strains, *in vivo* cloning was carried out by expression of a recombinase, RecET. However, *in vivo* cloning in iVEC3 is dependent on XthA, a 3' to 5' exonuclease and is independent of RecET. The mechanism is considered to be as follows.

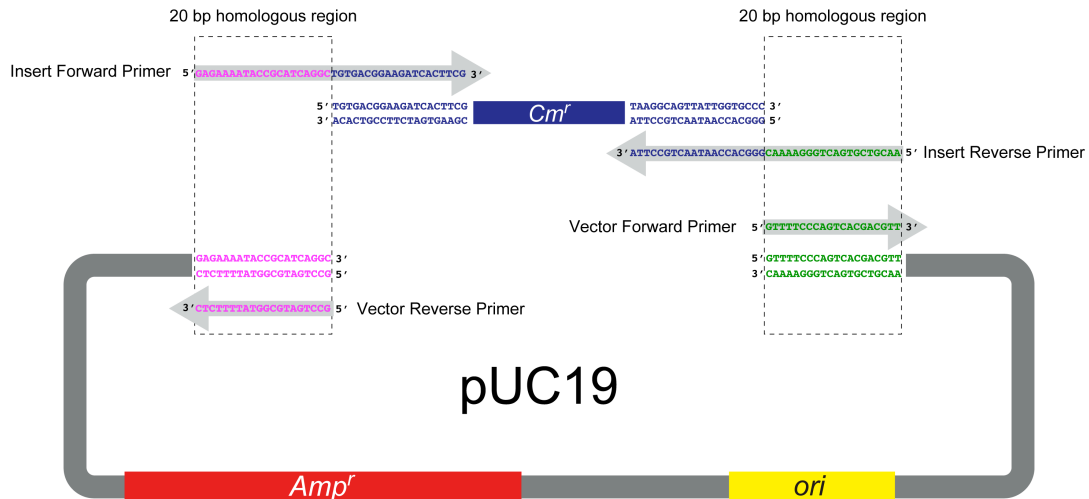
1. Ends of DNA fragments introduced into *E. coli* cells are resected from 3' to 5' by XthA and 5'-protruding single-stranded ends are produced.
2. Homologous single-stranded ends of vector and insert anneal, gaps are filled by DNA polymerases and nicks are repaired by DNA ligases.



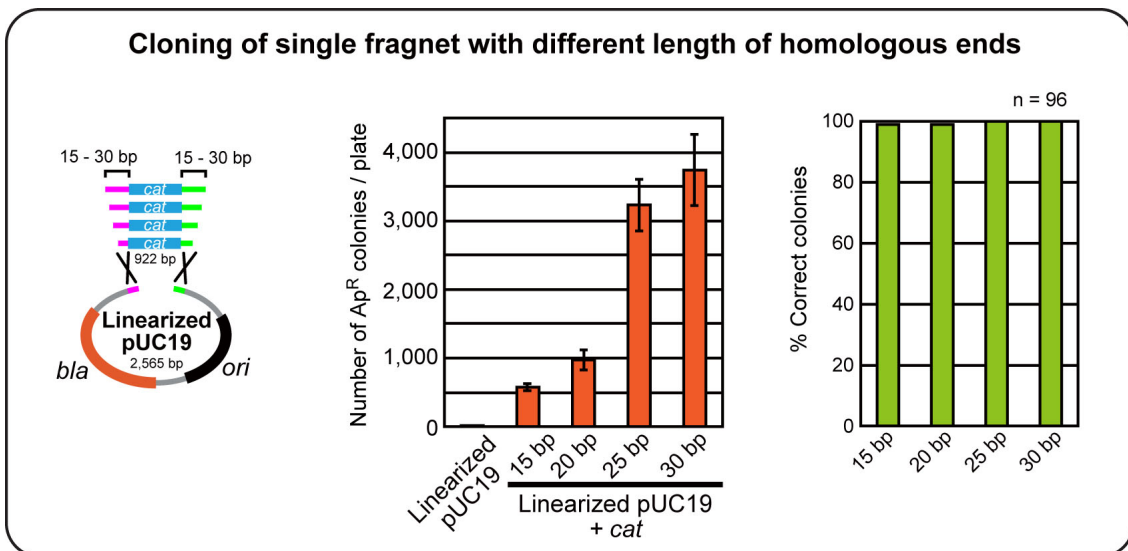
The efficiency of the *in vivo* cloning has improved compared to previous iVEC strains, making it very easy to use. The detailed information about iVEC3 will be described in elsewhere (Nozaki *et al.*, in preparation).

Genotype of iVEC3: MG1655 Δ hdsR Δ endA Δ recA

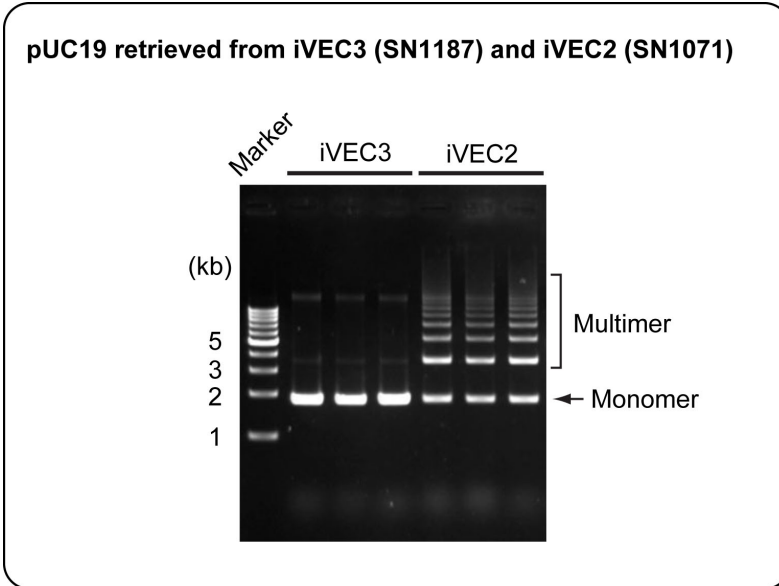
The primer DNAs are designed to have 20 - 40 additional ends which are homologous to the corresponding vector (or insert) sequences. For example, primers used for cloning of chloramphenicol resistant gene from pACYC184 into pUC19 vector are shown in the figure below.



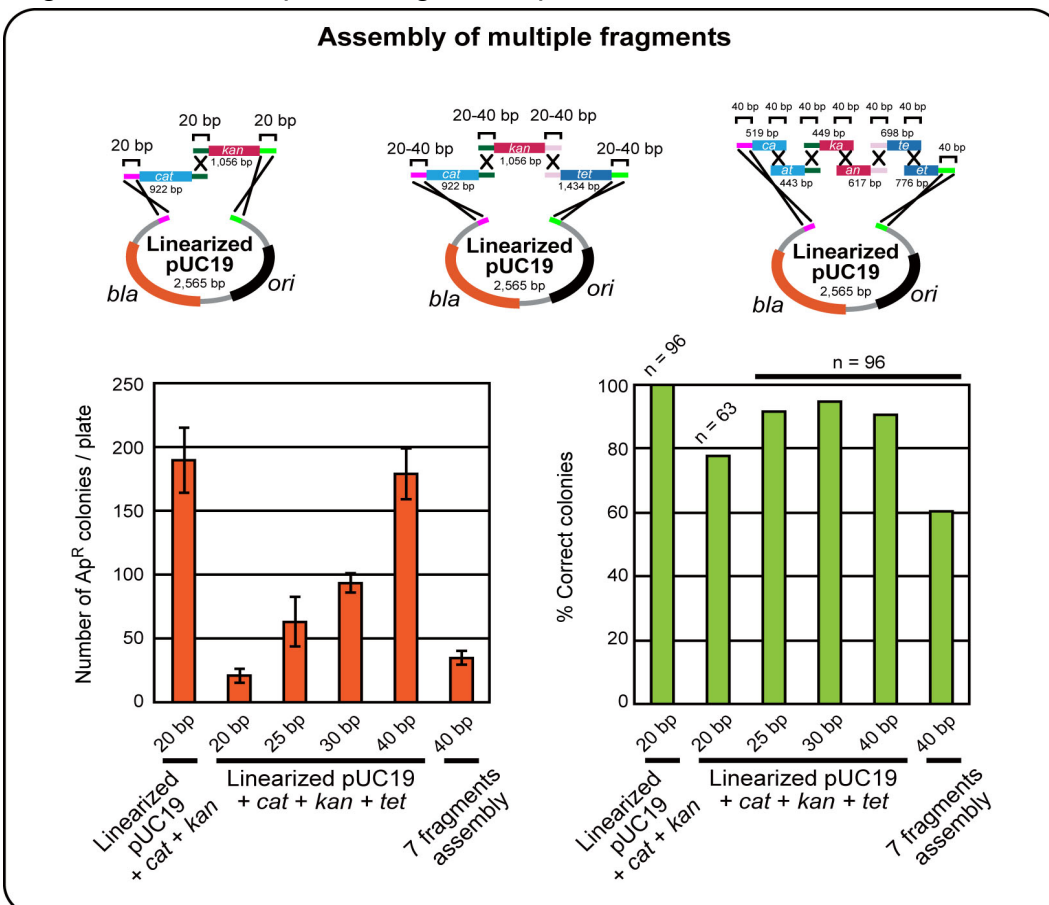
After transformation using 0.15 pmol (100ng) of insert DNA and 0.05 pmol (90 ng) vector DNA with 20 bp homologous sequences at their ends, about 1,000 colonies appeared. In addition, almost 100 % of the colonies had correctly assembled plasmids. If you clone DNA fragment that is not too long up to several kb, enough number of colonies can be obtained with 15 bp of homologous sequences.



Multimerization of the plasmids doesn't occur using iVEC3. Plasmids (pUC19) retrieved from iVEC3 and iVEC2 are shown below.



Multi-fragments assembly is also available. It was able to assemble 7 fragments with 40 bp homologous sequences.



Sometimes it is difficult to construct bigger plasmids that is longer than 10 kb by decrease of efficiency of DNA uptake. This problem can be overcome by increasing the amount of DNA to introduce or lengthen the homologous sequences.

iVEC3 as well as previous iVEC strains shows highest performance using transformation by TSS method (Chung *et al.*, 1989, PNAS). We modified this method to carry out preparation of competent cell and transformation only in one tube.

Transformation for iVEC

Reagent

- TSS (Transformation and Stock Solution) 50 ml (about 50 transformation)

L broth	25 ml
2xTSS	20 ml
DMSO	5 ml

Store at 4 °C.

- 2xTSS (20 ml)

PEG8000	4 g
1M MgSO ₄	2 ml
80 % glycerol	5 ml
L broth	to 20 ml

Autoclave for 15 min at 120 °C.

Mix well before use.

Store at 4 °C.

- L broth (1,000 ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
H ₂ O	to 1,000 ml

Adjust to pH 7.0 with 5N NaOH.

Autoclave for 15 min at 120 °C.

Preparation of DNAs for transformation

- 15 - 40 bp of the homologous sequences at the end of insert and vector is required. The number of colonies after transformation increases with longer homologous sequences.
- 100 ng/ μ l of PCR products are enough. However, when introducing longer DNA which exceeds 5 kb, the thicker one is preferred.
- You can use PCR products without purification, if the vector template DNA in PCR reaction is less than 50 pg/ μ l.
- When the amount of vector template DNA can't be reduced, inactivation of vector template by treatment of DpnI after PCR increases proportion of hit colonies.

Preparation of competent cells and transformation

Day 1: Preparation of competent cells (Required time: 3 min)

- L broth
- Sterilized tooth pick or sterilized tip
- Incubator at 37 °C

1. Pick colony of iVEC strain on plate or glycerol stock of iVEC strain with a sterilized toothpick. (A little is all fine.)
2. Suspend cells in 1.5 ml tube that is filled with 1 ml of L broth.
3. Stand the tube for overnight (about 20 hours, 16 - 24 hours are also fine) at 37 °C with the tube lid closed.

Day2: Transformation (Required time: 80 min, Actual working time: 5 min)

- Cooled centrifuge
- Water bath incubator at 37 °C
- **Liquid Nitrogen**
- Linearized vector and insert DNA by PCR. Purification of the PCR products is not essential.

1. Chill the overnight culture in 1.5 ml tube on ice for 5 - 10 min.
2. Centrifuge the tube at 5,000 g for 1 min at 4 °C to spin down the cells.
3. Remove the supernatant and stand on ice.
4. Add 1 - 2 µl each of PCR-amplified insert and vector DNAs into 100 µl of ice-cold TSS (Transformation and Stock Solution) and mix well. This step is recommended to be carried out between operation 1 and 2.
5. Resuspend the cells with TSS-DNA solution (100 µl) by pipetting.

6. Immediately after resuspension, freeze in liquid nitrogen.
※ **Important** Freeze in liquid nitrogen is recommended. Freezing in -80 or -30 °C reduces the number of transformants.
7. Put the frozen tube on ice and incubate for 10 min on ice.
8. Shortly mix by vortex (for 1 sec) and put back on ice and incubate for further 10 min on ice.
9. Add 1 ml of L broth (room temperature) and mix by inversion.
10. Incubate in water bath incubator at 37 °C for 45 min. (Heat shock is not needed.)
11. Centrifuge at 5,000 g for 1 min to spin down the cell. Remove 1 ml of the supernatant and resuspend the cells with 100 µl of remained supernatant.
12. Spread the cells on agar plate with appropriate antibiotics, and incubate at 37 °C for overnight.

Protocol for preparation of freezing stock competent cell

Reagents

- 2xTSS (20 ml)

PEG8000	4 g
1M MgSO ₄	2 ml
80 % glycerol	5 ml
L broth	to 20 ml

Autoclave at 120 °C for 15 min.

Mix well before use.

Store at 4 °C.

- DMSO

Preparation of DNAs for transformation

- 15 - 40 bp of the homologous sequences at the end of insert and vector is required. The number of colonies after transformation increases with longer homologous sequences.
- 100 ng/μl of PCR products are enough. However, when introducing longer DNA which exceeds 5 kb, the thicker DNAs are preferred.
- You can use PCR products without purification, if the vector template DNA in PCR reaction is less than 50 pg/μl.
- When the amount of vector template DNA can't be reduced, inactivation of vector template by treatment of DpnI after PCR increases proportion of hit colonies.

Preparation of competent cells

Day 1

1. Pick colony of iVEC strain on plate or glycerol stock of iVEC strain into 3 ml of L broth.
2. Incubate with shaking at 37 °C for overnight (about 16 hours).

Day 2

1. Add 1 ml of overnight culture into 60 ml of L broth preheated to 37 °C and incubate for 90 min. Usually, after 90 min incubation, OD₆₀₀ become 0.4 - 0.6.
2. Stand the flask on ice for 5 - 10 min. From this step, manipulate the cells on ice.
3. Centrifuge at 5,000 g for 5 min at 4 °C and discard the supernatant.
4. Resuspend the cells with 2 ml of ice-cold L broth.
5. Add 1.6 ml of ice-cold 2xTSS and mix.
6. Add 0.4 ml of DMSO (room temperature) and mix.
7. Aliquot 0.1 ml into sterilized microcentrifuge tubes.
8. Freeze in liquid nitrogen and store at -80 °C.

Transformation

1. Melt the frozen competent cells on ice.
2. Add 1 - 2 µl of PCR products of insert and vector into competent cell and mix.
3. Incubate on ice for 20 min.
4. Add 1 ml of L broth (room temperature) and mix by inversion.
5. Incubate in water bath incubator at 37 °C for 45 min.
6. Centrifuge at 5,000 g for 1 min to spin down the cell. Remove 1 ml of the supernatant and resuspend the cells with 100 µl of remaining supernatant.
7. Spread the cells on agar plate with appropriate antibiotics, and incubate at 37 °C for overnight.