Establishment of a rapid method to assemble and transfer DNA fragments into the JCVI-syn3B minimal synthetic bacterial genome

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Abstract

JCVI-syn3B (syn3B), a minimal synthetic bacterium that only possesses essential genes, facilitates the examination of heterogeneous gene functions in minimal life. Conventionally, *Escherichia coli* is used to construct DNA fragments for gene transfer into the syn3B genome. However, the construction process is challenging and time-consuming due to various issues, including the inhibition of *E. coli* growth and unexpected recombination, especially with AT-rich DNA sequences such as those found in *Mycoplasma* genes. Therefore, in this study, we aimed to develop a new transformation method to overcome these issues. We assembled the vector and target DNA fragments using an in vitro homologous recombination system and subsequently transferred the products into the syn3B genome. We obtained approximately 5,000 recombinant colonies per milliliter of the original culture in eight days, which is four days shorter than the conventional period, without any recombination issues, even for AT-rich DNA.

Key words

Homologous recombination, *loxP*, Transformation

Significance

The minimal synthetic bacterium JCVI-Syn3B is a useful tool for basic and applied biology. Plasmids for transformation are generally constructed using *Escherichia coli*; however, unexpected recombination occurs in some cases. To prevent such issues, this study developed a new method without any in vivo *E. coli* system.

Introduction

JCVI-syn3B (syn3B) is a minimal synthetic bacterium whose genome only consists of essential genes derived from the small bacterium, *Mycoplasma mycoides* [1-3]. Its genome contains a locus of *loxP* sites to which external genes are transferred via the Cre recombinase activity (Fig. 1) [2]. It is useful in both basic and applied biology [4-8]. Transformation requires a plasmid containing the target DNA, Cre recombinase, *loxP* sites, and puromycin resistance gene [2]. We previously constructed a plasmid using *Escherichia coli* (Fig. 2) as described below. Day 1: Polymerase chain reaction (PCR)-amplified vector and target DNA fragment were assembled via homologous recombination-based methods using the NEBuilder® and In-Fusion® systems. The constructs were then used to transform *E. coli* cells. Day 2: Transformed colonies were inoculated into a liquid medium. Days 3–5: Plasmids were isolated and sequenced. Days 6–9: Syn3B was transformed with the plasmid. Days 10–12: Colonies were cultured in a liquid medium and target gene transfer was confirmed via direct PCR. This method exhibited some drawbacks, including poor *E. coli* growth and unexpected recombination. Moreover, unexpected gene recombination was observed in *Mycoplasma* and related species, possibly due to their high AT content (67–76%) [9,10]. Therefore, in this study, we aimed to explore four transfer methods without *E. coli* to develop a new *E. coli*-free approach that is four days faster than the conventional method for gene transfer.

Materials and Methods

Bacterial strains and culture conditions

JCVI-syn3B (GenBank, CP069345.1) and *E. coli* (DH5α) were cultured in the SP4 [1,2] and Luria–Bertani media at 37°C, respectively.

Preparation of DNA fragments

Mycoplasma mobile genome was isolated as previously described [11]. Vector- and focused *M. mobile* DNA fragments were amplified from pSD128 DNA [4] and *M. mobile* genomic DNA using primers P1–P2 and P3–P4 (Table 1), respectively. The DNA fragments were assembled using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Inc., MA, USA) and In-Fusion HD® Cloning Kit (Takara Bio Inc., Kusatsu, Japan). Then, the assembled product was amplified via PCR using

74 primers P5 and P6 and circularized via self-assembly with NEBuilder®.

75 **Transformation and direct PCR**

76 Transformation of JCVI-syn3B was performed as previously described
77 [5]. Transformed colonies were picked, inoculated into 100 μL of SP4 medium
78 containing puromycin (3 μg/mL), cultured for 18–24 h, and confirmed via PCR using
79 primers P7 and P8.

80

81 **Results and Discussion**

82 **Transformation using assembled and amplified linear DNA**

83 For transformation, we assembled the plasmid using an in vitro homologous
84 recombination system and amplified it using PCR. The amplified DNA fragments (Fig.
85 3(i)) were transferred into the syn3B genome. As our target DNA, we used a 1,916-bp
86 fragment containing the 1,536-bp gene encoding phosphoglycerate kinase from *M.*
87 *mobile* (MMOB4530) and its flanking region (at 565,247–567,161 nt on the genome)
88 [12-16]. Initially, we performed assembly using the In-Fusion® system, but the
89 resulting target fragments could not be successfully amplified via PCR, possibly due to
90 the absence of DNA polymerase and ligase for the gap-filling activity of the In-Fusion®
91 system that prevents mutagenesis during filling [17,18]. Subsequently, we used
92 NEBuilder® with an in-built gap-filling activity [19]. Using NEBuilder®, we
93 successfully amplified the DNA fragments (Fig. 4A). Upon transforming syn3B with
94 100 ng of the PCR product, 392 ± 124 colonies (n=6) were obtained. However, the
95 efficiency was approximately 1/20 of that achieved with plasmid pSD128 of *E. coli*
96 (Fig. 5(i)). We investigated the cause for this low efficiency by performing
97 transformation using circularized DNA.

98 **Transformation using assembled, amplified, and circularized DNA**

99 PCR products were circularized by NEBuilder® (Fig. 3(ii)). The amount of DNA
100 converted to a form other than the original linear form was estimated from the agarose
101 gel electrophoresis band intensity (Fig. 4B), which indicated a 94.6% conversion. The
102 DNA mixture was used for transformation (Fig. 5(ii)). Notably, transformation
103 efficiency was similar to that achieved with the *E. coli* plasmid. These results suggest
104 that circularized DNA achieves a transformation efficiency similar to that achieved with

105 the supercoiled plasmids of *E. coli*. Next, we examined the products assembled without
106 PCR amplification.

107 **Transformation using assembled circular DNA**

108 The product assembled using NEBuilder®, which was expected to be circular, was
109 directly integrated into the syn3B genome (Fig. 3(iii)). The DNA product assembled
110 using 30 ng vector and 28 ng target DNA was transferred into syn3B, resulting in 2,067
111 ± 262 colonies from 425 μL cultures (n=6; Fig. 5(iii)). This shows the advantage of
112 circular DNA in the uptake process. Next, we assembled the product using In-Fusion®
113 (Fig. 3(iv)) and obtained 1,282 ± 418 colonies from 425 μL cultures (n=6; Fig. 5(iv)),
114 indicating plasmid instability caused by the gap region (P = 0.005 via Student's t-test).
115 Here, the transformation efficiency could not be directly compared with that of the
116 conventional method as the precise amount of assembled DNA was unknown. However,
117 sufficient number of colonies were obtained for gene transfer using the NEBuilder® and
118 In-Fusion® products.

119 **Unexpected recombination in the transformants**

120 We examined the transfer of the target DNA into *loxP* via direct PCR using primers
121 P7 and P8 (Fig. 1) for 18 transformants, and the process was repeated six times (Fig. 6).
122 For the colonies obtained using the conventional method, we observed PCR bands at the
123 correct positions. However, compared to that of the conventional method, success rates
124 (74, 75, 93, and 92%) were low for the linear (Fig. 3(i)), circular (Fig. 3(ii)),
125 NEBuilder®-assembled (Fig. 3(iii)), and In-Fusion®-assembled (Fig. 3(iv)) DNAs,
126 respectively. This decrease in success rate was due to unexpected amplification during
127 PCR. Additionally, transformation efficiency of our direct transformation method was
128 lower than that of conventional transformation using *E. coli* plasmids. This was
129 expected, as the isolated plasmids were cloned into *E. coli* colonies.

130 To date, we have constructed 58 syn3B constructs with AT content of 65–76%.
131 Moreover, using the established method, we successfully transferred a 14.5-kb fragment
132 consisting of eight genes of *M. mobile* into the syn3B genome. We also transferred the
133 genes of *Haloplasma contractile* into syn3B using the new method [20,21], which is
134 impossible with *E. coli*-based gene manipulation systems. Approximately 58 kb of the
135 transferred DNA region was sequenced from 18 transformants. Two clones each with

single-nucleotide deletions and substitutions were observed. These mutations were possibly caused by PCR, as similar mutations have been reported in *E. coli*-based recombination PCR products.

Conclusion

Overall, we developed a direct rapid method for gene transfer to the JCVI-syn3B strain, particularly useful for DNA sequences unstable or harmful to *E. coli*.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

AU performed all of the experiments. AU and MM wrote an early version of the manuscript. All authors contributed to the project and manuscript.

Data Availability Statement

All data generated/analyzed in this study are included in this article.

Acknowledgements

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Figure Legends

Fig. 1 Schematic of DNA transfer into the JCVI-syn3B (syn3B) genome. Plasmid pSD128 used for gene transfer contained a Cre/*loxP*-related region and puromycin resistance gene. Target DNA was transferred into the syn3B genome via the Cre recombinase activity. Blue arrows indicate the primers used for direct polymerase chain reaction (PCR).

Fig. 2 Comparison of the conventional method with the new established method. The new method was four days shorter than the conventional method.

168

169 **Fig. 3** DNA preparation for transformation. (i) Linear DNA, including the target DNA,
170 Cre/*loxP* system, and puromycin resistance gene. (ii) Circular DNA prepared using
171 NEBuilder®. (iii) DNA assembled from two fragments using NEBuilder®. (iv) DNA
172 assembled from two fragments using In-Fusion®. Four types of DNA were used for
173 syn3B transformation.

174

175 **Fig. 4** DNA fragments used for transformation. Size markers are shown in the left lanes.
176 (A) Three PCR products used in this study. (B) DNA fragments used for transformation.
177 (i) Linear and (ii) circular DNA fragments, as indicated in Fig. 3, are shown.

178

179 **Fig. 5** Transformation efficiencies with various DNA fragments. Amount of circular
180 DNA was estimated based on the electrophoresis band intensity. DNA amounts
181 obtained with (iii) NEBuilder® and (iv) In-Fusion® accounted for the total DNA
182 amount used for assembly.

183

184 **Fig. 6** Direct PCR analysis of the transformants. PCR was performed on 18 colonies
185 obtained using each DNA plasmid.

186

187 **Table 1. Primers used in this study**

Name	Sequence
P1	5'-GTCAAAGATCATACCGTTCG-3'
P2	5'-AGCTAGCTCAGTCCTTATTT-3'
P3	5'-AAATAAGGACTGAGCTAGCTTTGAATCGAACTGAATATGC-3'
P4	5'-CGAACGGTATGATCTTTGACTACTCATAATATGCTACCTC-3'
P5	5'-GGTTGGACTCAAGACGATAG-3'
P6	5'-GTCTTGAGTCCAACCGGCCGGCCCGGAGGCGTCCC-3'
P7	5'-GTAAATTCCCAAATTATTCCATCA-3'
P8	5'-TATGTGATAATGCCAATCGCTAAG-3'

188

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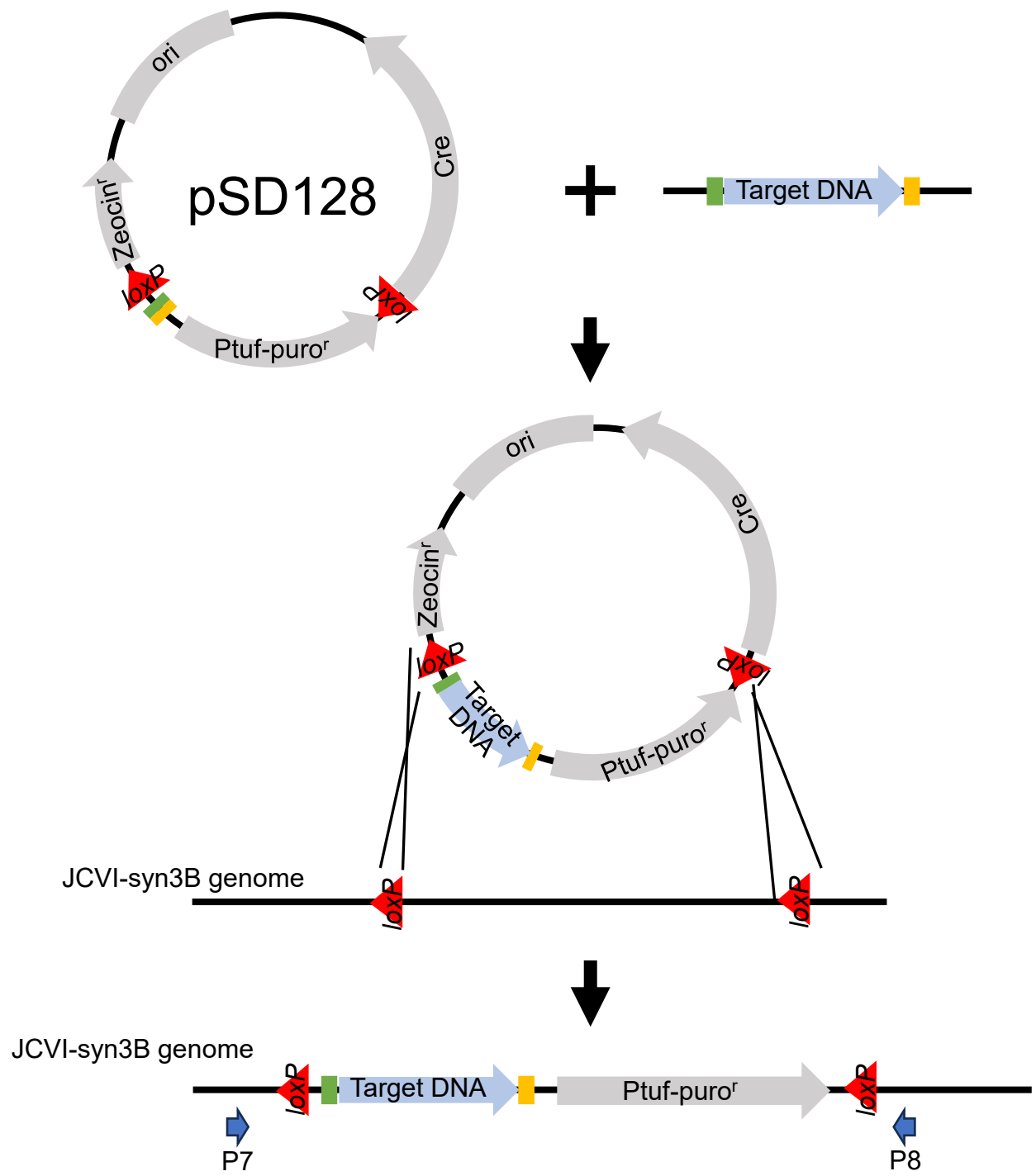
Fig. 1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Ptuf-puro^r
JCVI-syn3B genome
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loxP
JCVI-syn3B genome
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Target DNA
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loxP
P7
P8

Fig. 2

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・Transformation into E. coli</td><td>1</td><td rowspan="4">・Assembly of vector and target DNA
・Transfer into JCVI-syn3B cell</td></tr>
<tr><td>・Colony pick up and cultivation</td><td>2</td></tr>
<tr><td rowspan="3">・Plasmid isolation
・Sequencing</td><td>3</td></tr>
<tr><td>4</td></tr>
<tr><td>5</td><td rowspan="4">・Colony pick up and directPCR
・Sequencing</td></tr>
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<tr><td>7</td></tr>
<tr><td>8</td></tr>
<tr><td>9</td><td rowspan="4"></td></tr>
<tr><td rowspan="3">・Colony pick up and directPCR</td><td>10</td></tr>
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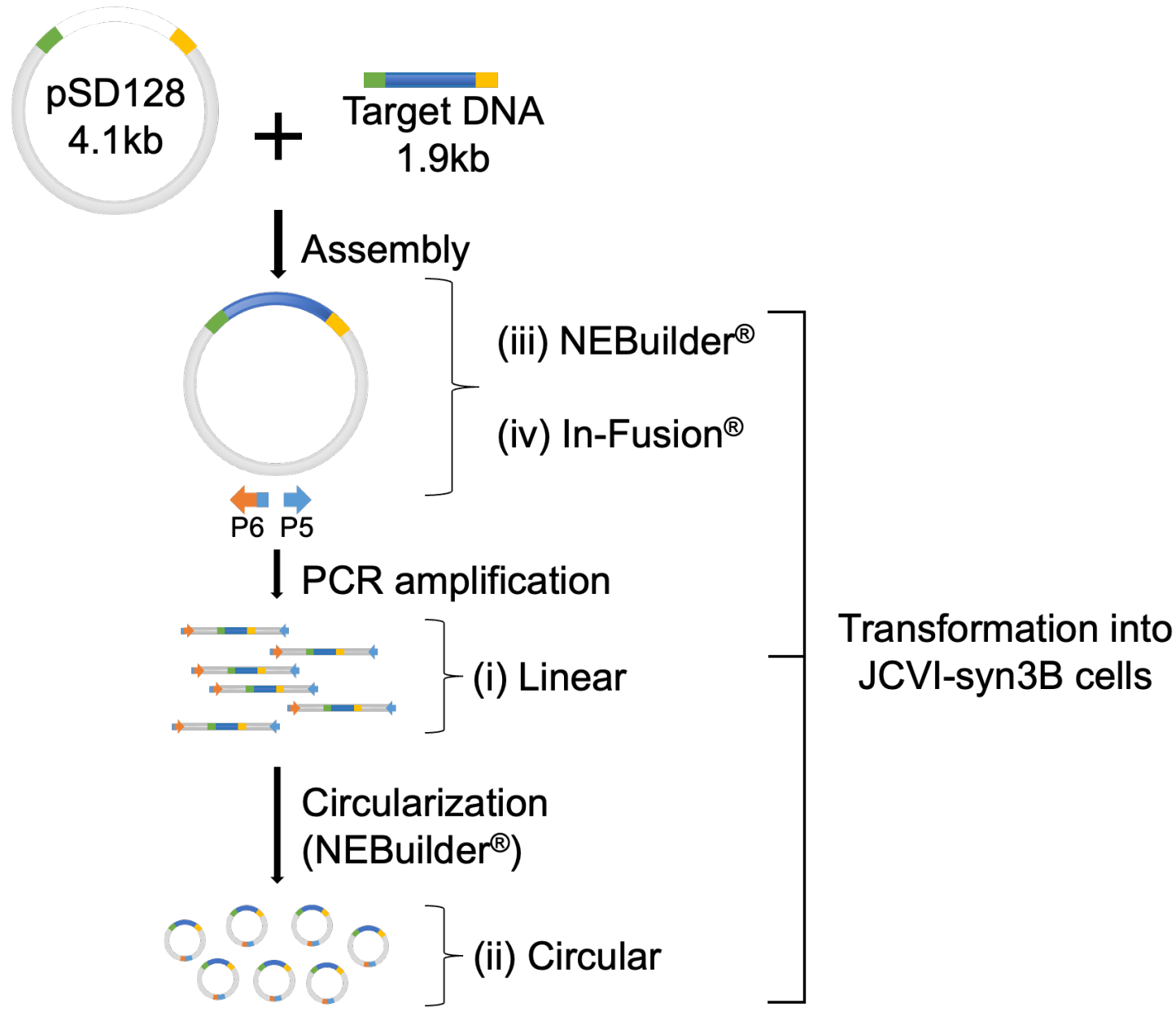
Fig. 3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Target DNA
1.9kb
Assembly
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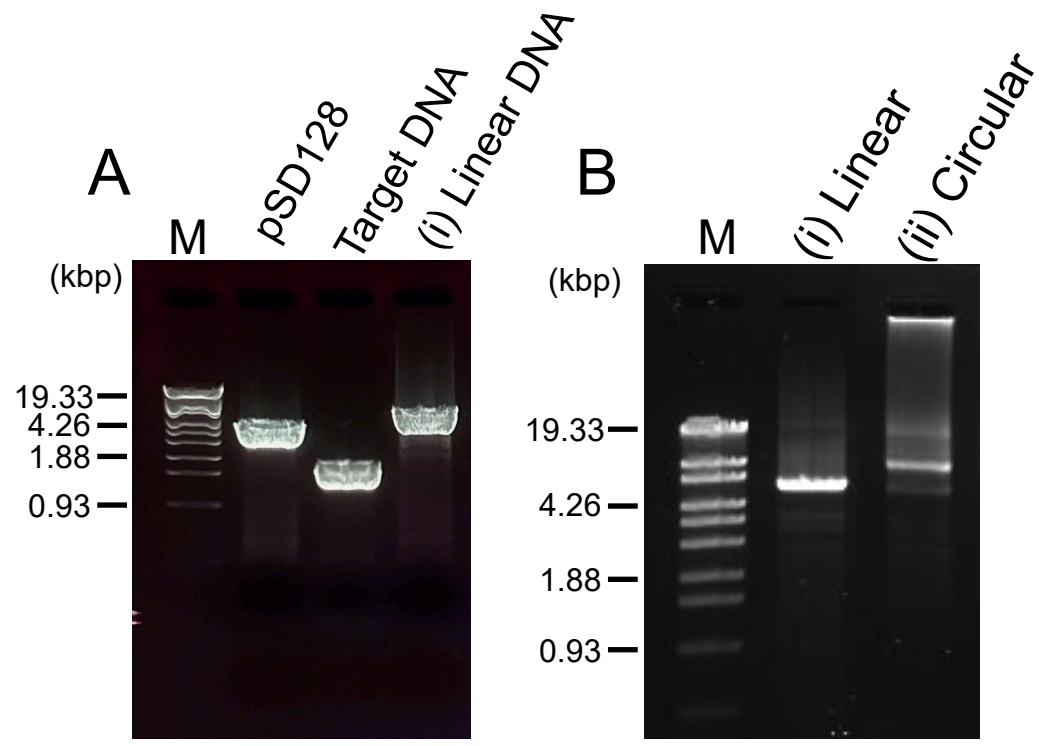
(iv) In-Fusion®
P6
P5
PCR amplification
(i) Linear
Circularization
(NEBuilder®)
(ii) Circular
Transformation into
JCVI-syn3B cells

Fig. 4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4.26
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(kbp)
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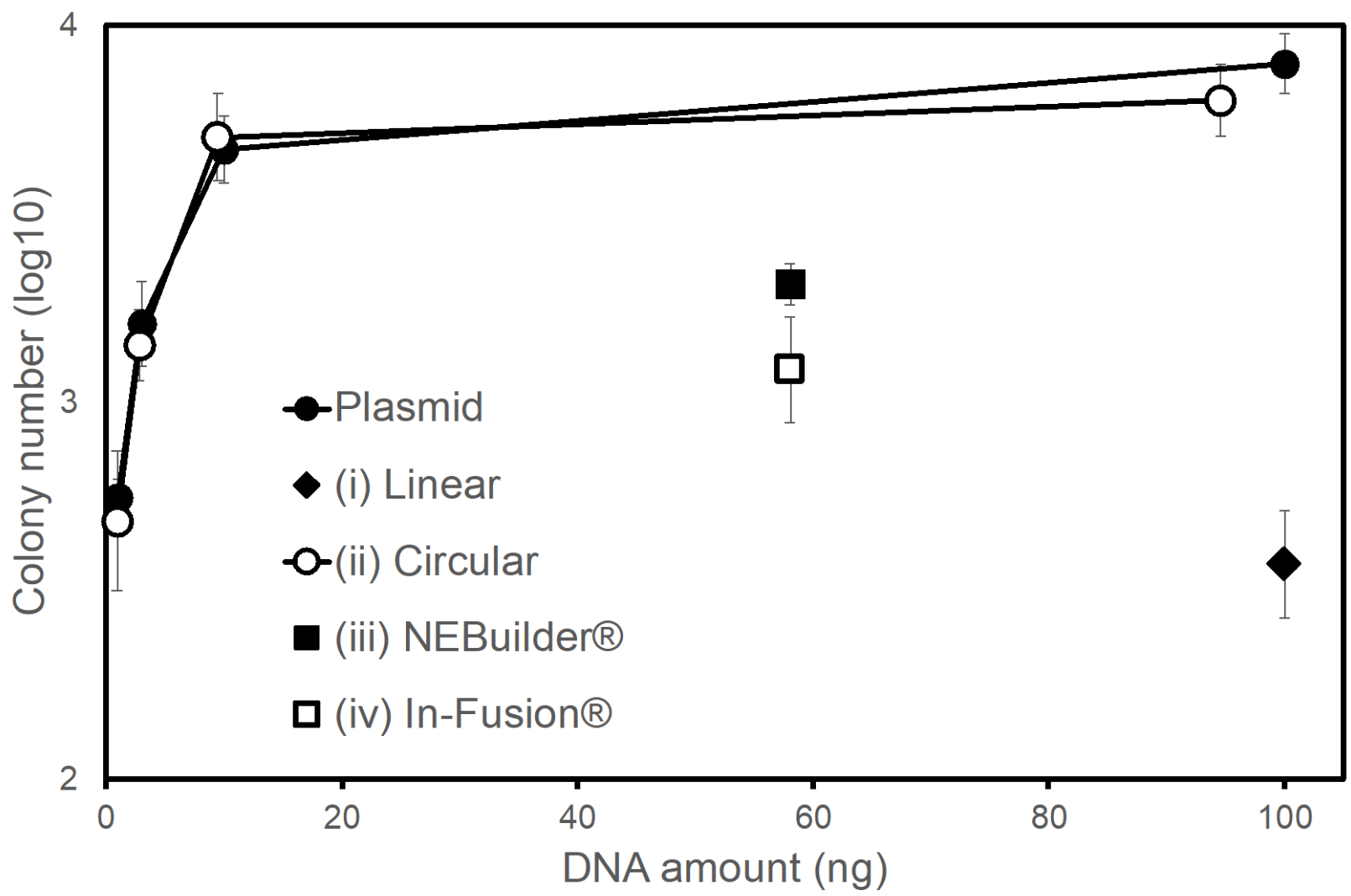
4.26
1.88
0.93

Fig. 5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3
2
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20
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DNA amount (ng)
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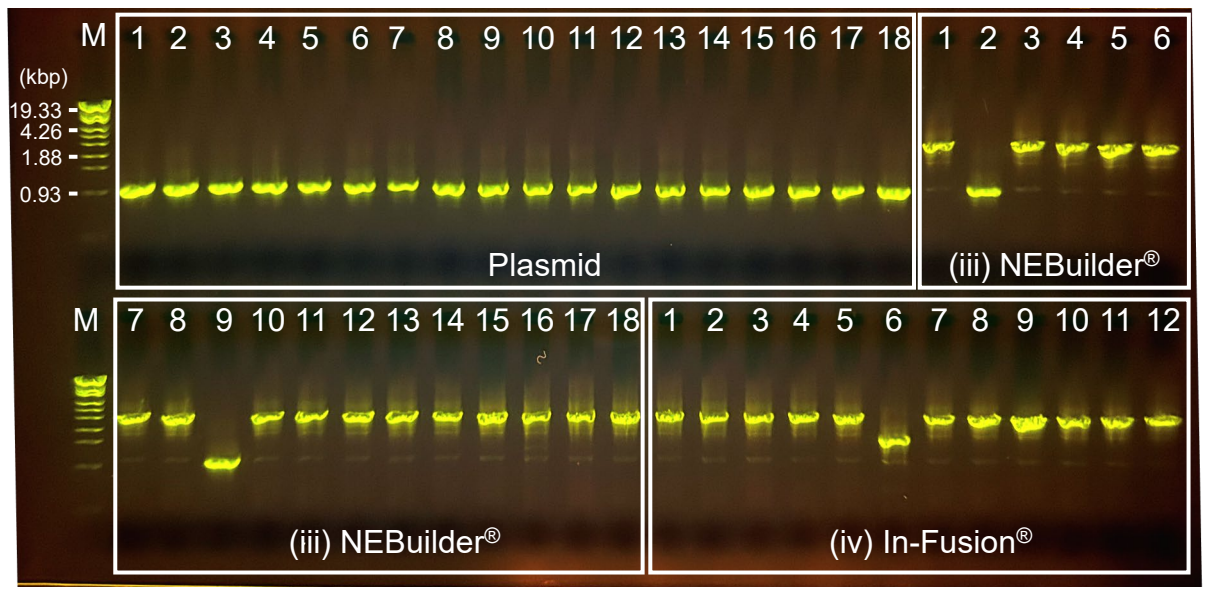
(iv) In-Fusion®

Fig. 6


M
(kbp)
19.33
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Plasmid
1 2 3 4 5 6
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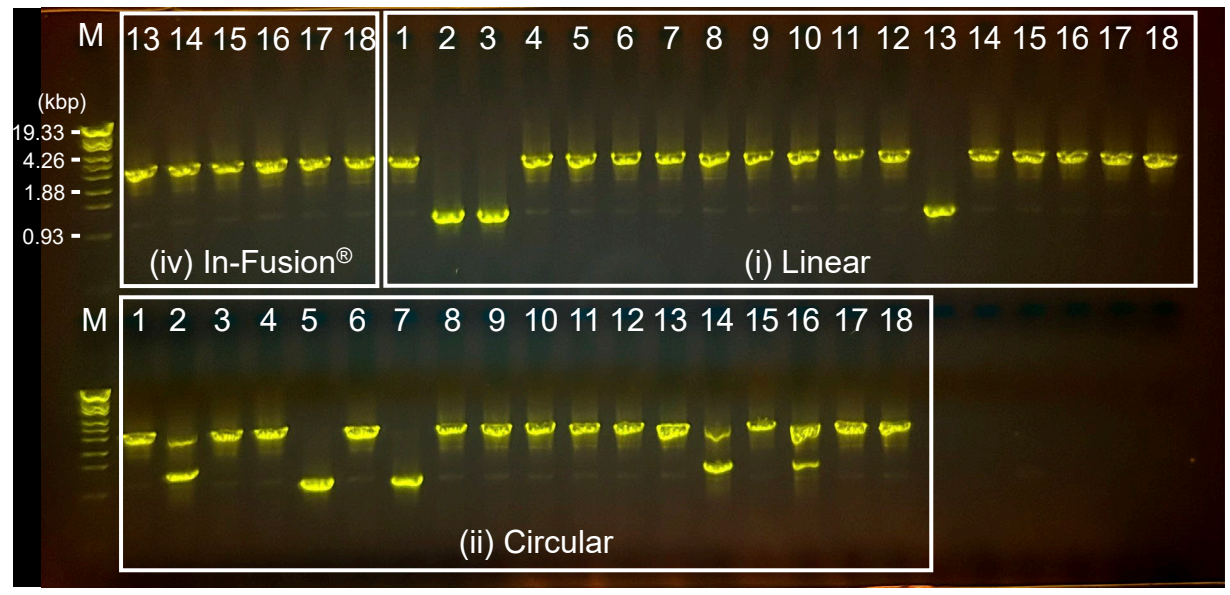
(iv) In-Fusion®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19.33
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(iv) In-Fusion®
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
(i) Linear
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
(ii) Circular