AEM Accepts, published online ahead of print on 18 May 2012
Appl. Environ. Microbiol. doi:10.1128/AEM.00844-12
Copyright © 2012, American Society for Microbiology. All Rights Reserved.

AEM Accepts published online ahead of print

1 # One-step sequence- and ligation-independent cloning (SLIC): Rapid and versatile cloning method for functional genomics studies
2
3

4

5 Jae-Yeon Jeong[a], Hyung-Soon Yim[a], Ji-Young Ryu[a], Hyun Sook Lee[a], Jung-Hyun Lee[a],
6 Dong-Seung Seen[b], Sung Gyun Kang[a,*]

7

8 [a]Marine Biotechnology Research Center, Korea Ocean Research & Development Institute, PO Box 29,
9 Ansan 425-600, Korea
10 [b]Macrogen Inc., World Meridian Venture Center, Geumchun-Gu, Seoul 153-781, Korea

11

12

13

14 [*]Address correspondence to Sung Gyun Kang, Marine Biotechnology Research Center, Korea
15 Ocean Research & Development Institute, PO Box 29, Ansan 425-600, Korea. Email:
16 sgkang@kordi.re.kr

17

18

19 Keywords: recombinant DNA, ligation-independent cloning, T4 DNA polymerase, single-
20 strand annealing, homologous recombination, multiple fragment assembly

21

22

23 Running title: One-step sequence- and ligation-independent cloning

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

24 ## Abstract

25

26 We developed one-step sequence- and ligation-independent cloning (SLIC) as a simple, cost-
27 effective, time-saving, and versatile cloning method. Highly efficient and directional cloning
28 can be achieved by direct bacterial transformation 2.5 min after mixing any linearized vector,
29 insert(s) prepared by PCR, and T4 DNA polymerase in a tube at room temperature.

30

31

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

32 The need for high-throughput recombinant DNA technology is demanding due to the
33 rapidly increased interest in functional genomics studies following the surge of data
34 generated by next generation sequencing. Construction of library or converting existing
35 library into different context requires high-throughput gene cloning method, but conventional
36 methods suffer from high cost, prolonged manipulation, or sequence restriction.

37 Ligation-independent cloning (LIG) is based on the 3' to 5' exonuclease activity of T4
38 DNA polymerase and has been used for two decades as a high-throughput method due to its
39 uniformity and cost-effectiveness, but requires a specifically designed vector containing a
40 long stretch of sequence that is lacking a particular dNTP (1, 3-5, 8, 11). Sequence- and
41 ligation-independent cloning (SLIC) overcomes sequence restraint of LIG and allows the
42 assembly of multiple overlapping fragments simultaneously, but the cloning efficiency of
43 SLIC in the absence of RecA is rather low (9). Various recombinase-based cloning methods
44 including Gateway cloning (Invitrogen), Cre-lox recombination, Red/ET recombination
45 (Gene Bridges), In-Fusion (Clontech), Cold Fusion (System Biosciences), and CloneEZ
46 (GenScript) have been developed, but general use of these methods has been hampered by
47 high cost and restrictions in the sequence or hosts (2, 12-15)

48 We optimized SLIC to make it comparable to commercial ones in terms of simplicity,
49 time-saving, and cloning efficiency. One-step SLIC utilizes only T4 DNA polymerase but
50 shows similar cloning efficiencies to those of the original SLIC in the presence of RecA and
51 commercial ones. The overview of one-step SLIC is illustrated in Fig. 1A. First, vector needs
52 to be linearized either by restriction enzyme digestion or inverse PCR. Insert(s) are prepared
53 by PCR with primers with 15 bp or more extension homologous to each end of the linearized
54 vector. Second, vector and insert(s) are mixed and incubated at room temperature for 2.5 min
55 with T4 DNA polymerase to generate 5' overhangs. For optimal results, 1:2 to 1:4 molar ratio
56 of vector to insert is desirable. Third, the reaction mixture is placed on ice for 10 min for

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

57 single strand annealing, and then, competent *E.coli* cells were transformed with the annealed
58 DNA complex directly. The annealed complex turns into seamless recombinant DNA through
59 homologous recombination *in vivo* with high efficiency. A detail method is provided as a
60 Supplementary Protocol.

61 pUC118-HMG (10) was cleaved with *Bam*HI and 1 kb insert containing Ton_0709 was
62 amplified by PCR from the genomic DNA of *Thermococcus onnurineus* NA1 (7) with
63 forward (blue arrow) and reverse (red arrow) primer that has 22 bp homology to the *Bam*HI-
64 cleaved vector end (Fig. 1B; Supplementary Table S1). Both the linearized vector and PCR
65 product were purified by commercial PCR purification kit and eluted in 10 mM TrisCl, pH
66 8.5. One hundred ng of the vector and 40 ng of the insert were mixed with Buffer 2 plus BSA
67 (NEB) in a 10 μl reaction and 0.6 U of T4 DNA polymerase (NEB) was treated at room
68 temperature for 2.5 min. The mixture was put on ice immediately to stop the reaction. After
69 10 min of annealing on ice, 1 μl of the reaction was added directly to home-prepared
70 competent cells of *E.coli* strain DH5α (100 μl) or TOP10 (50 μl, Invitrogen). *E.coli* cells
71 were incubated on ice for 20 min, heat shocked at 42°C for 45 sec, returned to ice for 2 min,
72 added with 0.9 or 0.95 ml of LB, and recovered at 37°C for 1 hr. Ten to 100 μl of the cells
73 were plated on 100 μg/ml ampicillin plate, and incubated at 37°C for 16 hr. The gaps
74 generated by excessive exonuclease activity are repaired and annealed strands are joined
75 efficiently by homologous recombination *in vivo* (6, 8, 9). The recombination efficiency was
76 100% when 22 colonies were randomly picked and analyzed by *Eco*RI digestion (Fig. 1C and
77 D). Incubation on ice for more than 10 min or storage at -20°C did not alter transformation
78 efficiency, but shorter incubation on ice did result in reduced efficiency (data not shown).

79 In original SLIC, vector and insert were treated with T4 DNA at room temperature for 30
80 min to 1 hr in separate tubes, added with dCTP to stop the reaction, and annealed at 37°C for
81 30 min (9). To optimize one-step SLIC, incubation time of the vector and insert with T4 DNA

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

82 polymerase was varied from 0 to 60 min at room temperature. We found 2.5 min is sufficient
83 to generate 5' overhangs for single strand annealing and incubation for more than 5 min
84 severely impairs the cloning efficiency (Fig. 2A). When T4 DNA polymerase was treated
85 with various concentrations of the vector with a vector to insert molar ratio of 1:2, as low as
86 0.5 ng/μl vector gave sufficient number of colonies (Fig. 2B). To evaluate the effect of vector
87 to insert molar ratio, the vector (100 ng) was mixed with appropriate amounts of the insert
88 and treated with T4 DNA polymerase for 2.5 min. The cloning efficiency was the highest
89 when vector to insert molar ratio was 1:4 (Fig. 2C). One-step SLIC was efficient when 0.3 U
90 or more T4 DNA polymerase was used to treat 100 ng of the vector in a vector to insert molar
91 ratio of 1:2 (Fig. 2D).

92 To determine the minimal length of homology for one-step SLIC, primers with different
93 homology length were used for PCR. Cloning efficiency was proportional to the length of
94 homology and primers with 10 bp or longer homology gave sufficient number of colonies
95 (more than 5×10^5 cfu/μg vector) when the homologous region matches the end of the
96 linearized vector perfectly (Fig. 3). In many cases, the endonuclease site of the insertion point
97 in the vector needs to be deleted or switched to a different restriction site. LIC was shown to
98 be tolerant to these changes (8). When 4 bp of *Bam*HI sequence (Fig. 3B, underlined) were
99 deleted in the insert, the minimal length of homology required for one-step SLIC increases to
100 15 bp (Fig 3A).

101 To test if multiple fragments can be cloned simultaneously using one-step SLIC, 1 kb
102 fragments flanking Ton_1323 were amplified by PCR from *T. onnurineus* genomic DNA with
103 primers that have a 21 ~25 bp homology at their ends. pUC118-del-HMG, a vector designed
104 to construct markerless deletion mutants of *T. onnurineus*, was digested with *Sma*I and mixed
105 with the left and right arm of Ton_1323 in various vector to inserts molar ratios at vector
106 concentration of 10 ng/μl (Fig. 4A-C). T4 DNA polymerase was treated for 2.5 min and

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

107 competent TOP10 cells were transformed with the DNA. Vector to inserts molar ratio of 1:2:2
108 gave the highest number of recombinant clones (Fig. 4B). When 8 colonies from each group
109 were randomly picked and analyzed by restriction digestion, 87.5% resulted in positive
110 recombinant clones (Fig. 4C). To further check if four fragment assembly is possible with this
111 technology, pUC118-HMG vector was digested with *Bam*HI and *Pst*I to produce a 3.1 kb
112 backbone and 1.8 kb HMG cassette. Digested fragments were co-purified by a commercial
113 PCR purification kit and mixed with left and right flanking region of Ton_0707 in 1:1:1:1 of
114 backbone : HMG cassette : left arm : right arm molar ratio (Fig. 4D). Restriction digestion
115 analysis of the colonies resulted in 95.8% correctly recombined clones (Fig. 4E and F),
116 clearly demonstrating one-step SLIC can be also used as a multiple fragment assembly
117 technology. It is worth mentioning that HMG cassette shown in Fig. 4D is not produced by
118 PCR, implying that multiple fragment assembly can be used widely to construct a composite
119 vector with multiple fusion tags or regulatory elements, or to clone large cDNAs or genes in a
120 single cloning.

121 We routinely use this technology to produce gene knockout constructs for *T. onnurineus*
122 as a single or multiple fragment assembly. Sequencing analyses revealed 100% correctly
123 recombined clones at the junctions of the recombinant DNA even when homologous regions
124 at the ends of insert did not match exactly with the ends of linearized vector as shown in Fig.
125 3. One-step SLIC is highly versatile since you can insert your gene of interest in any vector at
126 any desired position without additional sequences or suitable restriction site. By modulating
127 the junction between the homologous and gene specific region of PCR primers for insert, you
128 can delete or add any additional sequences such as restriction enzyme site or tag sequence (8).
129 In case there is no restriction enzyme site available, vector can be linearized by reverse PCR
130 with subsequent *Dpn*I treatment. Same as LIC, one-step SLIC also offer a uniform
131 conversion of genes into one context to another regardless of sequence variations of genes of

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

132 interest since there is no need to digest inserts with restriction endonucleases, providing
133 robust basis for high-throughput gene cloning. Together with cost- and time-saving properties,
134 one-step SLIC makes high-throughput gene cloning practical for functional genomics studies.
135 Although we only tried up to four fragment assembly, we believe more than four fragment
136 assembly can be possible with one-step SLIC. It would also greatly facilitate synthetic
137 biology as well as cloning long DNA with multiple restriction enzyme sites.
138

139

140 Acknowledgements

141 This work was supported by KORDI in-house program (PE98802), the Marine and Extreme
142 Genome Research Centre and the Development of Biohydrogen Production Technology using
143 the Hyperthermophilic Archaea program of the Ministry of Land, Transport, and Maritime
144 Affairs, Korea.
145

146 Competing Interests

147 The authors declare no competing interests.
148

149

150

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

151 # References

152

153 1. **Aslanidis C, de Jong PJ.** 1990. Ligation-independent cloning of PCR products (LIC-
154 PCR). Nucleic Acids Res **18:**6069-6074.
155 2. **Cheo DL, Titus SA, Byrd DR, Hartley JL, Temple GF, Brasch MA.** 2004. Concerted
156 assembly and cloning of multiple DNA segments using in vitro site-specific
157 recombination: functional analysis of multi-segment expression clones. Genome Res
158 **14:**2111-2120.
159 3. **de Jong RN, Daniels MA, Kaptein R, Folkers GE.** 2006. Enzyme free cloning for high
160 throughput gene cloning and expression. J Struct Funct Genomics **7:**109-118.
161 4. **Hamilton MD, Nuara AA, Gammon DB, Buller RM, Evans DH.** 2007. Duplex strand
162 joining reactions catalyzed by vaccinia virus DNA polymerase. Nucleic Acids Res
163 **35:**143-151.
164 5. **Kuijper JL, Wiren KM, Mathies LD, Gray CL, Hagen FS.** 1992. Functional cloning
165 vectors for use in directional cDNA cloning using cohesive ends produced with T4 DNA
166 polymerase. Gene **112:**147-155.
167 6. **Kuzminov A.** 1999. Recombinational repair of DNA damage in Escherichia coli and
168 bacteriophage lambda. Microbiol Mol Biol Rev **63:**751-813, table of contents.
169 7. **Lee HS, Kang SG, Bae SS, Lim JK, Cho Y, Kim YJ, Jeon JH, Cha SS, Kwon KK,
170 Kim HT, Park CJ, Lee HW, Kim SI, Chun J, Colwell RR, Kim SJ, Lee JH.** 2008.
171 The complete genome sequence of Thermococcus onnurineus NA1 reveals a mixed
172 heterotrophic and carboxydotrophic metabolism. J Bacteriol **190:**7491-7499.
173 8. **Li C, Evans RM.** 1997. Ligation independent cloning irrespective of restriction site
174 compatibility. Nucleic Acids Res **25:**4165-4166.
175 9. **Li MZ, Elledge SJ.** 2007. Harnessing homologous recombination in vitro to generate
176 recombinant DNA via SLIC. Nat Methods **4:**251-256.
177 10. **Matsumi R, Manabe K, Fukui T, Atomi H, Imanaka T.** 2007. Disruption of a sugar
178 transporter gene cluster in a hyperthermophilic archaeon using a host-marker system
179 based on antibiotic resistance. J Bacteriol **189:**2683-2691.
180 11. **Travis H, Hileman TH, Santangelo TJ.** 2012. Genetic techniques for *Thermococcus
181 kodakarensis*. . Front Microbiol **In Press**.
182 12. **Walhout AJ, Temple GF, Brasch MA, Hartley JL, Lorson MA, van den Heuvel S,**

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

183 **Vidal M.** 2000. GATEWAY recombinational cloning: application to the cloning of large
184 numbers of open reading frames or ORFeomes. Methods Enzymol **328:**575-592.

185 13. **Xu R, Li QQ.** 2008. Protocol: Streamline cloning of genes into binary vectors in
186 Agrobacterium via the Gateway(R) TOPO vector system. Plant Methods **4:**4.

187 14. **Zhang Y, Buchholz F, Muyrers JP, Stewart AF.** 1998. A new logic for DNA
188 engineering using recombination in Escherichia coli. Nat Genet **20:**123-128.

189 15. **Zhu B, Cai G, Hall EO, Freeman GJ.** 2007. In-fusion assembly: seamless engineering
190 of multidomain fusion proteins, modular vectors, and mutations. Biotechniques **43:**354-
191 359.

192

193

194

195

196

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

197 ## Figure Legends

198

199 **FIG. 1.** Overview of one-step SLIC. (A) Schematic diagrams of one-step SLIC. (B) Partial
200 sequences of the vector and insert. Underlying arrows indicate forward and reverse primer
201 used to amplify the insert. Homologous regions were marked in color. *Bam*HI site is in bold.
202 (C) Restriction map of the vector and insert. (D) Analysis of the recombinants. Plasmid
203 DNAs purified from twenty-two independent colonies (marked as 1-22) derived from Fig. 2A
204 (from 2.5 min treated sample) were digested with *Eco*RI and analyzed on an agarose gel.
205 *Eco*RI-digested vector as a control (V) yields 4.8 and 0.1 kb fragments while that of correctly
206 recombined clone yields 4.25, 1.6, and 0.1 kb fragments.

207

208 **FIG. 2.** Optimization of one-step SLIC. (A) Effect of the duration of T4 DNA polymerase
209 treatment on one-step SLIC. One hundred ng of *Bam*HI-digested pUC118-HMG (4.9 kb) was
210 mixed with insert (Ton_0709, 1 kb, 22 bp homology) in 1:2 vector to insert molar ratio in 10
211 μl reaction. The mixture was incubated with T4 DNA polymerase (0.6 U) at 22°C for the
212 indicated time. Then, the reaction was incubated on ice for 10 min, and competent TOP10
213 cells were transformed with the annealed DNA complex. (B) Effect of the vector DNA
214 concentrations on one-step SLIC. Various concentrations of the vector were mixed with the
215 insert in 1:2 vector to insert molar ratio. T4 DNA polymerase (0.6 U) was treated at 26°C for
216 2.5 min. After 10 min on ice, competent DH5α cells were transformed with the annealed
217 DNA complex. (C) Effect of the vector to insert molar ratio on one-step SLIC. (D) Effect of
218 the amount of T4 DNA polymerase on one-step SLIC. Each bar represents the mean ± SD of
219 triplicates.

220

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

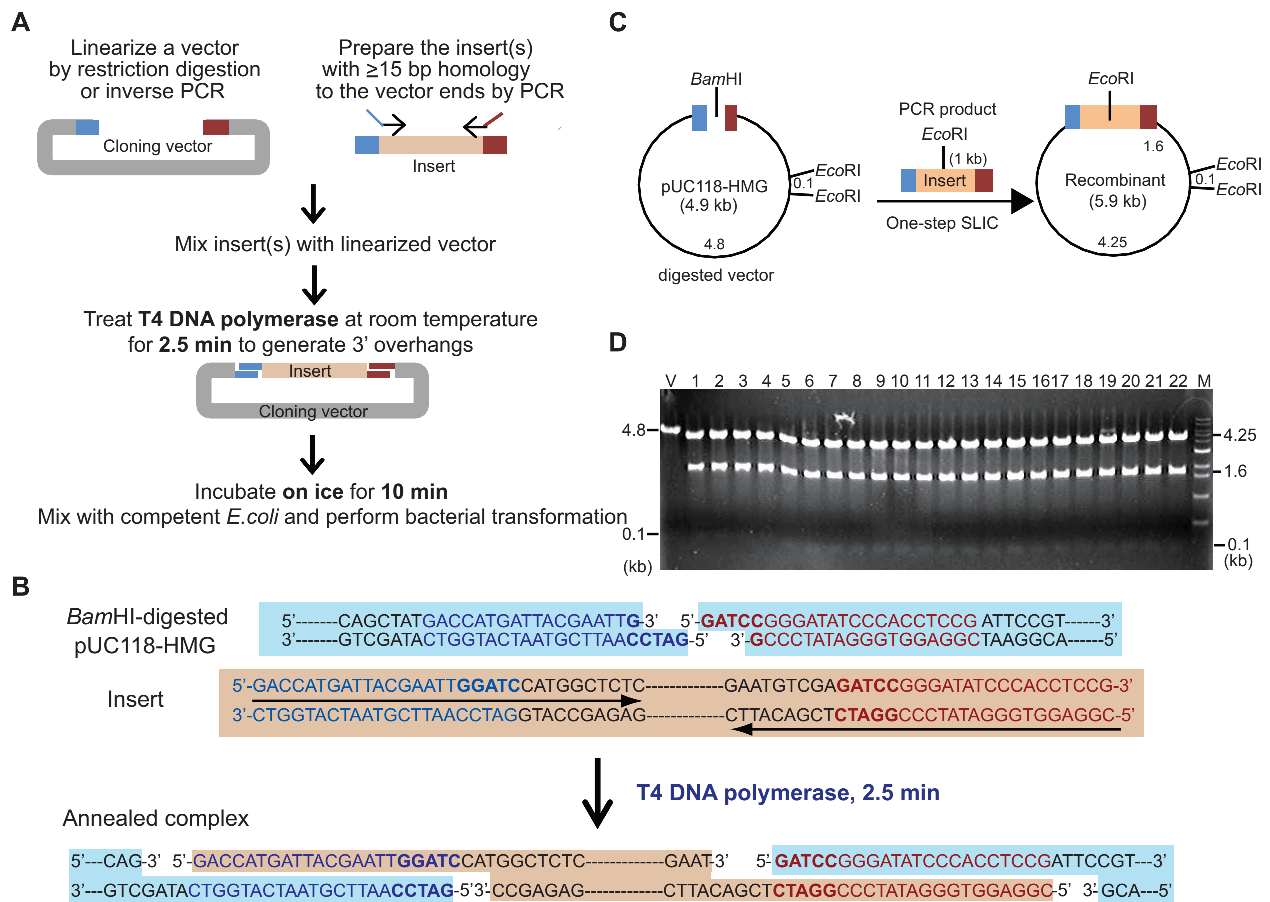
AEM Accepts published online ahead of print

221 **FIG. 3.** Comparison of the cloning efficiencies between inserts with perfect match and those
222 with 4 bp deletion. (A) Insert (Ton_0709, 1 kb) was amplified by PCR with primers with
223 various lengths of homology. *Bam*HI-digested pUC118-HMG was mixed with the insert in a
224 1:2 molar ratio at 10 ng vector/μl, treated with T4 DNA polymerase (0.6 U) for 2.5 min, and
225 competent TOP10 cells were transformed with the DNA. Each bar represents the mean ± SD
226 of triplicates. (B) Partial sequences of the vector and insert. Homology regions are in bold
227 and deleted region is underlined.
228
229 **FIG. 4.** Simultaneous assembly of multiple fragments using one-step SLIC. (A) Schematic
230 diagram of three fragment assembly. (B) Cloning efficiencies of three fragment assembly. (C)
231 Analysis of the recombinants from three fragment assembly with *Nco*I. Aberrant clones are
232 indicated in red. (D) Strategy of four fragment assembly. pUC118 backbone (3.1 kb) and
233 HMG cassette (1.8 kb) were co-purified after digestion of the pUC118-HMG with *Bam*HI
234 and *Pst*I, mixed with the left and right flanking regions of Ton_1323 generated by PCR, and
235 one-step SLIC was performed. (E) Cloning efficiencies of four fragment assembly. (F)
236 Analysis of the recombinants from four fragment assembly with *Hind*III. Aberrant clone was
237 marked in red. LA, left arm; RA, right arm. Each bar represents the mean ± SD of triplicates
238 (B, E).

Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

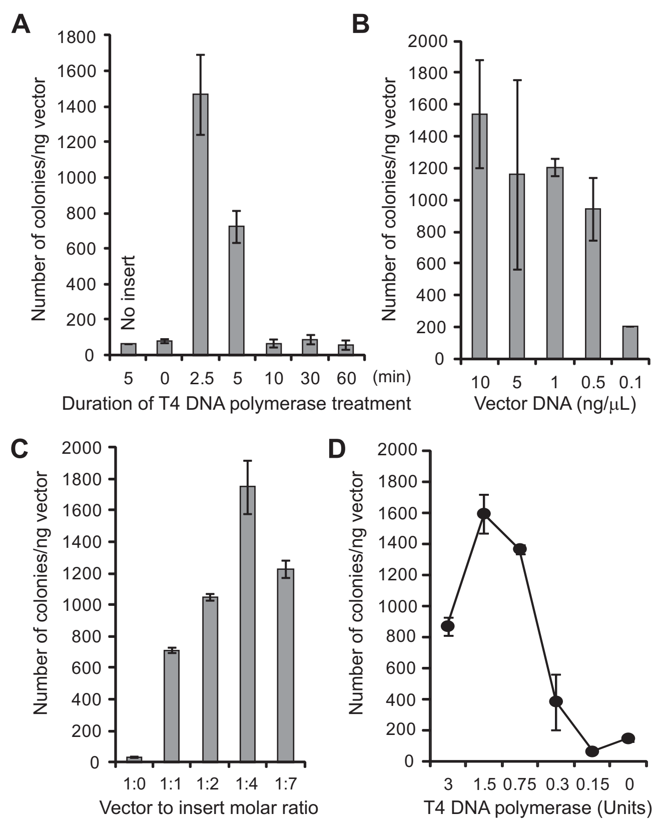
Figure 1



Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

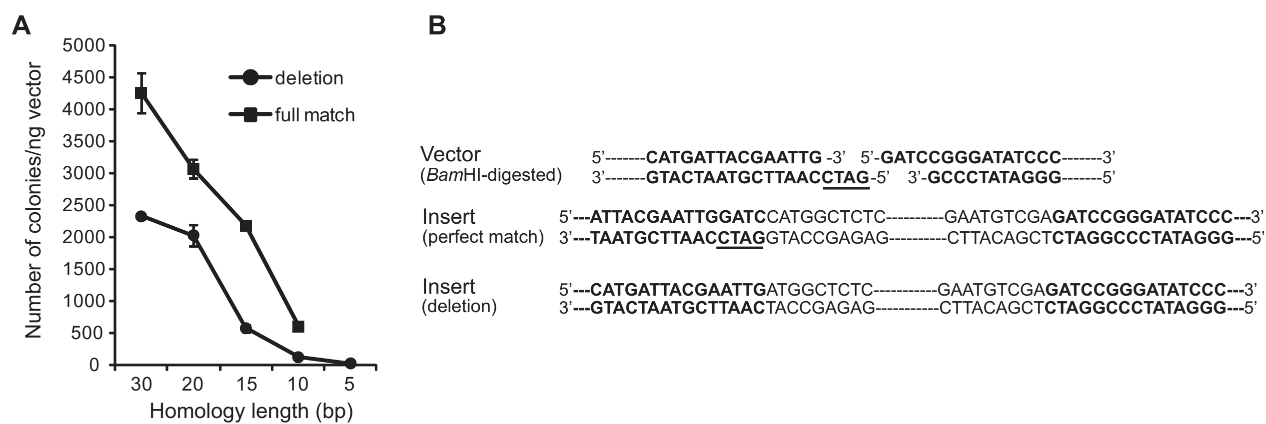
Figure 2



Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

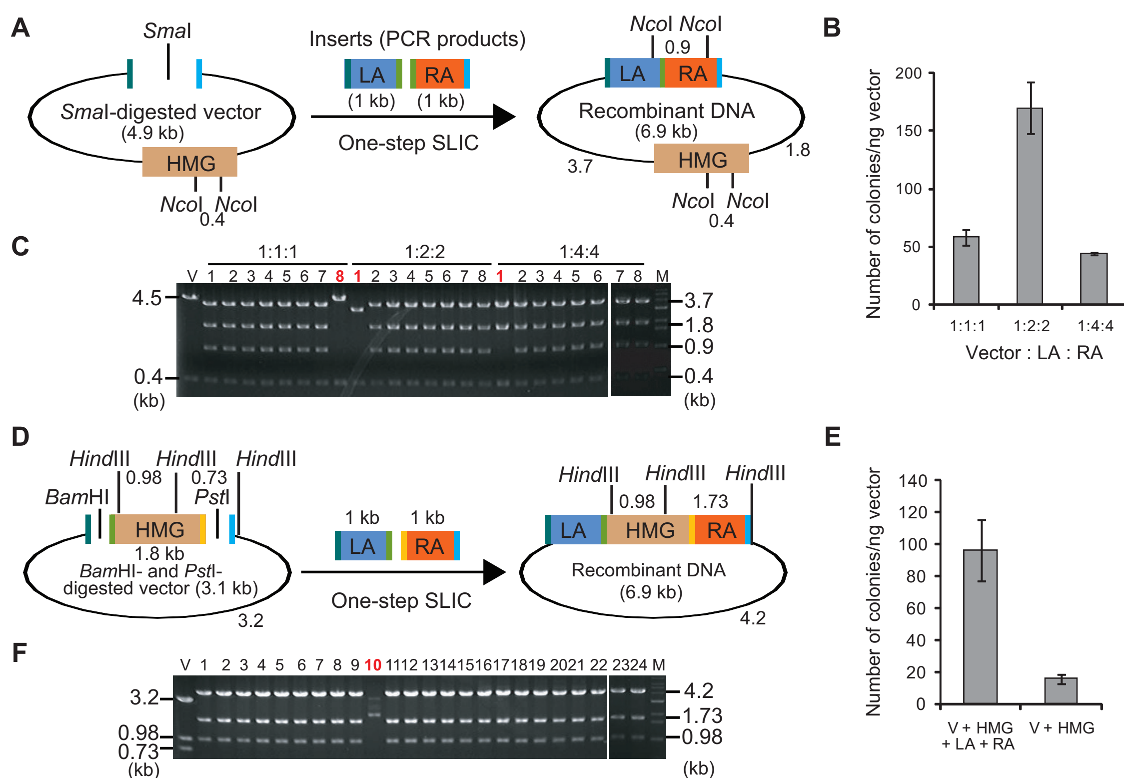
Figure 3



Downloaded from http://aem.asm.org/ on January 24, 2019 by guest

AEM Accepts published online ahead of print

Figure 4



Downloaded from http://aem.asm.org/ on January 24, 2019 by guest