In-Fusion™ assembly: seamless engineering of multidomain fusion proteins, modular vectors, and mutations

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In-Fusion™ can join any two pieces of DNA that have a 15-bp overlap at their ends. The result is equivalent to a recombination event at the ends of the DNAs. The 15-bp overlap may be engineered by inclusion in primers used to PCR amplify a segment of DNA. Originally described for inserting one piece of DNA into a restriction enzyme-digested plasmid, we have found In-Fusion can join four or more pieces of DNA in a single reaction. We used this insight to construct seamless fusion proteins, modular vectors with readily interchangeable segments, and novel mutagenesis strategies. Replacement In-Fusion can be used to delete any desired DNA segment in a plasmid and replace it with any desired new DNA segment without limitations on position or size.

INTRODUCTION

DNA constructs are typically joined by ligation at restriction enzyme sites and construct options are limited by the available unique sites in the vector and gene. In contrast, an In-Fusion™ enzyme reaction can join any two pieces of DNA that have 15 bp of identity at their ends. The 15-bp overlap may be engineered by inclusion in primers used to PCR amplify a segment of DNA. The pieces of DNA may be generated by PCR and have blunt ends or by restriction digest of plasmid DNA and have sticky or blunt ends depending on the enzyme used. The In-Fusion mechanism is ligation-independent and while proprietary, likely uses the unique properties of the 3′–5′ exonuclease activity of poxvirus DNA polymerase (1–3). When incubated with linear duplex DNAs with homologous ends in the presence of Mg^{2+} and low concentrations of dNTP, the 3′–5′ proofreading activity of poxvirus DNA polymerase progressively removes nucleotides from the 3′ end. This exposes complementary regions on substrate DNAs that can then spontaneously anneal through base pairing, resulting in joined molecules containing a hybrid region flanked by nicks, 1–5 nucleotide gaps, or short overhangs (Figure 1A). The annealed structures are metastable because the poxvirus DNA polymerase has a lower affinity for nicked or gapped DNA ends than for duplex ends. Introduction into *Escherichia coli* repairs any single-stranded gaps. Thus, one copy of the overlap is present in the final DNA product, and the result is equivalent to a recombination event at the ends of the DNAs. Originally described for inserting one piece of DNA into a restriction enzyme-digested plasmid, we have found that In-Fusion can join four or more pieces of DNA. We used this insight to develop seamless fusion proteins, modular vectors, and novel mutagenesis strategies.

MATERIALS AND METHODS

Design of a construct. The desired pieces of a DNA construct are assembled in a DNA manipulation program such as Sequencher™ (Gene Codes, Ann Arbor, MI, USA). For example, as shown in Figure 1B, DNA segments encoding the interleukin-2 (IL-2) signal sequence (4,5), the extracellular domain of CD101 minus its endogenous signal sequence (6), and the fragment crystallizable (Fc) domain of murine immunoglobulin G3 (IgG3) (7) are assembled with a mammalian expression vector.

Design of overlap primers. Sense and antisense PCR primers are designed, which contain a 15-bp overlap with the adjacent segment of the construct and 20–30 bp of segment-specific sequence. The junction between two pieces of DNA can be made seamless by including no additional DNA sequence. Alternatively, short pieces of DNA such as restriction sites, translation initiation sites, linkers, or epitope tags can be added by inclusion in the primer sequences. Table 1 and Table 3 give the primers for the constructs in Figure 1B and Figure 3, A and B. Vector segments can be generated by restriction enzyme digest of a plasmid or by PCR. Where a primer is designed to overlap a restriction-digested DNA fragment, the 15-bp overlap is counted from the cleavage site on the antisense DNA strand as described in the Clontech In-Fusion user manual. Most primers are 35–55 bp, and we find quality control by mass spectroscopy to provide sufficient purity (Midland Certified, Midland, TX, USA).

Generation of DNA segments. The DNA segments were PCR amplified from appropriate templates with overlap primers (designed as described in the section entitled Design of overlap primers) and *PfuUltra*® II Fusion Hot Start polymerase (Stratagene, La Jolla, CA, USA), gel-purified, and quantitated. The use of a high-fidelity polymerase reduces errors, but *Taq* polymerase PCR products will also work.

In-Fusion reaction. Twenty-five to one hundred nanograms of restriction enzyme-digested, gel-purified vector were mixed at a molar ratio of 1 vector to 2 of each DNA segment in a total of 10 µL water in one tube of In-Fusion Dry-Down reaction mix (Clontech, Mountain View, CA, USA). The reaction was incubated at 42°C for 30 min, transferred to ice, and 40 µL Tris EDTA (TE) were added. Four microliters were transformed into One Shot® TOP10 competent *E. coli* (1×10^9 cfu/µg; Invitrogen, Carlsbad, CA, USA), miniprepped, and characterized by restriction enzyme digest and sequencing.

RESULTS AND DISCUSSION

DNA constructs often include undesired amino acids encoded by restriction sites engineered to provide a joining point, or seam at which two DNAs can be ligated. This is particularly undesirable for fusion proteins or recombinant antibodies, since the undesired amino acids may perturb structure, reduce expression, or be antigenic. We have found that In-Fusion will join multiple pieces of DNA together, thereby facilitating the design of seamless fusion protein constructs that contain only the desired protein sequence and are not constrained by the presence or absence of restriction enzyme sites. We designed a seamless CD101-IgG3 fusion protein by joining in silico four pieces of DNA encoding a secretory signal, CD101, and IgG3 Fc with an expression vector. No restriction sites are incorporated between the fusion protein domains, and *Nco*I and *Sal*I sites are present at the insertion site into the vector. PCR primers were designed to amplify each segment and contain a 15-bp overlap with the adjacent segment (Table 1). As shown in Figure 1B, DNAs encoding (*i*) three different secretory signal sequences [IL-2, erythropoietin (EPO), IgG1) to optimize protein expression; (*ii*) the extracellular domain of CD101 minus its endogenous signal sequence; and (*iii*) the Fc domain of murine IgG3 were generated by PCR using primers that contained a 15-bp overlap with the adjacent segment and 20–30 bp of segment-specific sequence.

The three DNA segments and gel-purified expression vector were joined in an In-Fusion reaction by mixing at a molar ratio of 1 vector to 2 of each signal-CD101-Fc DNA segment in one tube of In-Fusion Dry-Down reaction mix (Table 2). The reaction was transformed into TOP10 *E. coli*; 10 colonies per construct were miniprepped and characterized by *Nco*I + *Sal*I restriction enzyme digest and sequencing (Figure 2). All 10 minipreps of each construct contained all four pieces of DNA. Of note, the insert contains two internal *Nco*I sites, but since only the vector and not the inserts need to be digested with *Nco*I, internal restriction sites in PCR products do not limit the design of the construct. Sequencing of six minipreps was sufficient to identify error-free isolates of the 3.6-kb coding region containing IL-2 signal-CD101-Fc, EPO signal-CD101-Fc, and IgG1 signal-CD101-Fc. Of the three unsuccessful isolates, two contained 1- or 2-bp deletions in a junction region, and one contained two copies of the IgG1 signal sequence. When transiently transfected into COS cells, the IL-2 signal-CD101-Fc construct was expressed well [optical density (OD) 0.09], but the EPO signal-CD101-Fc (undetectable) and IgG1 signal-CD101-Fc (undetectable) were not thus identifying the IL-2 signal sequence as one that could successfully direct the secretion of this large, difficult to express, protein.

An *Nco*I site (CCATGG) in a vector is a universal cloning site for the start of a coding region, as it includes an ATG as well as the end of a Kozak consensus translational start site (GCCACCATG) (8). Since the 15-bp overlap for In-Fusion is counted from the restriction enzyme cut site on the antisense DNA strand, the 15-bp overlap for an *Nco*I

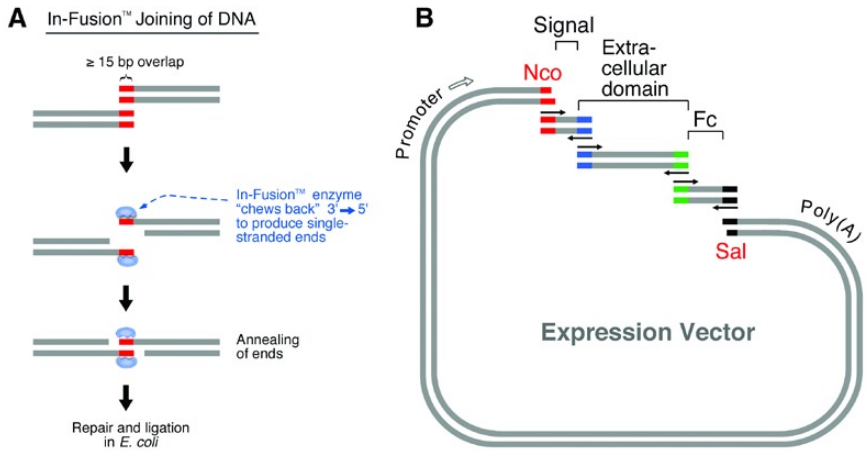

Figure 1. Mechanism of an In-Fusion reaction and its use in constructing a three-piece fusion protein. (A) In-Fusion reaction mechanism. (B) Seamless construction of an immunoglobulin fusion protein. Colored regions indicate overlap regions with 15 bp of identity. Arrows indicate PCR primers. Each segment is generated by PCR with primers that include the overlaps and joined to *Nco*I-*Sal*I-digested vector in an In-Fusion reaction.

Table 1. Primers for PCR Amplification of IL-2 Signal, CD101, and Murine IgG3 Segments

IL-2 Signal with Overlaps to *Nco*I-Digested Vector and CD101, 88-bp PCR Product
Sense (*Nco*I underlined)
5′-TTCAAATCCACCATGGATAGAATGCAATTGTTG-3′
Antisense
5′-CTGTTACTTCTCTCTGAGAATTCGTAACCAAAGCCAAAGACAAAGCAATCA-3′
CD101, 2799-bp PCR Product
Sense
5′-CAGAGAGAAGTAACAGTTCAGAAA-3′
Antisense
5′-GGCCGAGGAGCAGATCCTGGAA-3′
Murine IgG3 with Overlaps to CD101 and *Sal*I-Digested Vector, 771-bp PCR Product
Sense
5′-ATCTGCTCCTCGGCCCCTAGAATACCCAAGCCCAGTACC-3′
Antisense (*Sal*I underlined)
5′-**AGTAACGTTAGTCGAC**TCAGTGTCTTGTAAGACCCGAGGA-3′
Overlaps are colored to match Figure 1B. IL-2, interleukin 2; murine IgG3, murine immunoglobulin G3.

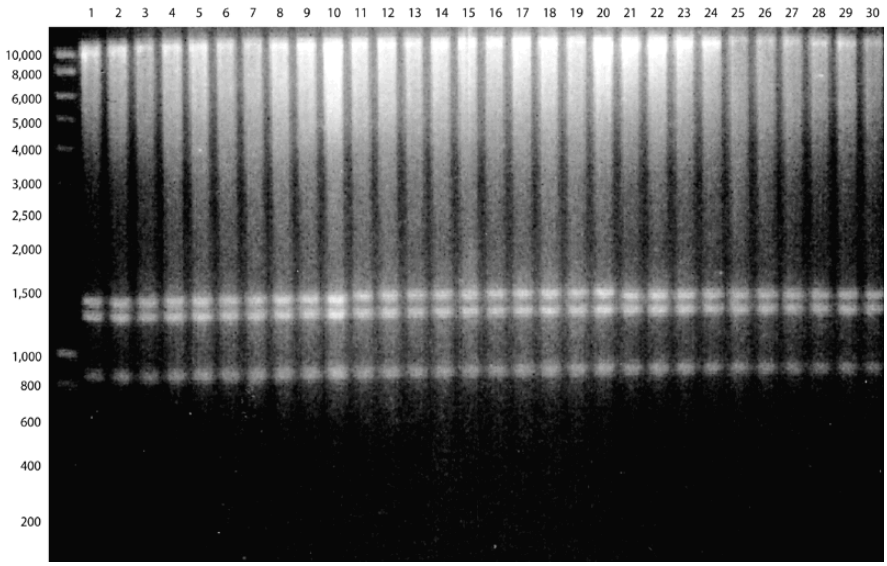

Figure 2. Plasmid minipreps of an In-Fusion reaction. Individual plasmids from the four-piece In-Fusion reaction illustrated in Figure 1B were digested with NcoI-*Sal*I and separated by electrophoresis on an agarose gel. Lanes 1–10, IL-2 signal-CD101-murine IgG3 Fc-vector; lanes 11–20, EPO signal-CD101-murine IgG3-vector; lanes 21–30, murine IgG1 signal-CD101-murine IgG3-vector. All plasmids had the 11.4-kb vector band and three other bands (there are two internal *Nco*I sites in CD101), indicating all pieces of DNA were incorporated into the construct. IL-2, interleukin 2; IgG, immunoglobulin G; EPO, erythropoietin; Fc, fragment crystallizable.

site is 5′-10 bp of vector-CCATG-3′, and the next nucleotide in the gene-specific region of the primer can match the natural nucleotide in the gene, thus requiring no change in the natural coding sequence. An *Nco*I site will be recreated if the next nucleotide is a G.

Technically, we see fewer background colonies if the insertion site in a plasmid vector is created by digestion with two restriction enzymes rather than one. In-Fusion may inefficiently facilitate the joining of the homologous ends of a singly digested vector. Phosphatasing of the vector is unnecessary and reduces efficiency. Accurate quantitation and ratios of vector and DNA segments are critical for success of multipiece constructs. Of 17 recent constructs, 28 of 38 minipreps sequenced were error-free and none required sequencing of more than 3 isolates to obtain the desired sequence. Of these 17 constructs, two-, three-, and four-piece In-Fusion reactions resulted in an average of 671, 539, and 56 colonies, respectively, on a plate spread with 0.1 mL of a total 0.3-mL transformation reaction. The use of highly competent *E. coli* (1×10^9 cfu/µg) is recommended for multipiece In-Fusion reactions as the number of transformants decreases as the number of pieces of DNA in the In-Fusion reaction increases. The size of the DNA segments appears to be unimportant and has varied from 83–12,000 bp. The length of the overlap between segments should be at least 15 bp, and slightly longer also works. The overlap sequence in the primers is not limited by G/C content or DNA sequence and may engineer in new restriction sites, but should avoid long hairpins at the end.

In-Fusion can be used to facilitate mutation of DNA via two strategies. As illustrated in Figure 3A, two unique restriction sites are identified that flank a desired mutation in a plasmid. Ideally, these are located 200–400 bp from the desired mutation so PCR products are >200 bp. The desired mutation is designed in silico and sense and antisense primers that incorporate the mutation (base pair changes, deletions, additions) and 20–30 bp of gene-specific priming sequence are synthesized. The mutagenic primers must overlap by 15 bp at their 5′ ends. Primers are also made that have 15 bp of identity flanking the unique restriction sites and 20–30 bp of gene-specific priming sequence. The two DNA segments are PCR amplified and joined with restriction enzyme-digested plasmid by In-Fusion, thereby incorporating the mutation.

As an example, primers for mutation W45A of human TIM-4 (hTIM-4) (9) are shown in Table 3. A TIM-4 template for PCR was made by *Xho*I digest of an hTIM-4 cDNA clone in pEF6 and gel purification of a 1566-bp fragment containing the cDNA. A 206-bp 5′ PCR product containing the W45A mutation was made using the antisense mutant and 5′ sense primers of Table 3. A 509-bp 3′ PCR product containing the W45A mutation was made using the sense mutant primer and 3′ antisense primers of Table 3. The hTIM-4 cDNA clone in pEF6 was digested with *Spe*I and *Bsp*EI, the 6375-bp fragment containing the vector and 3′ end of hTIM-4 was gel-purified, and 24 ng was combined with 1.6 ng 206-bp 5′ mutant PCR product and 3.8 ng of 509-bp 3′ mutant PCR product in a 10 µL In-Fusion reaction (1:2:2 molar ratio). Of five TIM-4 mutants made in this fashion, sequencing revealed 3 of 3, 3 of 3, 2 of 3, 2 of 3, and 1 of 8 to contain an error-free sequence.

An alternative strategy (Figure 3B) for mutagenesis is to use the mutagenic sense and antisense primers in a PCR with circular plasmid as template. A linear PCR product is generated that

Table 2. Four-Piece In-Fusion Reaction of Figure 1B and Figure 2

Secretory Signal	CD101 Domain	Murine IgG3 Fc Tail	Vector	Total Transformants	Error-Free/ Sequence (No.)
IL-2, 88 bp, 1.6 ng	2799 bp, 49.3 ng	771 bp, 13.6 ng	11,365 bp, 100 ng	690	1/2
EPO,108 bp, 1.9 ng	2799 bp, 49.3 ng	771 bp, 13.6 ng	11,365 bp, 100 ng	1150	1/2
Murine IgG1, 84 bp, 1.5 ng	2799 bp, 49.3 ng	771 bp, 13.6 ng	11,365 bp, 100 ng	2130	1/2

IgG1, immunoglobulin G1; Fc, fragment crystallization; IL-2, interleukin 2; EPO, erythropoietin.

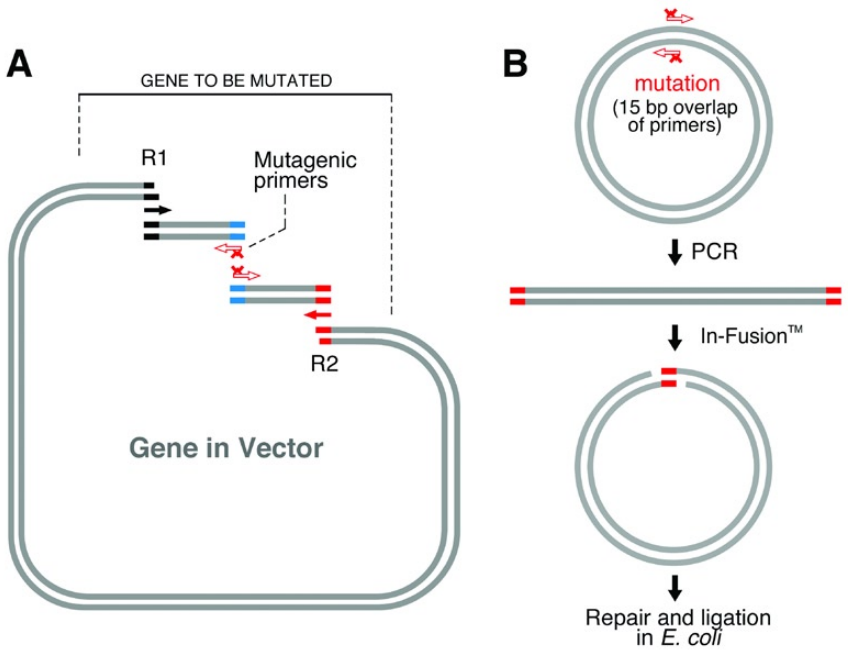

Figure 3. DNA mutagenesis strategies. (A) R1 and R2 indicate unique restriction sites flanking the desired mutation. Two DNA segments are amplified with the mutation incorporated at the DNA end via mutagenic primers and re-introduced into R1 + R2 digested plasmid via In-Fusion. (B) The mutagenic primers are used in PCR with circular plasmid as template to generate a linear molecule that is gel-purified and re-circularized via In-Fusion. Colored regions indicate overlap regions with 15 bp of identity.

Table 3. Primers for Mutagenesis

Primers for PCR Amplification to Make Human TIM-4 W45A Mutant (for Figure 3A)
5′ Sense Primer (Overlap with Vector Underlined, SpeI Site Bold)
5′-GCTCGGATCC**ACTAGT**CCAGTGTG-3′
Antisense Mutant Primer (Mutation Underlined)
206-bp PCR Product
5′-GAAGCGGATGAGTACAGACAGGGCAAAGTC-3′
Sense Mutant Primer (Mutation Underlined)
5′-TGTACTCATCCGCTTCTCACAACAGCAACAGC-3′
3′ Antisense Primer (BspEI Site Bold)
509-bp PCR product
5′-TGTGGCTTCC**TCCGGA**AGGGTGCTTGGGGTTA-3′
Primers for PCR Amplification to Make Human IgG1 Hinge C103S Mutant (for Figure 3B)
4609-bp PCR Product
Sense Mutant Primer (Mutation Underlined)
5′-AGTTGAGCCCAAATCTTCCGACAAAACTCACACA-3′
Antisense Primer
5′-GATTTGGGCTCAACTTTCTTGTCCACCTTGGTGT-3′
Overlaps are colored to match Figure 3. IgG1, immunoglobulin G1.

can then be recircularized in an In-Fusion reaction. As an example, 0.1 ng of a supercoiled plasmid containing human IgG1 cDNA in pCR-Blunt II was used as a template in a PCR with the human IgG1 sense mutant and antisense primers shown in Table 3. Cycling conditions were one cycle of 95°C for 2 min; 30 cycles of 95°C for 20 s, 59°C for 20 s, and 72°C for 2 min; and one cycle of 72°C for 3 min. The 4609-bp linear PCR product was gel-purified, and 25 ng were incubated in a 10-µL In-Fusion reaction, resulting in circularization of the plasmid via the 15-bp overlap at the ends. One of three plasmids sequenced had the desired sequence. The strategy of Figure 3A is more labor intensive but requires re-sequencing of a smaller region of DNA and is very reliable. The strategy of Figure 3B may be limited by the difficulty of PCR amplifying a large segment of DNA and likely requires re-sequencing of a larger region; however, it does not require the identification of unique restriction sites.

In-Fusion can also be used to replace any DNA segment in a plasmid with any desired new DNA segment. We term this strategy replacement In-Fusion, and two variations are illustrated in Figure 4. In the first strategy (Figure 4A), two unique restriction sites are identified that flank the desired replacement in a plasmid. Ideally, these are located 200–400 bp from the desired replacement, so PCR products are >200 bp. Primers are designed to amplify the two regions between the boundary of the replacement and each unique restriction site plus 15-bp overlaps. The replacement segment is amplified from an appropriate template using primers with a 15-bp overlap to the ends of the plasmid PCR product and 20–30 bp of segment specific sequence. The two PCR products, the replacement segment, and plasmid digested with the two unique restriction enzymes are joined in an In-Fusion reaction.

An alternative strategy has been described (10) and is shown in Figure 4B. An antisense primer is designed at the 5′ boundary of the segment of plasmid to be replaced, and a sense primer is designed at the 3′ boundary of the segment of plasmid to be replaced. Using the circular plasmid as a template, these primers are used to PCR amplify the desired region of the plasmid as a linear molecule lacking the segment to be replaced. The replacement segment is amplified from an appropriate template using primers with a 15-bp overlap to the ends of the plasmid PCR product and 20–30 bp of

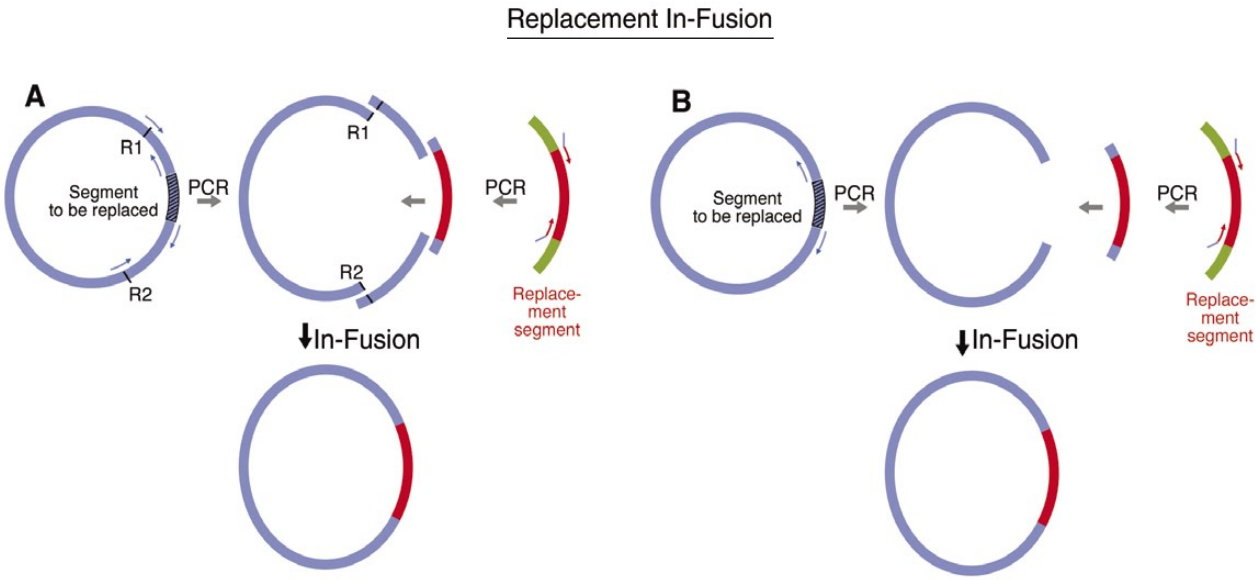

Figure 4. Replacement In-Fusion. The segment to be replaced is indicated by hatched lines. Sense and antisense primers are designed at the boundaries of the DNA segment to be replaced. The 3′ ends of the primers define the point of deletion. The replacement segment, indicated in red, is PCR amplified from an appropriate template using primers with a 15-bp overlap to the ends of the plasmid PCR product and 20–30 bp of replacement segment-specific sequence. Primers are indicated by arrows and are color-matched to their regions of identity. (A) Primers are designed to amplify the regions between the boundaries of the replacement and unique restriction enzymes sites R1 and R2 plus a 15-bp overlap. The two PCR products, the replacement segment, and plasmid digested with R1 and R2 are joined in a four piece In-Fusion reaction. (B) Sense and antisense primers at the boundaries of the DNA segment to be replaced are used to PCR amplify the desired region of the plasmid as a linear molecule lacking the segment to be replaced. The linear plasmid and replacement segment DNAs are joined by an In-Fusion reaction.

segment-specific sequence. The two DNAs are then joined by an In-Fusion reaction. No restriction sites are used in this strategy. Difficulty in PCR amplifying a very large segment of a plasmid suggests the use of the strategy shown in Figure 4A.

In-Fusion facilitates the construction of modular vectors and synthetic genes (Figure 5). One develops a toolbox of DNA segments that can be joined as desired, including fusion protein partners [Fc, green fluorescent protein (GFP), etc.], epitope tags, linkers, promoters, poly(A) sites, selectable markers, and origins of replication. A modular vector with the desired DNA segments can be designed in silico. Restriction enzymes that do not cut are identified, and a unique site is incorporated in silico between each segment. The toolbox DNAs can be used as templates for PCR with primers that include 15-bp overlaps at the desired junctions. The 15-bp overlaps may incorporate a different unique restriction enzyme site in each junction. Up to four pieces at a time may be joined by In-Fusion reaction, creating a modular vector whose individual components may be readily excised and replaced.

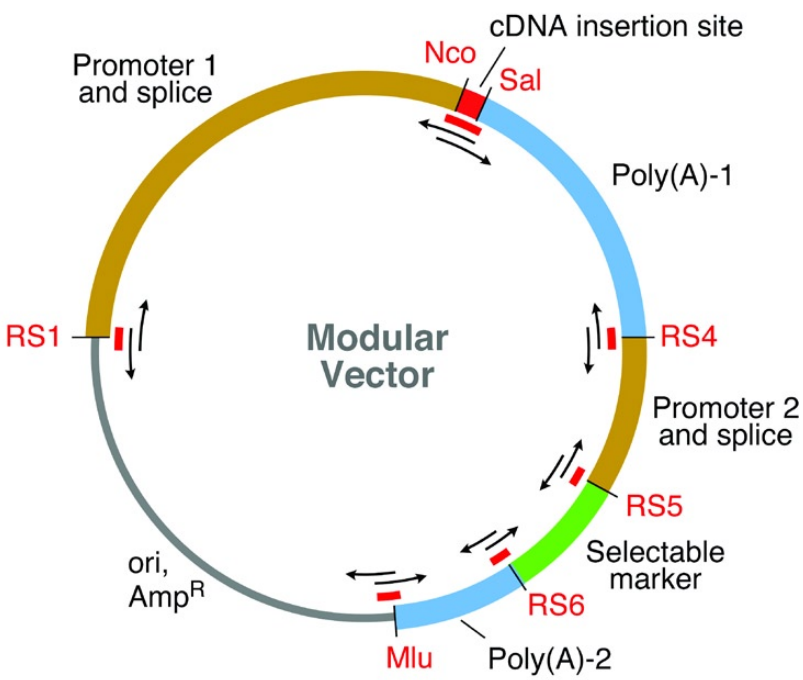

Figure 5. Modular vector. Red bars indicate junctions between segments. Different unique restriction sites (RS) may be engineered into the junctions via the primers used to amplify the segment. Each segment is amplified with primers that include 15 bp of overlap with the adjacent segment. DNAs are joined via In-Fusion reaction so no restriction digest is required. *Nco*I is a useful site as it is a universal acceptor for a coding region. *Mlu*I is a convenient site for linearization of vector for transfection.

Artificial genes can be constructed via PCR with overlapping sets of primers; however, this method is difficult for sequences longer than a few hundred base pairs (11,12). The construction of larger artificial genes can be facilitated by PCR amplifying the gene in 300-bp segments with 15-bp overlaps at the DNA ends and joining the pieces and vector via an In-Fusion reaction.

The use of two-piece In-Fusion reactions in high-throughput applications has been described (3,13) and additional work is needed to test whether multipiece In-Fusion reactions are adaptable to high-throughput. In-Fusion assembly provides a powerful way to join multiple pieces of DNA and facilitates the construction of seamless fusion proteins and modular vectors with readily interchangeable segments. Replacement In-Fusion can be used to replace any DNA segment in a plasmid with any desired new DNA segment without limitations to position or size.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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