Benchmarks

Self-assembly cloning: a rapid construction method for recombinant molecules from multiple fragments

Akira Matsumoto[1] and Taichi Q. Itoh[2]
[1]*Department of Biology, Juntendo University School of Medicine, Inzai, Chiba, Japan and* [2]*Graduate School of Systems Life Sciences, Kyushu University, Hakozaki, Fukuoka, Japan and Research Fellow of the Japan Society for the Promotion of Science, Japan*

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Enzyme-free cloning (EFC) can rapidly produce an in-frame fusion gene with multiple fragments. To practically apply EFC, we investigated the extent and sequence of complementary staggered overhangs necessary to direct self-assembly of multiple fragments as well as a size limitation of the constructed DNA molecule. Six-base pair overhangs with 50% GC content were sufficient to direct self-assembly. A functional plasmid that exceeded 10 kb, which includes an in-frame fusion domain, was efficiently constructed from four PCR fragments in one step by our improved method.

Traditional cloning procedures based on restriction enzymes and DNA ligase are inefficient when constructing recombinant molecules with multiple fragments. Another limitation is the dependence upon restriction enzymes; a restriction site in the multicloning site of a plasmid often cannot be used when the insert contains a site for the same enzyme. This problem can be overcome by subcloning, but this is a time-consuming procedure. Enzyme-free cloning (EFC) (1–3) is a good alternative for producing in-frame fusion genes from multiple fragments, because it is simple, versatile, and cost-effective.

In this study, we modified the original EFC procedure (1) to clone multiple fragments in a single step (Supplementary Figure S1). Briefly, this was accomplished by performing two PCRs (one with the tailed forward primer and the untailed reverse primer, and the other with the untailed forward primer and the tailed reverse primer; Supplementary Table S1) to create the insert fragment. These two PCR products were treated with *Dpn*I to completely digest the template plasmid (4), mixed, denatured, and reannealed to create fragments having single-stranded overhangs. Using the same procedure, vector fragments with staggered overhangs complementary to those of the insert were generated. A portion of the reactions for the inserts and the vector were mixed at room temperature, allowed to self-assemble, and used to transform competent cells. Our modified method depends upon *Dpn*I to digest template molecules after PCR, so it does not qualify as an EFC method; it is similar to EFC for self-assembly of multiple fragments, so we decided to call it self-assembly (SA) cloning.

We first identified the minimum length and optimal GC content necessary for the overhangs with one insert and one vector. We synthesized tailed primers to amplify P_{gpt}::*lacZ* fragment as an insert with 4- to 12-nucleotide overhang ends. The former length corresponds to that of overhangs created by restriction enzymes generally used in molecular cloning, and the latter was commonly used for previous EFC procedure (1–3) and ligation-independent cloning (LIC) (5). To adjust GC content of the tail region in each primer to 50% in this primary experiment, the length of the region was set as an even number (Supplementary Table S1). Cloning efficiency correlated to the length of the overhangs, except in the case of four-nucleotide overhangs, which was not successfully cloned (Figure 1). We obtained a satisfactory number of blue colonies for common molecular cloning with inserts carrying greater than six-nucleotide tails. Thus six-nucleotide tails are the minimum length needed for successful SA cloning, which is the shortest tail length among ligase-free cloning methods for multiple inserts (6–9). There were <20 false positive white colonies among a huge number of blue ones. Gel electrophoresis analysis revealed that white colonies had a very short insert (data not shown), which we assume was derived from a nonspecific PCR product. The number of white colonies dramatically increased when we omitted digestion of the template plasmid by *Dpn*I (data not shown), suggesting that this step is indispensable for efficient cloning.

We then tested whether GC content in eight-nucleotide tails affected SA (Supplementary Table S2). GC contents of 75% decreased the cloning efficiency to 50%, and GC content of 25% severely disrupted cloning (Supplementary Table S3). Thus, GC contents near 50% within the complementary region are optimal for SA cloning.

Since SA cloning is PCR-based, it is imperative that high-fidelity polymerases are used, particularly when amplifying coding sequences to avoid PCR errors. However, SA cloning is expected to be more resistant to such errors than traditional cloning methods, because heterozygous PCR fragments are reannealed to create inserts with single-stranded overhangs. Instead of sequence analysis of the entire plasmids purified from all blue colonies, we checked the effect of mutation in the overhang region with newly designed primers having various mutation types (Supplementary Table S2). Because any mutation we introduced decreased the cloning efficiency (Supplementary Table S3), we conclude that the efficiency of SA cloning is highly sensitive to mutations within the complementary tails. This is likely to warrant the fidelity of nucleic acid sequence of constructed plasmid at least in overhang regions.

We applied SA cloning to construct a functional expression plasmid with multiple fragments (Figure 2). The plasmid contains two promoters, one for the expression of *EGFP-Zeo* and the other for the expression of *DsRed*, in cultured insect cells. We placed the nuclear localization signal (NLS) (10) in-frame with *EGFP-Zeo* (Figure 2 and Supplementary Table S3), so the difference in subcellular localization between EGFP-Zeo and DsRed could be observed by fluorescence microscopy when expressed in insect cells. The plasmid was composed of four fragments, and was >10 kb in length (Figure 2). Since

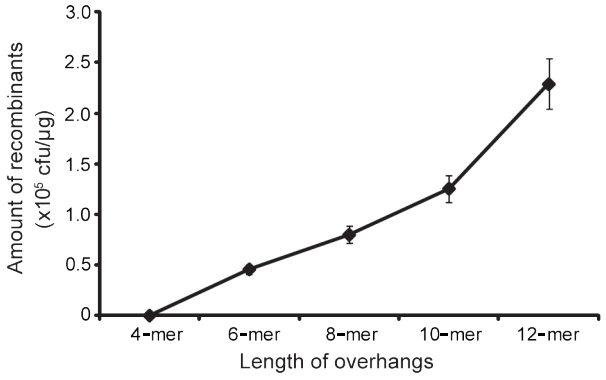

Figure 1. Cloning efficiencies depending on the length of overhangs. Two PCR fragments with various lengths of complementary overhangs. The insert fragment is 3.9 kb from P_{gpt}::*lacZ*, and the vector is 4.3 kb from *pGL3-basic*. The number of blue colonies obtained per 1 μg *pGL3-basic* PCR fragment is represented as colony formation units (cfu). Error bars represent SEM ($n = 3$).

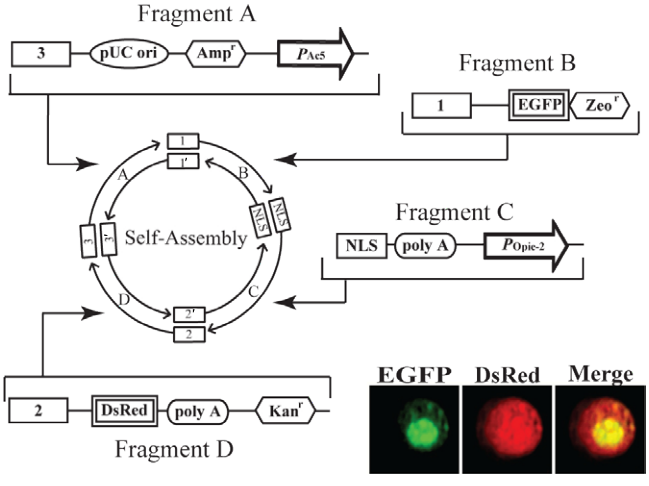

Figure 2. A multifunctional plasmid created by SA cloning that expresses reporter proteins in different subcellular compartments in insect cells. The plasmid, designed to self-assemble with four fragments, has several functional units: a replication origin (represented as an oval); three antibiotic resistance genes for amplicillin, zeocin, and kanamycin (a hexagon); two genes encoding the fluorescent reporter proteins enhanced GFP (EGFP) and DsRed (a double box) are driven by P_{Ac5} and P_{Opie-2} promoters (an arrow) and terminated by the polyA signal sequences (a round-edged rectangle), respectively; an NLS (box) fused in-frame to *EGFP-Zeo*, in which the NLS sequence was also used as a complementary overhang to self-assemble between fragments B and C. Only the sense strand of each fragment with the 5′ overhang region (a numbered box) is illustrated around the designed plasmid. The signal of EGFP is shown as green in nucleus (left panel of insets) and red in cytoplasm (middle). The right panel represents the merge of EGFP and DsRed signals.

this plasmid encodes antibiotic resistance to ampicillin, zeocin, and kanamycin carried in fragments A, B, and D, respectively (Figure 2), we selected transformants of bacterial colonies on culture plates containing these antibiotics. We picked 13 colonies and checked that the plasmids purified from each colony were the expected size of 10.5 kb. We also confirmed that each plasmid carried fragment C, which did not contain an antibiotic selection marker (Figure 2), by PCR amplification with the primer sets used in the SA cloning procedure. Two plasmids were independently transfected into insect cells. In both cases, we observed red fluorescence in the cytoplasm and green fluorescence in the nucleus (insets in Figure 2), suggesting that the desired plasmid was produced and functioned properly in cultured cells.

Our SA cloning method largely follows the original EFC procedure (1) and does not need any additional enzymes or specific materials, with the exception of *Dpn*I to digest template molecules after the PCR step (4). However, this digestion step does not decrease the ease of the original EFC procedure, and SA does not depend on the activity of *Dpn*I. The efficiency, flexibility, and cost-effectiveness of SA cloning make it a suitable method for constructing recombinant DNA molecules from multiple fragments.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Akira Matsumoto, Department of Biology, Juntendo University School of Medicine, 1-1 Hiraga Gakuendai, Inzai, Chiba 270-1695, Japan. e-mail: akirarcb@sakura.juntendo.ac.jp

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