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SMC, a simple method to rapidly assemble multiple fragments into one construct

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1. ABSTRACT

We describe a simple method, split-marker-mediated multiple-piece cloning (SMC), to rapidly assemble multiple DNA fragments into one construct in yeast. In this approach, a selectable marker is split into two non-functional, overlapping halves, of which one half is on the plasmid backbone. Homologous recombination reconstitutes the marker gene and assembles all DNA fragments in the desired order. This method allows rapid one-step fusion of various DNA fragments that contain ~30 base pair overlaps in yeast using raw PCR and/or restrict enzyme-digested products. We assembled seven DNA fragments into one contig in a single step by SMC in eight days.

2. INTRODUCTION

Gene cloning by restriction enzyme cutting and ligation revolutionized molecular biology in the 20th century (1). However, this method is limited by the availability of restriction enzyme sites. In addition, using this traditional cloning method to assemble multiple pieces of DNA requires multiple laborious steps. Recombination-based methods eliminate the requirement of restriction enzymes. Bacteria recombineering has gained some momentum in cloning long DNA pieces in recent years, but only one piece of DNA can be cloned at each step (2, 3). A few *in vitro* recombination methods, using single strand DNA terminals generated by enzyme digestion, have recently been developed to assemble several DNA pieces in

Table 1. Screening of pTS-GAL4-UAS-attB1

Exp. No.	Screening				
	SD/-Ura	PCR	Enzyme digestion	Sequencing	Positive colony (%)
1	102	92/96	8/8	2/2	95.8
2	116	91/96	8/8	NA	94.7
3	893	91/96	8/8	NA	94.7

Column 1: the number of independent experiments; Column 2: total number of colonies that grew on SD-Ura plate (1/10 of the transformants were plated in experiment 1 and 2, while all transformants were plated in experiment 3); Column 3: colony PCR results, positives/total PCRed colonies; Column 4: enzyme digestion results of rescued plasmids from PCR positive clones, correct digestions/total digestions; Column 5: sequencing results, correct/total sequenced; Column 6: the percentage of positive colonies were based on colony PCR results. NA: no sequencing was attempted for experiment 2 or 3

one step (4-9), though the efficiency drops rapidly with increasing numbers of, as well as the lengthening of the DNA fragments to be assembled (4-6). In addition, some of the *in vitro* methods necessitate generating all of the DNA fragments via PCR (6, 7), or are limited by the uniqueness of nucleotide sequences at the junctions (8). Homologous recombination in yeast has been used for cloning since 1987 (10), and for assembling the complete *Mycoplasma genitalium* genome from 25 DNA fragments with overlaps of 80 to 360 base pair (bp) in one reaction (6). Recently, Gibson (11) has shown that 38 oligos containing 30 bp overlaps can be co-transformed and assembled into a dsDNA vector in yeast. For recombination between two DNA fragments, a 40 bp overlaps yields an efficiency of about 90%, while 30 bp overlaps yields an efficiency of about 80%, which drops to 3.4% with a 20 bp overlap (12). Thus, for simple DNA cloning a 30 bp overlap is enough. In fact, we perform three-piece DNA cloning routinely with the convention yeast homologous recombination. However, when the number of DNAs to-be-joined increases, the success rate drops rapidly (13). We developed the split-marker-mediated multiple-piece cloning method (SMC), which allows for rapidly assembling of multiple DNA pieces containing 30 bp overlaps in a single step with high efficiency.

3. MATERIAL AND METHODS

3.1. Materials

Yeast strain *MATα leu2Δ0 lys2Δ0 ura3Δ0 YAR047CΔ::KanR*. *KanR* is a gift from Chris Foote (University of Missouri-Columbia). All amino acids were purchased from Sigma and yeast nitrogen base was from Clontech. Phusion™ Hot Start High-Fidelity DNA polymerase and Taq DNA polymerase were bought from New England Biolabs Inc. and TaKaRa Bio Inc. respectively. Synthetic-drop-out media of yeast, SD-ura and SD-leu were made following the instruction in Clontech's Yeast Protocols Handbook (http://www.clontech.com/).

3.2. Preparation of DNA fragments

All DNA fragments, except plasmid backbones, were generated by high fidelity PCR using Phusion™ Hot Start High-Fidelity DNA polymerase on Autorisierter Thermocycler (Eppendorf). The cycle parameters are: 30 cycles of 98°C for 7 s, 58°C for 15 s, and 72°C for 40 s for URA3-C, KanR, intein and GAGA-UAS, 72°C for 90 s for GAL4-C, and 72°C for 10 s for GAL4-N, attB-SA, and SD-attB; 30 cycles of 98°C for 7 s, 62°C for 15 s, 72°C for 35 s for LEU2-N and LEU2-C. URA3-C and the TS intein were gel-purified and re-amplified by high fidelity PCR (25 cycles of 98°C for 7 s, 68°C for 60 s). Oligos and templates are listed in table 2. pREC was constructed by digesting p416Met25 (14) with *Sac* I and *Stu* I and releasing the Met25 promoter and URA3-C-containing fragment followed by the addition of three restriction enzyme sites (*EcoR* I, *Hind* III and *Cla* I) (Figure 2). To construct pREC-1, p416Met25 was digested with *Sac* I, and LEU2-N was subsequently added by homologous recombination in yeast (Figure 5). The homologous regions were engineered into the PCR primers (Table 2). pREC and pREC-1 were linearized by *Hind* III and *EcoR* I, respectively, and the digestion products were used directly.

3.3. Yeast transformation

Unpurified PCR products and linearized plasmids were co-transformed into yeast as described by Schiestel and Gietz (15) with some modifications. Briefly, we picked two to six fresh yeast colonies (less than 2 weeks) from a plate and resuspended in 4 ml of YPD medium. After two hours' culturing, the cells, with an OD_{600} density of about 0.6, were harvested by centrifugation and were washed in 10 ml of sterile distilled water. The washed cells were resuspended in 1 ml of sterile 2X TE/LiOAc (prepared from 10X stocks: 10X TE=0.1 M tris.HCl/0.01 M EDTA, pH 7.5; 10X LiOAc=1M LiOAc, pH7.5). The cells were spun down (1 minute, 3000 rpm, Eppendorf Centrifuge 5415D), and the supernatant was poured off. The cell pellet was resuspended in the residual liquid. About 100 ng of linear plasmid and inserts, at a molar ratio of about 1:5, were added in a 1.5 ml eppendorf tube, mixed thoughrouly and dried on CentriCap Concentrator (LABCONCO). To this DNA mixture, we added 3.8 µl of denatured Sonicated Salmon Sperm DNA (10mg/ml, Stratagene), 21.2 µl of competent cells, and 150 µl of sterile PEG 4000/TE/LiOAc (to make 1.5 ml of PEG 4000/TE/LiOAc, mix 1.2 ml of 50% polyethylene glycol 4000, 150 µl of 10X TE, and 150 µl of 10X LiOAc). The cells were incubated for 30 minutes at 30°C, and then 17.5 µl DMSO was added and mixed with the cells gently but thoroughly. We heat-shocked the cells at 42°C for 15 min and incubated them on ice for 2 minutes, spun the cells down in a microcentrifuge for 5 sec, washed them once with 0.5 ml of TE or sterile water and resuspended the cell pellet in 1 ml of YPD. After 2 hours of incubation at 30°C with constant agitation, cells were washed once with 0.5 ml of TE or sterile water and were resuspended in 1 ml of TE or sterile water. 100 µl of the suspension were plated on selection plates, which were incubated at 30°C until colonies appeared (about two days).

Table 2. Templates and primers for PCR

Fragments	Template	Primer	Sequence
Ura3C	p416Met25(14)	Ura3C-F	5'-GGCAGAAGAAGTAACAAAGG-3'
		Ura3C-R	5'-AAGCTTGGGTAATAACTGATATAATTAAATTG-3'
KanR	pFA6a-GFP(S65T)-kanMX6(19)	KanR-F	5'-ATTTAATTATATCAGTTATTACCCAAGCTTAGCTTGCCTCGTCCC-3'
		KanR-R	5'-TGGATGGCGGCGTTAGTATCG
attB-SA	pBS-attB-SA-SD-attB[1]	attB-SA-F	5'-CTGTCGATTCGATACTAACGCCGCCATCCAACTAGTAAACGCTAGCGATGTAG-3'
		attB-SA-R	5'-GACCTGCGGAAGAGATAAATC-3'
Gal4N	Yeast genomic DNA	Gal4N-F	5'-ATCAACCGATTTATCTCTCTTCCGCAGGTCTTCTACAAAATGAAGCTACTGTC-3'
		Gal4N-R	5'-CATTAAAACATTGGTACCCTTGGCAAAGCACTTAAGCTTTTTAAGTCGGC-3'
TS-Intein	p416Met25-GFP-S18(14)	Intein-F	TGCTTTGCCAAGGGTACCAATG
		Intein-R	5'-CTTGGCGCACTTCGGTTTTTCTTTGGAGCAATTATGGACGACAACCTGGTTG-3'
Gal4C	Yeast genomic DNA	Gal4C-F	5'-TGCTCCAAAGAAAAACCGAAGTGCG-3'
		Gal4C-R	5'-TGACCATGGGTTTAGGTATAATGTTATCAAGGTACCCTCTTTTTTTGGGTTTGGTGGGGT-3'
tK10	pUASp(18)	tK10-F	5'-TTGATAACATTATACCTAAACCCATG
		tK10-R	5'-TGCAGCCAATCCGCCGCACC-3'
SD-attB	pBS-attB-SA-SD-attB[1]	SD-attB-F	5'-GGG**GTTTAAAC**GTAAGTTATTGAACAATGGCATC-3'
		SD-attB-R	5'-ATATAT**GCGGCCGC**ATGCATGATGTAGGTCACGGT-3'
GAGA-UAS	pUASp(18)	UAS-F	5'-TGGAGCTGAGGGTGCGGCGGATTGGCTGCAAATTGGCCGCTCTAGCCC-3'
		UAS-R1[2]	5'-AATTAACCCTCACTAAAGGGAACAAAAGCT**GCGGCCGC**'**GTTTAAAC**'GATCCCCGGGCGGGTACCAATGAA-3'
		UAS-R2[3]	TAGTTCATAGGGTAGGGGAATTTCGACCG**GCGGCCGC**'**GTTTAAAC**'GATCCCCGGGCGGGTACCAATGAA-3'
LEU2N	pGAD424	LEU2N-F	5'-ATTTAATTATATCAGTTATTACCCAAGCTAACTGTGGGAATACTCCAGGT-3'
		LEU2N-R	5'-AATTAACCCTCACTAAAGGGAACAAAAGCT**GAATTC**'GGACCAAATAGGCAATGGTG-3'
LEU2C	pGAD424	LEU2C-F	5'-CAACATGAGCCACCATTGCCT-3'
		LEU2C-R	5'-TGACCATGGGTTTAGGTATAATGTTATCAACCGGTCGAAATTCCCCTACCTATG-3'

[1] Hugo Bellen, personal communication, [2] for construction of pTS-GAL4-UAS-attB1 using pREC, [3] for construction of pTS-GAL4-UAS-attB1' using pREC-1, [4] *Pme* I, [5] *Not* I, [6] *EcoR* I, The underlined were homologous region for recombination. Two restriction enzyme sites (*Pme* I and *Not* I) were incorporated into UAS-R1 and UAS-R2, and then were introduced behind GAGA-UAS (Figure 9A). *Pme* I and *Not* I were introduced into SD-attB-F and SD-attB-R seperatly to clone SD-attB into pTS-Gal4-UAS-attB2 (Figure 9B). *EcoR* I site was introduced into LEU2N-R, and incorporated into pREC-1 to facilitate linearizing of pREC-1.

3.4. Yeast plasmid rescue

Single-colonies of yeast were streaked onto selective media plate with toothpicks and incubated at 30°C for 24 hours. The yeast was used directly to do colony PCR or plasmid rescue. Colony PCRs were done using the primers specific for tK10, and DNA polymerase ExTaq (TaKaRa). PCR parameters were: denaturing at 95°C for 90 s; 35 cycles of 94°C for 20 s, 62°C for 15 s, and 72°C for 120 s; with a final extension at 72°C for 5 minutes. For plasmid rescue, total yeast DNA was extracted as described by Hoffman (16) and transformed into Top10 electroporation competent cell using the Gene Pulser Electroporator (Bio-Rad) (2 mm cuvette, 2.5 kV, 25 uF, 200 ohms).

4. RESULTS

4.1. Principle of SMC

Two key factors contribute to the success of SMC. One is the powerful homologous recombination ability of yeast. The other is the use of a split selectable marker gene, which can be an auxotrophic gene or an antibiotic resistant gene. In this approach (Figure1), the selectable marker is split into two non-functional halves with a 30 bp overlap, one of which is on the plasmid backbone. Only via homologous recombination will a functional selection gene be regenerated. This essentially eliminates the false positives (or background clones) resulting from plasmid self-circularization and the random insertion of the plasmid backbone into the yeast genome.

4.2. Developing of SMC

Split-marker-mediated multiple-piece cloning originated from our need to generate a complex construct, pTS-GAL4-UAS-attB1, to use a temperature-sensitive (TS) intein (14, 17) to control gene expression in *Drosophila melanogaster*. Besides the plasmid backbone, this construct contains seven pieces, KanR, attB-SA, the N-terminus of *GAL4* (GAL4-N), a temperature sensitive intein allele, the C-terminus of *GAL4* (GAL4-C), a K10 terminal signal sequence (tK10), and GAGA-UAS (Figure 3 A-C). High fidelity PCRs with primers containing 30 bp overlaps were used to obtain attB-SA from pBS-attB-SA-SD-attB (a gift from K. Venken and H. Bellen at Baylor College of Medicine), GAL4-N and GAL4-C from yeast genomic DNA, the intein allele from p416Met25-GFP-intein (14), tK10 and GAGA-UAS from pUASp (18), and the KanR gene from pFA6a-GFP(S65T)-kanMX6 (19). Oligos and templates are listed in Table 2.

We used a low-copy number (in yeast) yeast-bacteria shuffle plasmid, p416Met25 (14), as the parental plasmid. In the beginning, we tried to assemble all seven fragments directly by co-transforming them with the linearized p416Met25 into yeast competent cells. As mentioned above, the background was very high; we obtained one true positive clone out of 96 colonies.

To increase the cloning efficiency, we deleted the C-terminus of *URA3* (URA3-C) and its terminator and the Met25 promoter of p416Met25 to generate pREC that contains the N-terminus of *URA3* (URA3-N) but no functional yeast selectable marker (Figure 2). Neither the intact, nor the linearized pREC sustained yeast growth on uracil-drop out synthetic medium (SD-Ura). We reasoned that co-transformation and homologous recombination of URA3-C, linear pREC, and the seven DNA fragments-to-be-assembled should lead to circularization of the plasmid and reconstitution of an intact *URA3,* thus should allow the yeast to grow on SD-Ura media. URA3-C was generated by high fidelity PCR using p416Met25 as a template, gel-purified, and re-amplified using the purified PCR product as a template. To screen for colonies that contain the desired construct, colony PCR was performed using primers specific to tK10 (Figure 4). To our surprise, more than 95% (92/96) of clones had incorporated tK10 (Figure 3 and Table 1). We randomly chose eight clones for plasmid rescue. All eight generated the expected enzyme

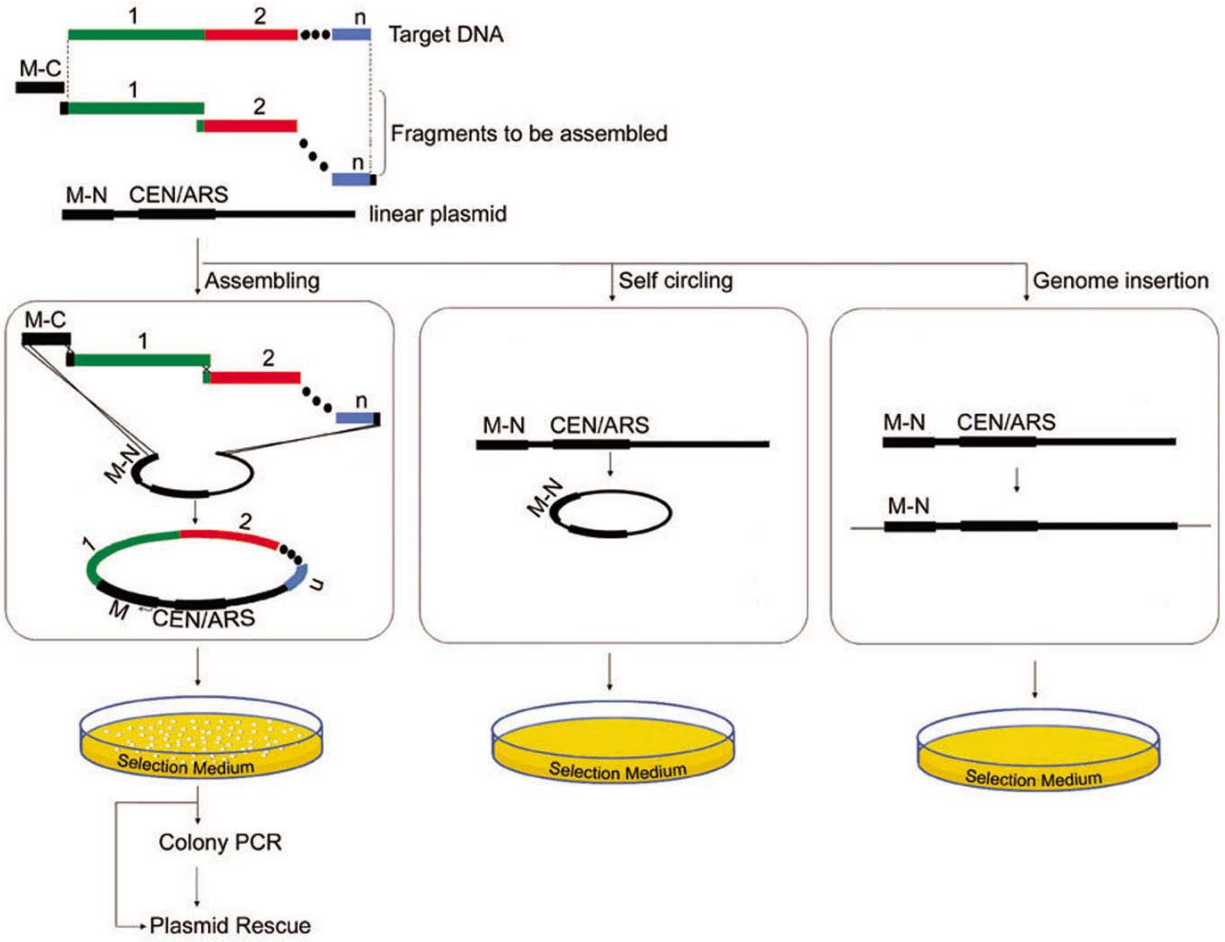

Figure 1. Principle of split-marker mediated multiple-piece cloning. A selectable marker (M) is split into two non-functional halves, M-N and M-C. The N-terminal half, M-N, is located on the plasmid backbone. Adjacent DNA fragments, such as M-N and M-C, M-C and DNA fragment 1, *etc.*, share 30 bp overlaps. Co-transformation of the linearized plasmid, M-C, and the DNA fragments to-be-joined into yeast results in a number of outcomes, including the reconstitution of the selectable marker and the simultaneous fusion of the DNA fragments (left), self circularization of the plasmid backbone (middle), and random insertion of the backbone or the selectable marker gene into the yeast genome (right). Only the clones containing a reconstituted selectable marker can grow on the selection media. Positive clones can be screened using colony PCR followed by plasmid rescue or directly using plasmid rescue.

digestion profiles (as shown in Figure 3D). We sequenced the isolated plasmids from two clones; no rearrangement or point mutation was observed (Data not shown). To assess the efficacy, we repeated the cloning process twice, and obtained very similar results (Table 1).

4.3. Comparison of split-marker-mediated versus intact-marker-mediated screening

We reasoned that the increase in efficiency may have resulted from eliminating the majority of unwanted products produced by illegitimate end-joining, including plasmid self circularization, incomplete digestion of plasmid backbone, and genome random insertion (Figure 1). To test this hypothesis directly, we constructed pREC-1 (Figure 5), which has two selectable marker genes; a split marker, *LEU2*, and an intact marker, *URA3*. We then used pREC-1 to assemble the same set of DNA inserts, generating pTS-GAL4-UAS-attB1' (Figure 6A). The split *LEU2* marker allows for the selection of only the desired recombinants, while the intact *URA3* selects for three groups of products: the desired recombinants, the self-circularized plasmid and random chromosome insertions of the *URA3* gene. As expected, all of the sixty-one randomly selected colonies from the split selectable marker grew on both SD-Ura and SD-Leu plates (Figure 6B). It is highly likely that the vast majority, if not all, of the colonies are true positive clones because every clone of the five randomly chosen colonies generated plasmids with the expected enzyme digestion profiles (Figure 7, lane 1-5). In contrast, more than 90 percent (71/76) of colonies from the intact selectable marker did not grow on SD-Leu plates and, thus were false positives for the desired homologous recombination (Figure 6C). The five colonies that did grow on the SD-Leu plates contained the desired construct as shown by plasmid rescue and enzyme digestion (Figure 7, lane 6-10).

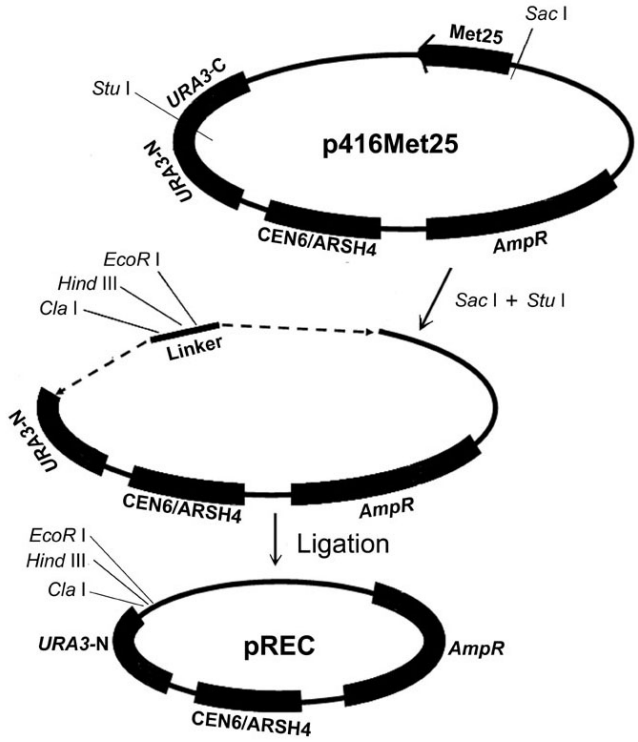

Figure 2. Construction of pREC. p416Met25 was digested by *Sac* I and *Stu* I, and then three restriction enzymes sites were added using a linker (annealed by two oligos: 5'-aattcaagcttatcgat-3' and 5'-atcgataagcttg-3') between *Sac* I and *Stu* I.

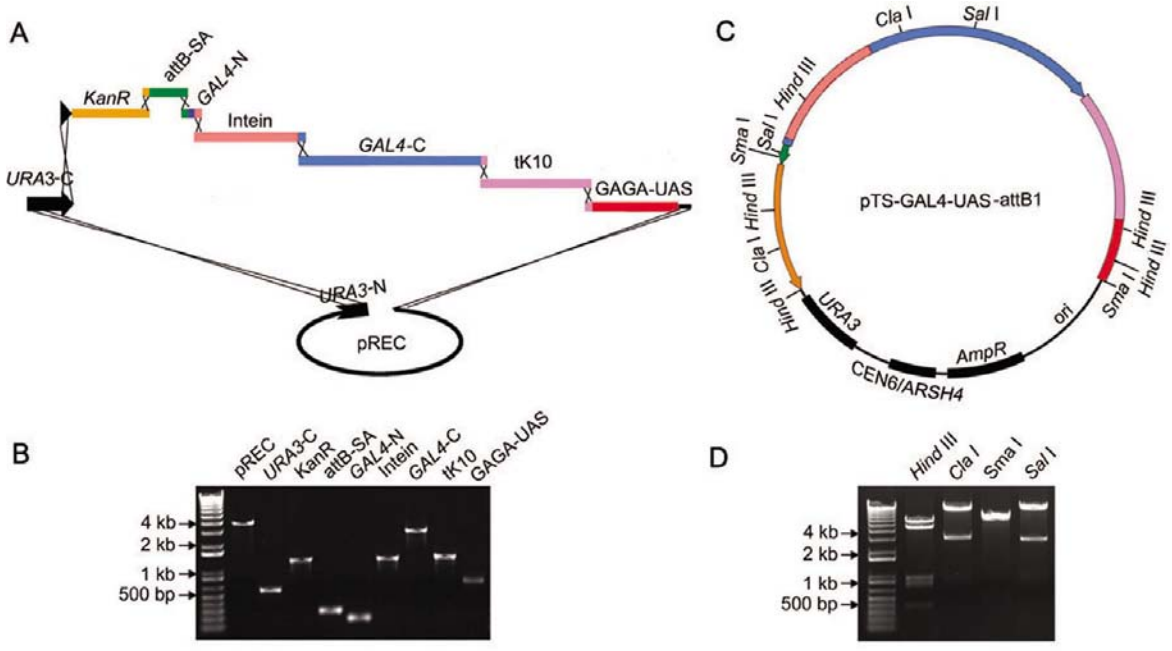

Figure 3. Cloning of pTS-GAL4-UAS-attB1. (A) Schematic representation of the DNA fragments to be assembled and the linearized plasmid backbone. The selectable marker *URA3* is split into Ura3-N, which is located on the plasmid backbone, and Ura3-C. Ura3-C and the seven DNA fragments to be joined, KanR, attB-SA, GAL4-N, intein, GAL4-C, tK10, and GAGA-UAS, were generated by high fidelity PCR. Ura3-N-pREC was a Hind III-digested product of vector pREC. Both the PCR and restriction enzyme digestion products were used without purification. (B) DNA fragments to be assembled. (C) The map of the resulting plasmid. (D) Restriction enzyme digestion profile of a rescued plasmid from one Ura^+ clone.

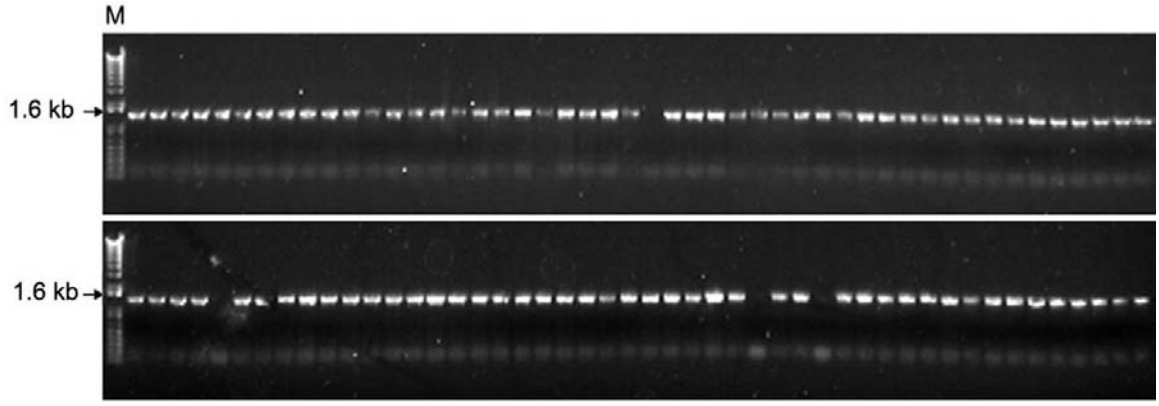

Figure 4. The results of colony PCR of experiment 1. The top panel shows clones 1 to 48, and the bottom panel clones 49 to 96. The resulting PCR fragments of tK10 from the positive clones are ~1.4 kb, as expected.

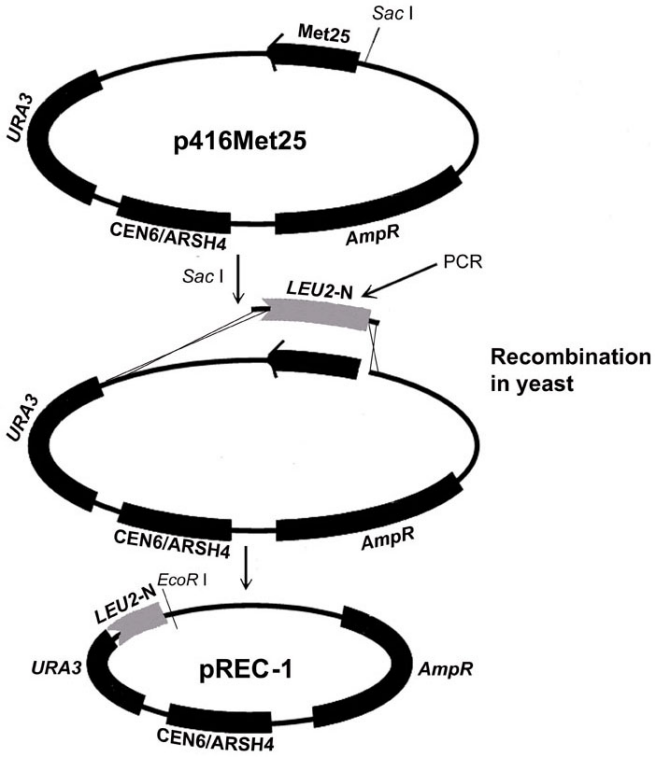

Figure 5. Construction of pREC-1. p416Met25 was digested by *Sac* I, and then LEU2N was added by recombination in yeast. LEU2N was amplified with High-Fidelity PCR. The homologous regions have been added into primers (Table 2).

4.4. Direct usage of raw PCR products and restriction enzyme digestion products

In the assembling of both pTS-GAL4-UAS-attB1 and pTS-GAL4-UAS-attB1' described above, raw PCR products were used directly without purification, demonstrating that raw PCR products can be used directly without adversely affecting cloning efficiency. The only time that PCR products need to be purified is when the PCR template is a plasmid that can propagate in yeast and contains the same selectable marker as the intended plasmid backbone. To demonstrate that all DNAs can be raw PCR products, we performed the second round of PCR for URA3-C and the TS intein using purified first-round PCR products as templates. In addition to raw PCR products, restriction enzyme digested DNA fragments can also be used without purification. For instance, the plasmid backbones used during our cloning were directly from restriction enzyme digestions. The needlessness of purification makes split-marker-mediated multiple-piece cloning convenient to use.

5. DISCUSSION

Our results demonstrate that SMC allows for facile assembling of complicated constructs, with high efficiency. The homologous regions can be located not only at the DNA termini but also within internal regions of the DNA fragments (Figure 5). The limitation on the number or size of DNA fragments that can be assembled is not known. Using this method, we have successfully and efficiently assembled seven fragments and the plasmid backbone. This method will facilitate the engineering of sophisticated knock-in, knock-out, or gene tagging constructs.

Single-piece cloning with a split marker containing 271 to 750 bp overlaps has been reported previously (20). Such long overlaps are not easy to generate and may even be longer than certain DNA fragments to be cloned. In contrast, SMC uses 30 bp overlaps, which can be incorporated easily in the PCR primers. To link two fragments without overlaps, an oligonucleotide linker of 60 bp (30 bp homology to each fragment) can be used. For multi-piece DNAs cloning, SMC is much less time-consuming and much simpler than the traditional DNA cloning method (Figure 8). For example to clone 7 fragments, 7 rounds of seven or eight steps of manipulation and four days for each round are needed using traditional cloning, while only five steps and a total of seven to eight days using SMC.

Shao *et al* found that, homologous recombination with 50 bp overlaps in yeast, allowed for cloning of three

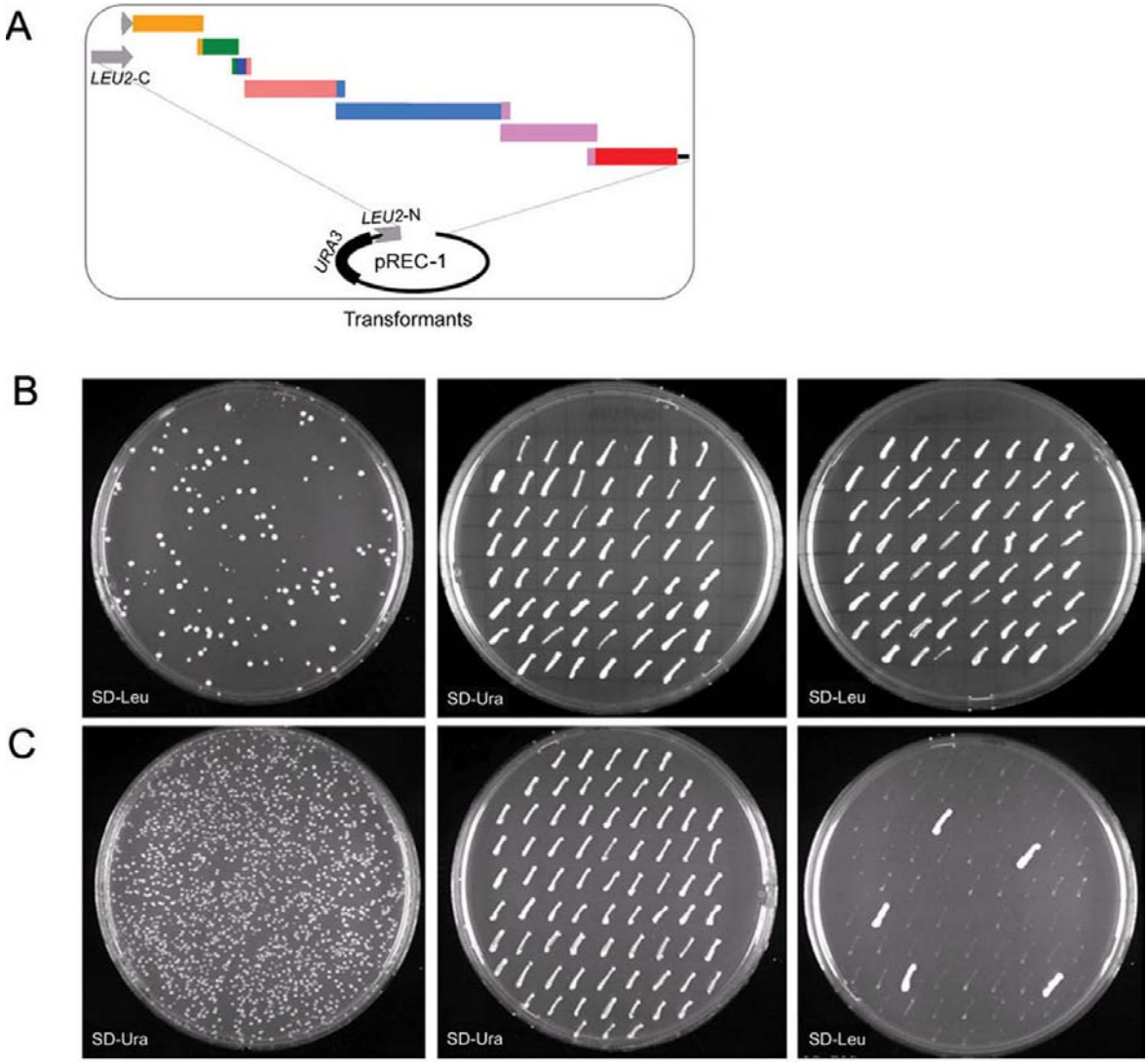

Figure 6. Comparison of split marker versus an intact marker screening. (A) Vector design. The same seven DNA fragments shown in Figure 3 were assembled using a different plasmid backbone, pREC-1, which contains an intact selectable marker, *URA3*, and the N-terminus of a split marker (LEU2-N). The C-terminus of the split marker (LEU2-C), which was generated by high fidelity PCR, was co-transformed with the linearized plasmid and the seven DNA fragments to be assembled into yeast. Equal amounts of the transformation mix were plated onto SD-Leu (B, left) and SD-Ura plates (C, left). (B) Split-marker-mediated screening is highly efficient. Sixty-one colonies were randomly picked from the plate using the spit marker as the selectable agent (B, left) and streaked in duplicate onto SD-Ura (B, middle) and SD-Leu (B, right) plates. All sixty-one grew on both SD-Leu and SD-Ura plates, thus, were likely true positives. (C) Most of the colonies containing the intact selectable marker were false positives. Seventy-six colonies were picked from the plate using the intact marker as the selectable agent (C, left) and streaked in duplicate onto SD-Ura (C, middle) and SD-Leu (B, right) plates. Although all seventy-six grew on the SD-Ura plate, only five of them grew on the SD-Leu plate and were thus true positives. This demonstrates that SMC greatly reduces background clones.

DNA fragments with 100% efficiency. However, the efficiency dropped to only 20% when eight pieces were assembled (13). According to the observation of Hua *et al* (12), one would expect a more than 10% decrease in efficiency with a 30 bp overlap than that with a 50 bp overlap, even with two-fragment cloning. When we used raw PCR products with 30 bp overlaps, the efficiency to clone eight-pieces is 6.6% (5 out of 76 clones, Figure 6C). Amazingly, with split marker, this efficiency is increased to ~100% (61 out of 61 clones, Figure 6B). This demonstrates that the SMC has dramatically increased the power of yeast homologous cloning. Furthermore, no customized optimization is needed and raw PCR and/or restriction enzyme digestion products can be used directly.

Two things are worth noting when using the SMC method. Firstly, as very short regions of homology are sufficient for the recombination, manipulating

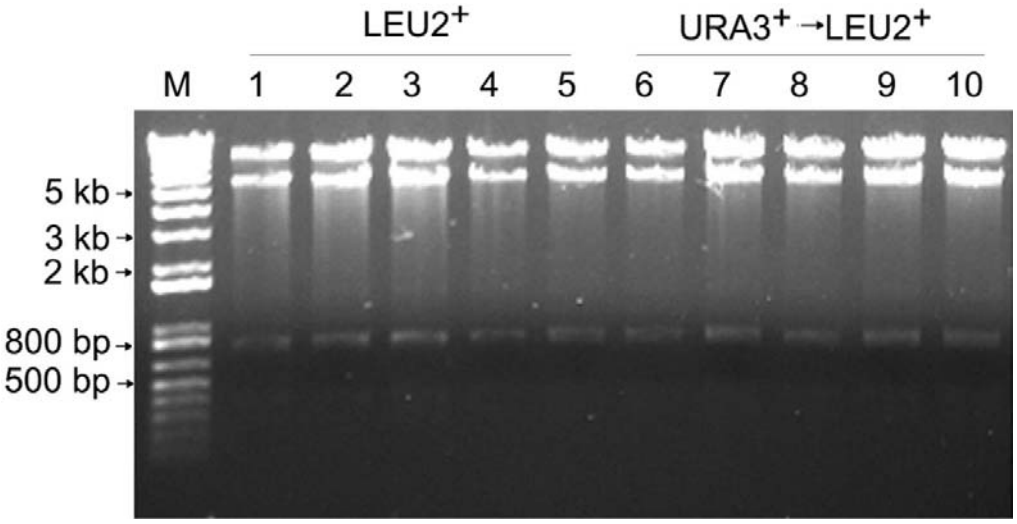

Figure 7. Restriction enzyme digestion profiles of the resulting pTS-GAL4-UAS-attB1'. Lanes 1 to 5: *Hind* III digestion profiles of the resulting pTS-GAL4-UAS-attB1' from five randomly-chosen clones that grew on SD-Leu plate. Lanes 6 to 10: *Hind* III digestion profiles of the resulting pTS-GAL4-UAS-attB1' from the five clones that grew on both SD-Ura and SD-Leu plates.

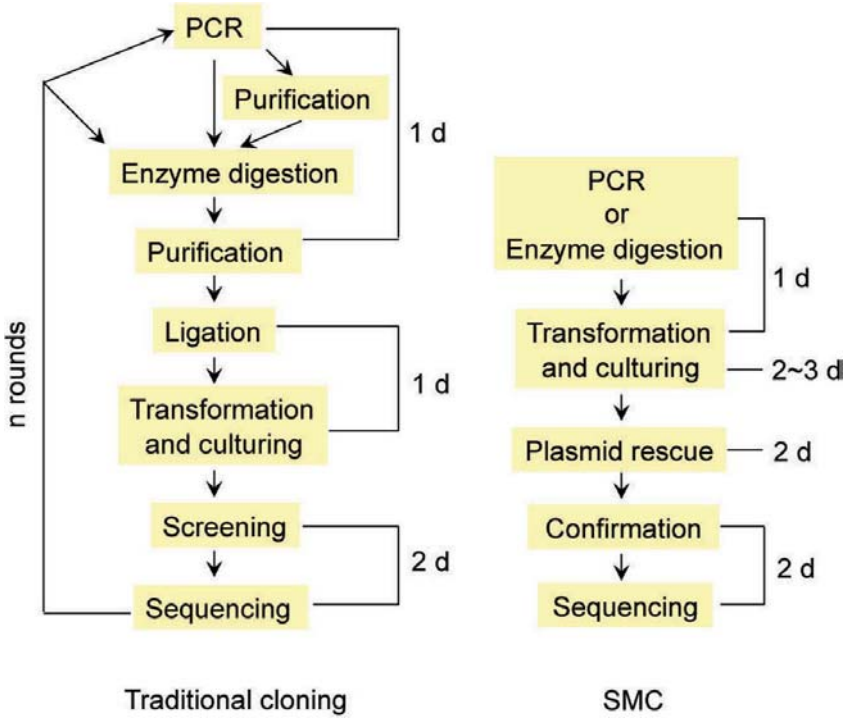

Figure 8. Comparison of traditional cloning method and SMC. With traditional restriction-enzyme-digestion-ligation cloning method, it takes about four days to clone one DNA fragment. To clone multiple pieces, it needs at least several cycles of four-days' cycling. The traditional cloning method is also limited by the availability of restriction enzymes. In addition, the introducing of restriction enzymes sites between DNAs will result in unnecessary, extra bases. On the other hand, SMC clones multi-piece DNAs in one cycle, no need of restriction enzyme sites, no purification, no customized optimization.

fragments sharing the same or highly homologous regions can be problematic. This can be addressed by a combination of SMC and the traditional gene-cloning method. For example, to clone pTS-GAL4-UAS-attB2 (Figure 9), which contains two attB sites, we first generated pTS-GAL4-UAS-attB1 using SMC as described above. Subsequently, the second attB containing fragment, SD-attB, was inserted by restriction enzyme digestion and ligation. Secondly, the recombinant products may occasionally acquire mutations because the PCR

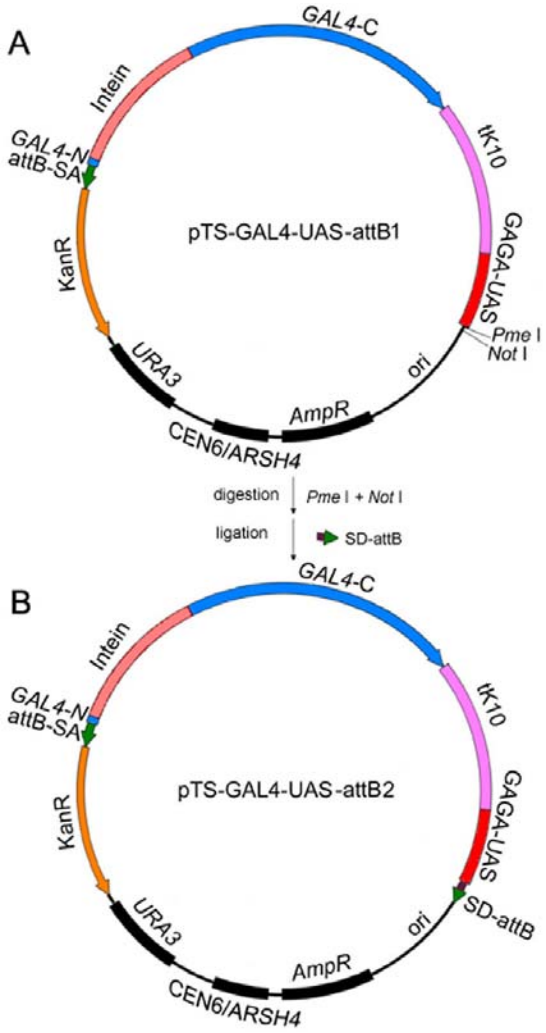

Figure 9. Cloning of pTS-GAL4-UAS-attB2. (A) The map of pTS-GAL4-UAS-attB1, showed two restriction enzymes site, *Pme* I and *Not* I. (B) The map of pTS-GAL4-UAS-attB2. By restriction enzyme digestion and ligation, SD-attB was introduced.

polymerase is not completely error-free. This problem can be greatly decreased by using high fidelity DNA polymerase. We used Phusion™ Hot Start High-Fidelity DNA Polymerase (New England Biolabs) to amplify our DNA fragments. No point mutations were introduced during cloning, even though we re-amplified the intein fragment.

6. ACKNOWLEDGEMENTS

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