Recombination-mediated PCR-directed plasmid construction *in vivo* in yeast

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ABSTRACT

We have extended the technique of PCR-directed recombination in *Saccharomyces cerevisiae* to develop a simple method for plasmid or gene construction in the absence of suitable restriction sites. The DNA to be cloned is PCR-amplified with 30–40 bp of homology to a linearized yeast plasmid. Co-transformation into yeast results in homologous recombination at a position directed by the PCR oligonucleotides.

The observation that linear DNA fragments can efficiently stimulate recombination in *Saccharomyces cerevisiae* (1) has led to the rapid development of powerful methods for DNA manipulation in yeast (2). Widely used recombination-based DNA manipulation methods include integrative DNA transformation, which is used to produce a chromosomal null allele of a gene of interest (3), and allele rescue which involves the transplacement of a mutation from the chromosome onto a plasmid-borne copy of that gene (1). A third use for recombination-based DNA manipulation in yeast is for straightforward cloning purposes. In this case, a restriction fragment whose ends bear homology to plasmid sequences can be directly 'ligated' into a linearized vector by *in vivo* recombination, alleviating the need for an *in vitro* ligation reaction (4). The three recombinational gene manipulation methods described above rely on sufficient homology at the cut ends of a restriction fragment or plasmid to engage the recombination machinery. Until recently, it was assumed that the amount of homology necessary to mediate such events was significant (>100 bp, but see below).

An important recent advance in the use of recombination-based methods for gene manipulation in yeast involves the use of PCR, rather than restriction enzymes, to generate the DNA fragment to be manipulated. Baudin (5) and Weber (6) demonstrated that the amount of homology necessary to promote efficient recombination-mediated gene disruption in yeast is actually quite small (in the order of 30–50 bp), indeed small enough to be synthesized as part of a PCR primer. Schiestl and co-workers (7) systematically examined the length of homology necessary to mediate such events and found that as few as 15 bp was sufficient to mediate homologous integration.

We have found that the recombination-mediated PCR targeting method can also be effectively used to (i) clone a desired gene onto a plasmid, and (ii) to generate chimeric genes with precise fusion junctions. We systematically examined the amount of homology needed to efficiently carry out such PCR fragment-directed homology-mediated cloning experiments. The general procedure is outlined in Figure 1. Basically, a linearized target plasmid containing a selectable marker (e.g. *URA3*) is co-transformed with a PCR fragment. By homologous recombination a subset of the cut plasmids are recircularized and simultaneously acquire a DNA segment of interest; recombinants are selected as Ura+ transformants.

In order to evaluate the use of PCR-directed homologous recombination for gene construction, and to compare it with direct ligation and transformation into yeast, a two-color reporter system was developed. When yeast cells express the *xylE* gene (8) from *Pseudomonas putida*, which encodes catechol 2,3 dioxygenase (C23D), they convert the colorless membrane-permeable substrate catechol into a bright yellow membrane-impermeable product, 2-hydroxymuconic semialdehyde. A second reporter, green fluorescent protein (GFP) fluoresces upon exposure to 365 nm light. The starting plasmid for this work was pYCAT1, which contains the *xylE* gene expressed from the yeast *GAL1* promoter in the 2μ plasmid pYES2 (Invitrogen). *GFP* was obtained by PCR from pBAD-GFP (9). The PCR primers added either 20, 30 or 40 nt of homology to regions 5′ and 3′ of *xylE*, such that homologous recombination would generate a precise replacement of the *xylE* coding sequence with that of *GFP*, as shown in Figure 1. The parental plasmid, pYCAT1, was digested with *Sal*I at a unique restriction site located near the middle of *xylE*. Yeast were co-transformed with the PCR fragments plus the *Sal*I-digested plasmid, or uncut plasmid as a control. As a comparison, GFP was also ligated upstream of *xylE* by conventional cloning methods (i.e. restriction digestion, followed by T4 DNA ligase), followed by direct transformation of the ligation mix into yeast. The desired class of transformants, in which *GFP* has replaced *xylE* via recombination, will be colorless and capable of fluorescence. Table 1 shows the results of the recombination and ligation experiments.

Table 1 shows that recombination-mediated PCR cloning requires that the plasmid be linearized, as no recombinants were obtained from uncut plasmid (frequency <0.25%; see Table 1 legend for experimental details). The length of the homologous region also has a significant effect on recombination frequency. The highest frequency of recombinants is obtained with at least 40 nt of homology, and a vector:insert ratio of 1:3. Under these

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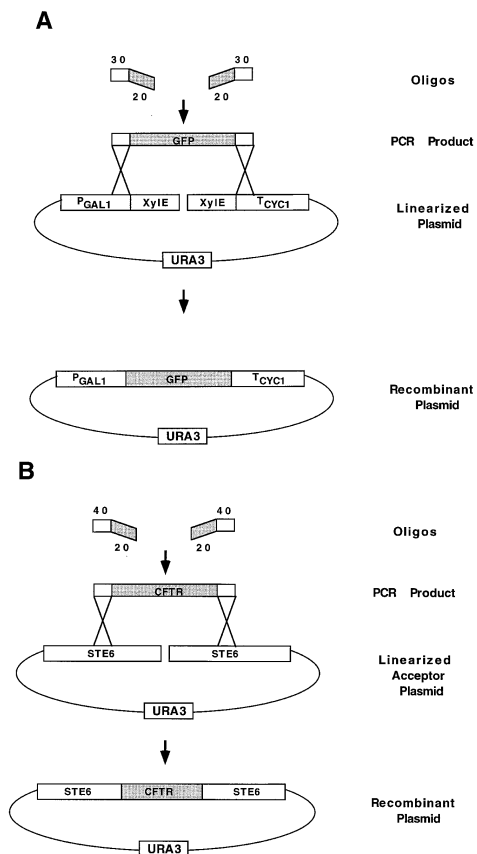[+] Present address: Dupont Merck, Experimental Station E-400/5207, Wilmington, DE 19880-0400, USA

A

B

Figure 1. Schematic representation of micro-homology mediated PCR cloning for plasmid construction. (**A**) Precise replacement of one gene by another. The PCR product encoding *GFP* at the top has 30–40 nt of homology at each end to the region of the plasmid at which crossing-over is to occur, which in this case are the promoter and terminator regions of pYCAT1. Recombination results in replacement of the region between the homologous sequences with the region carried by the PCR fragment. In the model system used here, this results in the elimination of *xylE* and its replacement by *GFP*. For details, see Table 1. (**B**) An example of chimera construction. The first nucleotide-binding domain (NBD1) of yeast STE6 was replaced with NBD1 of the human cystic fibrosis transmembrane conductance regulator (CFTR) to generate an in-frame fusion encoding a *STE6/CFTR* protein chimera (10). *STE6* was digested at a unique restriction site (*Pml*I) which lies within NBD1. The positioning of the in-frame fusion was dictated by the 40 bp *STE6* homology region at the ends of the PCR fragment.

conditions 69% of all transformant colonies expressed GFP. It should also be noted that while 40 nt of homology produced optimal results, 30 nt resulted in only a modest reduction of recombinants, and even 20 nt of homology is sufficient to generate the desired product, at least under certain conditions (vector:insert 1:6). By comparison, direct ligation resulted in over 90% of all colonies expressing GFP, although this method requires the presence of suitable restriction sites.

Homologous recombination is a convenient cloning alternative when suitable restriction sites are not available. Our results show that a 20–40 bp region of homology at the desired crossover region and a unique restriction site within the region to be deleted are the sole requirements. This method allows for the replacement of entire genes (above) or small regions within a single gene to form chimeric genes. In particular, the capacity to encode precise fusion junctions within a PCR oligonucleotide primer allows one to produce chimeric genes with specific joints. An example of the latter application is shown in Figure 1B, where an in-frame substitution was made in the yeast *STE6* gene (10). While this example used substitution within a yeast gene, any gene could be used with the procedures described above, following subcloning into a yeast shuttle vector; thus the method has general utility for tailor-made gene construction. In the absence of selection, or a phenotypic marker, recombinants can be tested by yeast colony PCR (11) or by extraction of plasmid from pooled clones, transformation into *Escherichia coli* and bacterial colony PCR (10). It should be noted that the inserted DNA may contain PCR-generated mutations. To minimize the frequency of errors it is important to include (or solely use) a high-fidelity polymerase such as Pfu, and to use the minimum possible number of PCR cycles.

Table 1. Percentage of yeast colonies expressing GFP following ligation or recombination

| Vector:Insert | Ligation | Recombination | | | |
| | | Linearized vector | | | Uncut |
		40 nt	30 nt	20 nt	40 nt
1:0	0	0	–	–	0
1:1	41	61	33	0	0
1:3	82	69	54	0	0
1:6	93	66	48	12	0

Of the remaining colonies the majority express CAT. A low percentage of colonies express neither, possibly reflecting aberrant re-circularization of the cut plasmid or PCR errors. The size of the homology region for recombination into either linearized or uncut vector is shown. pYCAT1 was linearized with *Sal*I. *GFP* was amplified by PCR (92°C, 30 min; 50°C, 30 min; 72°C, 1 min, 30 cycles using 5 U *Taq* polymerase and 0.125 U *Pfu* polymerase, 100 μl reaction) with either 20, 30 or 40 nt of homology to the regions immediately upstream and downstream of *xylE*. The 40 nt homology primers were GCGGCCGCTCTAGAGGATCCAGATCTGGTACCGC-AAGCTTatggctagcaaaggagaagaact (upstream) and GTGACATAACTAATTACA-TGATGCGGCCCTCTAGGAGCTCttatttgtagagctcatccatgccat (downstream), with the regions of homology to pYCAT1 shown in capitals. The 30 nt homology primer lacked the first 10 bases in both primers, and the 20 nt primer lacked the first 20 nt. Linearized plasmid and PCR-amplified gel-purified *GFP* were co-transformed into yeast in the ratios shown using 100 ng of plasmid. *S.cerevisiae* INVSC1 (Invitrogen) was grown in YP-galactose prior to transformation, then plated directly onto SC medium containing galactose and lacking uracil (3) to select for transformants and induce expression from the *GAL1* promoter. After colonies had formed (50–400 colonies per transformation; for instance 40 nt homology gave 200 colonies at 1:1 cut plasmid:insert, and 378 colonies at 1:6) GFP and C23D were assayed as described above. Transformation frequency with uncut pYCAT1 was 8800 colonies/μg DNA.

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