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PCR-Synthesis of Marker Cassettes with Long Flanking Homology Regions for Gene Disruptions in *S. cerevisiae*

ACHIM WACH

Institut für Angewandte Mikrobiologie, Biozentrum, Universität Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland

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A PCR-method for fast production of disruption cassettes is introduced, that allows the addition of long flanking homology regions of several hundred base pairs (LFH-PCR) to a marker module. Such a disruption cassette was made by linking two PCR fragments produced from genomic DNA to *kanMX6*, a modification of dominant resistance marker making *S. cerevisiae* resistant to geneticin (G418). In a first step, two several hundred base pairs long DNA fragments from the 5′- and 3′-region of a *S. cerevisiae* gene were amplified in such a way that 26 base pairs extensions homologous to the *kanMX6* marker were added to one of their end. In a second step, one strand of each of these molecules then served as a long primer in a PCR using *kanMX6* as template. When such a LFH-PCR-generated disruption cassette was used instead of a PCR-made disruption cassette flanked by short homology regions, transformation efficiencies were increased by at least a factor of thirty. This modification will therefore also help to apply PCR-mediated gene manipulations to strains with decreased transformability and/or unpredictable sequence deviations.

KEY WORDS — Yeast genome analysis; Functional analysis; G418 resistance; PCR-based gene disruption

INTRODUCTION

PCR-generated DNA molecules consisting of a marker cassette flanked by short homology regions to the target locus (Baudin *et al.*, 1993) are now used in several laboratories for directed gene alterations in *S. cerevisiae*. Systematic studies on the length of the flanking regions added to the marker have shown that 30 bp of homology at each side are sufficient for successful targeting (Manivasakam *et al.*, 1995). The use of direct repeat-containing oligonucleotides for PCR-amplification of the heterologous *Kluyveromyces lactis URA3* marker cassette has been reported and it was shown that such a construct can be excised from the genome by mitotic recombination (Laengle-Ronault & Jacobs, 1995). Problems with high background of false positive transformants (marker reversion) using auxotrophic markers were solved when *kanMX*, a completely heterologous dominant resistance marker, was used as selector module (Wach *et al.*, 1994). These *kanMX* modules are hybrids of the coding sequence of the *kan*^{r} gene of transposon *Tn*903 coding for aminoglycoside phosphotransferase (Oka *et al.*, 1981) and transcriptional and translational control sequences from the *TEF* gene of the filamentous fungus *Ashbya gossypii* (Steiner, 1991; Steiner & Philippsen, 1994). Aminoglycoside phosphotransferase activity renders *S. cerevisiae* resistant to the drug geneticin (G418) (Jimenez & Davies, 1980). Due to the heterology of the *kanMX* selector module, correct disruptions of *S. cerevisiae* genes with PCR-generated *kanMX* molecules flanked by 35 bp of yeast homologous DNA at either side, occur with a very low level of illegitimate integrations (Wach *et al.*, 1994).

Successful targeting of these PCR-products is dependent on the perfect homology within a small region at both ends of the transforming DNA to

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Table 1. Sequences of the deoxy-oligonucleotides used in this study.

Oligonucleotide	Sequence	Restriction site(s)
1	5′-GCCAGATCTGTTTAGCTTGCC-3′	*Bgl* II
2	5′-GCGGTTTAAACTGGATGGCGGCGTTAGTATC-3	*Pme*I
3	5′-CGGCAGCTGCACACAACAGCGTCAAAAATTGATTAAAAGGTAAGTTATCATGCGTACGCTGCAGGTCGAC-3′	*Pvu*II, *Sun*I, *Pst*I, *Sal*I
4	5′-GGCACTAGTTATGTATACAACAAATCTGCGTATACAAATGGCACATTTCTCACATCGATGAATTCGAGCTCG-3′	*Spe*I, *Cla*I, *Eco*RI, *Sac*I
$P_{5'\text{-}720}$	5′-GCCGTACGGAGTTACAAGACCATTTC-3′	*Sun*I
$P_{5'\text{-}400}$	5′-AGTGCGGCCGCGTAGGACATTGATTTCCTG-3′	*Not* I
$P_{5'L}$	5′-GGGGATCCGTCGACCTGCAGCGTACGCATGATAACTTACCTTTTAATCAATTTTTG-3′	*Bam*HI, *Sal*I, *Pst*I, *Sun*I
$P_{3'L}$	5′-GTTTAAACGAGCTCGAATTCATCGATTGAGAAATGTGCCATTTGTATACGCAGATTTG-3′	*Pme*I, *Sac*I, *Eco*RI, *Cla*I
$P_{3'}$	5′-AAACCTGGTAATCAGTGGCTCCTTG-3′	*Pme*I half site
10	5′-GCGCGCCATGATTTCGCATCATGTT-3′	*Bss*HII
11	5′-CCTCGACATCATCTGCCC-3′	
12	5′-GGGGCAAAGCGTGTACCAC-3′	

Underlined sequences in oligonucleotides mark those parts of the sequence that are complementary to the pFA6-MCS in plasmid pFA6-kanMX6.

the target locus. Thus, sequence polymorphism in different strain backgrounds will restrain homologous recombination when PCR-made molecules with small homology regions are used. Elongation of the homology region flanking the marker module to several hundred base pairs would solve this problem and an easy method to obtain such DNA molecules would be advantageous for fast and high-throughput gene disruptions.

In this paper a PCR-technique to generate selector modules for yeast transformation with long flanking sequences homologous to the yeast genome is reported together with a modified *kanMX* selector module and an updated transformation and selection procedure to obtain G418-resistant *S. cerevisiae.*

MATERIALS AND METHODS

Strains and Media. The *E. coli* strain XL1-blue (Bullock *et al.*, 1987) served as plasmid host. For selective growth, the bacteria were grown either on 16 g bacto-tryptone, 10 g yeast extract, and 5 g NaCl (2 × YT) containing 100 mg/l ampicillin or 50 mg/l kanamycin (Fluka AG, Buchs, Switzerland). As *S. cerevisiae* strain, FY1679 (Winston *et al.*, 1995) (*MAT* **a**/α; *ura3-52/ura3-52; trp1-Δ63/+; leu2Δ1/+; his3Δ200/+)* was used. *S. cerevisiae* was grown on 2% yeast extract, 1% peptone, and 2% glucose (YPD) (Treco, 1989). Solid media contained, in addition, 2% agar (Difco Laboratories). G418 resistant strains were grown on YPD plates containing 200 mg/l of G418 (geneticin, Gibco BRL, Gaithersburg, MD). Routine *E. coli* and DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989).

Thermostable DNA polymerases. Thermostable DNA polymerase from *Thermus brockianus* (DyNAzyme II) was purchased from Finnzymes Oy, Espoo, Finland. Thermostable DNA polymerase from *Thermococcus litoralis* (Vent DNA polymerase) was obtained from New England Biolabs, Beverly, MA, U.S.A. *Taq* DNA polymerase (*Thermus aquaticus*) was from Boehringer, Mannheim, Germany).

Construction of pFA6-kanMX6 plasmid. The *kanMX4* selector module was amplified by PCR using pFA6-kanMX4 (Wach *et al.*, 1994) as template and oligonucleotides 1 and 2 (Table 1) as primers. The 1·4 kb PCR product was then treated with phenol/chloroform, ethanol precipitated, and digested with *Bgl*II and *Pme*I. This fragment was isolated by agaraose gel electrophoresis and ligated into pFA6a, also cut with *Bgl*II and *Pme*I, yielding plasmid pFA6-kanMX6. This procedure created a *kanMX*

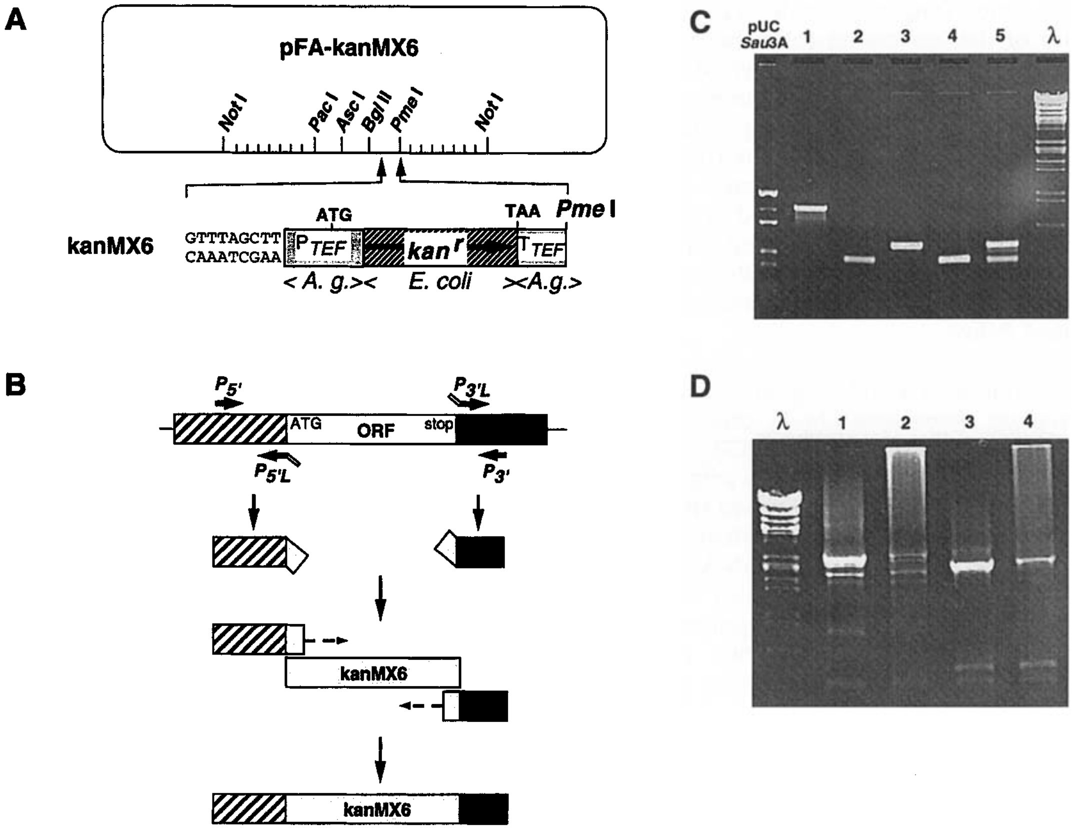

Figure 1. Two-step PCR-synthesis of disruption cassettes with long flanking homology regions. (A) Map of pFA-kanMX6. P_{TEF}, T_{TEF} indicates promotor terminator of the *Ashbya gossypii (A.g.) TEF* gene. Only relevant restriction enzyme sites are labeled. A detailed description of the pFA polylinker and the sequence of the *kanMX* module can be found elsewhere (Wach *et al.*, 1994). (B) Schematic view of the two-step PCR-synthesis of the DNA molecules used for transformation of *S. cerevisiae*. Four primers were designed that bind either to the 5′-region of the target gene ($P_{5'}$), directly upstream of its start codon ($P_{5'L}$), immediately downstream of its stop codon ($P_{3'L}$), or in the 3′-region of the target gene ($P_{3'}$). In the first PCR the primer pair $P_{5'}$-$P_{5'L}$ ($P_{3'L}$-$P_{3'}$) amplifies the DNA region directly upstream (downstream) of the target gene's coding region yielding fragment 1 (fragment 2). Primer $P_{5'L}$ ($P_{3'L}$) was designed in such a way that 26 bases extensions homologous to the 5′-(3′-) side of the marker gene (open boxes in primer symbols) were added to the 3′-(5′-) end of fragment 1 (fragment 2). In the subsequent PCR, fragment 1 and 2 are used as primers and marker DNA as template. (C and D) Ethidium bromide-stained agarose gels with *Hin*dIII/*Hin*dIII, *Eco*RI fragments of λ-DNA and pUC19, *Sau*3AI fragments as marker. For analysis of synthesized PCR products, 5 μl aliquots out of 50 μl total volume were loaded on the gel. (C) Fragments synthesized in the first PCR with genomic DNA as template and following primer combinations (lane 1: $P_{5'-720}$ and $P_{5'L}$; lane 2 and 4: $P_{3'L}$ and $P_{3'}$; lane 3: $P_{5'-400}$ and $P_{5'L}$; lane 5: $P_{5'-400}$ and $P_{5'L}$ together with $P_{3'L}$ and $P_{3'}$). (D) Fragments obtained in the second PCR with the PCR fragments produced in the first reaction (lane 1 and 2: 720 bp 5′- and 300 bp 3′-fragment as primers; lane 3 and 4: 400 bp 5′- and 300 bp 3′-fragment as primers) and different quantities of *kanMX6* template (lane 1: 0·1 μg; lane 2: 0·5 μg; lane 3: 0·1 μg; lane 4: 0·5 μg).

module in which the five nucleotides (GTCGA) from the former *Sal* I site were replaced by the hexa-nucleotide TTAAAC creating a *Pme*I site immediately downstream of the selector module (see Fig. 1A).

PCR-synthesis of kanMX6 DNA with short flanking regions homologous to S. cerevisiae *genomic DNA (SFH-PCR).* A 1·5 kb PCR fragment was generated by using pFA6-kanMX6 as template and two primers (Table 1, oligonucleotides 3 and 4) with 18-19 nucleotide homology to the multiple cloning site (MCS) of pFA6-kanMX6 (underlined sequences in Table 1). These primers have 45 additional nucleotides which were either homologous to the region immediately upstream of the second codon (oligonucleotide 3) or to that downstream of the last codon (oligonucleotide 4) of a 456 codon ORF (ORF456) on yeast chromosome XIV (A. Düsterhöft, personal communication). To facilitate PCR product cloning, either a *Pvu*II- (oligonucleotide 3) or a *Spe*I site (oligonucleotide 4) were introduced at the 5′-ends of the oligonucleotides. PCR was performed in 50 µl reaction volume with 500 ng of template DNA. Other conditions were as described (Wach *et al.*, 1994). PCR-products were purified and quantified as outlined below.

PCR-synthesis of DNA fragments with long flanking regions homologous to S. cerevisiae *genomic DNA (LFH-PCR).* In this PCR a mixture of thermostable DNA polymerases with and without 3′–5′ exonuclease activity was used (Barnes, 1994). Good results were obtained when per reaction 1 U of exonuclease-free DNA polymerase (DyNAzyme or *Taq* polymerase) was used together with 0·1–0·4 U of DNA polymerase exhibiting 3′–5′ exonuclease activity (Vent DNA polymerase). The sequences of the four primers used here were derived (i) from the DNA sequence 720 bp or 400 bp upstream of the start codon ($P_{5'\text{-}720}$ and $P_{5'\text{-}400}$ primers), (ii) from the DNA sequence 300 bp downstream of the stop codon ($P_{3'}$ primer), (iii) from the DNA sequence immediately upstream of the second codon ($P_{5'L}$ primer), and (iv) from the DNA sequence immediately downstream of the last codon ($P_{3'L}$ primer) of ORF456 (see Fig. 1B for schematical depiction of primer binding sites).

(A) First PCR: Approx. 50–100 ng of template DNA were used and either two separate PCRs with one primer pair in each reaction or one PCR with two primer pairs per reaction were made. The final concentration of each primer was 1 µM in 50 µl standard reaction mix supplemented with 0·2 mM of dATP, dCTP, dGTP, and dTTP. The mixture was overlayed with light mineral oil and PCR executed under following conditions: 30 cycles of 30 sec at 92°C, 30 sec at 55°C, and 45 sec at 72°C. In these PCRs the following fragments from the 5′- and 3′-region of ORF456 were generated (see Table 1 for primer sequences): (i) a 720 bp fragment (5′-region) with primer combination $P_{5'\text{-}720}$ and $P_{5'L}$, (ii) a 400 bp fragment (5′-region) using primers $P_{5'\text{-}400}$ and $P_{5'L}$, (iii) a 300 bp DNA fragment (3′-region) when $P_{3'L}$ and $P_{3'}$ were used as primers, and (iv) when all four primers were used in a single reaction ($P_{5'\text{-}400}$ and $P_{5'L}$ together with $P_{3'L}$ and $P_{3'}$) a 400 bp fragment (5′-region) and a 300 bp DNA fragment (3′-region) were synthesized simultaneously. PCR-products were quantified by analyzing 5 µl of the PCR-mix by agarose gel electrophoresis.

(B) Second PCR: Here variable amounts of *Not* I digested pFA6-kanMX6 (0·01–0·5 µg) were used as template and 300–400 ng of each product from the first PCR together with 1 µM of each of the outermost 5′-($P_{5'\text{-}720}$ or $P_{5'\text{-}400}$) and 3′-primers ($P_{3'}$) (see Fig. 1B and Table 1). The reaction mix was overlayed with light mineral oil, heated to 92°C for 120 sec, and then 1·0+0·4 U of DNA polymerase mix were added (hot-start). The reaction was performed for 25 cycles of 1 min at 92°C, 30 sec at 50°C, and 120 sec at 72°C. For final polishing of the product, the mix was incubated for 4 min at 72°C. PCR-products were purified and quantified as described below.

Purification, concentration, and quantitation of PCR products. All PCR products were phenol/chloroform extracted and subsequently ethanol-precipitated in the presence of 0·3 M Na-Acetate. Resulting DNA pellets were washed with 70% ethanol (v/v), dried for 10 min and then resuspended in 10 µl deionized water. The amount of DNA was quantified by analyzing 1 µl sample by standard agarose gel electrophoresis.

Transformation of Yeast. 0·5–5 µg of PCR-synthesized DNA molecules were used to transform LiAc-treated cells (Schiestl & Gietz, 1989; Gietz & Woods, 1994). As additional step, transformed cells were resuspended in 2 ml YPD and pre-incubated for 3–4 hours at 30°C. Subsequently cells were collected by centrifugation, resuspended in 1·1 ml deionized water and plated in 350 µl aliquots on YPD-G418 (200 mg/l).

Cloning of the PCR products. One µl of the concentrated PCR product solution was ligated into agarose gel-purified pFA6a digested with *Pvu*II and *Eco*RV. During ligation 1 U of T4 polynucleotide kinase was added and ligation was performed at 23°C for 12 hours. Transformed *E. coli* cells were selected on 2 × YT-kan plates. Recombinant plasmids were purified and analyzed by restriction enzyme mapping.

Verification of G418^r transformants by PCR was performed analogous to the method introduced by Huxley *et al.* (1990) as described (Wach *et al.*, 1994) in a PCR with yeast cells and three primers per reaction (Table 1, oligonucleotides 10, 11, and 12).

RESULTS

Construction of pFA6-kanMX6

A slightly modified *kanMX* module, *kanMX6*, was constructed (see Materials and Methods for details). This dominant selection marker is flanked by eight-base restriction enzyme recognition sites (see Fig. 1A) in pFA vectors allowing convenient replacement or excision of marker modules in cloned disruption cassettes.

Two-step PCR to synthesize long flanking homology regions to kanMX6

The synthesis of enlarged homology regions flanking the new *kanMX6* marker module was achieved by two-step PCR (Fig. 1B). In a first round of PCR two fragments of yeast genomic DNA were amplified carrying 26 bases of homology to the 5′- or 3′-side of the marker module which were introduced by two of the four primers used in this reaction (Table 1, oligonucleotides $P_{5'L}$ and $P_{3'L}$, underlined sequences). The PCR products were either produced in two separate reactions, so that adjustments of the primer concentration for the following PCR was feasible, or simultaneously in a single reaction. As template for this first PCR either a plasmid containing a 8 kb *Eco*RI fragment of genomic DNA, a cosmid with 41 kb genomic DNA insert, or total yeast genomic DNA were used. The sizes of the generated PCR fragments observed by agarose gel electrophoresis (Fig. 1C) were in good agreement with the theoretical sizes of the fragments predicted from the primer binding sites on the chromosomal DNA, *i.e.* 720 bp ($P_{5'\text{-}720}$, $P_{5'L}$ primer pair) or 400 bp ($P_{5'\text{-}400}$, $P_{5'L}$ primer pair) for the 5′-region of ORF456 and 300 bp ($P_{3'L}$, $P_{3'}$ primer pair) for its 3′-region.

In the second PCR, these products were linked to the *kanMX6* marker due to the presence of the 26 bp overlapping regions of homology between each of the PCR products from the first reaction with one or the other end of the marker DNA template. Best results were obtained when 200–300 ng of each product from the first PCR were used. When higher amounts of these fragments were taken only a minor quantity of correct final PCR product formed. It was essential that all PCRs were performed in the presence of a thermostable DNA polymerase with 3′–5′ exonuclease activity to allow for cleavage of unspecific single nucleotide 3′-overhangs from the PCR products. Suitable amounts of the 2·4 kb and 2·1 kb disruption cassettes were obtained in the second PCR (Fig. 1D), the size of which being in agreement with the expected length of a molecule consisting of joined 5′-fragment (720 bp or 400 bp), *kanMX6* (1400 bp), and 3′-fragment (300 bp).

As seen in Fig. 1D, the amount of the marker fragment used in the second PCR was critical. Obviously, competition between the complementary strands of the PCR product from the first reaction and the complementary region on the marker fragment was overcome only when the ratio of the different components was well balanced. It also helped, when the two outside primers ($P_{5'}$ and $P_{3'}$, Fig. 1B) were included in the second PCR. Under such conditions those DNA strands that were compatible with the marker fragment were also amplified.

S. cerevisiae *transformation with* kanMX6 *flanked by long homology regions*

The efficiency of transformation of *S. cerevisiae* cells with PCR-made disruption cassettes carrying short flanking homology regions (SFH-PCR product) was compared to that of PCR-synthesized disruption cassettes with long flanking homology regions (LFH-PCR products). For this purpose, *kanMX6* disruption cassettes were made for deletion of the same target locus (ORF456) using both PCR techniques. The results of this comparison are listed in Table 2. An approximately 30- to 50-fold increase in transformation efficiency was observed when LFH-PCR products were used instead of SFH-PCR products. However, it should be mentioned that nine base pairs introduced by the primers at every end of the SFH-PCR disruption cassette were non-homologous to the target locus, because they carried restriction sites for convenient cloning of the SFH-PCR products. In our experience, additional nucleotides, non-homologous to the targeted gene at the 5′-end of the SFH-PCR products, reduce the efficiency of homologous recombination at the target locus.

Some modifications of the protocol used for selection of G418-resistant *S. cerevisiae* clones were necessary since the procedure originally described using G418-containing top-agar overlay (Wach *et al.*, 1994) did not give fully satisfactory results with the strain FY1679, a S288C derivative

Table 2. Comparison of transformation efficiencies.

PCR product used for transformation	Total number of transformants[1]	Transformants per µg of DNA
ORF456, SFH-PCR	17	3
ORF456, LFH-PCR (720 bp at 5′)[2]	167	86
ORF456, LFH-PCR (400 bp at 5′)[3]	231	138

[1] Three independent transformations were made with independently generated PCR products. Control transformation with a CEN-ARS plasmid carrying *kanMX* yielded 2×10^5 transformants per µg DNA.

[2] See Figure 1D, lane 1.

[3] See Figure 1D, lane 3.

(Winston *et al.*, 1995), of which the complete genomic sequence will soon be known and which will be used as reference strain for functional analysis. With this strain best efficiencies were obtained when LiAc treated cells (Schiestl and Gietz, 1989) were used for transformation followed by a 3–4 hours incubation in liquid YPD and then plating on selective medium. The 3–4 hours non-selective pre-incubation period in liquid YPD gave rise to the appearance of many micro-colonies which were not observed when transformed cells were immediately plated on YPD-G418 or in the wild type control. It can be speculated that sufficient amounts of aminoglucoside phosphotransferase to inactivate G418 during several rounds of cell divisions could be produced from the non-integrated DNA fragment during pre-incubation. However, positive transformants were clearly distinguishable by colony size after 48 hours of selective growth. Transformed cells can also be plated on YPD and then, after 12 hours of non-selective growth, replica-plated on YPD-G418 (J.H. Hegemann, personal communication) which lead to an increase in transformation efficiency by a factor of two to three.

DISCUSSION

A new method for PCR-synthesis of disruption cassettes (LFH-PCR) is introduced in which the length of the homology region flanking the marker is defined by the choice of the primer binding sites at the target locus. Different templates (plasmid insert, cosmid insert, genomic DNA) have been tested and it was demonstrated, that genomic DNA can be used as template to produce these homology regions simultaneously in a single reaction. In the second PCR the dominant resistance marker *kanMX6* was used as template. The synthesis of disruption cassettes in a single LFH-PCR, *i.e.* by using genomic DNA, *kanMX6* template, and all four primers in a single reaction, should be possible but a substantial contamination with wild type DNA, amplified from the genomic locus by the outermost primers, must be considered.

Any selectable marker that functions in *S. cerevisiae* can be utilized in the PCR-method described above. Although not tested here, it should be possible to synthesize DNA molecules with this PCR procedure that can be used to replace an integrated selectable marker from a genomic locus (Vidal & Gaber, 1994). It can also be envisaged, that complete marker removal may be conceivable by linking the two genomic fragments obtained in the first PCR via a DNA fragment containing only the two overlapping homology regions, *e.g.* the pFA polylinker in this case. Thus, in a scenario in which the *URA3* selectable marker is first used for a gene disruption followed by a second transformation with marker-free DNA (s.a.), transformants which have excised the marker from the genome could be selected by their ability to grow on 5-fluoroorotic acid.

In pFA6-kanMX6 the *kanMX* marker module is flanked by eight-base restriction enzyme recognition sites resulting in a full compatibility to previously constructed pFA vectors with reporter-marker double-modules (Wach *et al.*, 1994). In addition to convenient marker replacement, a LFH-PCR product that was cloned into a yeast centromeric vector can be useful for different types of experiments: (i) for future gene disruptions by using the excised DNA fragment containing the *kanMX6* marker together with the flanking homology regions for transformation, and (ii) for gap repair cloning of the wild type allele by transformation with a linear plasmid fragment from which the *kanMX6* marker but not the flanking homology regions have been removed.

Presently many laboratories have begun to investigate the disruption phenotypes of genes discovered by systematic sequencing of the *S. cerevisiae* genome. Comparable to disruptions with PCR-made markers flanked by short homology regions, the PCR technique presented here, should allow successful and high-throughput gene inactivations in probably any *S. cerevisiae* strain (including those of industrial importance). Also with well characterized *S. cerevisiae* laboratory strains longer homology regions flanking the marker in a disruption cassette might be advantageous, *e.g.* to avoid mis-integrations which occur with high frequency when homologous selection markers with small flanking homology regions are used (Baudin *et al.*, 1993). The PCR-method described here is faster and more versatile compared to conventional cloning techniques, when disruption cassettes with long flanking homology regions are required, and PCR-made disruption cassettes produced by this technique are most promising for gene modifications in many different *S. cerevisiae.*

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