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# A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast

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## ABSTRACT

**Heterologous markers are important tools required for the molecular dissection of gene function in many organisms, including *Saccharomyces cerevisiae*. Moreover, the presence of gene families and isoenzymes often makes it necessary to delete more than one gene. We recently introduced a new and efficient gene disruption cassette for repeated use in budding yeast, which combines the heterologous dominant *kan*$^r$ resistance marker with a Cre/*loxP*-mediated marker removal procedure. Here we describe an additional set of four completely heterologous *loxP*-flanked marker cassettes carrying the genes *URA3* and *LEU2* from *Kluyveromyces lactis*, *his5*+ from *Schizosaccharomyces pombe* and the dominant resistance marker *ble*$^r$ from the bacterial transposon Tn*5*, which confers resistance to the antibiotic phleomycin. All five *loxP*–marker gene–*loxP* gene disruption cassettes can be generated using the same pair of oligonucleotides and all can be used for gene disruption with high efficiency. For marker rescue we have created three additional Cre expression vectors carrying *HIS3*, *TRP1* or *ble*$^r$ as the yeast selection marker. The set of disruption cassettes and Cre expression plasmids described here represents a significant further development of the marker rescue system, which is ideally suited to functional analysis of the yeast genome.**

## INTRODUCTION

In 1996 the complete sequence of the genome of the budding yeast *Saccharomyces cerevisiae* was published (1) and functional analysis of the more than 6000 genes found in this organism entered a new phase. Subsequently new tools were developed to speed up this process. Gene disruption is one of the most powerful techniques available for the study of gene function, and a breakthrough was achieved when it became apparent that only very short sequences of yeast DNA on either side of a marker gene are needed for efficient integration into the yeast genome by homologous recombination. This permits the use of gene disruption cassettes created by PCR (2,3). Thus, for the first time, large-scale, systematic gene disruption projects became feasible (4,5). The next important step was the introduction of the completely heterologous *kan*$^r$ marker, which confers resistance to the antibiotic G418 (6). This dominant marker dispenses with the need for yeast strains that are auxotrophic for the markers normally used for gene disruption. Moreover, because the *kan*$^r$ gene (which is driven by the *Ashbya gossypii TEF2* promoter and terminated by the *A.gossypii TEF2* terminator) shows no homology to the yeast genome, recombination between the yeast genome and internal parts of the *kanMX* disruption cassette (which would result in misintegration) is minimal, thus maximising the frequency of correct integration. Consequently, the *kanMX* gene disruption cassette was chosen for large-scale gene disruption projects, as well as for use in the genome-wide gene knockout project (5,7,8).

In functional studies in *S.cerevisiae* it is often necessary to delete more than one gene. This is partly due to the fact that substantial portions of the yeast genome are duplicated (9,10). Moreover, as many cellular functions are maintained by more than one isoenzyme, it is often necessary to create multiple gene disruptions to uncover gene function (11–13). One prominent example is the hexose transporter family: in this case, concurrent knockout of at least 20 transporter genes was required to block uptake of hexose completely (14).

Marker rescue and reuse offers a convenient and efficient way to introduce multiple gene disruptions. Removal of a previously integrated disruption cassette is most easily achieved if the respective cassette is flanked by directly repeated sequences 400–500 bp in length. Mitotic recombination between the repeated sequences results in excision of the marker and leaves a single repeat in the genome (15). This principle has been used with a variety of marker genes (16–20). The main problems encountered with this approach are: (i) the low rate of mitotic recombination, which makes it necessary to screen for rare instances of marker loss (except in cases where

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+AF298780, AF298782, AF298785, AF298788–AF298790, AF298792–AF298794

counter-selection is possible, e.g. *URA3*; 21); (ii) the fact that each marker rescue event leaves an additional repeat sequence in the genome which serves as an unwanted recombination target for integration of the gene disruption cassette in the next round of gene deletion.

Recently we designed a new and efficient gene disruption cassette for repeated use by combining the advantages of the *kanMX* gene disruption cassette with those of the Cre/*loxP* recombination system of bacteriophage P1 (22). The *kanMX* cassette is flanked by two direct repeats of the 34 bp *loxP* sequence, yielding the *loxP–kanMX–loxP* cassette, which can be converted into a highly efficient gene disruption cassette by the addition of short DNA sequences homologous to the genomic locus of interest using PCR (Fig. 1). Once correctly integrated into the genome the *kanMX* marker can be efficiently rescued by transformation with a plasmid carrying the gene for Cre recombinase under control of the *GAL1* promoter and induction of Cre expression by shifting the cells to galactose-containing medium. Cre-induced recombination results in loss of the *kanMX* cassette, leaving behind a single *loxP* site at the original integration site (Fig. 1) (22). Since its introduction the *loxP–kanMX–loxP* cassette has been widely used (see for example 23–26). Many gene families have now been analysed in this way: for example, the above mentioned hexose transporter family (14) and a series of gene families of unknown function investigated within the framework of the EUROFAN project (27; J.H.Hegemann and U.Gueldener, in preparation). Moreover, a modified *loxP–kanMX–loxP* cassette has been used to control gene expression in a novel way. For this purpose the *loxP–kanMX–loxP* cassette is embedded in the *ACT1* intron and, after genomic integration of this 'maxi-intron' into the 5′-terminal region of the gene of interest, expression of this gene is completely inhibited. Gene expression is restored only after Cre-mediated site-specific recombination has deleted the *kanMX* unit and thus created a small spliceable intron carrying a single *loxP* site (28). In a different application the *loxP–kanMX–loxP* marker cassette was used to repeatedly add an epitope to different gene products at their C-termini (29). Finally the *loxP–kanMX–loxP* marker has been incorporated into an integration cassette that can be used to fuse GFP to any protein of interest (30; U.Gueldener and J.H.Hegemann, in preparation). Meanwhile the *loxP–kanMX–loxP*/Cre system has also been adapted successfully for use in the yeast *Kluyveromyces lactis* (31).

One limitation of the Cre/*loxP* system so far has been its restriction to the *kan*$^r$ marker gene. This has meant that the system could not be used in the background of the *kanMX* deletion strains created in the genome-wide knockout project (8). Furthermore, it would be interesting in certain cases to follow a deleted gene or genes in genetic crosses (by following the disruption cassette markers) before the markers are excised by inducing Cre expression.

In this paper we describe the construction of additional *loxP*–marker gene–*loxP* cassettes for repeated gene disruption. Most importantly we introduce a new dominant drug resistance cassette carrying the *ble*$^r$ gene from the bacterial transposon Tn*5*, which confers resistance to the antibiotic phleomycin (Phleo). Finally, construction of additional Cre expression plasmids carrying *HIS3*, *TRP1* or the dominant *ble*$^r$ marker gene allows a much wider application of this powerful marker rescue system and significantly increases the number of new markers of the Cre/*loxP* system (22,27,31), thus further enhancing the utility of the Cre/*loxP* system in functional genome research.

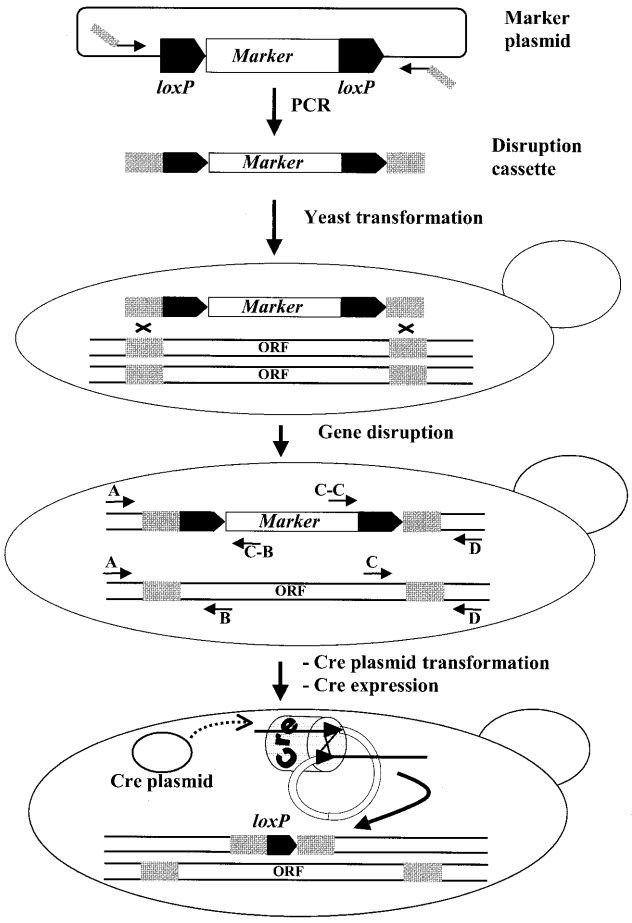


**Figure 1.** The *loxP*/Cre gene disruption and marker rescue procedure. The gene disruption cassette consists of a selection marker gene (marker), usually conferring drug resistance or prototrophy, flanked by two 34 bp *loxP* sequences as direct repeats located adjacent to 45 bp of sequence flanking the chromosomal target sequence (ORF) to be deleted. The disruption cassette is produced by PCR using oligonucleotides comprising 19 or 22 3′ nucleotides complementary to sequences in the template (marker plasmid) flanking the disruption cassette and 45 5′ nucleotides that anneal to sites upstream or downstream of the genomic target sequence to be deleted. After transformation of the linear disruption cassette into yeast cells, selected transformants are checked by PCR for correct integration of the cassette and concurrent deletion of the chromosomal target sequence. The verification PCRs are done using combinations of primers complementary to sequences within the cassette (C-B, cassette B primer; C-C, cassette C primer) and to sequences within or flanking the target sequence (A, B, C and D). In a diploid yeast cell (shown here) in which one allele of the target sequence has been replaced by a disruption cassette, all PCRs indicated will produce products of the expected size. Finally, expression of the Cre recombinase results in removal of the marker gene, leaving behind a single *loxP* site at the chromosomal locus.

## MATERIALS AND METHODS

### Strains and media

*Escherichia coli* strain XL1-Blue was obtained from Stratagene (Heidelberg, Germany). The yeast strain CEN.PK2-1C was used for *in vivo* recombination to generate plasmid pSH65 and

for gene disruption experiments. All culture media were prepared as described previously (32). For selection of G418 resistance after yeast transformations, cells were plated onto YPD plates containing 200 µg/ml G418 sulphate (Gibco BRL, Germany; batch 11811-031, activity may be batch-dependent) (dissolve in water and add to autoclaved YPD cooled to 60°C, to a final active concentration of 200 µg/ml) after an initial period of growth (2 h) in non-selective medium. Selection for phleomycin resistance was performed on YPD plates containing 7.5 µg/ml phleomycin (PHLEL0100; Cayla, France) (dissolve in water and add to autoclaved YPD cooled to 60°C). Systematic testing revealed that in this case preincubation in non-selective medium did not enhance transformation efficiency. For selection for the *kan*$^r$ gene in *E.coli*, bacteria were plated on YT plates containing 50 µg/ml kanamycin sulphate (M6750; Sigma, Germany) (6). *Pfu* polymerase (M7741) was obtained from Promega (Germany); *Taq* polymerase was prepared as described (33).

## Plasmid construction

*Positioning of new heterologous marker genes between two loxP sites.* Plasmid pUG6 carrying the *loxP–kanMX–loxP* module has been described previously (22). For construction of plasmid pUG27 (carrying *loxP–his5*$^+$*–loxP*) the *Bgl*II–*Sac*I DNA fragment from plasmid pFA6a-HIS3MX6 (18), carrying the *Schizosaccharomyces pombe his5*$^+$ ORF (which complements the *S.cerevisiae his3* mutation) plus the *A.gossypii TEF2* promoter and terminator, was cloned into *Bgl*II + *Sac*I-cleaved plasmid pUG6, replacing the *kanMX* unit between the two *loxP* sites. For construction of plasmid pUG66 (carrying *loxP–ble*$^r$*–loxP*) the vector pUT332, containing the *ble*$^r$ gene from transposon Tn*5* (34), was used as the template in a PCR with the oligonucleotide primers 1104 (5′-GCCGTAAGCCATGGCCGACCAAGCGACGCCCAAC-3′) and 1106 (5′-GGCGCCGGAGTACTGATCATGAGATGCCTGCAAGC-3′) (restriction sites underlined) to amplify the *ble*$^r$ open reading frame. The resulting PCR product was cut with *Nco*I and *Sca*I and ligated with *Nco*I + *Sca*I-cleaved pUG6, thus replacing the *kan*$^r$ coding sequence. For construction of plasmid pUG72 (carrying *loxP–URA3–loxP*; originally named pJJH726) the *K.lactis URA3* gene including the promoter and terminator was amplified from genomic DNA of the *K.lactis* type strain CBS2359 with oligonucleotides KlURA3-*Sac*I (5′-GGCGAGCTCGTTTTATTTAGGTTCTATCGAGG-3′) and KlURA3-*Bam*HI (5′-CCGCGGATCCCAATACAACAGATCACGTG-3′). The resulting PCR product of ~1.4 kb was cleaved with *Sac*I and *Bam*HI and ligated to *Bgl*II + *Sac*I-cleaved pUG6, thus replacing the complete *kanMX* unit. For construction of plasmid pUG73 (carrying *loxP–LEU2–loxP*; originally named pJJH727) the *K.lactis LEU2* gene plus promoter and terminator sequences was amplified by PCR using *K.lactis* genomic DNA and the oligonucleotides KlLEU2-*Sac*I (5′-GGCGAGCTCGCTGTGAAGATCCCAGCAAAGG-3′) and KlLEU2-*Bam*HI (5′-GGCGGGATCCGCAGGCTAACCGGAACCTG-3′). The PCR product of ~2.2 kb was digested with *Sac*I and *Bam*HI and ligated into *Bgl*II + *Sac*I-cut pUG6.

*Construction of new Cre expression plasmids.* Plasmid pSH47 (*GAL1-cre*, *URA3*) has been described previously (22). For construction of plasmid pSH62 (*GAL1-cre*, *HIS3*) a 1.4 kb *Eco*RI–*Xho*I DNA fragment of pSH47 carrying the *cre* open reading frame was ligated with *Eco*RI + *Xho*I-cleaved p413GAL1 (35). For construction of plasmid pSH63 (*GAL1-cre*, *TRP1*) the 1.4 kb *Eco*RI–*Xho*I DNA fragment of pSH47 carrying the *cre* gene was ligated to *Eco*RI + *Xho*I-cut p414GAL1 (35). For construction of plasmid pSH65 (*GAL1-cre*, *ble*) the vector pUT332, containing the *ble*$^r$ gene from transposon Tn*5* plus the *S.cerevisiae TEF1* promoter (34), was used as template in a PCR with the oligonucleotide primers 1095 (5′-AGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCGGCCGCGTTGTAAAACGACGGCCAG-3′) and 1096 (5′-AGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGCGTACACGCGTCTGTACAG-3′). At their 3′-ends the oligonucleotides (underlined) are homologous to plasmid pUT322, while the 5′-ends of the oligonucleotides are homologous to the sequences to the left and right of the *Kpn*I restriction site in pSH47. The ~1.0 kb PCR product carrying the *ble*$^r$ gene plus the *S.cerevisiae TEF1* promoter was transformed into yeast strain CEN.PK2 together with the *Kpn*I-cleaved plasmid pSH47, selecting for uracil prototrophy after homologous recombination of both DNA molecules in yeast. Genomic DNA from individual yeast transformants was isolated (32), transformed into *E.coli* and plasmid DNA was prepared. The correct plasmid, called pSH64, was identified by restriction enzyme and sequence analysis. Subsequently pSH64 was cut with *Nco*I (site located within the *URA3* coding sequence) and the ends were filled-in with *Pfu* polymerase and religated to inactivate the *URA3* gene, yielding pSH65.

The complete DNA sequences of all pUG and pSH plasmids have been deposited in GenBank and the corresponding accession numbers can be found in Figures 2 and 5.

## Plasmid requests

The vectors pUG6 (*kan*$^r$), pUG27 (*his5*$^+$), pUG66 (*ble*$^r$), pUG72 (*URA3*) and pUG73 (*LEU2*) carrying the various *loxP*–marker gene–*loxP* gene disruption cassettes as well as the Cre expression plasmids pSH47 (yeast selection marker *URA3*), pSH62 (*HIS3*), pSH63 (*TRP1*) and pSH65 (*ble*$^r$) can be obtained individually (pUG6, accession no. P30114; pUG27, accession no. P30115; pUG66, accession no. P30116; pUG72, accession no. P30117; pUG73, accession no. P30118; pSH47, accession no. P30119; pSH62, accession no. P30120; pSH63, accession no. P30121; pSH65, accession no. P30122) or as a package (DEL-MARKER-SET) from Euroscarf (Frankfurt, Germany; http://www.uni-frankfurt.de/fb15/mikro/euroscarf/). The package includes the heterologous gene disruption cassettes from the McCusker group (19,20). Companies should contact Johannes H. Hegemann.

## PCR-mediated generation of *loxP*–marker gene–*loxP* gene disruption cassettes and their integration into the yeast genome

All *loxP*–marker gene–*loxP* disruption cassettes presented in this work can be generated by PCR using oligonucleotides carrying the same 19 and 22 3′ nucleotides, while the 40 5′ nucleotides must be homologous to sequences to the left or right of the gene to be deleted (see Fig. 2 and Table 1). As a test case for the new disruption cassettes the *ADE2* and *YOR387c* genes were deleted using the oligonucleotides listed in Table 1. For each disruption cassette one preparative PCR was performed as described previously (22). The PCR conditions were: 95°C for 5 min (hot start); 25 cycles of 94°C for 40 s,

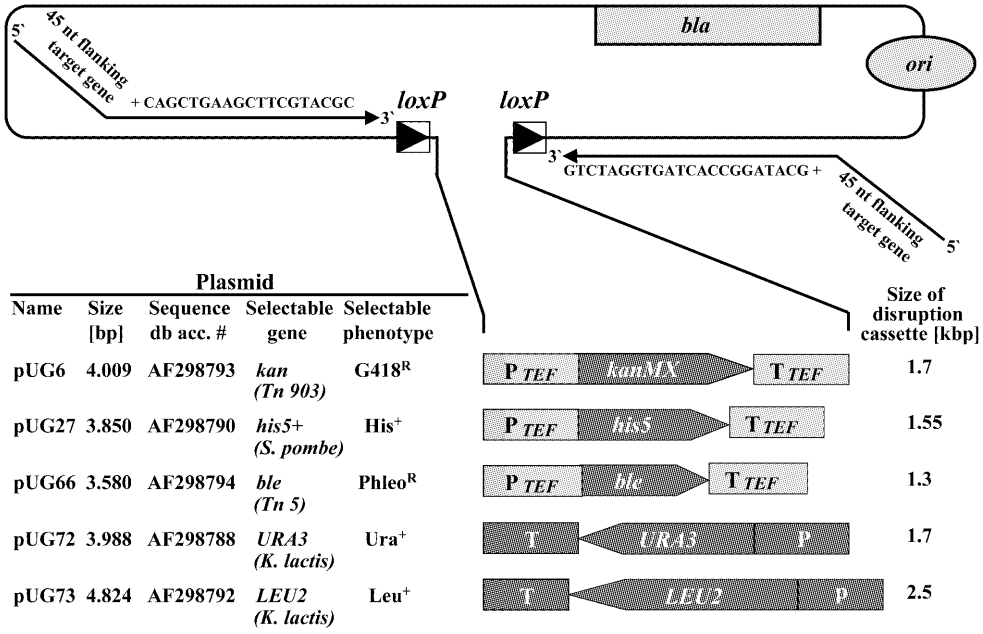


**Figure 2.** The series of *loxP*–marker gene–*loxP* gene disruption cassettes. The marker plasmids pUG6, pUG27, pUG66, pUG72 and pUG73 serve as templates for PCR to generate the five different gene disruption cassettes. All marker genes (selectable genes) are derived from organisms other than *S.cerevisiae* and are expressed from the *A.gossypii TEF2* promoter and terminated by the *TEF2* terminator (6), except for the two *K.lactis* genes, *URA3* and *LEU2*, which retain their own regulatory sequences. *kan*$^r$ and *ble*$^r$ are dominant resistance marker genes conferring resistance to G418 and phleomycin (selectable phenotype), respectively. Each marker gene including promoter and terminator is flanked by *loxP* sites. Since the vector backbone is the same in all cases, each of the five disruption cassettes can be amplified by PCR using the same two primers. The complete vector sequences have been deposited at GenBank under the accession numbers listed in the figure (Sequence db acc. #).

58°C for 1 min and 68°C for 2 min; a final step at 68°C for 15 min. The PCR product was purified and the cassettes used for yeast transformation as described before (22).

## RESULTS AND DISCUSSION

### Construction of new *loxP*-flanked gene disruption cassettes

The recently introduced *loxP–kanMX–loxP* gene disruption cassette combines the advantages of the heterologous, dominant *kan*$^r$ marker with the capacity for efficient gene disruption by Cre-mediated recombination between the two *loxP* sites, resulting in efficient marker rescue (22) (Fig. 1). In order to expand the Cre/*loxP* technology and maximise the utility of this system, we have constructed additional *loxP*–marker gene–*loxP* cassettes. The four newly created *loxP*-flanked marker cassettes, like the original *loxP–kanMX–loxP* cassette, completely lack homology to the genome of the budding yeast, which maximises the probability of recovering the desired homologous integration event (Fig. 2). Construction of the new gene disruption cassettes started from the widely used *loxP–kanMX–loxP* cassette cloned in plasmid pUG6 (22). For construction of the *loxP–his5*$^+$*–loxP* cassette the *kanMX* unit located between the two *loxP* sites in plasmid pUG6 was replaced by the *S.pombe his5*$^+$ unit obtained from plasmid pFA6a-HIS3MX6 (18), yielding plasmid pUG27. The *S.pombe his5*$^+$ gene complements the *S.cerevisiae his3* mutation. The overall degree of identity between the *S.pombe his5*$^+$ and *S.cerevisiae HIS3* DNA sequences is 59%, with several blocks exhibiting higher homology (70–76% identity). This, however, is too low to allow efficient homologous integration of the *S.pombe* gene at the *HIS3* locus (18).

For construction of the heterologous *loxP–URA3–loxP* and *loxP–LEU2–loxP* cassettes, both genes plus their promoter and terminator sequences were isolated by PCR from *K.lactis* genomic DNA and cloned between the two *loxP* sequences in plasmid pUG6, replacing the *kanMX* unit and resulting in the plasmids pUG72 and pUG73. The two *K.lactis* genes complement the corresponding *ura3* and *leu2* mutations in *S.cerevisiae* (36,37). The *K.lactis* genes are 73 (*URA3*) and 77% (*LEU2*) identical to their *S.cerevisiae* homologues. This is too low to permit efficient recombination between the corresponding *K.lactis* and *S.cerevisiae* genes, in particular as the longest stretch of DNA sequence identity between corresponding genes is 17 bp in the case of *URA3* and 18 bp for *LEU2*. The *loxP–URA3–loxP* cassette should be particularly useful, because (i) most *S.cerevisiae* laboratory strains are *ura3*$^-$ and (ii) 5-fluoroorotic acid-containing media can be readily used for counter-selection (21).

Finally, the *ble*$^r$ gene from transposon Tn*5* was used to create the new heterologous dominant drug resistance marker cassette *loxP–ble*$^r$*–loxP*. The *ble*$^r$ gene renders prokaryotes and lower eukaryotes resistant to phleomycin, which belongs to the metallo-glycopeptide group of antibiotics of the bleomycin family (38). Cells expressing the *ble*$^r$ gene become resistant to phleomycin as a result of tight binding of the antibiotic by the Ble protein. The Ble protein appears not to be toxic to *S.cerevisiae*. Phleomycin is produced in fermentation cultures by *Streptomyces verticillus* (39). It has previously been shown that expression of the *ble*$^r$ gene from an episomal or an integrative plasmid allows direct selection of phleomycin-resistant

**Table 1.** List of oligonucleotides (5′→3′)[a]

| Target gene / Disruption marker | | Gene disruption [b] | # of Oligonucleotide |
|---|---|---|---|
| *ADE2* | | CAATCAAGAAAAACAAGAAAATCGGACAAAACAATCAAGTcagctgaagcttcgtacgc | 1134 |
| | | ATAATTATTTGCTGTACAAGTATATCAATAAACTTATATAgcataggccactagtggatctg | 1135 |
| *YOR387c* | | GCAGCCACTAGCTTATAATTATTTGAACTATTTGTCGAACcagctgaagcttcgtacgc | 1017 |
| | | GTTCAAAAAAGAATCATTATTATGATCCAATGACAGTGACgcataggccactagtggatctg | 1018 |
| | | **Verification primers / target gene - specific** | |
| *ADE2* | A | CTTGACTAGCGCACTACC | 1102 |
| | B | TTACAACGAAGTTACCTCTTCCATC | 1182 |
| | C | AACAAAGGCTGAACTACATTACAGG | 1183 |
| | D | GACGCTTTATAATTTGGC | 1103 |
| *YOR387c* | A | GCATAATCACAACTGATC | 1025 |
| | B | ATGCCATAGTGCCACTTC | 1227 |
| | C | CTCAGTATGGCGTTCAAG | 1228 |
| | D | ATGTTGAGAATGGACCAC | 1026 |
| | | **Verification primers / disruption cassette - specific** [c] | |
| *Tn903 kan*$^r$ *S.p. his5*$^+$ *Tn5 ble*$^r$ | kan-B | GGATGTATGGGCTAAATG | 363 |
| | kan-C | CCTCGACATCATCTGCCC | 335 |
| *K.l. URA3* | Ura-B | CAGACCGATCTTCTACCC | 1115 |
| | Ura-C | TTGGCTAATCATGACCCC | 1127 |
| *K.l. LEU2* | Leu-B | AGTTATCCTTGGATTTGG | 1136 |
| | Leu-C | ATCTCATGGATGATATCC | 1137 |

[a]For nomenclature of verification primers A–D see Figure 1.
[b]Lower case letters indicate nucleotides homologous to sequences left and right of the cloned disruption cassettes (see Fig. 2).
[c]Primers kan-B and kan-C can be used for the disruption cassette markers *kan*$^r$, *his5*$^+$ and *ble*$^r$.

*S.cerevisiae* transformants (34,38,40). The *ble*$^r$ gene was isolated by PCR from plasmid pUT332 (34) and placed between the *A.gossypii TEF* promoter and terminator in plasmid pUG6, yielding plasmid pUG66 (Fig. 2).

## Gene disruption properties of the new *loxP*–marker gene–*loxP* cassettes

An important property of the new cloned gene disruption cassettes is that, like the original *loxP–kanMX–loxP* cassette (22), each of the marker genes plus regulatory elements is flanked by two *loxP* sequences embedded in the same vector backbone (see Fig. 2 and Materials and Methods). This allows the use of the same primer sequences to amplify all of the gene disruption cassettes.

To test the fidelity of the new cassettes for targeted gene disruption all cassettes were used to delete two genes: *ADE2* and *YOR387c*. The 2 × 5 gene disruption cassettes were amplified in parallel using primers 1134 and 1135 (for *ADE2*) and 1017 and 1018 (for *YOR387c*) (Table 1). The 5′-ends of the primers contain 45 nt stretches that are homologous to sequences upstream of the ATG start codon and downstream of the stop codon, respectively, of the target genes, followed by a 19 nt segment homologous to sequences to the left of the *loxP* site and a 22 nt motif that anneals to the right of the *loxP* site of the disruption cassettes (Figs 1 and 2). Comparable amounts of the various disruption cassettes were generated by PCR and purified (Fig. 3). Approximately 2 µg of the gene disruption cassettes were transformed into strain CEN.PK2-1C and transformants were selected on the corresponding SD dropout plates, on YPD + 7.5 µg/ml phleomycin (for the *loxP–ble*$^r$*–loxP* cassette) or on YPD + 200 µg/ml G418 (for the *loxP–kanMX–loxP* cassette) plates, as described before (22). Transformation of the five different disruption cassettes in all cases yielded comparable numbers of transformants.

For selection of phleomycin-resistant colonies we first determined the usable range of antibiotic concentrations and found that 7.5 µg/ml phleomycin (in YPD) was optimal for selection of *loxP–ble*$^r$*–loxP* transformants. At this concentration no spontaneous phleomycin-resistant colonies were found. In contrast to what has been described for transformation with a *ble*$^r$ gene-carrying multicopy plasmid (40), we could not improve the transformation efficiency of the *loxP–ble*$^r$*–loxP* cassette further by incubating the transformation mix in liquid YPD medium for several hours (up to 10 h was tested) prior to plating on YPD plates containing phleomycin. However, we did not try to enhance the transformation efficiency of the *loxP–ble*$^r$*–loxP* cassette further by adding glycerol to the transformation plates (38).

To confirm correct integration of the various disruption cassettes into the *ADE2* and *YOR387c* loci, 24 transformants from each plate were subjected to colony PCR using combinations of the corresponding target gene-specific (primers A–D) and disruption cassette-specific primers (primers C-B and C-C) (Fig. 1 and Table 1). A typical example of the results of the PCR analysis of putative *yor387c* deletants using target gene-specific primer A and one of the three cassette-specific primers

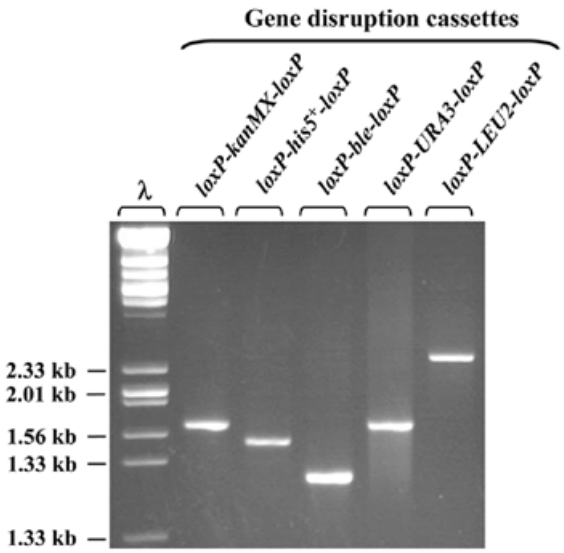


**Figure 3.** For deletion of the *ADE2* gene, five different gene disruption cassettes were produced in parallel PCRs using the primers 1134 and 1135 (see Table 1). The sizes of the cassettes vary between 1300 and 2500 bp (2% of each PCR product was loaded).

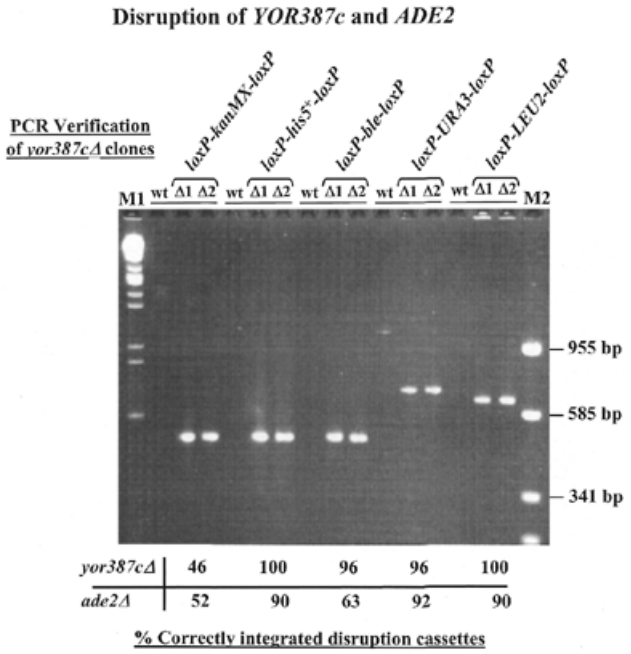


**Figure 4.** PCR analysis to confirm correct integration of the gene disruption cassettes at the *YOR387c* and *ADE2* loci (see Fig. 1). PCR was carried out on transformants obtained in the *YOR387c* disruption experiment, using target gene-specific primer A (primer 1025) and the disruption cassette-specific primer C-B (for *kan*$^r$, *his5*$^+$ and *ble*$^r$ the kan-B primer 363; for *URA3* the Ura-B primer 1115; for *LEU2* the Leu2-B primer 1136). The products obtained from two *yor387c*Δ mutants (Δ1 and Δ2) disrupted with each of the various disruption cassettes and from wild-type (wt) cells were then subjected to agarose gel electrophoresis. For both genes 24 transformants obtained with each of the gene disruption cassettes were analysed in similar experiments. The percentages of correctly integrated cassettes at *YOR387c* and *ADE2* are indicated below the gel. DNA fragment size markers: M1, *Eco*RI + *Hin*dIII-cleaved λ DNA; M2, *Bsp*143I-cleaved pUC19 (the sizes of selected marker DNA fragments are shown on the right).

C-B is shown in Figure 4. The PCR products obtained from the various deletion strains are between 350 and 600 bp in length and thus exhibit the calculated cassette-specific sizes predicted for correct integration. The frequency of correctly integrated cassettes at the *YOR387c* locus varied between 46% for the *loxP–kanMX–loxP* cassette and 100% for the *loxP–his5*$^+$*–loxP* and *loxP–LEU2–loxP* cassettes. Analysis of the *ade2* transformants revealed a similar level of correctly integrated cassettes: between 52% (*loxP–kanMX–loxP*) and 92% (*loxP–URA3–loxP*) of the transformants had undergone the correct homologous recombination event (Fig. 4). As expected, all *ade2* deletion strains characterised by PCR also produced red colonies on indicator plates due to accumulation of a biosynthetic intermediate product, thus confirming the PCR results (data not shown). In summary, PCR analysis of putative *yor387c* and *ade2* deletants revealed that all five cassettes disrupted their target genes with similar efficiency: at least 50% of the transformants had undergone the predicted homologous recombination event. Similar efficiencies for this type of gene disruption have been observed by others, mainly due to the fact that the gene disruption cassettes used in these cases consist of completely heterologous DNA sequences (6,19,22).

Our results show that the two heterologous *K.lactis* genes *URA3* and *LEU2* exhibit excellent selection marker qualities for this type of gene deletion. In fact, the *URA3* gene from this yeast has previously been used for gene disruption experiments by others (27,41). Likewise, the *his5*$^+$ gene from *S.pombe* proved to be an efficient marker for gene disruption, as has been reported by others for a non-*loxP* version of the *his5*$^+$ gene (18,42).

The results of the gene knockout experiment show that the *ble*$^r$ gene is an efficient heterologous dominant resistance marker: 63 and 96% of the transformants proved to have correctly integrated the *loxP–ble*$^r$*–loxP* gene disruption cassette at the *ADE2* and *YOR387c* loci, respectively. The transformation efficiency of this cassette is comparable to that of the second dominant resistance marker, *loxP–kanMX–loxP*, and to that of the other heterologous auxotrophic markers used here (data not shown). Moreover, *ade2*Δ strains carrying the *ble*$^r$ marker showed no obvious growth disadvantage when compared with *ade2*Δ strains containing one of the other marker genes, suggesting that these markers are probably phenotypically neutral. However, this needs to be tested more thoroughly in the future. To exclude the possibility that high level expression of the phleomycin resistance protein has adverse effects on cellular functions in *S.cerevisiae*, the marker can be easily removed by activating the Cre-mediated recombination process to delete the *ble*$^r$ gene.

Finally, it is important to note that no cross-resistance with other currently used dominant resistance markers has been reported. Therefore, phleomycin can be used to select cells that are already resistant to other selective agents, for example G418. This makes the dominant *loxP–ble*$^r$*–loxP* cassette a very useful tool when working with the *kan*$^r$-based KO strain collection produced by the Transatlantic Yeast Consortium during the EU project EUROFAN 2. Our laboratory routinely uses both dominant markers to produce double deletion strains.

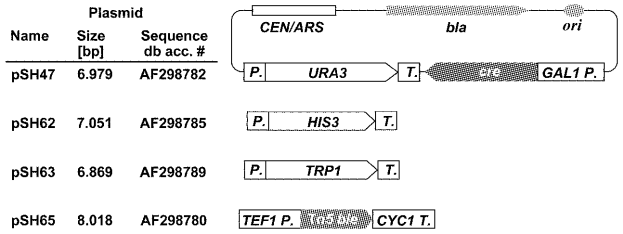


**Figure 5.** Cre expression plasmids. All plasmids carry the *CEN6/ARSH4* module to ensure stability in yeast and a Cre expression cassette consisting of the *cre* open reading frame flanked by the galactose-inducible *GAL1* promoter and the *CYC1* terminator from *S.cerevisiae*. The vectors differ in the type of selection marker used. The *URA3*, *HIS3* and *TRP1* marker genes are from *S.cerevisiae* and retain their own regulatory sequences, while the *ble*$^r$ gene from transposon Tn*5* is expressed from the *S.cerevisiae TEF1* promoter.

### New Cre expression plasmids for marker rescue

Expression of Cre recombinase in yeast strains carrying one (or more) of the *loxP*–marker gene–*loxP* cassettes results in efficient recombination between the *loxP* sites, removing the marker gene and leaving behind a single *loxP* site at the chromosomal locus (22). To increase the flexibility of this system, we have exchanged the *URA3* selection marker in the original *cre* expression vector pSH47 for *HIS3* (resulting in plasmid pSH62) and *TRP1* (pSH63), as well as for the dominant resistant marker *ble*$^r$ (pSH65) (Fig. 5). The *cre* expression cassette consisting of the inducible *GAL1* promoter, the *cre* gene and the *CYC1* terminator is not affected by these modifications. The new vectors were tested for efficiency of marker rescue and were found to induce the same rates of marker loss as described for the original pSH47 vector (22; data not shown). In the meantime these new *cre* expression vectors have also been successfully used by others (see for example 28). In our routine work we no longer induce Cre expression by shifting *loxP*–marker gene–*loxP* marked cells transformed with a *cre* plasmid to galactose medium. Instead, we grow these cells overnight in liquid YPD medium (with glucose as carbon source) and then plate ~200 cells on YPD plates. Replica plating onto YPD and YPD plus the drug (or onto the corresponding selective medium in the case of the auxotrophic markers) results in the identification of cells which have lost the marker gene from the disruption cassette (~5% of the total). This is probably due to residual activity of the *GAL1* promoter in the presence of glucose.

Several *loxP*-flanked marker genes can be removed from the genome simultaneously (data not shown). However, care must be taken not to induce Cre expression too strongly (e.g. by using multicopy plasmids or inducing Cre expression for prolonged times), as this will result in unintended recombination events between various *loxP* sites (27).

### Swapping of cassette markers

An additional advantage of the *loxP*-flanked *kanMX*, *ble*$^r$ and *his5*$^+$ marker genes is their use for marker cassette exchange. The presence of completely identical DNA sequences of ~300 bp to the left and right of the marker gene in each cassette (see Fig. 2) allows for easy replacement of each of the marker genes by any of the others. For this purpose only two oligonucleotides have to be designed which allow amplification of the marker gene of interest plus the promoter, terminator and *loxP* sequences. Transformation of this cassette into a yeast strain carrying one of the other two cassettes integrated into the genome replaces the marker gene within the integrated cassette. Successful cassette swapping can be verified by PCR analysis using target gene-specific (A and D) and new cassette-specific primers. To exclude unintended targeting events, swapping experiments should be restricted to single deletion strains that do not carry plasmid pSH65 (carrying *S.cerevisiae TEF1* sequences).

### Conclusion

We have constructed four new gene disruption cassettes with the marker genes *ble*$^r$, *his5*$^+$, *URA3* and *LEU2*, consisting of completely heterologous DNA flanked by *loxP* sequences. In addition, the Cre expression plasmid has been equipped with three other selection markers (*HIS3*, *TRP1* and *ble*$^r$). The set of five different gene disruption cassettes and four different Cre expression vectors presented here will significantly extend the tool kit for functional analysis of the *S.cerevisiae* genome. In fact, some of the new vectors have already been used successfully in a variety of experiments (see for example 28,43; J.Heinisch, unpublished results).

The availability of the two dominant marker genes *kan*$^r$ and *ble*$^r$ and a Cre expression vector carrying *ble*$^r$ as the yeast selection marker facilitates the creation of multiple gene knockouts not only in commonly used laboratory strains, but also in prototrophic industrial or wild-type strains of *S.cerevisiae*.

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