Cloning-free genome alterations in *Saccharomyces cerevisiae* using adaptamer-mediated PCR

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Running title: Adaptamer-mediated genome alterations in *Saccharomyces*

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Introduction

The budding yeast *Saccharomyces cerevisiae* is an appealing eukaryotic model system in part due to its amenability to genome manipulation. Since linear DNA readily undergoes homologous recombination in yeast, precise alterations of genes in the chromosome can be done with relative ease.[1] For example, targeted gene disruptions to produce null alleles are performed routinely in yeast and provide a vital tool for understanding gene function.[2,3] Allele replacement methods allow the introduction of novel mutations into any gene of interest.[4] Similarly, gene fusions that append functional epitopes such as green fluorescent protein (GFP) or an immunogenic tag are another important tool for studying gene function.[5] These methods all rely on the efficient homologous recombination of yeast to make specific changes to genes at their endogenous chromosomal loci.

In recent years, many genome manipulation techniques have been revised to take advantage of PCR. Below we describe PCR-based techniques for gene disruption, allele replacement and epitope fusions using special primers we call adaptamers. Adaptamers are chimeric primers containing sequences at their 5' end that facilitate the fusion of any two pieces of DNA by PCR. This principle is illustrated in Figure 1. The two DNA fragments to be fused are each amplified with an adaptamer and a standard PCR primer. The 5' sequence tags on the adaptamers are reverse and complementary to each other. In a second round of PCR, the two amplified DNA fragments are mixed, the complementary ends anneal and are then extended by the polymerase. Primers are also included that bind at the termini of the fused DNA fragments leading to exponential amplification of the fused product.

In each of the techniques described below, adaptamer technology is used to mediate the fusion of a dominant selectable marker to DNA fragments that provide homology for chromosomal targeting. Upon transformation of these PCR products into yeast, homologous recombination yields the targeted genomic integration with high efficiency. Thus adaptamers obviate the need for traditional cloning in *Escherichia coli* to build integrating vectors and offer a considerable savings of time. In addition, these methods each incorporate directly repeated sequences flanking the selectable marker. This permits reuse of the genetic marker after its deletion from the genome by direct repeat recombination.

Gene disruption strategies

One-step gene disruption in yeast is a technique used to replace a functional gene with a dominant selectable marker in a process requiring homologous recombination.[3]

Traditionally this involves interrupting a cloned gene on a plasmid by inserting a dominant selectable marker using standard cloning techniques (Figure 2A). The plasmid is cut with restriction enzymes to produce a linear DNA containing the selectable marker and several hundred base pairs of DNA on each side of the selectable marker. Upon transformation into yeast, the homologous DNA flanking the marker promotes two recombination events that replace the copy of the gene on the chromosome with the inactive copy in a single step (Figure 2B). The dominant marker allows selection of recombinants after transformation. Although the recombination events are efficient, this method requires time consuming cloning steps in *E. coli* to produce the desired integrating DNA. In addition, interruption of the gene depends on available restriction sites. In many cases this leaves a partial fragment of an ORF and may not provide a true null allele. PCR methods significantly expedite construction of integrating DNA.

PCR-based methods of gene disruption streamline the traditional approach by replacing cloning in *E. coli* with PCR amplification. The simplest of these approaches is sometimes referred to as a "microhomology" or "long primer" method. The homologous DNA is synthesized as 40 to 60 nucleotide 5' extensions on primers designed to amplify a selectable marker (Figure 3A). After amplification, the PCR products are transformed into yeast and the 40 to 60 base pairs of homology on each end of the DNA promotes recombination with genomic DNA to disrupt the gene (Figure 3B).[6-8]

This method greatly simplifies one-step gene disruptions since no cloning is necessary. In addition, it is possible to create precise deletions of ORFs to ensure production of the null alleles. However, the length of homology to the genome is limited by the amount of DNA that can be reasonably synthesized on the 5' ends of the primers. Recombination is relatively inefficient with such short homology regions requiring several micrograms of DNA to produce only a few transformants.[7] Increasing the length of homologous DNA to $\geq$ 600 bp on each side of a selectable marker increases recombination efficiency producing 100- to 1000-fold more transformants per microgram of DNA.[8]

The adaptamer-directed gene disruption method described below combines the efficient genome integration made possible by longer homologous DNA fragments and the rapid construction methods facilitated by PCR.

Adaptamer-directed gene disruptions

The adaptamer-directed gene disruption method relies on the same principles of homologous recombination as the gene disruption methods described above. However, in this strategy yeast intergenic regions are amplified to provide 200 to 500 bp of

homology on each side of the gene to be disrupted. The homologous DNA fragments are then fused to a selectable marker via complementary adaptamers. Upon transformation into yeast, these DNA fragments recombine with the homologous genomic locus to disrupt the gene. A set of 6361 primer pairs was designed to amplify yeast intergenic regions. These contain common 20 bp 5' sequence tags that are not homologous to *S. cerevisiae* genomic DNA.[9] These intergenic adaptamers can be specifically suited for the task of gene disruptions and are available from Research Genetics (Huntsville, AL). Figure 4 shows a map of intergenic adaptamers on an 8 kb segment of yeast chromosome IV. The intergenic adaptamers are uniformly oriented, independent of gene orientation, so that all forward primers contain the same 5' sequence tag called C and all reverse primers contain a different 5' sequence tag called D (see Table 1 for sequences).

As outlined in Figure 5, two rounds of PCR are necessary to create a gene disruption cassette by this method. In the first round, intergenic DNA fragments and the selectable marker are amplified in four PCR amplifications. Amplification of intergenic regions using the C and D adaptamer incorporates the sequence tags into the ends of those PCR products (Figure 5A). Approximately 2 ng genomic DNA is added to 20µl reactions containing 0.5 µM of the specific intergenic adaptamers, 200 µM of each dNTP, and 1.5 units *Taq* polymerase. Genomic DNA is prepared by standard methods for use as template DNA.[10] Intergenic adaptamers were designed to have a minimal annealing temperature of 52°C.[11] Amplification is performed using the following cycle conditions: 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute and finally 72°C for 5 minutes. In rare cases where amplification using intergenic adaptamers has given poor or inconsistent yields using the above conditions, the annealing temperature was reduced to 45°C for the first eight cycles followed by 22 cycles using the 52°C annealing temperature.

The selectable marker is amplified in two overlapping segments from plasmid pWJ1077 (Figure 5B and 5C). We use the orthologous *URA3* gene from *Kluyveromyces lactis* as a selectable marker because it complements a *S. cerevisiae ura3* mutant and can also be counter-selected using 5-fluoro-orotic acid (5-FOA).[12] Additionally, the sequence homology between the *K. lactis URA3* and the *S. cerevisiae URA3* is approximately 70% at the nucleotide level.[13] Therefore, recombination of *K. lactis URA3* sequence with the endogenous *URA3* allele is greatly decreased.[14,15] We incorporated 143 bp direct repeats into the *URA3* plasmid so the marker can be recycled after integration (see below). The 3' section of *URA3* is amplified with adaptamer d and internal primer kli5', while the 5' section of *URA3* is amplified with adaptamer c and internal primer kli3' (see Table 1 for

sequences). Adaptamers c and d have 5' sequence tags that are the reverse and complement of the 5' tags on the C and D intergenic adaptamers. PCR of selectable marker segments is performed using 2 ng pWJ1077 plasmid DNA as a template with the same conditions described above, and substituting 55°C for the annealing temperature.

A second round of PCRs is required to fuse the selectable marker fragments to the amplified intergenic regions via the complementary sequence tags on the adaptamers. The left intergenic region is fused to the 3' *URA3* segment through annealing of the d and D sequence tags, while the right intergenic region is fused to the 5' *URA3* segment by the annealing of the c and C sequence tags (Figure 5D). Conditions for these PCR fusions are as follows: 10 to 25 ng of the intergenic PCR amplified DNA are mixed in a 50 µl reaction with approximately equimolar amounts of the appropriate *K. lactis URA3* DNA fragment. Typically, the template DNA fragments for fusion are simply diluted 50- to 100-fold from the first round of PCRs into the fusion reaction mixture and do not require purification. Fusion PCRs also contain 0.5 µM primers, 200 µM of each dNTP and 3.8 units of Taq polymerase. Cycle conditions are 94°C followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1.5 or 2 minutes (depending on total length) and finally 72°C for 10 minutes.

DNA fragments for a gene disruption are produced in two overlapping parts for several reasons. First, every amplified intergenic region is bounded by the same C and D sequence tags, thus the split marker approach isolates the complementary c and d ends of the *URA3* gene into two separate PCR fusion reactions ensuring proper orientation of the fused product. Second, PCR fusions can be accomplished using the 20-mer C or D sequences as primers rather than the specific adaptamers for each intergenic region (Figure 5D). This simplifies the setup of PCRs when DNAs for many gene disruptions are produced at once. Finally, while the length an integrating DNA sequence can be long, the split marker approach generates shorter individual fusion PCR fragments thereby increasing the reliability of the amplifications.

Typical products produced from first round of PCR and subsequent PCR fusions are shown in Figure 5E. The fused DNA fragments can be taken directly from the PCR amplification mixtures and used for yeast transformations. The three recombination events required to reconstitute the selectable *URA3* marker and replace the targeted ORF are illustrated in Figure 6. The homologous intergenic regions target recombination to the intended locus while the *URA3* segments reconstitute the selectable marker[16] resulting in a precise disruption of the ORF. Yeast transformations are performed using the standard lithium acetate procedure as described.[17] Approximately 300 ng of each fusion DNA fragment is added to competent cells using conditions that give 10^6 transformants/µg of

circular plasmid DNA. The number of transformants in various gene disruption experiments ranges from 10 to over 200 using these amounts of DNA.

Often the integrated selectable marker is used to follow segregation of the null mutant during crosses to other strains. In this case, it is best to verify gene disruptions using two PCRs. Internal *K. lactis URA3* primers are paired with primers that bind in the left and right genomic DNA outside of the intergenic regions to amplify across each recombination junction (gray arrows in Figure 6A). However, it may also be desirable to recycle the selectable marker. This is achieved by recombination between the 143 bp direct repeats flanking URA3 which leads to a pop-out (Figure 6B). This is typically done by growing overnight cultures of a yeast gene disruption strain without selection for the *URA3* marker and then plating 100-200 µl of the saturated culture onto 5-FOA plates to select for the pop-out recombinants. Verification of marker-less gene disruptions can be done using a single PCR reaction that amplifies across the disruption since this is now a short DNA sequence. For the gene disruptions performed thus far using this method, 11 of 14 yielded a gene disruption efficiency of 70-90%. Lower gene disruption efficiencies (about 20%) occur when a particular gene disruption produces a slow growth phenotype. In such cases, it is more efficient to perform gene disruption in a diploid strain.[18]

Intergenic PCRs and PCR fusions are simple to perform. Disruption of any yeast gene requires four specific intergenic adaptamers to amplify the flanking DNA, but all other components are common. The *K. lactis* DNA fragments can be amplified in quantity and used as a resource for many different PCR fusions. Purification of intergenic PCR products is not necessary between the first and second rounds of PCR except in rare cases where PCR products contain contaminating non-specific DNA fragments. In addition, PCR products from the fusion reactions can be added directly to yeast transformation reactions. Thus all PCRs and the yeast transformations for a gene disruption experiment can usually be accomplished in a day. While this method incurs an added cost for the extra PCRs compared to the “long primer” gene disruption method, an overall increase in efficiency can be realized because fewer transformants need to be screened to identify successful gene disruptions. This becomes important when multiple gene disruption experiments are carried out at once.

Intergenic adaptamers have standard names based on the name of the adjacent chromosome feature to the “left” of the intergenic region. This is illustrated in Figure 5 where the adaptamers to amplify the "left" intergenic region for *ILV1* (iYER085C-F and -R) are named after the YER085C ORF and the adaptamers to amplify the "right" intergenic region (iYER086W-F and -R) are named after the systematic name of the *ILV1* ORF. While the nomenclature is standard, it is complicated by the fact that adaptamer pairs do not exist for

every intergenic region due to overlapping ORFs. In addition, intergenic regions ≥1.5 Kb are split into sections of ≤1.2 Kb amplicons.

Lists of all the intergenic adaptamers and sequences are available by contacting Research Genetics. In addition, maps of all 16 yeast chromosomes showing intergenic adaptamer binding sites, the systematic name for each set of intergenic primers and the length of the amplified DNA can be viewed on our website (http://rothsteinlab.hs.columbia.edu/projects/primersearch.html).

Moving "barcoded" gene disruptions into a new strain

A world-wide consortium of yeast labs recently completed construction of an arrayed library of yeast gene disruption strains for more than 5000 genes.[19] This library represents a unique resource for an experimental organism and is a valuable addition to yeast researchers' toolbox. The deletion consortium strains were constructed by the "microhomology" PCR technique using kanamycin resistance as the selectable marker. The kanMX4 cassette was PCR amplified and attached to 45 bp of homology from each side of the targeted coding region. The 5' homology is adjacent to and includes the ORF start codon while the 3' homology is adjacent to and includes the ORF stop codon. Thus a precise disruption of each ORF was made. Additionally, the long primers contain two unique gene-specific sequence tags on the 5' (UPTAG) and 3' (DOWNTAG) ends of each disruption cassette providing distinct "barcode" identifiers for each gene disruption. As an example, the structure of the *ILV1* locus is shown in Figure 7A along with the structure of the *ilv1* deletion strain produced by the consortium.

The UPTAG (checkered box) and DOWNTAG (black box) contain a unique 20 bp sequence specific to each strain flanked by standard 18 bp primer binding sites for amplification of these tags so that all the barcodes in a population can be PCR-amplified using the same pairs of primers. Hybridization of the amplified DNA to a microarray gene chip that contains the barcodes for all the gene disruption strains can be used to monitor the presence or absence of a particular disruption strain in a population of cells. This has been used to identify gene disruptions in a population that are enriched for or selected against in response to various growth conditions.[19]

These types of population biology experiments are likely to be useful to a number of researchers that work with other yeast strain backgrounds. Therefore we have developed an adaptamer gene disruption method with the specific goal of transferring gene disruptions containing the unique tags into a new strain. We have moved a number of these tagged

gene disruptions into the W303 strain background[20] and find that the method is as efficient as the general method described above.

A simple way to transfer the gene disruptions from the consortium strains to a new strain is to choose primers that PCR amplify across the gene disruption to produce a DNA molecule containing the selectable marker, the unique tags, and sufficient homologous DNA to effect recombination. The DNA can then be transformed to select for G418-resistant recombinants. However, the KanMX selectable marker is not recyclable and thus cannot be reused if additional disruptions need to be performed in that strain.

A simple adaptation of the adaptamer-directed gene disruption method described above allows transfer of the barcoded gene disruptions into a new strain while replacing the kanamycin resistance cassette with the recyclable *K. lactis URA3* marker As shown in Figure 7B, the intergenic regions flanking the gene disruption are amplified using intergenic adaptamers and the common primers flanking the UPTAG and DOWNTAG, U2 and D2 respectively. *K. lactis URA3* is amplified in overlapping sections as described above (see Figure 4B). In this case, the 5' sequence tags on the adaptamers are the reverse and complement of U2 and D2 and are referred to as u2 and d2, respectively. Figure 7C shows PCR fusion using these adaptamer sequences to generate DNA fragments for gene disruption.

UPTAG and DOWNTAG sequences in the deletion strains have the same orientation as the start and stop codons of the disrupted gene and the C and D intergenic adaptamers all have the same orientation along the chromosome. Therefore amplification of a Watson strand gene disruption (e.g. *MCD1* in Figure 4) is accomplished using the C intergenic adaptamer with the U2 adaptamer and the D intergenic adaptamer with the D2 adaptamer. For a Crick strand gene disruption (e.g. *NTH1* in Figure 4), the C intergenic adaptamer is used with the D2 adaptamer and the D intergenic adaptamer is used with the U2 adaptamer.

Amplification of intergenic regions from the consortium strains is performed using the same PCR conditions described above. Most of the amplification of yeast intergenic regions in our lab have been performed using relatively clean DNA preparations.[10] However, it is also possible to perform these amplifications by colony PCR.[21] This is useful where gene disruptions are moved from many different strains.

Allele replacements using adaptamers

The ability to introduce a specific mutant allele into the yeast genome is an invaluable genetic tool. A mutant allele in one strain can be moved into a different strain background by

a standard genetic cross. However, this requires successive backcrosses to ensure that the new mutant strain becomes congenic to the desired background. Allele replacement by homologous recombination obviates the need for multiple backcrosses because the new allele is introduced directly into the desired strain. For novel mutations constructed *in vitro*, allele replacement methods facilitate the stable integration of the mutant allele into the genome.

Traditional allele replacement methods require that the mutant allele be subcloned into a yeast integrating vector that contains a selectable/counter-selectable marker such as *URA3*. The integrating plasmid is linearized with a restriction enzyme that cuts once within the mutant allele. Transformation of this linear DNA into yeast effects homologous recombination to yield an integrated direct repeat in which a mutant and a wild-type copy of the targeted gene are separated by *URA3*. Direct repeat recombination pops out the marker leaving a single allele. The efficiency of recovering the mutant allele is 50% or less and depends on the location of the mutation with respect to the ends of the DNA repeats. We have developed an adaptamer-based allele replacement technique in which mutant alleles are PCR amplified and fused to a selectable marker for genome integration.[22] A major advantage of the adaptamer method is the high probability that both integrated repeats contain the mutant lesion. This increases the efficiency with which the mutant allele is recovered after the selectable/counter-selectable marker is recycled.

The adaptamer-based allele replacement method described below uses the complete ORF for DNA homology to target integration. This was designed to take advantage of the complete set of commercially available adaptamers that can amplify every yeast ORF (Research Genetics, Huntsville, AL).[23] All forward ORF adaptamers contain the 19 bp A sequence tag followed by 20 to 25 nucleotides of homology to a specific ORF starting at the ATG (See Table 1). All reverse adaptamers contain the 20 bp B sequence tag followed by 20 to 25 nucleotides of homology at the 3' end of the ORF and includes the stop codon. Amplification using these adaptamers produces a precise copy of the ORF with the A and B tags appended to the 5' and 3' ends, respectively (Figure 8A).

The *K. lactis URA3* selectable marker is amplified in two overlapping sections as described earlier (Figure 5C). Fusion of the selectable marker to the amplified ORF is performed in two parallel reactions. The 5' *K. lactis URA3* fragment is fused to the ORF via the complementary b and B sequence tags while the 3' *URA3* fragment is fused via the complementary a and A sequence tags (Figure 8B). Integration into the genome is mediated by the homologous ORF DNA, while the *URA3* segments recombine to reconstitute the selectable marker (Figure 8C).

There are three possible recombination products depending on the position of the crossovers between the incoming DNA and the chromosome. Either both alleles are incorporated (as shown in Figure 8D) or one mutation is incorporated and the other is not (there are two types, one of which is shown in Figure 8E). In fact it is most common to recover the class in which both integrated direct repeats contain the mutation. We have shown that when the transferred mutation lies ≥ 80 bp from the end of the fragment, the crossover occurs between the mutation and the end of the DNA greater than 95% of the time.[22] This is most likely due to the fact that crossing over is stimulated at or near the ends of the incoming DNA.[24]

This adaptamer-based allele replacement method is an efficient way to introduce an allele into a new strain background without cloning in *E. coli*. The method as presented above uses complete ORFs as homologous DNA to promote genome integration. This is advantageous since the pre-made adaptamers for allele replacement of any ORF can be obtained at a modest cost. Furthermore, the *URA3*-marked intermediate maintains gene function as the ORF is not disrupted. This is important when alleles of essential genes must be transferred. The drawback to this allele replacement method is that random mutations can be introduced by PCR. Thus, it is important to limit the number of cycles of amplification and to use a high fidelity polymerase. In cases where ORFs are long, it is prudent to design new adaptamers that limit the total length of amplified DNA. This is not necessarily problematic for allele replacement of essential genes. As long as the homologous DNA includes the N-terminus and the promoter or the C-terminus with the stop codon, a functional copy of the gene is maintained upon integration. Here as well, there is a high probability of incorporating the mutation into both repeats.

In-frame gene fusions using adaptamers

Attaching functional epitopes to a gene can be an important route to understanding protein function. Fusion of immunogenic tags can facilitate protein purification and biochemical analysis of protein-protein interactions. Indirect immunofluorescence methods using such epitopes makes it possible to study subcellular localization of a protein and even co-localization with other proteins provided that distinct immunogenic tags are available. Microhomology-based integration methods have been used to introduce such changes directly into the yeast genome, obviating the need for a cloned gene.[7] In fact, a set of plasmids has recently been constructed containing several kinds of useful epitopes adjacent to selectable markers.[5,25] Amplification of the epitopes and markers in these plasmids

with primers containing homology to a gene can be used to integrate and construct epitope fusions in the yeast genome.

One drawback to introducing epitopes by the microhomology methods is that, along with the desired epitope, most of these protocols also integrate a selectable marker and affect gene structure. This imposes limits on how these epitopes can be used. For instance, insertions on the 5' end of an ORF to produce an N-terminal protein fusion must also include a heterologous promoter to drive gene expression. If expression from the endogenous promoter is important, then it is necessary to use a C-terminal epitope fusion - which may or may not compromise protein function. This problem is solved by integrating the epitope as a direct repeat flanking a selectable marker.[26] Recombination between the repeats deletes the marker and other heterologous sequences. However, integrating epitopes using microhomology is still inefficient.

We have developed an adaptamer-directed method in which fusions can be made to any functional sequence without leaving a selectable marker in the genome. This is accomplished by using the epitope sequence as a direct repeat flanking the selectable marker. The epitope is fused to genomic DNA by first amplifying this DNA with appropriate adaptamers. We have thus far applied these techniques to produce fusions of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to Rad52 to investigate its relocalization in response to DNA damage.[27] Below we describe the method for adding C-terminal or N-terminal CFP and YFP tags to any gene.

The cloning of GFP from *Aequorea victoria* has provided a useful tool for cell biological studies in *S. cerevisiae* and other organisms.[28] Subsequent mutagenesis of GFP has resulted in a number of enhanced variants of the protein having altered fluorescent properties. In particular, blue- and red-shifted versions, CFP and YFP respectively, that have non-overlapping excitation and emission spectra have been produced. These fluorophores can be visualized independently in a living cell, thus facilitating co-localization experiments. In addition, with appropriate filters, CFP and YFP can potentially be used to study protein-protein interactions *in vivo* by means of Fluorescence Resonance Energy Transfer (FRET).[29] Here we utilize the W7 (CFP) and 10C (YFP) clones obtained from R. Tsien, University of California, San Diego.[29,30]

We have constructed two plasmids each for CFP and YFP that can be used to create fusions to any gene of interest (Figure 9). One plasmid contains YFP next to the first two-thirds segment of *URA3*. The second plasmid contains the last two-thirds of *URA3* followed by the YFP ORF such that when the overlapping segments of *URA3* recombine, the YFP sequences are oriented as direct repeats (Figure 10A). The method as illustrated

in Figure 10 shows an in-frame fusion of YFP to the 3' end of the ORF. Two PCRs are performed to amplify the YFP-*URA3* DNA fragments. The first plasmid is amplified with the forward YFP primer GFPstart-F and the internal kli3' *URA3* primer. The second plasmid is amplified with the internal kli5' *URA3* primer and the reverse YFP primer GFPend-R. Parallel PCRs are performed to amplify a 200 to 300 bp section of the 3' end of a coding region and 200 to 300 bp directly adjacent to that ORF. Adaptamers are used for each of these PCRs in order to fuse these segments to the YFP-*URA3* fragments in the second round of PCRs (Figure 10B). When C-terminal fusions are made, the stop codon from the gene is included in the DNA amplified from the intergenic region and omitted in the DNA amplified from the ORF (see DFxTag2 adaptamer in Table 1). PCR conditions are identical to those used for the gene disruption methods described above. Co-transformation and integration of these sequences produces a precise in-frame fusion of YFP to the targeted ORF. Subsequent recombination between YFP direct repeats pops-out the marker leaving only the inserted YFP coding sequences (Figure 10C).

The above method describes C-terminal YFP fusions, but can, in principle, be used to fuse any C-terminal epitope. For N-terminal or internal epitope fusions, the adaptamers must be designed so that fusion to the ORF maintains the desired reading frame. The PCRs and transformations described can easily be performed in 1 to 2 working days. In general, the success-rate of the procedure is high with approximately 50% of the candidate clones being correct after pop-out of the *URA3* marker. Among the incorrect clones, about half lack an integration at the target site and the other half have acquired mutations during the PCRs, which underscores the importance of using a high fidelity Taq polymerase. We also applied this method to short tags such as 6xHIS and FLAG. Because of the small size of these tags, a region of the flanking non-coding genomic DNA was incorporated as part of the direct repeat since efficient recombinational pop-out of the *URA3* marker requires a minimum of approximately 100 bp of DNA homology [31,32].

Summary

Each of the adaptamer-directed genome manipulation methods is predicated on the fact that recombination between two DNAs is enhanced by increasing the length of homology. Many of the current PCR-based genome manipulation techniques rely on very short homologies to promote recombination. In these cases homology length is dictated by the technical limits of oligonucleotide synthesis. Adaptamers circumvent this problem since long homology regions are produced in a first round of PCR, and then fused to the selectable marker in a second round of PCR via complementary sequence tags on the adaptamers. Furthermore, many of the techniques described here rely on pre-existing and commercially

available adaptamer sets that can be obtained inexpensively rather than designing new primers for every experiment. Although a cost is incurred by performing multiple PCR amplifications, the increase in recombination efficiency is dramatic. Finally, the adaptamer-mediated PCR fusion methodology is versatile and can be applied to varied genome manipulations.

Acknowledgements

We thank Marisa Wagner, Bilyana Georgieva and Uffe Mortensen for critical reading of the manuscript. This work was supported by National Institutes of Health grant HG00193 to R. Reid, a grant from the Alfred Benzon Foundation to M. Lisby, National Institutes of Health grants HG01620 and GM50237 to R. Rothstein and Merck Genome Research Institute grant 5 to R. Rothstein.

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Table 1. PCR primers and adaptamers.

Primer Sequence	Name	Description
ccgctgctaggcgcgccgtg...	C	5' nonhomologous sequence tag used for all forward intergenic adaptamers.
gcagggatgcggccgctgac...	D	5' nonhomologous sequence tag used for all reverse intergenic adaptamers.
CTTGACGTTCGTTCGACTGATGAGC	kli5'	*K. lactis URA3* internal 5' primer.
GAGCAATGAACCCAATAACGAAATC	kli3'	*K. lactis URA3* internal 3' primer.
gtcagcggccgcatccctgcCCTCACTAAAGG GAACAAAAGCTG	d-Kl	3' *K. lactis URA3* "d" adaptamer. Nonhomologous region is reverse and complement of D.
cacggcgcgcctagcagcggTAACGCCAGGG TTTTCCCAGTCAC	c-Kl	5' *K. lactis URA3* "c" adaptamer. Nonhomologous region is reverse and complement of C.
GTCGACCTGCAGCGTACG	U2	5' primer for amplification of intergenic DNA from the yeast deletion strains.
CGAGCTCGAATTCATCGAT	D2	3' primer for amplification of intergenic DNA from the yeast deletion strains.
cgtacgctgcaggtcgacgggcccGTGTCACCA TGAACGACAATTC	u2-Kl*	5' *K. lactis URA3* "u2" adaptamer. Nonhomologous region is reverse and complement of U2.
atcgatgaattcgagctcgatcgatGTGATTCTGG GTAGAAGATC	d2-Kl†	3' *K. lactis URA3* "d2" adaptamer. Nonhomologous region is reverse and complement of D2.
ggaattccagctgaccaccatg…	A¥	5' nonhomologous sequence tag included on all forward ORF adaptamers.
gatccccgggaattgccatg…	B	5' nonhomologous sequence tag included on all reverse ORF adaptamers.
catggcaattcccggggatcGTGATTCTGGGTA GAAGATCG	b-Kl	5' *K. lactis URA3* "b" adaptamer. Nonhomologous region is reverse and complement of B.
catggtggtcagctggaattccCGATGATGTAG TTTCTGGTT	a-Kl	3' *K. lactis URA3* "a" adaptamer. Nonhomologous region is reverse and complement of A.
gttcttctcctttactcat…	g1	5' nonhomologous sequence tag of the GFP 5' end adaptamer.
ggatgaactatacaaaTAA…	g2#	5' nonhomologous sequence tag of the GFP 3' end adaptamer.
ATGAGTAAAGGAGAAGAAC	GFPstart-F	5' GFP primer. Reverse and complement of the g1 sequence tag.
TTTGTATAGTTCATCCATGC	GFPend-R	3' GFP primer. Underlined sequence is reverse and complement of the nonhomologous 5' section of the g2 sequence tag.

All sequences are listed in the 5' to 3' direction. Lowercase sequences denote nonhomologous 5' segments or sequence tags on adaptamers

* The underlined sequence is a *Apa*I restriction site

† The underlined sequence is a *Cla*I restriction site

¥ The underlined sequence is the ORF start codon.

The underlined sequence is a stop codon necessary for C-terminal GFP fusions

Figure Legends

Figure 1. Adaptamer-directed PCR fusions. Two different double-stranded DNA sequences are represented by thick gray and black lines. PCR primers are illustrated by arrows. Adaptamers are shown as arrows with triangles at their 5' ends representing the complementary sequence tags A and a. PCR amplification with these adaptamers incorporates the 5' sequence tags into double-stranded DNA (diamonds). In a second PCR reaction the first PCR products are mixed, the incorporated complementary sequence tags anneal and are extended by the polymerase (dashed lines). In successive PCR cycles, distal primers (arrows) amplify the fused product.

Figure 2. One-step gene replacement. A. Interruption of a gene cloned on a plasmid with a selectable marker using standard cloning techniques is represented by dashed lines. ORFs are indicated by open arrows, yeast intergenic sequences are illustrated by thick black lines, and plasmid DNA is indicated by a thin line. The restriction sites to linearize the gene disruption fragment are marked R. **B.** Two homology-directed recombination reactions (X) occur to produce a gene disruption in a single step.

Figure 3. "Microhomology" one-step gene disruptions. A. A selectable marker is PCR amplified using chimeric primers containing 5' sequences with homology to genomic DNA immediately upstream and downstream of the ORF to be disrupted. **B.** Integration mediated by short homology regions flanking the selectable marker.

Figure 4. Orientation of adaptamers on an 8 kilobase pair region of yeast chromosome IV. A map of 15 kilobase pairs near the centromere of chromosome IV. The black circle represents the centromere. White arrows indicate known or predicted open reading frames. Adaptamers (not to scale) are

shown as black arrows with gray (C) or black (D) triangles representing the standard 5' sequence tags.

Figure 5. Adaptamer-directed gene disruptions. A. Amplification of intergenic regions flanking the *ILV1* gene on chromosome V. Two PCR reactions amplify intergenic DNA containing the adaptamer tags (diamonds). **B.** Plasmid pWJ1077 containing the *K. lactis URA3* gene and the 143 bp direct repeats. **C.** Direct repeats flanking the *K. lactis URA3* ORF are represented as hatched boxes and were made by PCR amplifying a 143bp sequence 5' to the *URA3* ORF and cloning it into *Cla*I and *Apa*I restriction sites on the 3' side of the ORF in plasmid pWJ1077. Amplification of overlapping segments in two PCR reactions is indicated by shading. **D.** Fusion PCR reactions using the left intergenic DNA and the 3' section of *URA3* in one reaction and the right intergenic region and the 5' portion of *URA3* in the second reaction. The upper cartoons illustrate annealing of single strands mediated by complementary sequence tags while the bottom cartoons illustrate fused PCR products. **E.** Example of PCR fragments used to produce an *ILV1* gene disruption. The 352 bp left and 380 bp right intergenic regions for the *ILV1* gene (lanes 1 and 2) were amplified using wild-type genomic DNA from the W303 strain background as a template. The 1095 bp *K. lactis URA3* 3' and 946 bp *URA3* 5' DNAs (lanes 3 and 4) were amplified using plasmid pWJ1077 as a template. 2µl of each reaction were loaded and run on a 0.8% electrophoresis gel. DNA was visualized by ethidium bromide staining. Fusion reactions were performed by diluting the PCR products from the first reactions 100-fold into a 50µl PCR. 2µl of the amplified 1447 bp *ILV1*-left fused to *URA3*-3' and the 1326 bp *ILV1*-right fused to *URA3*-5' were loaded and run on the same gel (lanes 5 and 6).

Figure 6. Integration of two PCR fragments to disrupt a gene. A. Three recombination events are required to replace a gene in a single step using two DNA fragments. Thick gray arrows represent primer binding sites for PCR-

analysis of integrations. **B.** Direct repeat recombination results in "pop-out" of the *URA3* marker leaving a small fragment of non *S. cerevisiae* DNA in place of the ORF.

Figure 7. Transfer of gene disruptions from consortium strains to a new strain. A. Construction of a gene disruption by the yeast deletion consortium was performed using 45 bp homology to a gene of interest. *ILV1* is used as an example. Unique identifiers on the 5' side of the ORF (UPTAG) and 3' side of the ORF (DOWNTAG) are indicated by the checkered and black boxes. Each tag contains a unique 20 bp identifying tag flanked by common 18 bp primer binding sites for amplification of the unique sequence from any deletion strain. This is shown in detail for the UPTAG sequence (also see Table 1 for primer sequences). **B.** The 18 bp primer binding sites flanking the unique identifying tags are used in combination with appropriate intergenic primers to amplify the identifying tag along with the intergenic DNA (see text). **C.** Fusion of selectable markers to intergenic regions is mediated by the 18 bp U2 or D2 adaptamers.

Figure 8. Allele replacment using adaptamers. A. Amplification of ORFs is accomplished using adaptamers designed to precisely amplify every yeast ORF from start to stop codons. The forward and reverse adaptamers contain 5' sequence tags referred to as A and B, respectively. A mutation in the amplified ORF is indicated by an asterisk. **B.** Fusion to a selectable marker is performed in two reactions generating DNAs with overlapping segments of the selectable marker. **C.** Genome integration is mediated by homologous ORF sequences. **D.** Integration producing two mutant copies of the ORF as direct repeats. Pop-out of the selectable marker results in a single mutated copy of the ORF in the genome. **E.** Integration resulting in one mutant and one wild-type copy of the ORF. Pop-out of the selectable marker can result in the mutant or the wild-type copy of the allele integrated into the genome.

Figure 9. Construction of CFP/YFP-tagging vectors. DNA sequences encoding either the blue- or red-shifted version (W7, 10C) of GFP were amplified by PCR from the corresponding pRSETB vectors [28,29]. These DNA fragments were fused by PCR to either the 5'- or 3'-two-thirds of *K. lactis URA3*, which was amplified from pWJ716 [21]. The resulting PCR products were cloned into the *Sac*II site of pRS423 [32] to make vectors for CFP/YFP-tagging. **A.** Vector maps of pWJ1162 and pWJ1163 for CFP-tagging. **B.** Vector maps of pWJ1164 and pWJ1165 for YFP-tagging.

Figure 10. General strategy for CFP/YFP-tagging of yeast proteins. Using appropriately designed primers, CFP and YFP can be targeted to any site in the yeast genome. This figure describes the fusion of YFP to the 3'-end of gene X. The procedure involves two rounds of PCR and a yeast transformation. **A.** PCR amplification of target sequences. The first round of PCR amplifies 300 to 500 bp DNA sequences upstream and downstream of the target site using primer pairs UF_X/UR_Xg1 and DF_Xg2/DR_X, respectively. The UR_Xg1 and DF_Xg2 primers contain 19 bp 5'-sequence tags complementary to the 5'- and 3'-ends of YFP, respectively. Since YFP is fused to the 3'-end of gene X in this example, a *TAA* stop codon has been added to g2. The stop codon is omitted when making N-terminal and internal fusions of CFP/YFP. YFP-*URA3* sequence cassettes were PCR amplified from pWJ1164 and pWJ1165 using primer pairs GFPstart-F/kli3' and kli5'/GFPend-R. **B.** Fusion of the YFP coding sequence to target sequences. The sequence tags (g1 and g2) facilitate the fusion of the target sequences to YFP-*URA3* sequences using adaptamer technology [21,22] and the primer pairs UF_X/kli3' and kli5'/DR_X. Approximately 100 ng of each PCR fragment was used in the fusion reactions. **C.** Integration by homologous recombination. The two PCR fragments (500 ng of each) were co-transformed into yeast for integration by homologous recombination using the lithium acetate method [16]. The recombination event results in a YFP direct repeat flanking an intact *K. lactis*

URA3 sequence which allow transformants to be selected on SC-Ura. Transformants were restreaked on SC-Ura and single colonies picked into 2 ml YPD and grown overnight before plating on 5-FOA to select for deletion of the *URA3* marker by pop-out recombination between the two flanking YFP sequences. A clean fusion of YFP to gene X is left in the genome. (Adapted from [26])

Figure 1.

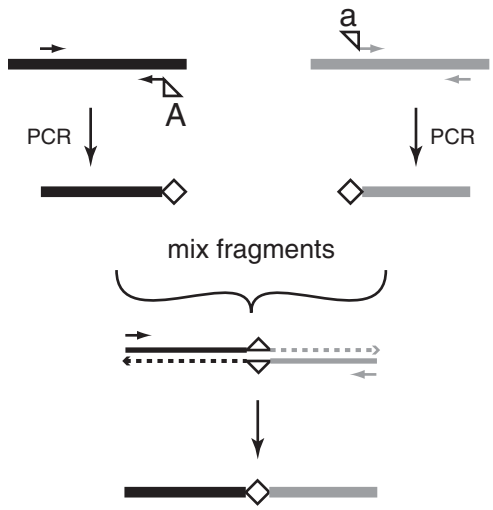


Figure 2.

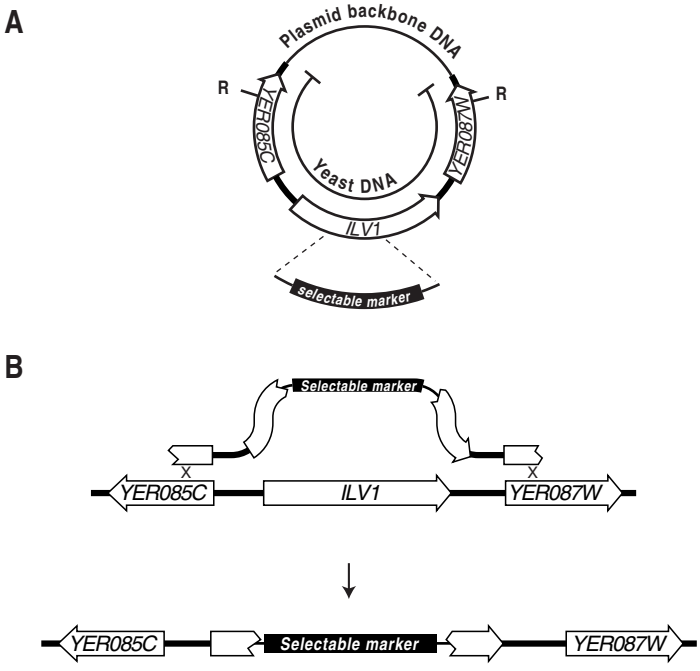

A
Plasmid backbone DNA
R
R
YER085C
YER087W
Yeast DNA
ILV1
selectable marker
B
Selectable marker
X
X
YER085C
ILV1
YER087W
YER085C
Selectable marker
YER087W

Figure 3.

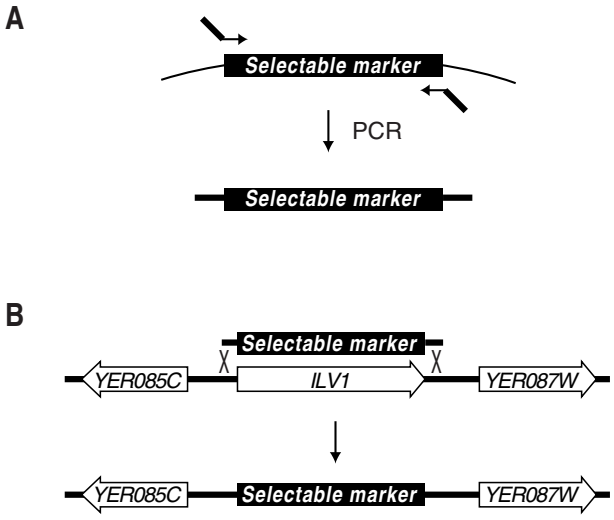

A
Selectable marker
PCR
Selectable marker
B
Selectable marker
YER085C
ILV1
YER087W
YER085C
Selectable marker
YER087W

Figure 4.

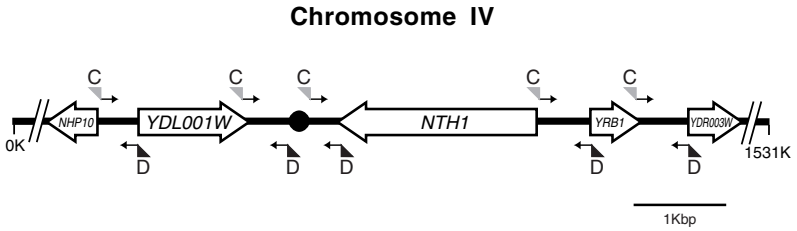

Chromosome IV
C
C
C
C
C
NHP10
YDL001W
NTH1
YRB1
YDR003W
0K
1531K
D
D
D
D
D
1Kbp

Figure 5.

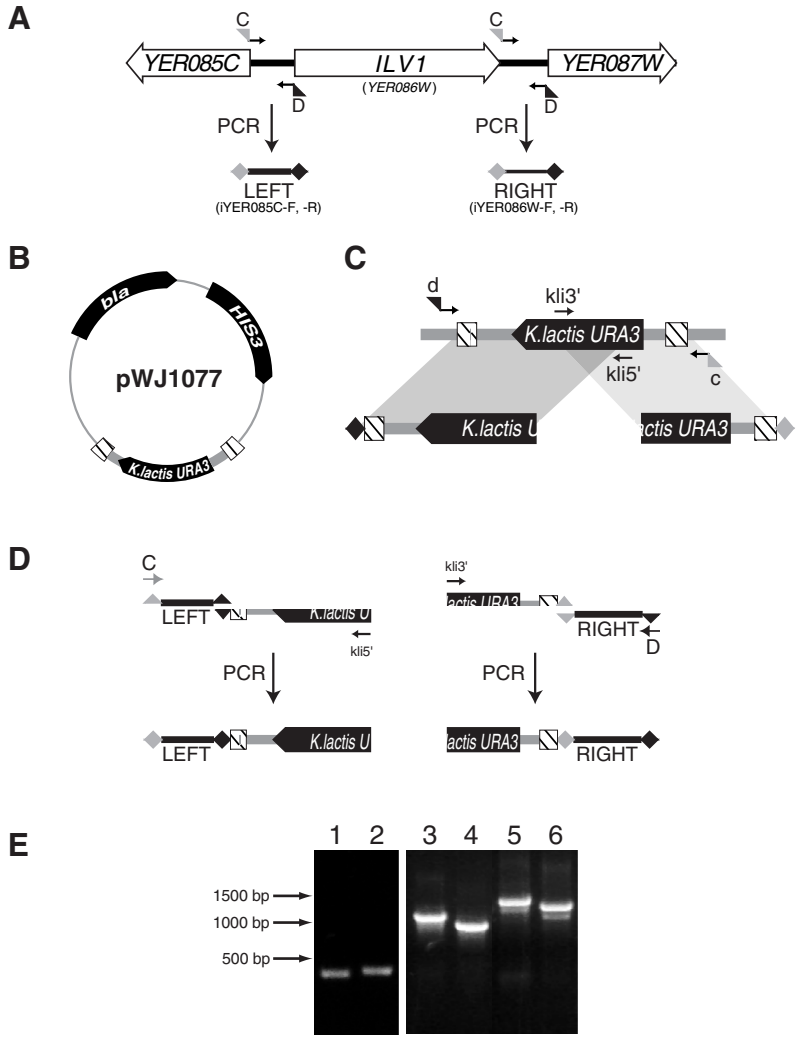

A
C
C
YER085C
ILV1
(YER086W)
YER087W
D
D
PCR
PCR
LEFT
(iYER085C-F, -R)
RIGHT
(iYER086W-F, -R)
B
bla
HIS3
pWJ1077
K.lactis URA3
C
d
kli3'
K.lactis URA3
kli5'
c
K.lactis U
ctis URA3
D
C
LEFT
K.lactis U
kli5'
PCR
LEFT
K.lactis U
kli3'
RIGHT
D
PCR
actis URA3
RIGHT
E
1 2 3 4 5 6
1500 bp
1000 bp
500 bp

Figure 6.

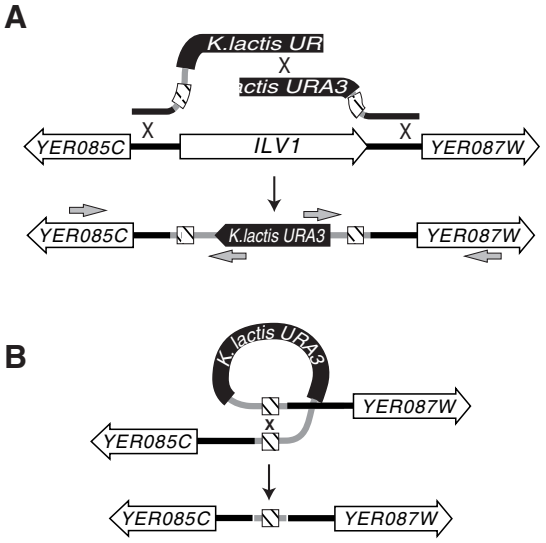

A
K.lactis UR
X
ctis URA3
X
X
YER085C
ILV1
YER087W
YER085C
K.lactis URA3
YER087W
B
K.lactis URA3
YER087W
X
YER085C
YER085C
YER087W

Figure 7.

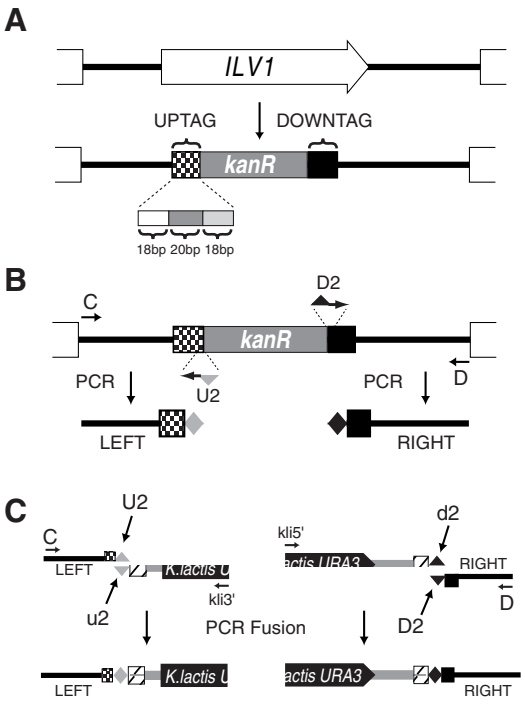

A
ILV1
UPTAG
DOWNTAG
kanR
18bp 20bp 18bp
B
C
D2
kanR
PCR
U2
PCR
D
LEFT
RIGHT
C
U2
LEFT
kli3'
u2
kli5'
d2
RIGHT
D
PCR Fusion
D2
LEFT
RIGHT

Figure 8.

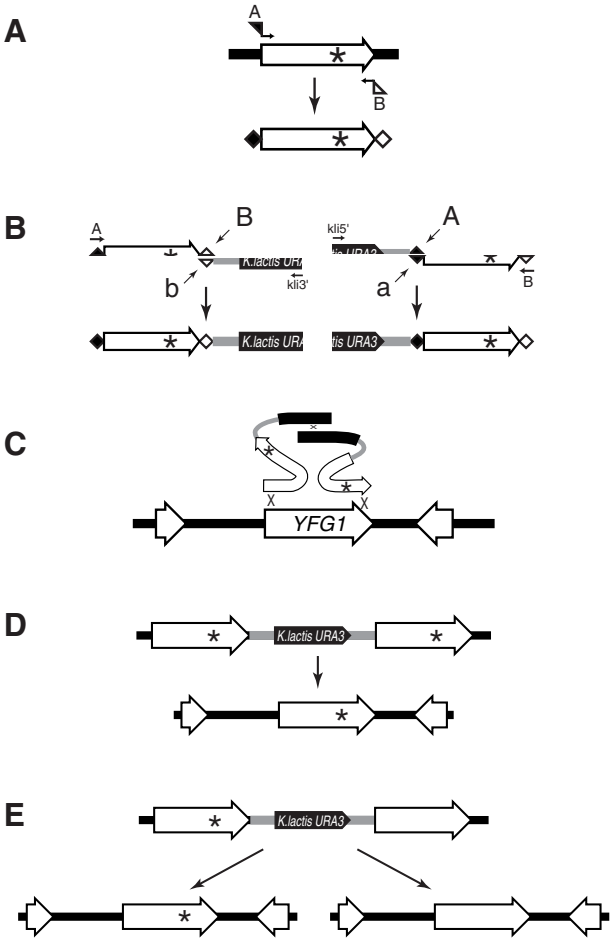

A
B
A
B
b
kli3'
kli5'
A
a
B
K.lactis URA3
C
X
X
YFG1
D
K.lactis URA3
E
K.lactis URA3

Figure 9.

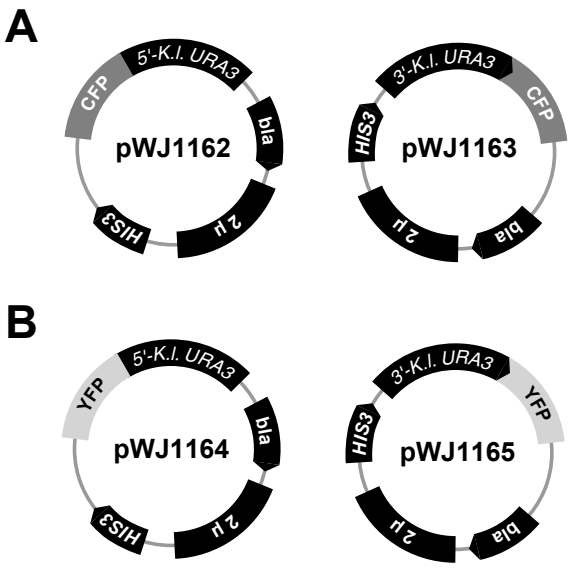
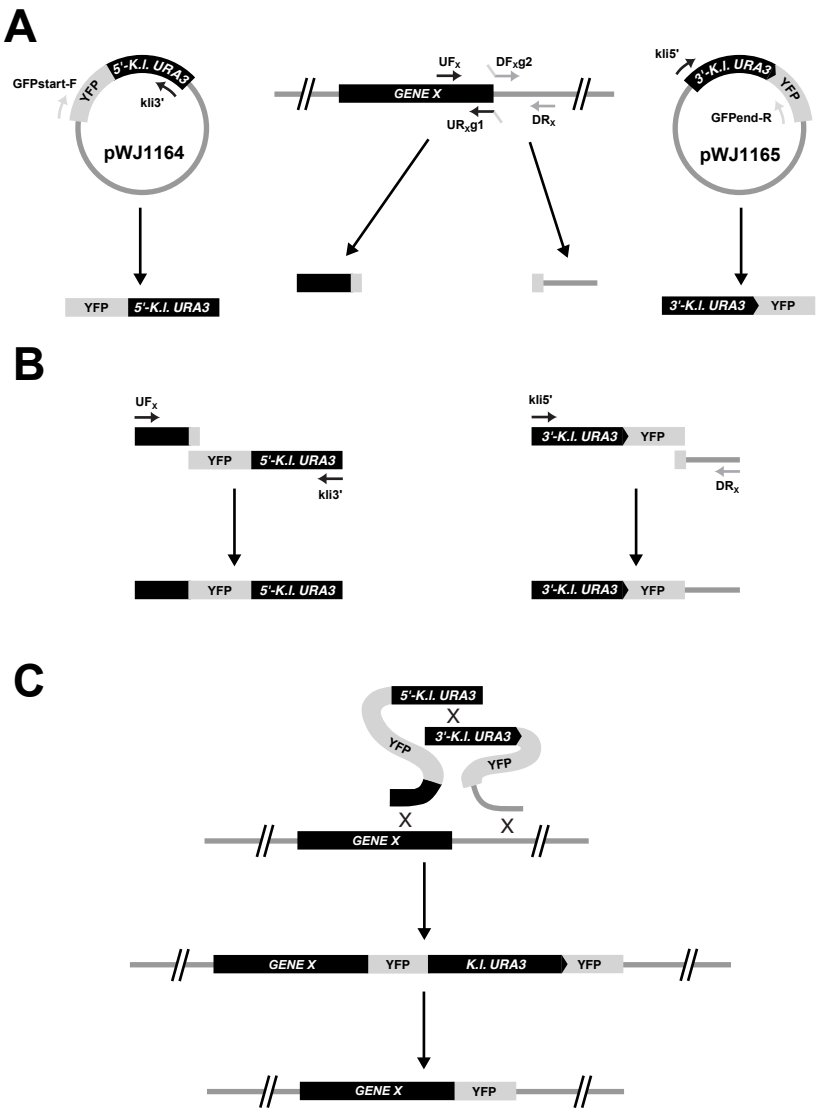

A
CFP
5'-K.I. URA3
bla
pWJ1162
2 µ
HIS3
3'-K.I. URA3
CFP
HIS3
pWJ1163
2 µ
bla
B
YFP
5'-K.I. URA3
bla
pWJ1164
2 µ
HIS3
3'-K.I. URA3
YFP
HIS3
pWJ1165
2 µ
bla

Figure 10.


A
GFPstart-F
YFP
5'-K.I. URA3
kli3'
pWJ1164
YFP
5'-K.I. URA3
UFx
DFxg2
GENE X
URxg1
DRx
kli5'
3'-K.I. URA3
YFP
GFPend-R
pWJ1165
3'-K.I. URA3
YFP
B
UFx
YFP
5'-K.I. URA3
kli3'
YFP
5'-K.I. URA3
kli5'
3'-K.I. URA3
YFP
DRx
3'-K.I. URA3
YFP
C
5'-K.I. URA3
X
3'-K.I. URA3
YFP
YFP
X
X
GENE X
GENE X
YFP
K.I. URA3
YFP
GENE X
YFP