

# In vivo site-directed mutagenesis using oligonucleotides

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Functional characterization of the genes of higher eukaryotes has been aided by their expression in model organisms and by analyzing site-specific changes in homologous genes in model systems such as the yeast *Saccharomyces cerevisiae*<sup>1</sup>. Modifying sequences in yeast or other organisms such that no heterologous material is retained requires *in vitro* mutagenesis together with subcloning<sup>2,3</sup>. PCR-based procedures that do not involve cloning are inefficient or require multistep reactions that increase the risk of additional mutations<sup>4,5</sup>. An alternative approach, demonstrated in yeast, relies on transformation with an oligonucleotide<sup>6</sup>, but the method is restricted to the generation of mutants with a selectable phenotype. Oligonucleotides, when combined with gap repair, have also been used to modify plasmids in yeast<sup>7</sup>; however, this approach is limited by restriction-site availability. We have developed a mutagenesis approach in yeast based on transformation by unpurified oligonucleotides that allows the rapid creation of site-specific DNA mutations *in vivo*. A two-step, cloning-free process, referred to as *delitto perfetto*, generates products having *only* the desired mutation, such as a single or multiple base change, an insertion, a small or a large deletion, or even random mutations. The system provides for multiple rounds of mutation in a window up to 200 base pairs. The process is *RAD52* dependent, is not constrained by the distribution of naturally occurring restriction sites, and requires minimal DNA sequencing. Because yeast is commonly used for random and selective cloning of genomic DNA from higher eukaryotes<sup>8</sup> such as yeast artificial chromosomes, the *delitto perfetto* strategy also provides an efficient way to create precise changes in mammalian or other DNA sequences.

## Results and discussion

We refer to our approach as *delitto perfetto* (Italian meaning “perfect murder” but used as an idiom for “perfect deletion”) because the introduction of the desired mutation involves the complete removal of a marker previously integrated at the target locus (Fig. 1). The first step involves integration of a counterselectable reporter (CORE) cassette using the highly proficient homologous recombination system of *S. cerevisiae*<sup>9–11</sup>. The mutation replacement step involves transformation by easily designed oligonucleotides that eliminate the CORE cassette.

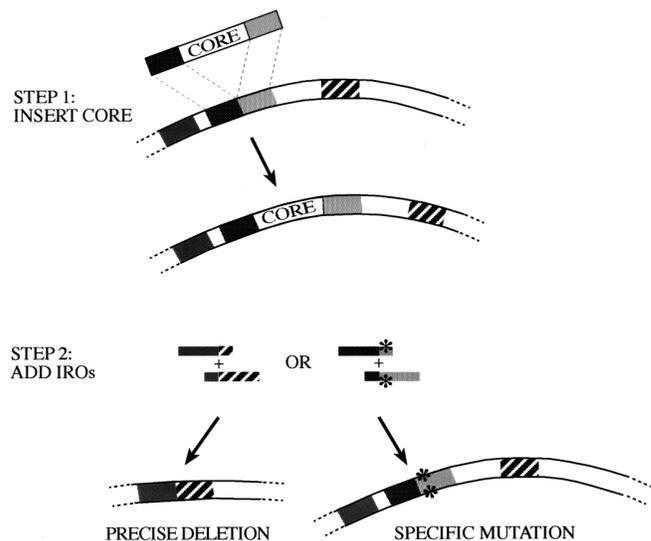
The strategy was applied initially to the deletion of the *MLP2* gene from the start to the stop codon (Fig. 2) such that no heterologous DNA was retained. The CORE cassette was targeted by standard homologous recombination techniques into three commonly used yeast strain backgrounds (BY4742, VL6 $\alpha$ , and E133). Two 80-nucleotide integrative recombinant oligonucleotides (IROs), *MLP2.a* and *MLP2.b*, were designed with a 20-base overlap at their 3' ends (Fig. 2). They were annealed and extended with DNA polymerase Pfx, resulting in a 140-base pair double-stranded molecule

that contains 70 base pairs homologous with the sequence upstream of and 70 base pairs downstream from the cassette. Cells were transformed with these molecules, and 5-fluoroorotic acid-resistant (5-FOA<sup>R</sup>) G418-sensitive (G418<sup>S</sup>) deletants were obtained easily, as shown in Table 1.

On the basis of these results, we expected that the *delitto perfetto* approach could also be applied to deletion of essential genes. To test this, we integrated the cassette beyond the stop codon of the *POL30* gene (*PCNA*) (Fig. 2) in strain E133, and a wild-type copy of the gene on a plasmid (pBL230) was introduced into the cell. Cells were then transformed with the 3'-overlapping IROs *PCNA.a* and *PCNA.b*, which were annealed and extended *in vitro*. They had homology upstream of the start codon and downstream from the CORE cassette (Fig. 2). Precise deletion of the chromosomal allele *POL30* was accomplished, though at a lower frequency than *MLP2*. This was probably due to competition for the plasmid-borne copy of *POL30* (Table 1).

We next applied the *delitto perfetto* strategy to the creation of site-specific mutations. This was demonstrated by the introduction of a silent mutation in *TRP5* that generated a new *Bam*HI site (Fig. 2). The CORE cassette was targeted into the *TRP5* gene of strain BY4742 (BY4742-TRP5-CORE). Cells were transformed with the 80-nucleotide IROs *TRP5.a* and *TRP5.b* (after annealing and extension) containing the *Bam*HI mutation site adjacent to the CORE-cassette insertion site, and 5-FOA<sup>R</sup> G418<sup>S</sup> colonies were isolated (Table 1). Among 50 clones tested, all had lost the cassette and acquired the desired mutation. Of 20 G418<sup>S</sup> clones sequenced, 19 did not have additional mutations.

Once the CORE cassette is integrated, many site-specific modifications can be generated simply by designing new oligonucleotides and repeating step 2 (Fig. 1). To investigate this, we applied the *delit-*



**Figure 1.** The *delitto perfetto* system using IROs. In step 1, a CORE cassette with *KIURA3* (counterselectable) and *kanMX4* (reporter) is inserted by standard DNA targeting procedures at a locus chosen to be deleted or close to a site where one or more changes are desired. In step 2, transformation of the cells with IROs leads to excision of the CORE cassette. Counterselection for loss of the *KIURA3* marker followed by testing for simultaneous loss of the *kanMX4* marker leads to deletion of the desired region or introduction of the desired mutation (asterisks), minimizing false positives. Generic DNA sequences are indicated as stippled or striped boxes. In this example, the IROs have a short overlap. The overlaps can be longer or can be extended, as described in the text.

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**Table 1. Accuracy of targeted changes with *delitto perfetto***

Strain	Locus	Designed change	IRO species used <sup>a</sup>	5-FOA <sup>R</sup> /10 <sup>7</sup> cells <sup>b</sup>	G418 <sup>R</sup> /10 <sup>7</sup> cells <sup>c</sup>	Correctly excised CORE <sup>d</sup>	Correctly targeted clones <sup>e</sup>	Clones without additional changes <sup>f</sup>
BY4742	<i>MLP2</i>	Δ ORF <sup>g</sup>	MLP2.a+b Pfx	225	97	18/18	3/3	2/3
VL6α	<i>MLP2</i>	Δ ORF	MLP2.a+b Pfx	215	118	6/6	ND <sup>h</sup>	ND
E133	<i>MLP2</i>	Δ ORF	MLP2.a+b Pfx	284	101	6/6	ND	ND
E133	<i>POL30</i>	Δ ORF	PCNA.a+b Pfx	70 <sup>i</sup>	4 <sup>i</sup>	9/12	ND	ND
BY4742	<i>TRP5</i>	A334A ( <i>Bam</i> HI)	TRP5.a+b Pfx	190	130	50/50	50/50	19/20
			TRP5.e+f	286	212	40/40	40/40	11/15
BY4742	<i>SIR2</i>	G270A	270.a+b Pfx	302	105	20/20	20/20	13/20
			270.a+b	151	27	20/20	20/20	17/20
			270.e+f	237	150	19/19	19/19	16/19
BY4742	<i>SIR2</i>	N345A	345.a+b Pfx	263	126	11/11	9/11	6/11
BY4742	<i>SIR2</i>	H364Y	364.a+b Pfx	210	42	14/14	12/14	6/14

<sup>a</sup>*In vitro* extension of IROs is indicated with Pfx. When Pfx is not mentioned, the IROs were added directly to the cells.

<sup>b</sup>Number of 5-FOA<sup>R</sup> clones per 10<sup>7</sup> viable cells transformed with 0.5 nmol of each IRO.

<sup>c</sup>Transformation frequency: number of G418<sup>R</sup> clones per 10<sup>7</sup> viable cells, per 0.5 nmol of each IRO.

<sup>d</sup>Number of clones with correct CORE-cassette excision/number of random, 5-FOA<sup>R</sup>, G418<sup>R</sup> samples tested by colony PCR.

<sup>e</sup>Number of clones that acquired the targeted change/number of samples sequenced, or digested with *Bam*HI.

<sup>f</sup>Number of targeted clones without additional mutations/number of samples sequenced. Additional mutations found within targeted clones were never detected outside the sequence covered by IROs. The spectrum of additional mutations within the sequence replaced by IROs included 70% deletions and was as follows: 46.7% 1 bp deletions, 26.7% 1 bp substitutions, 16.7% 2 bp deletions, 6.6% 1 bp deletions plus single-base pair substitutions and 3.3% duplications of part of an IRO sequence. Long, unpurified oligonucleotides are synthesized with a high proportion of truncated molecules, which may represent the source of the deletions observed.

<sup>g</sup>ORF, open reading frame.

<sup>h</sup>ND, not determined.

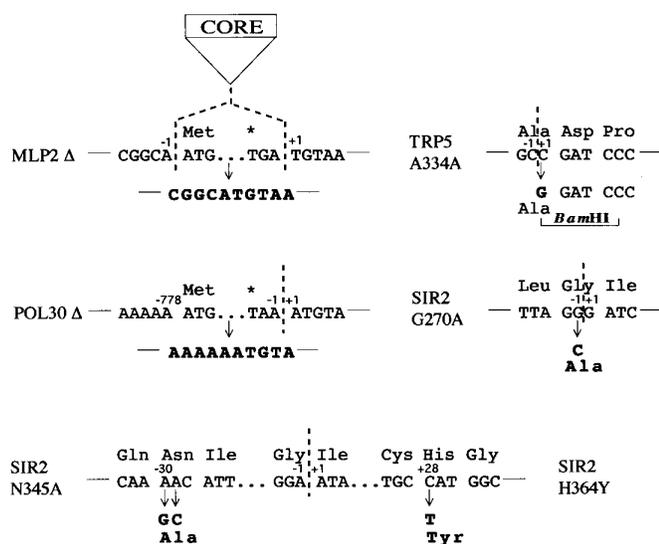
<sup>i</sup>Mean value of three experiments.

*to perfetto* strategy to site-specific changes in the regions surrounding two positions of CORE-cassette integration (codon 270 and between codons 354 and 355) in the *SIR2* gene (Fig. 2). Mutations were contained either in the overlapping regions (IROs 270.a+b) or in just one of the oligonucleotides (345.a+b and 364.a+b). After annealing and DNA synthesis, strains were transformed using these IRO pairs. All 5-FOA<sup>R</sup> G418<sup>R</sup> clones had lost the CORE cassette, based on colony PCR, and the site-directed mutation targeting was highly efficient (Table 1). In fact, infrequently occurring additional mutations all were found to reside in the region covered by IROs.

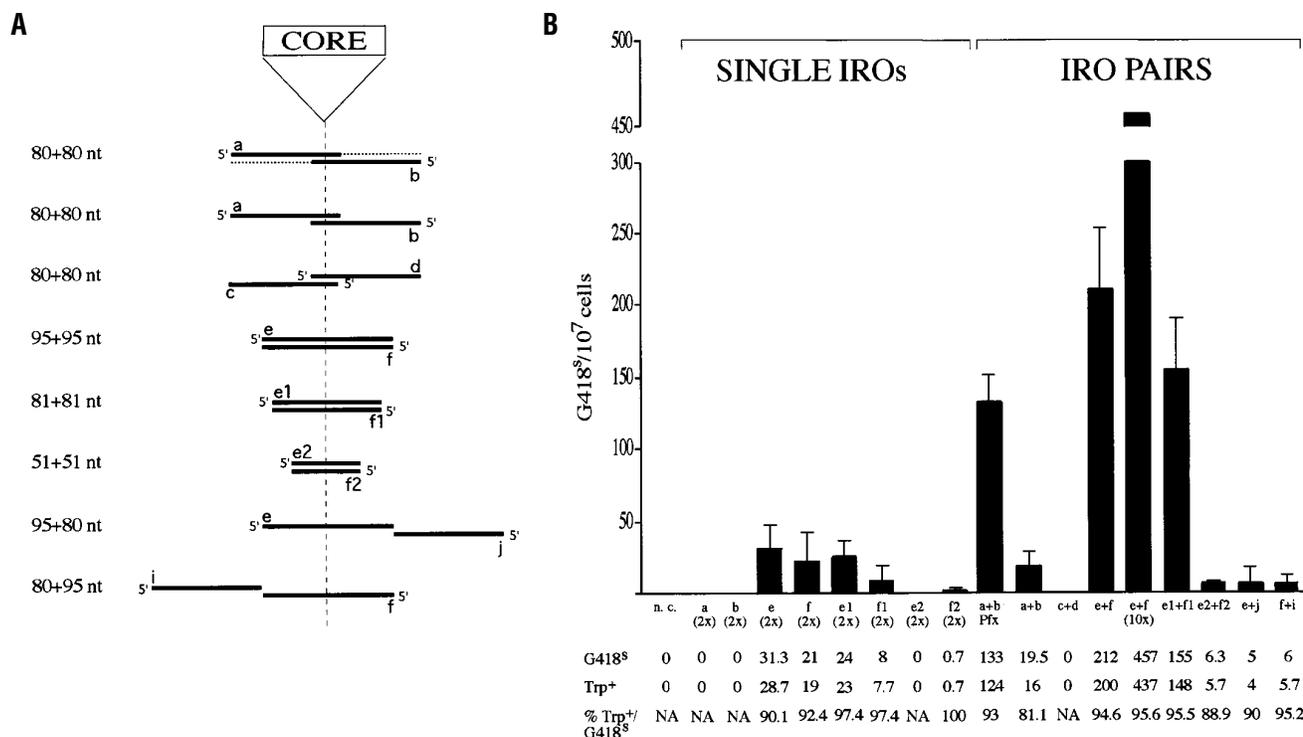
Site-directed mutagenesis can also be accomplished by adding individual oligonucleotides directly to cells. The strain BY4742-TRP5-CORE was transformed with several IROs (Fig. 3A)

that would yield the silent *Bam*HI mutation in *TRP5* (Fig. 2). The 95- (e or f) and 81-nucleotide (e1 or f1) single IROs that extend to either side of the CORE cassette were comparably effective at generating site-directed mutations when added individually (Fig. 3B). The minimum length of homology for efficient mutagenesis was between 25 and 40 nucleotides (compare the 51-mers e2 and f2 with e1 and f1).

Pairs of IROs were also examined for their ability to create the same site-specific mutation when added directly to cells without prior annealing or extension *in vitro*. As shown in Figure 3B, modification by the addition of unannealed pairs of 81- or 95-nucleotide IROs was highly efficient if they were fully complementary. Surprisingly, a pair of oligonucleotides with only a 20-base pair 3' overlap was also highly efficient at creating site-specific change. The efficiency with TRP5.a+b was about one-sixth of that when the pair was annealed and extended



**Figure 2.** Specific changes produced by IROs. Target loci in *MLP2*, *POL30*, *TRP5*, and *SIR2* are shown with their wild-type nucleotide and amino acid sequence. Asterisks indicate stop codons. DNA sequence changes and the corresponding amino acid substitutions are shown in boldface below the arrows. Dashed lines indicate the positions where the CORE cassette was integrated. The small numbers above each wild-type DNA sequence correspond to the base pair position relative to the place at which the CORE cassette was integrated. Integrative recombinant oligonucleotides (sequences listed in Supplementary Table 1 in Web Extras, *Nature Biotechnology* Online) are described with numbers indicating the DNA sequences spanned by each oligonucleotide, starting from the first 5' nucleotide and terminating with the last 3' base relative to the CORE cassette integration point. The IROs used to delete *MLP2* were *MLP2.a* (5' -70...-1, +1...+10) and *MLP2.b* (5' +70...+1, -1...-10); to delete *POL30*, *PCNA.a* (5' -848...-778, +1...+10) and *PCNA.b* (5' +70...+1, -778...-788); to generate the silent mutation in the codon 334 of *TRP5*, *TRP5.a* (5' -70...-1, +1...+10), *TRP5.b* (5' +70...+1, -1...-10), *TRP5.c* (5' +10...+1, -1...-70), *TRP5.d* (5' -10...-1, +1...+70), *TRP5.e* (5' -47...-1, +1...+48), *TRP5.f* (5' +48...+1, -1...-47), *TRP5.e1* (5' -40...-1, +1...+41), *TRP5.f1* (5' +41...+1, -1...-40), *TRP5.e2* (5' -25...-1, +1...+26), *TRP5.f2* (5' +26...+1, -1...-25), *TRP5.i* (5' -127...-47), and *TRP5.j* (5' +128...+48). The IROs used to generate the G270A mutation in *SIR2* were 270.a (5' -70...-1, +1...+10), 270.b (5' +70...+1, -1...-10), 270.e (5' -48...-1, +1...+47), and 270.f (5' +47...+1, -1...-48); to generate the N345A mutation in *SIR2*, 345.a (5' -85...-1, +1...+10) and 345.b (5' +70...+1, -1...-10); to generate the H364Y mutation in *SIR2*, 364.a (5' -70...-1, +1...+10) and 364.b (5' +85...+1, -1...-10).



**Figure 3.** Relationship between frequencies of site-directed mutations and structure of various IRO species used for transformation experiments. (A) Types of IROs examined. The IROs a and b overlap for 20 bases at their 3' ends, whereas c and d overlap for 20 bases at their 5' ends; e and f, e1 and f1, and e2 and f2 overlap completely. Pairs e+j or f+i do not overlap each other. The dotted lines indicate *in vitro* extension with Pfx. Dashes separate sequences in IROs that are homologous with the upstream or downstream regions of the CORE cassette integration point, respectively. The IRO nucleotide lengths are shown on the left. (B) The specific frequencies of 5-FOA<sup>R</sup> G418<sup>s</sup> clones per 10<sup>7</sup> cells in transformation experiments with single or pairs of IROs in the strain BY4742-TRP5-CORE are shown. Vertical bars represent the 95% confidence interval values from three to six determinations. The types of IROs analyzed are indicated below each bar. The amount of each oligonucleotide used was 0.5 nmol, unless otherwise indicated (2× or 10×). Transformation with 0.05 nmol of IROs reduced targeting efficiency by ~10-fold (data not shown). Pfx indicates that there was *in vitro* extension. Beneath each bar is the mean number of 5-FOA<sup>R</sup> G418<sup>s</sup> clones/10<sup>7</sup> cells followed by the mean number of Trp<sup>+</sup> clones and finally by the percentage of Trp<sup>+</sup> (% Trp<sup>+</sup>) among the 5-FOA<sup>R</sup> G418<sup>s</sup> clones. Among the G418<sup>s</sup> integrants, the proportion of Trp<sup>+</sup> clones was high, indicating that additional TRP5 inactivating point mutations were infrequent. Five random Trp<sup>+</sup> clones for each type of IRO used were tested and shown to contain the expected BamHI bands, demonstrating that the desired mutation was successfully inserted into the TRP5 gene. Of nine Trp<sup>+</sup> G418<sup>s</sup> clones randomly chosen for sequencing, all had a frameshift mutation confined to the IRO region (data not shown). n.c., negative control (no IRO DNA); NA, not applicable.

*in vitro* (TRP5.a+b Pfx). A similar pair of oligonucleotides, but with opposite polarity resulting in 5' overlaps (c+d in Fig. 3A), yielded no transformants. TRP5.e+j or TRP5.f+i, which do not overlap, showed no increase over either TRP5.e or TRP5.f alone. Similar results were obtained for modifications at SIR2 codon 270 (see Fig. 2) in strain BY4742 by using corresponding single and double IROs (270.a, b, c, d, e, f, e1, f1, e2, f2, j, and i; data not shown). Targeting efficiency was dependent on oligonucleotide concentration and size (Fig. 3B). Similar to the results with single IROs, the minimum homology required was between 25 and 40 nucleotides (e2+f2 vs. e1+f1). There appeared to be little DNA degradation within the cell, because protected oligonucleotides that were phosphorothioated at their 3' and 5' ends did not increase the transformation efficiency of the IRO pairs (TRP5.a+b, TRP5.c+d, or TRP5.e+f; data not shown).

The RAD52 gene, which is essential for nearly all types of homologous recombination, is essential for *delitto perfetto* mutagenesis. Wild-type and mutant strains (RAD52::LEU2; two independent isolates) were transformed with a control centromeric plasmid pLKL67Y (containing HIS3 and URA3) or the following TRP5 IROs: a+b Pfx, a+b, c+d, e+f, e, and f. No 5-FOA<sup>R</sup> G418<sup>s</sup> clones were recovered in the *rad52* strain with various IROs, even when a 10 fold greater amount of e+f DNA was used (data not shown). Comparable results were found for modification of the SIR2 codon 270. It is interesting that the single-oligonucleotide approach described by

Moerschell *et al.*<sup>6</sup> and Yamamoto *et al.*<sup>12</sup> does not require homologous recombination. Our system is different because small regions distant from each other (separated by the CORE cassette) must be brought into close proximity to be paired with the oligonucleotide sequences, a feature that may account for the dependence on RAD52.

The results presented here demonstrate the versatility of the *delitto perfetto* strategy. Pairs of IROs e+f (95 base pairs) and a+b Pfx (140 base pairs; Fig. 3) appear to be the most effective for introducing specific point mutations or insertions and for creating deletions. Integration by IROs is efficient provided there are 40 nucleotides of homology upstream of and downstream from the CORE-cassette integration site, whether within an IRO or at the ends of interacting IRO pairs. We suggest that because of the opportunity to use overlapping pairs of IROs, a locus of at least 58 (as shown in Fig. 2 for the SIR2-N345A and H364Y mutations) and probably up to ~200 nucleotides (using 100-nucleotide IROs with 20-nucleotide overlap) could be targeted for site-directed or random mutagenesis (using degenerate oligonucleotides). Mutagenesis efficiency may decrease as the site-directed mutation is placed more distal to the CORE because the IRO recombination event leading to excision of the CORE could occur without inclusion of the mutation in the IRO. The opportunity for multiple rounds of mutational changes over a large region is a significant advance; for other mutagenesis procedures, each step in the cre-



ation of site-specific mutations must be repeated from the beginning, even for additional modifications of the same residue.

Sequencing of the genomes of several higher eukaryotes, including humans, has produced a need for functional evaluation of genes within large DNA molecules. A disadvantage of present systems for modification of such large molecules<sup>13–15</sup> is the requirement for *in vitro* mutagenesis, which depends on laborious subcloning of the region to be mutagenized. If the mutation is created by transformation with a PCR product, extensive sequencing is required<sup>5,16</sup>. We propose that the *delitto perfetto* mutagenesis strategy presented here can be expanded to the functional analysis of genes contained within large yeast artificial chromosomes, such as human genes that are specifically isolated from the genome by TAR cloning<sup>8</sup>. The approach may also be applicable to other organisms in which homologous recombination is efficient, such as *Escherichia coli* (RecE/RecT recombination proficient)<sup>16</sup>, the moss *Physcomitrella patens*<sup>17</sup>, and chicken DT40 cells<sup>18</sup>.

### Experimental protocol

**Yeast strains and vectors.** Haploid strains BY4742 (*ura3Δ0*)<sup>19</sup>, VL6α (*ura3-52*), and E133 (*ura3-52*)<sup>20</sup> were used. Cells were grown in standard rich (YPD), glycerol (YPG), and synthetic minimal medium without uracil (SD Ura<sup>-</sup>), leucine (SD Leu<sup>-</sup>), or tryptophan (SD Trp<sup>-</sup>) and transformed as described<sup>9,20</sup>. Geneticin (G418) resistant cells were grown on YPD plates containing 200 μg/ml of G418 (Gibco BRL, Grand Island, NY). Ura<sup>-</sup> cells were selected on synthetic complete medium containing 5-FOA (Toronto Research Chemicals Inc., North York, ON, Canada) at 1 mg/ml. Genetic manipulations were conducted essentially as described<sup>9,20</sup>. The plasmid pCORE was constructed by cloning a 1.5 kb *Bam*HI-*Hinc*II fragment containing the *kanMX4* gene<sup>9</sup> into the *Bam*HI-*Ssp*I sites of pFA6aKIURA3 (ref. 21). Plasmid pBL230 (ref. 22) harbors genomic DNA containing the *POL30* gene (from 195 bp upstream of the ATG to 170 bp downstream from its stop codon). Deletion of *RAD52* was made using pΔ52Leu (ref. 20) cut with *Apal* and *NotI*. We created pLKL67Y by cloning a 1.2 kb *URA3* fragment from pRS315+*URA3* into the *Xho*I-*Sac*I sites of the centromeric vector pRS313 (ref. 20).

**PCR amplification and sequence analysis.** The *kanMX4* and *KIURA3* CORE cassette was amplified as a 3.2 kb DNA fragment from pCORE by using *Taq* DNA polymerase (Roche, Indianapolis, IN), with 32 cycles of 30 s at 94°C, 30 s at 56°C, and 3 min 20 s at 72°C. For integration of the cassette into chromosomal loci, chimeric 60- to 70-mers were designed, consisting of 40–50 nucleotides homologous with the appropriate flanking region of the genomic target locus and 20 nucleotides, shown here, which allow for the amplification of the CORE cassette: 5'...GAGCTCGTTCGACACTGG-3' for the *kanMX4* side and 5'...TCCTTACCATTAAGTTGATC-3' for the *KIURA3* side. To identify clones with correct CORE cassette integration, we performed colony PCR (ref. 9) using primers designed for annealing upstream of and downstream from the integration locus and within the marker cassette. Primers used for CORE cassette insertion and primers used in all verification experiments by colony PCR together with the complete sequences of all IROs used in this work are available on request and as Supplementary Table 1 in the Web Extras page of *Nature Biotechnology* Online.

Colony PCR fragments (~0.6 kb) for the new mutant constructs together with their corresponding wild-type loci were purified by phenol–chloroform extraction, purified using the QIAquick PCR Purification Kit (Qiagen USA, Valencia, CA), and used for DNA sequencing. Sequencing reactions were performed on both strands using the dRhodamine Terminator Cycle Sequencing Kit (ABI Prism, Applied Biosystems, Foster City, CA) and run in an automatic sequencing machine (ABI377; Applied Biosystems).

**Integrative recombinant oligonucleotide mutagenesis.** Integrative recombinant oligonucleotides used in this study differ by size and orientation with respect to the CORE cassette integration point. Gibco BRL (unpurified, unphosphorylated) oligonucleotides (designed as described in the legend to Fig. 2) were used in transformation experiments either singly or as pairs, as shown in Figure 3. To extend the sequence of the 3'-overlapping IROs (a+b) *in vitro*, we used a 50 μl reaction mix that contained 0.5 nmol (15–20 μg) of each primer, 4U *Platinum Pfx* (Gibco BRL), 5 μl 10 × buffer, 1 μl 50 mM

MgSO<sub>4</sub>, and 2 μl 10 mM dNTPs (Roche). We performed extension as follows: 1 min at 94°C and 30 s to 3 min at 68°C. Samples were ethanol-precipitated and resuspended in 10–20 μl water. The use of *Taq* (Roche) instead of *Platinum Pfx* DNA polymerase partially reduced the efficiency of oligonucleotide integration. Before adding IRO DNA to the cells, the single species or the two species combinations, except for the pair extended *in vitro* with *Pfx* DNA polymerase (a+b *Pfx*), were denatured at 100°C for 2 min and then placed immediately on ice to eliminate possible secondary structure. Cells from each IRO transformation were spread on a single YPD plate, incubated at 30°C, and replica-plated to 5-FOA the following day. After three days, colonies were replica-plated to YPD, G418, Trp<sup>-</sup> (for *TRP5* experiments), and YPG (to select against petite mutants) plates.

*Note: Supplementary information can be found on the Nature Biotechnology website in Web Extras ([http://www.biotech.nature.com/web\\_extras](http://www.biotech.nature.com/web_extras)).*

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