

One step construction of PCR mutagenized libraries for genetic analysis by recombination cloning

Al-Muataz Khalil, Jeffrey A. Julius and Jeff Bachant*

Department of Cell Biology and Neuroscience, University of California, Riverside, Riverside, California 92521, USA

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ABSTRACT

Recombination cloning encompasses a set of technologies that transfer gene sequences between vectors through site-specific recombination. Due in part to the instability of linear DNA in bacteria, both the initial capture and subsequent transfer of gene sequences is often performed using purified recombination enzymes. However, we find linear DNAs flanked by *loxP* sites recombine efficiently in bacteria expressing Cre recombinase and the lambda Gam protein, suggesting Cre/*lox* recombination of linear substrates can be performed *in vivo*. As one approach towards exploiting this capability, we describe a method for constructing large ($> 1 \times 10^6$ recombinants) libraries of gene mutations in a format compatible with recombination cloning. In this method, gene sequences are cloned into recombination entry plasmids and whole-plasmid PCR is used to produce mutagenized plasmid amplicons flanked by *loxP*. The PCR products are converted back into circular plasmids by transforming Cre/Gam-expressing bacteria, after which the mutant libraries are transferred to expression vectors and screened for phenotypes of interest. We further show that linear DNA fragments flanked by *loxP* repeats can be efficiently recombined into *loxP*-containing vectors through this same one-step transformation procedure. Thus, the approach reported here could be adapted as general cloning method.

INTRODUCTION

Recombination-based cloning was developed to address some of the limitations of conventional recombinant DNA techniques. In particular, using conventional methods, DNA sequences must be flanked by compatible restriction sites before they can be sub-cloned into

recipient vectors. It was recognized that genome-scale projects would require more efficient cloning methods (1). To address this problem, the strategy underlying all recombination-cloning systems is that DNA sequences—typically gene open reading frames—are first introduced into an entry vector to create master clones (2,3). Entry vectors are engineered to contain site-specific recombination sites that direct gene transfer from the master clone to a panel of expression plasmids. Recombinants are selected by appropriate genetic criteria, yielding constructs in which genes are juxtaposed to novel regulatory elements or fused to epitope or affinity tags.

The first recombination cloning method to be devised was the Univector system, in which Cre recombinase (4) is used to catalyze recombination between a *loxP* site on a master clone and a *loxP* site on an expression vector, fusing them to form a co-integrate plasmid molecule (1). It was also shown that a second recombination event could be employed to resolve plasmid fusions such that only sequences placed between two recombinase sites would be donated to the final recombinant. Subsequent cloning systems have featured this dual recombinase site strategy to effect precise gene transfer. For example, the Creator system offered by Clontech utilizes recombination between two *loxP* sites on the master clone and one *loxP* site on the expression vector to transfer gene sequences (5), while the Gateway system from InVitrogen employs two variant versions of lambda recombinase sites (6,7). One disadvantage of the Creator and Gateway systems as they are currently used is that both the initial capture of gene sequences and their subsequent transfer to expression vectors is catalyzed by purified recombinases, adding to the cost of these methods. To circumvent this, there has been an interest in developing cloning methods that can be performed directly in bacterial hosts. For example, a cloning system called MAGIC has recently been described that obviates the need for site-specific recombinases entirely, relying instead on endonuclease-targeted bacterial recombination to mobilize gene sequences (8). It can be anticipated that further development of *in vivo* cloning methods will improve the speed and efficiency of genome analysis. These methodological advances are

*To whom correspondence should be addressed. Tel: +1951 827 6473; Fax: +1951 827 3087; Email: jeffbach@citrus.ucr.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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important, because biologists are increasingly turning to recombination cloning as a means to systematically process and examine the function of the large numbers of genes. As a testament to this, multiple projects are underway to transfer complete sets of open reading frames from different organisms into recombination vectors to facilitate this experimentation (9–11).

We initiated the work in this report because, rather than just transferring genes to different expression plasmids, we wished to use recombineering to analyze large collections of gene mutations. Advances in functional genomics have opened up the possibility of using genome-wide RNAi or chemical genetics screens to assess null phenotypes for novel genes (12,13). In many cases, however, it would be beneficial to produce and analyze additional types of mutations, either for a more comprehensive genetic analysis or for other types of experiments. Towards this end, we describe a method for constructing large randomly mutagenized gene libraries in recombination entry vectors, and show this approach can be used effectively in conjunction with recombination cloning to identify allelic variants with novel phenotypic traits. In developing this method, it was necessary to establish conditions under which linear DNA molecules flanked by *loxP* could be stabilized for site-specific recombination *in vivo*. As an additional application, we show that the ability to stabilize linear DNAs for Cre/*lox* recombination could be exploited as a generalized cloning system.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strain BW23474 (1) [Δ *lac-169 rpoS* (14) *robA1 creC510 hsdR514 Δ uidA(MluI):pir-116 endA(BT333) recA1*] was used to propagate plasmids containing the R6K γ origin. DH5 α [*endA1 hsdR17 glnV44 thi-1 recA gyrA relA1 Δ (lacIZYA-argF)U169 deoR (ϕ 80*dlaC*(*lacZ*)M15*] was used for other plasmid manipulations. BUN10 (1) [*recB recC sbcA hsdR:pir-116*] and AK1 [*recB recC sbcB hsdR:pir-116*] were used to examine Univector re-circularization in *recB recC* mutants. AK1 was derived from JC6783 (15) by disrupting *hsdR* with a *pir-116-Cm^R* cassette.

To construct pAK047, a HindIII (filled with T4 DNA polymerase)-MscI fragment encoding Tc^R from pBR322 was cloned into XhoI treated (filled) pUNI-10 to form pAK004. pAK004 was treated with NdeI and KpnI, releasing a 1484 bp fragment containing a *loxP* site at the 3' end of *tet*. This fragment was blunted with T4 DNA polymerase, ligated to itself, and fused with pUNI-10 in an *in vitro* Cre/*lox* UPS reaction (1). The resulting recombinant, pAK005, contains *tet* flanked by *loxP*. The Kn^R region of pAK005 was replaced with *kanMX4* by cloning a SmaI/SacI fragment from pFA6a/*kanMX4* (16) into SacI/PvuII treated pAK005, yielding pAK046. pAK046 was PCR amplified with oligonucleotides 5'-AGCAGA TCAGATTACCCTGTTATCCCTAGGATTCACCACT CCAAGAATTGGAGC and 5'-TGCATGGCATTAGG GATAACAGGGTAATAACCAAGCCTATGCCTACA GCATCC, removing the majority of *tet* and introducing

I-SceI sites (underlined). The fragment was treated with I-SceI and re-ligated to form pAK047. To construct pJBN260, a 435 bp NotI-MscI fragment from pAK047 was cloned into HpaI/PspOMI-treated pDONR221. pJBN250 was constructed by PCR amplifying the *gam bet exo* region from lambda BstEII DNA standards (New England Biolabs) using oligos 5'-AGAAAGCTTT GTTGACAATTAATCATCCGGCTCGTATAATGTGT GGAATTGTGAGCGGATAACAATTTACCAAGGA GGTATACATATGGATATTAATACTGAAACTG and 5'-GCTAGTGCTAGCGACGAAAGTGATTGCGCCT ACCCG. The longer oligo anneals to the 5' end of *gam* (bold), and includes *trc* promoter (underlined) and Shine-Delgarno (italicized) sequences. The HindIII and NheI sites incorporated in the oligos were used to clone the resulting fragment into HindIII/NheI-treated pQL269. Details of constructing Univector plasmids for *URA3*, *DBF4* and *MIF2* mutagenesis are available upon request.

Mutagenic PCR

Error-prone PCR was performed essentially as described (17). Approximately 5 ng of template DNA was added to a reaction containing 5 μ l 10 \times Buffer B (10 mM Tris-HCl pH 9.0, 50 mM KCl final concentration), 5 μ l 10 pmol/ μ l JB.45 (final 1 pmol/ μ l), 5 μ l 10 pmol/ μ l JB.57 (final 1 pmol/ μ l), 3.5 μ l 25 mM MgCl₂ (final 1.75 mM), 0.25 μ l of 25 mM MnCl₂ (final 0.125 mM), 4.3 μ l of 10 mg/ml BSA, 1 μ l of 10 mM dNTPs, 1 μ l 10 mM dTTP, 1 μ l 10 mM dCTP (final 200 μ M dATP, 200 μ M dGTP, 400 μ M dTTP and 400 μ M dCTP), 1.5 μ l of 5 U/ μ l concentration Taq DNA polymerase (New England Biolabs), adjusted with H₂O to a volume of 50 μ l. For more biased nucleotide pools, the concentrations of dTTP and dCTP were adjusted to 600 μ M or 1 mM, with 2.0 mM Mg²⁺/0.25 mM Mn²⁺ and 2.5 mM Mg²⁺/0.5 mM Mn²⁺, respectively. Reactions were amplified using MJ Research PTC-100 thermal cyclers with an initial denaturation step of 2 min 92°C, followed by 35–40 cycles of 10 s 92°C, 1 min 30 s 65°C, 4₁/₂ min at 72°C and a final 15 min extension at 72°C. JB.45: 5'-TTTCATACACGGTGCCTGACTGCG. JB.57: 5'-AA CTGTGAATGCGCAAACCAACCC.

Preparation of competent bacterial cells

To prepare competent cells, 5 ml cultures of BW23474/pJBN250 or DH5 α /pJBN250 were incubated overnight in Luria-Bertani broth (LB) supplemented with spectinomycin (40 μ g/ml). Overnight cultures were diluted into 500 ml Super Broth (16 g BactoTryptone, 10 g Yeast Extract, 5 g NaCl, 5 ml 1 N NaOH, 500 ml dH₂O) containing spectinomycin and 300 μ M IPTG to induce expression of *cre* and *gam-bet-exo*. The cultures were then incubated at 37°C until they reached OD₅₉₅ 1.0. Culture flasks were cooled in an ice water bath and cells recovered by centrifugation (6000 r.p.m., 5 min, 4°C). The cell pellet was washed sequentially at 4°C with 500 ml 1 mM HEPES, pH 7.0, 250 ml 1 mM HEPES, pH 7.0 and 10 ml 10% glycerol in distilled H₂O. The final pellet was re-suspended in 1 ml 10% glycerol, frozen in 50 μ l aliquots in liquid nitrogen and stored at -80°C.

Library construction and analysis

We constructed mutagenized libraries by first precipitating PCR reactions (50 μ l) with 500 μ l of *n*-butanol. The pellet (10 min, 13 000 r.p.m.) was washed with 70% ethanol and re-suspended in 10–20 μ l distilled H₂O. 1–5 μ l was electroporated into competent BW23474/pJBN250 cells. Cells were plated onto LB/kanamycin (50 μ g/ml) media and incubated overnight at 42°C. For large libraries, pellets from 10 PCR reactions were pooled in 50 μ l H₂O and 10 separate transformations were plated onto ten 15 cm diameter LB/kanamycin plates. Transformants were scraped into 25 ml LB/kanamycin media and inoculated into two liters of Super Broth supplemented with kanamycin. After a 4 h amplification at 37°C, libraries were prepared using Qiagen Maxi-Prep kits. Fusion libraries were prepared using either *in vitro* UPS reactions (1) or by co-transformation of DH5 α /pJBN250 cells. In our hands, co-transformation typically yielded the largest number of transformants. Approximately 1 μ g each of mutagenized library and expression plasmid were electroporated directly into competent DH5 α /pJBN250 cells, and recombinants selected on LB/kanamycin plates at 42°C. We occasionally observed fusion libraries becoming contaminated with an apparent deletion form of correct recombinant plasmids. This variant retained the ColE1 origin, Ap^R and Kn^R regions, but removed expression plasmid sequences necessary to transform yeast hosts. To minimize this contamination, obviously faster growing colony regions were excised from transformation plates and libraries was prepared directly from recovered transformants without further amplification. Fusion libraries were analyzed in yeast strains derived from CRY1 [*MATa his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100*] using standard genetic techniques. Further yeast strain information is described in figure legends or is available upon request.

Cloning linear DNA fragments

For *in vivo* cloning experiments, 20 μ l of mini-prep DNA (~200–300 ng) was restricted to release antibiotic resistance markers flanked by *loxP*. The digests were combined with 5 μ l of mini-prep DNA for circular target vectors, and DNA mixtures were concentrated by *n*-butanol precipitation. The pellets were washed in 70% ethanol, re-suspended in 10 μ l of distilled H₂O, and 2 μ l was transformed into DH5 α cells harboring pJBN250. The transformations were allowed to recover for 1 h and plated on antibiotic-containing media at 42°C. Cultures for mini-prep analysis were also cultured at 42°C. *lox-kanMX-lox* was obtained by digesting pJBN240 (a pRS413-derived yeast mini-chromosome harboring *lox-kanMX-lox*) with DraIII and SacI. The *lox-tet-lox* cassette was derived by digesting pAK005 (an intermediate in constructing pAK047) with NotI and XhoI.

RESULTS

The strategy we developed for *in vivo* Cre/*lox* recombination and construction of mutagenized gene libraries is depicted in Figure 1A. In this procedure, the DNA

fragment to be mutated is first introduced into a recombination entry vector containing tandem *loxP* sites. The entire plasmid, including gene sequences of interest, is then mutagenized by error-prone PCR using primers binding between the *loxP* repeats. PCR-amplified linear molecules are converted into autonomously replicating, circular plasmids by transformation into bacteria harboring pJBN250. pJBN250 encodes two activities that were found to promote efficient plasmid re-circularization. One is Cre recombinase, which catalyzes recombination between the *loxP* sites at the ends of each PCR amplicon. The other is lambda *gam-bet-exo*, which stabilizes linear DNA sequences by blocking resection through the *recBCD* pathway. Plasmid libraries from these recombinants can then be used to transfer mutagenized gene variants into expression vectors using the appropriate recombination method (Figure 1B).

Entry vectors for constructing mutagenized gene libraries

Recombination entry vectors for this mutagenesis method are shown in Figure 2. pAK047 is an entry vector for the Univector plasmid fusion system (1). One of the *loxP* sites in pAK047 retains an orientation with the multiple cloning site as is found in other Univectors, ensuring mutagenized pAK047 constructs can recombine with compatible expression plasmids to create in-frame NH₂-terminal gene fusions. Our experience indicated PCR amplification could be enhanced in some cases by linearizing template DNA (data not shown). Accordingly, a recognition site for the I-SceI homing endonuclease was engineered between the PCR primer-binding sites in pAK047 for this purpose. pAK047 has also been modified such that the Univector Kn^R gene is replaced with *kanMX4*, conferring kanamycin resistance in *E. coli* and G418 resistance in yeast (16). pJBN260 is a modified donor plasmid for the Gateway system. Like pAK047, pJBN260 contains tandem *loxP* repeats, primer-binding sites for mutagenic PCR, and an I-SceI site.

Efficient plasmid re-circularization following transient cre and gam expression

Our approach to mutant library construction required that we establish conditions under which PCR-amplified linear DNA fragments could undergo Cre/*lox* recombination *in vivo*. Bacterial hosts to facilitate this type of cloning must meet several criteria. First, they must be *hsdR* deficient to prevent restriction of unmethylated DNA. In addition, in the case of pAK047-derived constructs, bacterial hosts must express the *pir* gene, which encodes a replication factor for the R6K γ origin used to propagate Univector plasmids (18). Most importantly, library construction requires expression of *cre* and RecBCD inhibition. In the original Univector report, *in vivo* fusion of circular plasmids was achieved by expressing *cre* under control of an IPTG-inducible promoter on a plasmid (pQL269) containing a temperature-sensitive replication origin (1). This approach made it possible to minimize further cycles of Cre/*lox* recombination following the initial fusion event. In preliminary attempts to re-circularize PCR amplicons, we transformed reaction

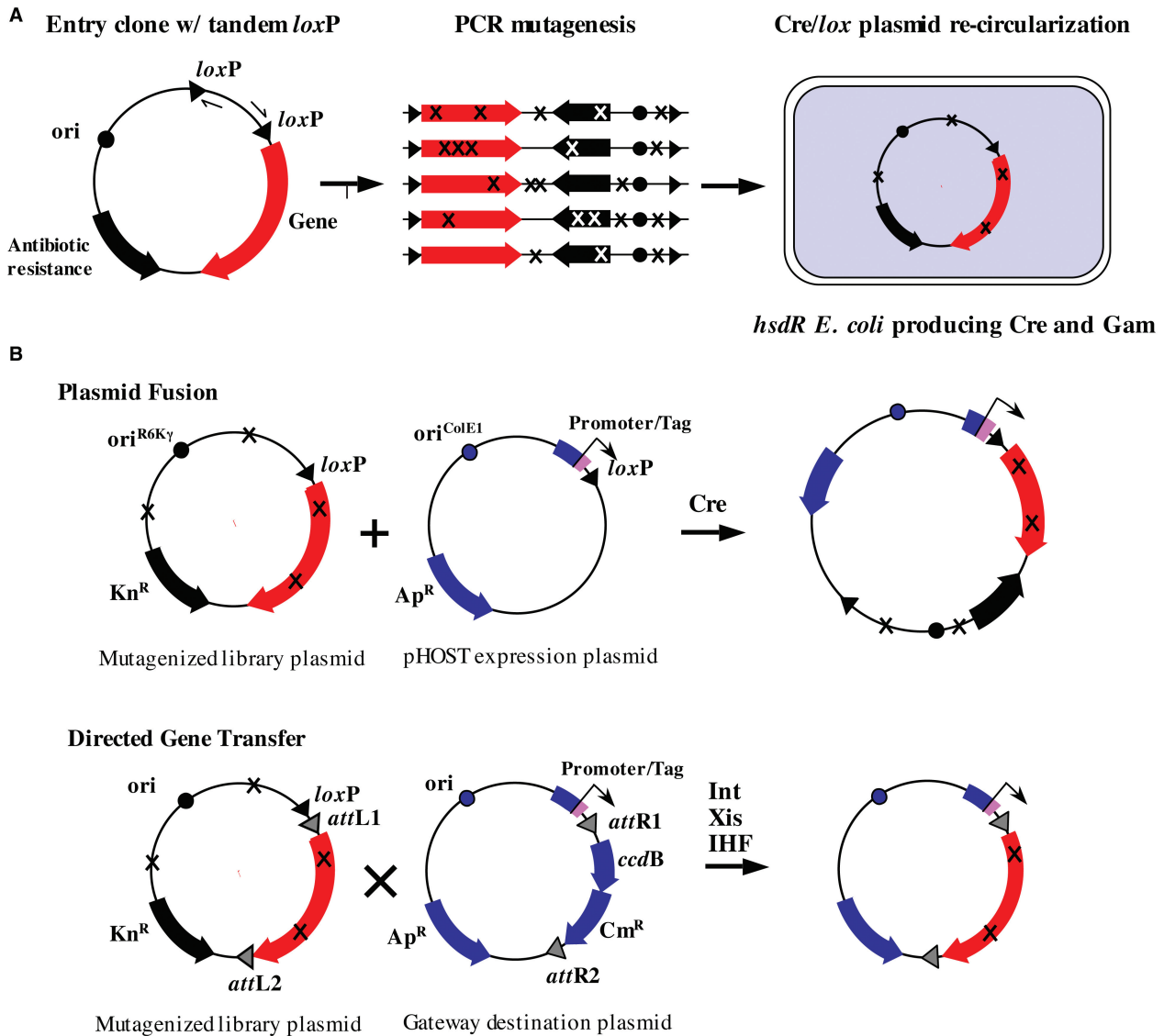


Figure 1. Schematic representation of mutant library construction and recombination. (A) Gene sequences are cloned into recombination entry vectors containing tandem *loxP* repeats. Plasmids are amplified by mutagenic PCR, producing linear amplicons flanked by *loxP*. Site-specific recombination between the *loxP* sites on each molecule is catalyzed in bacterial hosts expressing Cre and Gam, producing circular plasmid libraries. Amplicons containing mutations disrupting vector replication, selection or recombination will be eliminated during library construction. Other mutations that do not affect gene sequences of interest are likely to be phenotypically inert (in the case of expression using Univector plasmid fusion) or will not be transferred during recombination (for example, using Gateway cloning) (B) Mutagenized gene collections in these libraries are recombined with expression plasmids, converting the library into a form allowing gene mutations to be screened or assessed using a range of promoters or sequence tags. As depicted, this mutagenesis approach is compatible with Cre-catalyzed Univector plasmid fusion or with directed gene transfer methods such as Gateway recombination cloning.

products into *recB recC sbcA* or *recB recC sbcB* hosts harboring pQL269. Kn^R transformants were obtained with good efficiency, but, as reported previously (19), plasmid DNA was isolated from these transformants in a high molecular weight, presumably linear, form that was not compatible with subsequent plasmid fusion reactions (data not shown).

To address this problem, we examined whether transient RecBCD inhibition might provide a sufficient window of opportunity to permit efficient Cre/*lox* recombination of linear DNA substrates. The lambda Gam protein is a potent inhibitor of RecBCD (20).

Accordingly, the *gam-bet-exo* region of lambda, encoding Gam and recombination functions, was cloned into pQL269 under control of the *trc* promoter to create pJBN250, permitting IPTG-inducible expression of Cre and Gam (Figure 2). Mutagenized PCR products from Univector templates were transformed into *hsdR pir-116* hosts (BW23474) harboring pJBN250 and plated at 42°C to inactivate the pJBN250 origin of replication. Plasmid DNA from the resulting transformants migrated as an apparent series of circular multimers (Figure 3A), and restriction analysis yielded fragments of the predicted size for correctly re-circularized molecules (Figure 3B).

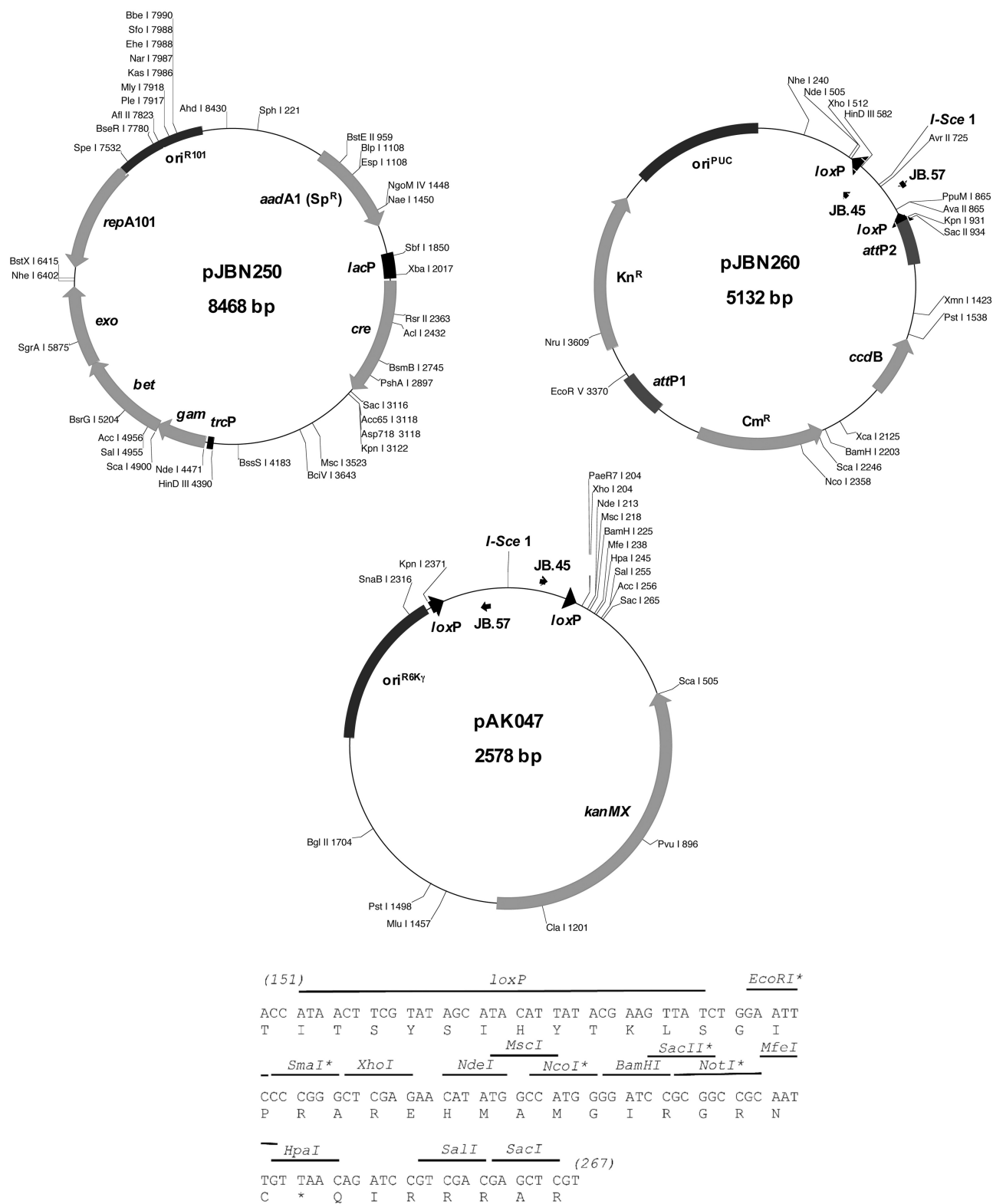


Figure 2. Plasmid maps for vectors created to support this mutagenesis method. Nucleotide positions of unique restriction sites are shown. Relevant genetic elements are displayed as bars or arrows to indicate the direction of transcription. In pJBN250, *cre* and *gam-bet-exo* are placed under control of IPTG-inducible promoters to catalyze Cre/*loxP* recombination of linear DNA. pJBN250 encodes Sp^R, and plasmid replication can be blocked by inactivating the temperature-sensitive R101 origin of replication at 42°C. pJBN260 and pAK047 are entry vectors for recombination cloning using the Gateway and Univector plasmid fusion systems, respectively. Both have been modified to contain tandem *loxP* repeats, an *I-SceI* site for template linearization and binding sites for JB.45 and JB.57 oligos to prime mutagenic PCR. Gene inserts can be introduced into pJBN260 through recombination with *attP* sites, while sequences are introduced into pAK047 using traditional cloning. The pAK047 polylinker is displayed as codons in frame with the *loxP* open reading frame. Restriction sites that are no longer unique are indicated (*).

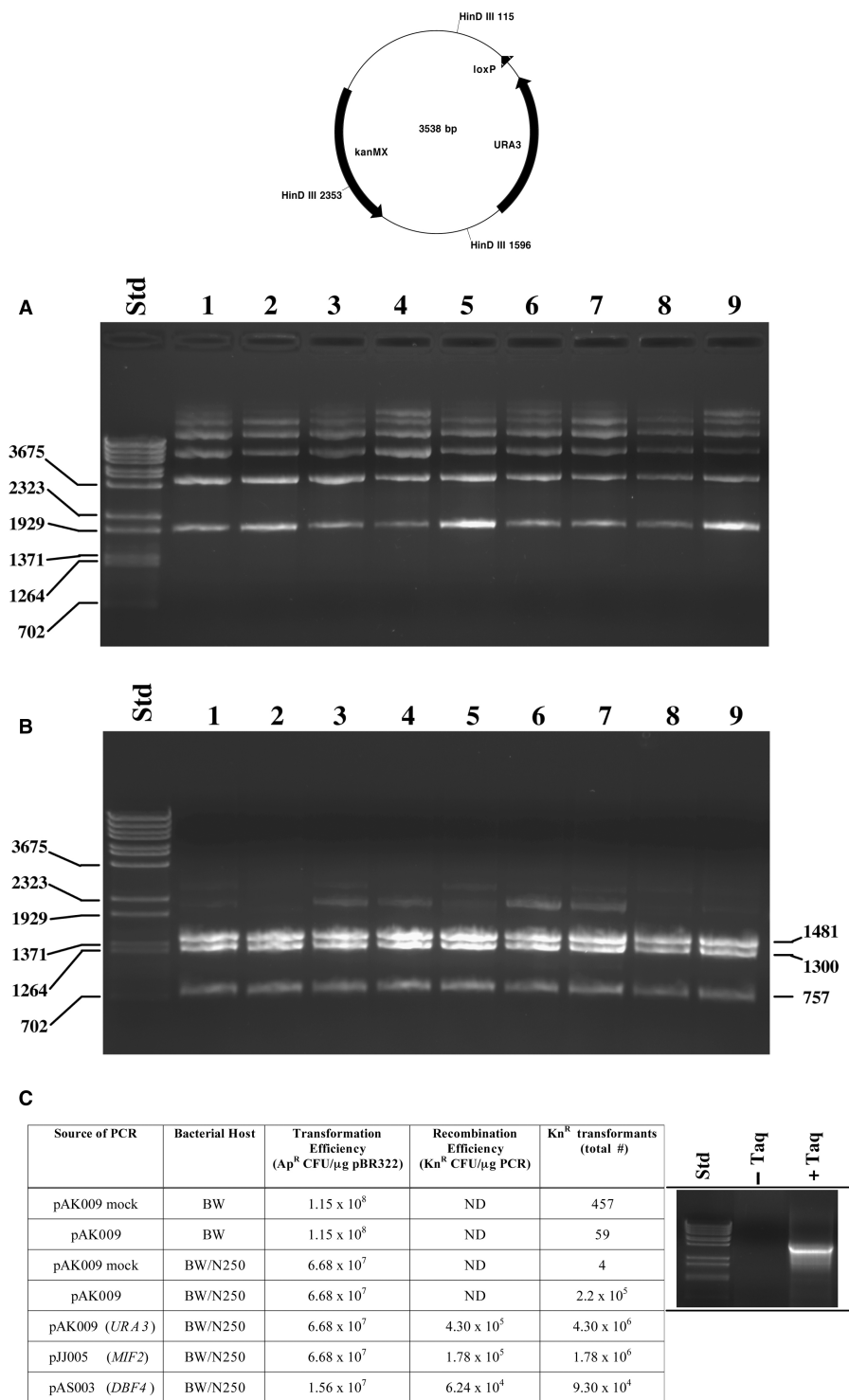


Figure 3. Plasmid re-circularization in pJBN250 transformed cells. Mutagenized PCR products from pAK009, (a pUNI10-*URA3* derivative), were transformed into BW23474 cells harboring pJBN250. Plasmid DNA from nine Kn^R transformants was analyzed before (A) or after (B) HindIII digestion, predicted to produce fragments of 1481, 1300 and 757 bp from correct recombinants (a map of the predicted recombinant is depicted above the gel panel). DNA was separated on 0.8% agarose gels and visualized by ethidium bromide staining. (C) Equal amounts of linearized pAK009 DNA (5 ng) were used as template for PCR reactions with or without (mock) Taq polymerase. The gel inset shows aliquots of the reaction products. Equivalent volumes of these reactions were transformed into BW23474 (BW) or BW23474/pJBN250 (BW/N250) strains, and dilutions were plated to calculate the total number of Kn^R colonies. In the bottom three rows of the table, mutagenized libraries were constructed using pooled PCR reactions from pAK009, pJJ005 and pAS003 (pUNI10 derivatives containing the indicated *S. cerevisiae* genes) as described in Methods section. In these cases, the concentration of the pooled PCR reaction products was quantified, and used to calculate recombination efficiency by dividing the total number of Kn^R colonies by the amount of PCR-amplified DNA used in the transformations. In all cases, transformation efficiency for circular plasmids was evaluated by determining the number of Ap^R transformants obtained from a known amount of pBR322.

Transformant recovery was dependent on successful PCR amplification and the presence of pJBN250 (~10 000-fold stimulation; Figure 3C). To determine the efficiency of this method, the *S. cerevisiae* *URA3*, *DBF4* and *MIF2* genes (including 5' promoter regions) were cloned into pAK047-related entry vectors and mutagenized by PCR. For each clone, the products from 10 separate PCR reactions were pooled, transformed into pJBN250-containing bacteria, and plated at 42°. Using competent cells with transformation efficiencies in the range of $2\text{--}7 \times 10^7$ CFU/ μg , we observed recombinants arising at frequencies of $2\text{--}6 \times 10^5$ / μg from PCR reactions (Figure 3C). With this efficiency, it was possible to construct libraries from greater than 10^6 recombinants for two of the libraries, and $\sim 10^5$ recombinants for the third. Thus, transient expression of Cre and Gam permits efficient intra-molecular Cre/*lox* recombination of linear plasmid molecules, and can be used to construct large mutagenized gene libraries.

Screening mutant libraries using univector plasmid fusion

To demonstrate that our plasmid libraries could be transferred by recombination, we fused our mutagenized *URA3* library (Figure 3C) to pHY314, a yeast minichromosome compatible with the Univector system. In addition to a *lox* site for Cre/*lox* recombination, pHY314 contains additional genetic elements necessary for minichromosome propagation in yeast. These include the *TRP1* gene, which allows the minichromosome to be maintained in yeast hosts by conferring the ability to grow in media lacking tryptophan. Library aliquots and pHY314 DNA were co-transformed into non-*pir* bacteria (DH5 α) harboring pJBN250, and 1.68×10^6 Kn^R transformants were recovered. Plasmid mini-preps from these transformants revealed the majority contained correct recombinants (data not shown). As a separate assessment of the composition of the fusion library, p*URA3*/pHY314 fusions were transformed into yeast, selecting for the minichromosome *TRP1* gene. We then determined how many of the Trp⁺ transformants were also resistant to G418. Of 500 Trp⁺ transformants analyzed, 79% were also able to grow on G418 media, indicating they harbored correct fusion clones. The 21% that could not grow on G418 could arise for several reasons. First, it is likely that un-recombined pHY314 is present in the library. Second, some fusion clones may harbor *kanMX* mutations that do not confer G418 resistance in yeast. Third, for 7% of Trp⁺ transformants, some colonies derived from the initial transformant could grow on G418 while others could not. Thus, one point to make from this analysis is that some level of fusion plasmid rearrangement may occur within the yeast host.

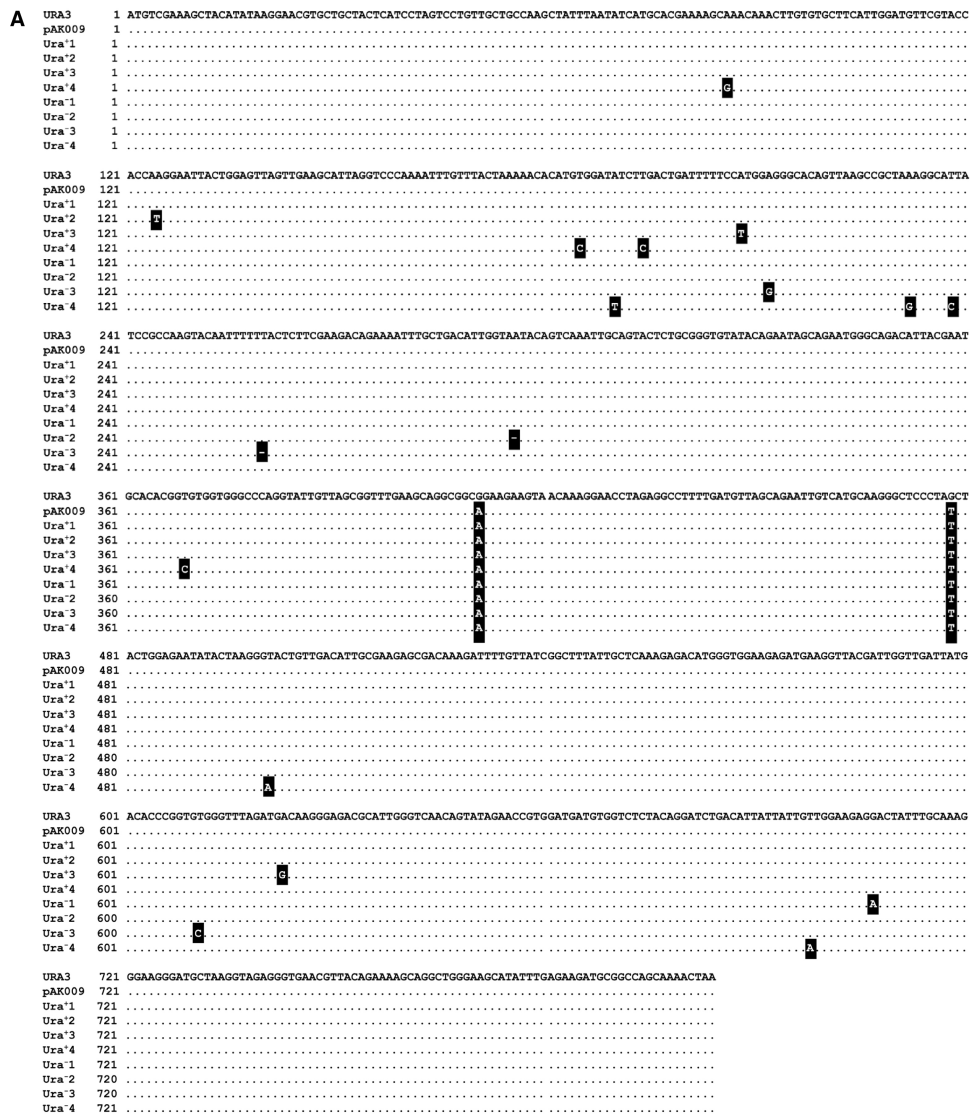
We next screened transformants harboring correct fusion clones (Trp⁺/G418^R) for inactivating *URA3* mutations. Of 395 clones screened, 9% exhibited a strong Ura⁻ phenotype. To analyze these mutants further, *URA3* coding region on fusion plasmids recovered from four Ura⁺ and four Ura⁻ transformants was sequenced. Compared to the parental *URA3* sequence, seven of the rescued plasmids contained additional mutations, most prominently A:T \rightarrow G:C transitions, and A:T \rightarrow T:A

transversions (Figure 4A). Corresponding Ura3 amino acid changes are summarized in Figure 4B. Two of the Ura⁻ clones had base-pair deletions that disrupted the *URA3* reading frame, while the third had a single G236E substitution and the fourth a closely positioned V233D alteration. An unusual feature of orotidine-5'-phosphate decarboxylase—the enzyme encoded by *URA3*—is that it makes contacts with the substrate phosphoryl group that are important for catalysis, even though this group does not participate in the decarboxylation reaction. In particular, the structure of Ura3 complexed with a putative transition state analog revealed that R235 and G234 make four hydrogen bonds with phosphoryl group oxygen atoms (21). From this structure, it is possible that negative charges introduced by the G236E and V233D substitutions may disrupt these contacts.

To further demonstrate the utility of these libraries, we used our mutagenesis technique to isolate conditional alleles of the yeast *DBF4* gene. *DBF4* encodes a regulatory subunit of the Cdc7 kinase, required to activate origins of replication during DNA synthesis (22,23). One temperature-sensitive allele, *dbf4-1*, has been described. At restrictive temperatures, *dbf4-1* mutants fail to initiate DNA synthesis (24), arrest in the cell cycle as large budded cells (24), and segregate unreplicated chromosomes on the mitotic spindle (25). This mitotic catastrophe is thought to occur because unduplicated chromosomes fail to biorient and restrain spindle extension (26). We postulated it might be possible to isolate less severe temperature-sensitive *dbf4* alleles that stabilized pre-anaphase spindle structure. To test this, we fused our mutagenized *DBF4* library (Figure 3C) to pHY314 and transformed the expression library into *dbf4-1* cells. Transformants were first selected for their ability to complement *dbf4-1* at 32°C, and then secondarily screened for a temperature-sensitive growth defect at 37°C. Of 500 transformants analyzed, three exhibited a temperature-sensitive phenotype that could be complemented by *DBF4*, indicating the fusion plasmids in these transformants harbored temperature-sensitive *dbf4* mutations (Figure 5A and B). Interestingly, two of these mutants were found to arrest as large budded cells that exhibited a greatly reduced frequency of aberrant spindle extension. (Figure 5C and D). Further analysis of these novel alleles is ongoing in our laboratory.

Cre/*lox* recombination of linear DNA fragments into plasmid vectors

The high efficiency of plasmid re-circularization in our library construction method suggested it might be possible to use Cre/*lox* recombination to directly clone DNA fragments *in vivo*. To determine the feasibility of this approach, a linear DNA fragment (*lox-kanMX-lox*) in which the *kanMX* cassette was flanked by *loxP* was co-transformed with the Ap^R plasmid pAS2*loxP* into bacterial hosts (DH5 α) harboring pJBN250. Transformants were selected on either kanamycin or ampicillin-containing media at 42°C, revealing an average frequency of 6.0×10^{-3} Kn^R to Ap^R transformants (three experiments; SD $\pm 2.2 \times 10^{-3}$). Restriction digests of plasmid DNA from Kn^R transformants revealed



B

pAK009-derived clone	Changes to Ura3p sequence
pAK009	A160S
Ura ⁺ 1	A160S
Ura ⁺ 2	K42M A160S
Ura ⁺ 3	M70L A160S D208G
Ura ⁺ 4	Q29R V62A K65S A160S
Ura ⁻ 1	A160S G236E
Ura ⁻ 2	Frameshift after G98
Ura ⁻ 3	Frameshift after F86
Ura ⁻ 4	I64F K78G A160S V233D

Figure 4. Analysis of fusion plasmids from mutagenized *URA3* libraries. (A) Recombinant plasmids were recovered from four Ura⁺ and four Ura⁻ strains following transformation of a mutagenized pAK009 + pHY314 fusion library into yeast. The *URA3* coding region was sequenced on both DNA strands and compared to the sequence of the *URA3* insert cloned into the parental pAK009 construct used as template for PCR mutagenesis. Differences from the Yeast Genome Database (www.yeastgenome.org) sequence for *URA3* (URA3) are depicted as highlighted nucleotides. (B) Summary of Ura3 amino acid alterations created by the mutations shown in (A).

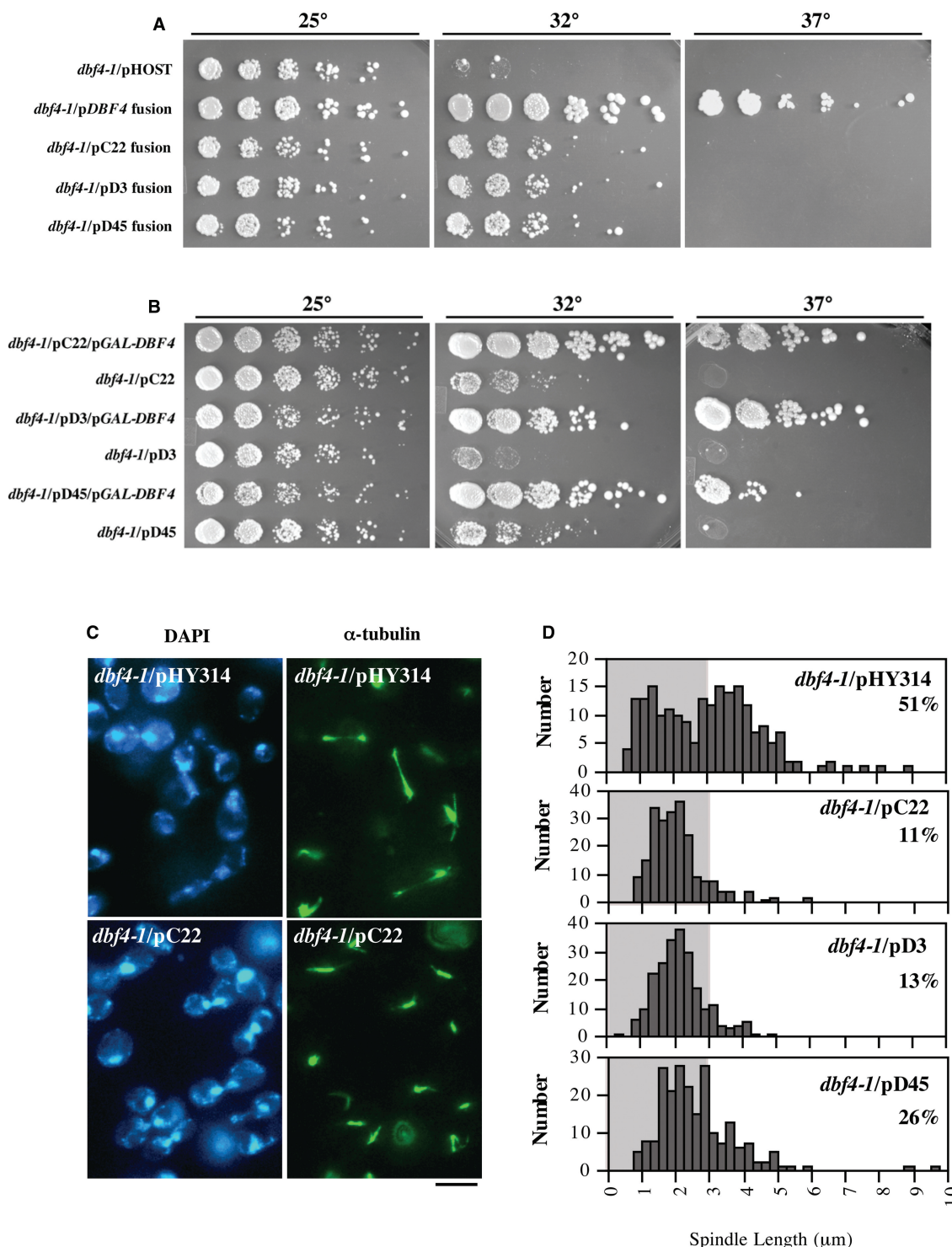


Figure 5. Isolation of temperature-sensitive conditional alleles from a mutagenized *DBF4* fusion library. A mutagenized pAS003 + pHY314 fusion library was used to select Trp^+ transformants that could complement JBY999, a *dbf4-1* temperature-sensitive strain, at 32°C. (A) Serial 10-fold dilutions were pronged onto Trp^- media for *dbf4-1* cells transformed with pHY314, an un-mutagenized pAS003 + pHY314 fusion plasmid, and three fusion library plasmids (pC22, pD3 and pD45) that were found to complement *dbf4-1* at 32°C but not 37°C. (B) *dbf4-1* strains containing pC22, pD3 and pD45 were transformed with a control plasmid expressing wild-type *DBF4* under control of the *GAL1* promoter (pJBN231). Transformants were assayed for growth on glucose media as in (A). *dbf4-1* transformed with pHY314, or the pC22, pD3 and pD45 temperature-sensitive fusion plasmids were arrested in G₁ with mating pheromone and released into fresh media at 34°. After 2_{1/2} h, cells were processed for DAPI staining and α -tubulin immunofluorescence microscopy to evaluate budding, nuclear morphology and spindle structure. (C) Micrographs depicting extended spindles in *dbf4-1*/pHY314 controls and a shorter, more normal pre-anaphase spindle structure in pC22 transformants. Bar, 5 μm . (D) Distribution of spindle lengths for each *dbf4-1* strain. Pole-to-pole distances were evaluated for 200 α -tubulin-stained cells using the measurement features of Meta-morph imaging software. The graphs also display the percentage of cells with spindle lengths of $\geq 3 \mu\text{m}$, which was used as a cutoff length for aberrant spindle extension.

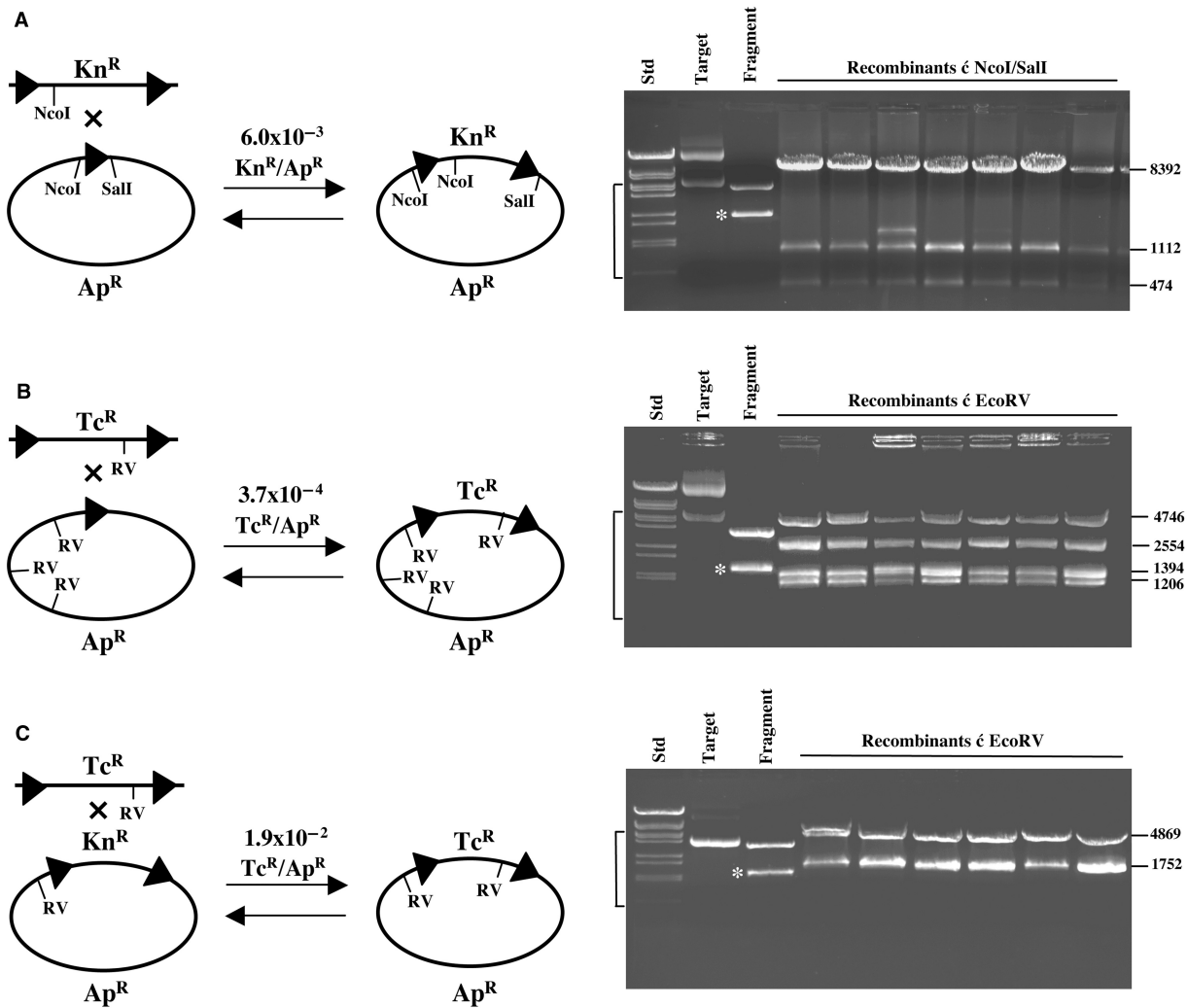


Figure 6. Cre/lox cloning of linear DNA *in vivo*. (A) A 2255-bp *lox-kanMX-lox* fragment was liberated by restriction digestion of pJBN240 (fragment lane; marked by *), and recombined with a circular pAS2loxP target plasmid (target lane) in Cre/Gam-expressing bacterial cells. The frequency of Kn^R to Ap^R transformants was determined. Mini-prep DNA was prepared from seven randomly chosen Kn^R clones and digested with NcoI and SalI, releasing fragments of 8392, 1112 and 474 bp in correct recombinants. Std lane: lambda DNA digested with BstEII. Within the bracketed range fragments are 4822, 4324, 3675, 2323, 1929, 1371, 1264 and 702 bp. (B) A 1550-bp *lox-tet-lox* fragment (fragment lane; *) from pAK005 was recombined with pAS2loxP (target). In this case, plasmid DNA from Tc^R clones was digested with EcoRV, releasing fragments of 4746, 2554, 1394 and 1206 for correct recombinants. (C) The same *lox-tet-lox* fragment in (B) was recombined with circular pJBN240 (target), and Tc^R clones were digested with EcoRV to determine if the *lox-kanMX-lox* region of pJBN240 had been replaced by *tet* sequences. Such recombinants exhibit restriction fragments of 4869 and 1752 bp following EcoRV digestion.

the predicted structure for correct recombinants (Figure 6A). Analogous experiments with a *lox-tet-lox* fragment resulted in a frequency of 3.7×10^{-4} Tc^R to Ap^R transformants (four experiments; SD $\pm 1.5 \times 10^{-4}$), and restriction analysis again confirmed the *tet* fragment had recombined in the expected configuration (Figure 6B).

We next examined whether it might be possible to impose an additional recombination event that would catalyze genetic exchange between linear and circular substrates. To assess this, the *lox-tet-lox* fragment was co-transformed into DH5 α /pJBN250 along with the Ap^R plasmid pJBN240. pJBN240 contains a *lox-kanMX-lox* cassette, and the goal of this experiment was to determine whether we could recover recombinants in which the *kanMX* sequences between the *lox* sites had been replaced

by *tet*. Tc^R recombinants were recovered at frequencies of 1.4×10^{-2} and 2.4×10^{-2} Tc^R / Ap^R transformants (two experiments), and restriction analysis confirmed marker replacement in $\text{Ap}^R\text{Tc}^R\text{Kn}^S$ clones (Figure 6C). Thus, at least under conditions where we can directly select for recombinants using antibiotic resistance markers, *in vivo* Cre/lox recombination allows direct cloning of DNA fragments flanked by *loxP* repeats.

DISCUSSION

Gam-mediated stabilization of linear DNA permits efficient Cre/lox recombination *in vivo*

This report describes a method for constructing randomly mutagenized gene libraries in a format compatible with

recombination cloning. Gene sequences are cloned into recombination entry vectors, and the entire master clone is PCR amplified under mutagenic conditions, producing linear plasmid amplicons flanked by *loxP*. The mutagenized plasmids are re-circularized through Cre/*lox* recombination by simply transforming them into Cre/*Gam*-expressing bacterial cells. The resulting libraries can then be processed by recombination cloning into desired expression formats in a similar fashion to other large collections of gene sequences. In the examples provided here, such libraries proved effective in conjunction with the Univector system to isolate mutant forms of the *S. cerevisiae* *URA3* and *DBF4* genes, suggesting it should be possible to apply this approach to other sets of mutations.

It is of course possible to construct PCR mutagenized libraries through a number of different approaches, some of which would also be compatible with library transfer by recombination cloning. For example, libraries could be constructed by directly cloning the mutagenized PCR fragments using conventional techniques or by using Topo cloning methods (In Vitrogen). Mutant libraries could also be constructed in Creator vectors using In-Fusion technology (In Vitrogen) or in Gateway constructs (Clontech) using *attB*-tailed PCR products. In comparison, our library construction method has at least two advantages. First, as an *in vivo* cloning procedure, it does not require purchasing charged vectors or recombinase mixtures, and should therefore be comparatively cost-effective for assembling large numbers of mutant libraries. Second, and more importantly from a genetic standpoint, our method is highly efficient, allowing libraries of over a million recombinants to be constructed. The ability to construct such deep libraries should greatly facilitate the identification of rare, informative mutant alleles, especially when a direct selection for the desired mutant phenotypes can be applied.

Although we have focused on the Univector system, our library construction method should also be compatible with other recombination cloning strategies. Towards this end, we have constructed a modified Gateway entry vector containing *attP* recombinase sites and tandem *loxP* sites. Gene sequences can be introduced into this vector by *attB/attP* recombination, mutagenized using Cre/*lox* re-circularization and transferred to Gateway destination plasmids (Figure 1B). Our method should also be compatible with the MAGIC cloning system (although it would be necessary to remove the I-SceI site between the *loxP* repeats) (8). In MAGIC, homing endonucleases are used to induce homologous recombination between 50 bp regions located on both recipient and donor vectors, effecting gap repair of the recipient plasmid with donor sequences. It was shown that PCR fragments flanked by these 50 bp regions could recombine with expression plasmids in MAGIC bacterial hosts (8). Thus, it is possible to construct PCR-mutagenized libraries in any desired MAGIC expression plasmid. However, if the intent is to create mutagenized libraries in MAGIC entry vectors so that libraries can be transferred to a range of expression constructs, other strategies must be employed. Of necessity, endonuclease sites on MAGIC entry vectors

are located outside the homology regions, preventing them from being gapped in a manner compatible with recombination. Therefore, it may be advantageous to use a method similar to ours to generate mutant libraries in MAGIC entry vectors.

In addition to intramolecular recombination, our results indicate *Gam*-mediated RecBCD inhibition enables linear DNA fragments to recombine with *loxP*-containing plasmids through a bimolecular reaction. We have also recently found that it is possible to amplify DNA fragments using PCR primers with appended *loxP* sites, allowing us to flank specific DNA segments with *loxP* repeats (J.A.J. and J.B., unpublished data). In theory it should therefore be possible to perform our mutagenesis procedure on any desired entry clone without having to introduce a tandem *loxP* cassette. It should also be possible to utilize *in vivo* Cre/*lox* recombination as a more general cloning procedure. What is principally required for this latter approach is an appropriately engineered capture vector that permits both positive selection for correct recombinants and counter-selection against un-recombined vector molecules. As we show here, the ability to replace genetic markers on the recipient plasmid should facilitate the design of such counter-selection strategies. Ultimately, a general *in vivo* recombination method for cloning linear DNA fragments is desirable, as it would provide an inexpensive alternative to some cloning techniques (such as capturing PCR products) currently performed using *in vitro* reagents.

Using recombination cloning for genetic analysis

Our intent in developing this library construction technique was to utilize recombineering to expedite the screening of gene mutations. It is worth considering what conditions must be met to perform this type of genetic analysis. Assuming an appropriate expression vehicle is available, a first condition is that recipient cells must be transformed/transfected efficiently. Second, if a direct selection cannot be applied, it must be possible to propagate transformants in a clonal fashion, and a screening procedure devised to identify transformants exhibiting desired phenotypes. Finally, a method must exist to recover mutations of interest. As we show for *S. cerevisiae*, both the budding yeast and fission yeast genetic systems meet all these criteria, and it should therefore be possible to use our mutagenesis system to isolate informative mutations from yeast cells under a wide variety of screening conditions. Furthermore, the ability to maintain a permanent mutant library, apply it under different expression conditions, and recover a virtually unlimited number of transformants are significant advantages compared to current yeast gap repair/plasmid shuffle mutagenesis techniques (27). Importantly, the use of recombination cloning in conjunction with these mutant libraries is also ideally suited for methodologies where yeast and bacteria are used as surrogate genetic systems, as in identifying interaction-defective alleles in reverse two-hybrid analysis (28) or proteins with altered properties in display or activity assays (29).

An ultimate goal would be to develop the procedures established here to a point where mutant libraries could also be productively screened in higher eukaryotes. There are reasons to believe that this should indeed be possible. Retroviral and adenoviral vectors provide extremely efficient gene delivery vehicles, and could presumably be engineered such that mutant libraries could be recombined into these vectors in a format allowing expression in mammalian cells. Cre treatment could then liberate master clones containing mutations of interest in a circular form suitable for recovery in bacteria. We also note that there has been significant progress in developing high-throughput screening methods for plant and animal cells (12). Thus, it may prove possible to screen recombination-based mutant libraries in metazoan cells for dominant phenotypic traits. Such mutations, especially in a conditional expression format, would provide valuable reagents for assessing gene function.

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REFERENCES

- Liu,Q., Li,M.Z., Leibham,D., Cortez,D. and Elledge,S.J. (1998) The univector plasmid-fusion system, a method for rapid construction of recombinant DNA without restriction enzymes. *Curr. Biol.*, **8**, 1300–1309.
- Marsischky,G. and LaBaer,J. (2004) Many paths to many clones: a comparative look at high-throughput cloning methods. *Genome Res.*, **14**, 2020–2028.
- Rual,J.F., Hill,D.E. and Vidal,M. (2004) ORFeome projects: gateway between genomics and omics. *Curr. Opin. Chem. Biol.*, **8**, 20–25.
- Sternberg,N., Hamilton,D., Austin,S., Yarmolinsky,M. and Hoess,R. (1981) Site-specific recombination and its role in the life cycle of bacteriophage P1. *Cold Spring Harb. Symp. Quant. Biol.*, **45**, 297–309.
- Siegel,R.W., Velappan,N., Pavlik,P., Chasteen,L. and Bradbury,A. (2004) Recombinational cloning using heterologous *lox* sites. *Genome Res.*, **14**, 1119–1129.
- Hartley,J.L., Temple,G.F. and Brasch,M.A. (2000) DNA cloning using *in vitro* site-specific recombination. *Genome Res.*, **10**, 1788–1795.
- Walhout,A.J., Temple,G.F., Brasch,M.A., Hartley,J.L., Lorson,M.A., van den Heuvel,S. and Vidal,M. (2000) GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol.*, **328**, 575–592.
- Li,M.Z. and Elledge,S.J. (2005) MAGIC, an *in vivo* genetic method for the rapid construction of recombinant DNA molecules. *Nat. Genet.*, **37**, 311–319.
- Gelperin,D.M., White,M.A., Wilkinson,M.L., Kon,Y., Kung,L.A., Wise,K.J., Lopez-Hoyo,N., Jiang,L., Piccirillo,S. *et al.* (2005) Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Dev.*, **19**, 2816–2826.
- Reboul,J., Vaglio,P., Rual,J.F., Lamesch,P., Martinez,M., Armstrong,C.M., Li,S., Jacotot,L., Bertin,N. *et al.* (2003) *C. elegans* ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. *Nat. Genet.*, **34**, 35–41.
- Temple,G., Lamesch,P., Milstein,S., Hill,D.E., Wagner,L., Moore,T. and Vidal,M. (2006) From genome to proteome: developing expression clone resources for the human genome. *Hum. Mol. Genet.*, **15**, R31–R43.
- Rines,D.R., Tu,B., Miraglia,L., Welch,G.L., Zhang,J., Hull,M.V., Orth,A.P. and Chanda,S.K. (2006) High-content screening of functional genomic libraries. *Methods Enzymol.*, **414**, 530–565.
- Boshoff,H.I. and Dowd,C.S. (2007) Chemical genetics: an evolving toolbox for target identification and lead optimization. *Prog. Drug Res.*, **64**, 49, 51–77.
- Allen,J.B., Zhou,Z., Siede,W., Friedberg,E.C. and Elledge,S.J. (1994) The *SAD1/RAD53* protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes Dev.*, **8**, 2401–2415.
- Luisi-DeLuca,C., Lovett,S.T. and Kolodner,R.D. (1989) Genetic and physical analysis of plasmid recombination in *recB recC sbcB* and *recB recC sbcA* *Escherichia coli* K-12 mutants. *Genetics*, **122**, 269–278.
- Wach,A., Brachat,A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.
- Leung,D.W., Chen,E. and Goeddel,D.V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique*, **1**, 11–15.
- Metcalf,W.W., Jiang,W. and Wanner,B.L. (1994) Use of the rep technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K gamma origin plasmids at different copy numbers. *Gene*, **138**, 1–7.
- Cohen,A. and Clark,A.J. (1986) Synthesis of linear plasmid multimers in *Escherichia coli* K-12. *J. Bacteriol.*, **167**, 327–335.
- Murphy,K.C. (1991) Lambda Gam protein inhibits the helicase and chi-stimulated recombination activities of *Escherichia coli* RecBCD enzyme. *J. Bacteriol.*, **173**, 5808–5821.
- Miller,B.G., Hassell,A.M., Wolfenden,R., Milburn,M.V. and Short,S.A. (2000) Anatomy of a proficient enzyme: the structure of orotidine 5'-monophosphate decarboxylase in the presence and absence of a potential transition state analog. *Proc. Natl Acad. Sci. USA*, **97**, 2011–2016.
- Jackson,A.L., Pahl,P.M., Harrison,K., Rosamond,J. and Sclafani,R.A. (1993) Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. *Mol. Cell. Biol.*, **13**, 2899–2908.
- Dowell,S.J., Romanowski,P. and Diffley,J.F. (1994) Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins *in vivo*. *Science*, **265**, 1243–1246.
- Johnston,L.H. and Thomas,A.P. (1982) A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **186**, 445–448.
- Toyn,J.H., Johnson,A.L. and Johnston,L.H. (1995) Segregation of unreplicated chromosomes in *Saccharomyces cerevisiae* reveals a novel G1/M-phase checkpoint. *Mol. Cell. Biol.*, **15**, 5312–5321.
- Bachant,J., Jessen,S.R., Kavanaugh,S.E. and Fielding,C.S. (2005) The yeast S phase checkpoint enables replicating chromosomes to bi-orient and restrain spindle extension during S phase distress. *J. Cell Biol.*, **168**, 999–1012.
- Muhrad,D., Hunter,R. and Parker,R. (1992) A rapid method for localized mutagenesis of yeast genes. *Yeast*, **8**, 79–82.
- Vidal,M., Brachmann,R.K., Fattaey,A., Harlow,E. and Boeke,J.D. (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. *Proc. Natl Acad. Sci. USA*, **93**, 10315–10320.
- Aharoni,A., Griffiths,A.D. and Tawfik,D.S. (2005) High-throughput screens and selections of enzyme-encoding genes. *Curr. Opin. Chem. Biol.*, **9**, 210–216.