slices using the QIAquick™ gel extraction kit (Qiagen, Valencia, CA, USA). The mixture of the isolated DNAs (each 100 ng) was incubated with 5 U T4 polynucleotide kinase (Promega, Madison, WI, USA) at 37°C for 30 min and kept at 65°C for 15 min to deactivate the enzyme. The DNAs phosphorylated at the 5’-end were then ligated using the Quick Ligation™ kit (New England Biolabs, Beverly, MA, USA) at 25°C for 10 min. The ligated DNA was transferred into competent E. coli (XL1-Blue; Stratagene) by a heat shock method. Colonies were formed with an efficiency of approximately 1.05 × 10⁶ cfu/mg DNA. The plasmids from 30 colonies were randomly chosen from those formed on the LB agar plate containing ampicillin (50 μg/mL), purified using the QIAprep® Spin Miniprep kit (Qiagen), run on 1% agarose gel impregnated with ethidium bromide, and visualized under UV light. Twenty-six out of the 30 plasmids were of the same size as pBluescript SK™ plasmid, and four plasmids were larger than that (data not shown). The larger plasmids were assumed to be products synthesized by an additional ligation after the recombination of two fragments. The mutated plasmid (Figure 2A, lane 2) had the same size as the template (Figure 2A, lane 1) and was resistant to HindIII restriction (Figure 2A, lane 3) but linearized by BamHI restriction (Figure 2A, lane 4). To confirm the mutation further, we sequenced the mutated HindIII-resistant plasmid using the ABI Prism® 3700 Automatic Sequencer (Applied Biosystems). As shown in Figure 2B, the adenosine of the HindIII recognition sequence of the plasmids was changed into thymidine.

Thus, the simple mutagenesis method described in the present study provides several advantages. First, our method allows us to obtain a clone with the desired mutation by the next day and to skip subcloning of the mutated gene. Second, compared with the protocol for the QuickChange Site-Directed Mutagenesis Kit, our protocol enables us not only to overcome the size limitation of the plasmid, a disadvantage frequently found when amplifying the entire plasmid in a single PCR (7), but also to remove the template plasmids without DpnI restriction. Third, deletion of a long DNA fragment or insertion of several bases can be made quite easily. Finally, we can apply our method to the vectors containing other antibiotic resistance genes such as kanamycin.

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High-fidelity Taq DNA polymerases have facilitated the development of PCR-mediated, site-directed mutagenesis (9). There are two common methods by which PCR can be used to introduce a point mutation in a gene. The first method is two-step (or double) PCR (25). The conditions for the second reaction of two-step PCR are often difficult to optimize, and the errors generated during this step are much more than “double”. Furthermore, two-step PCR requires laborious subcloning of the region to be mutagenized. The second method that can be used to introduce a single point mutation in a gene requires the use of the QuikChange™ kit (Stratagene, La Jolla, CA, USA) (8); this method requires only one-step PCR, but the whole plasmid must be amplified during the single step. Therefore, the QuikChange method often results in unsuccessful PCR using high-fidelity Taq DNA polymerases, especially when the template plasmid is large.

We have established a quick, convenient method of site-directed mutagenesis by combining the two-step PCR and the QuikChange methods with an in vivo homologous recombination system in yeast (13). As described in the

In Vivo Site-Directed Mutagenesis of Yeast Plasmids Using a Three-Fragment Homologous Recombination System

QuikChange protocol, we designed complementary primers in which one, two, or three desired nucleotide mutations were inserted. Fifteen base pairs of correct sequence flanked both sides of the mutated region (8) (Figure 1A).

Two PCR products were produced by using the high-fidelity Taq DNA polymerase Pfx (Invitrogen, Carlsbad, CA, USA), target wild-type DNA that had been cloned into a yeast vector (either the two-hybrid vector pOBD2 (13) or the general yeast CEN/ARS vector pRS414 (11)), and two primer pairs, each of which consisted of one mutated primer and a primer that was identical to a region 30 bp away from the multiple cloning site where the yeast vector was digested (Figure 1B). Unlike earlier methods, the new method next requires the direct transformation (1) of yeast cells (YPH499) with the two PCR products (1–2 μg), which did not undergo further purification, and a linearized yeast vector (0.5–1.5 μg). In yeast cells, these three fragments can undergo homologous recombination (Figure 1C). Only appropriately recombined and circularized plasmids or self-ligated plasmids were expected to be maintained in cells, and only cells containing appropriately recombined and circularized plasmids (Figure 1D) were expected to form colonies on plates containing selective medium.

The two-hybrid vector pOBD2 was linearized with Neol (New England Biolabs, Beverly, MA, USA), and pRS314 was linearized with BamHI (New England Biolabs). DpnI (10–20 U) was added directly to the PCR products (50 μL), and they were incubated at 37°C for 1 h to digest the template plasmids. Remaining enzyme activities were heat-inactivated by incubation at 68°C for 20 min. Transformed cells were plated onto synthetic minimal medium lacking tryptophan (Sc-Trp plates) and incubated for two days at 30°C.

We obtained a few hundred transformants in each experiment. Individual colonies were transferred to Sc-Trp medium (2–3 mL) or to a new Sc-Trp plate and incubated for 1–2 days at 30°C. The plasmids were then rescued from yeast cells by using a previously described yeast miniprep protocol (10) that had been modified. A cell pellet that was the size of a match head was collected in an Eppendorf® tube. The cells were resuspended in 200 μL yeast lysis buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% Triton® X-100, 1% SDS), and 200 μL 1:1 phenol:chloroform; approximately 0.1 g acid-washed glass beads were added. The tubes were vortex mixed for 10 min and then centrifuged at 20,817 × g for 5 min; both processes were done at room temperature. The water phase was removed and placed in a new Eppendorf tube; DNA was precipitated by the addition of ethanol. The DNA pellet was resuspended in 30 μL water. Electroporation was used to transform DH10B or DH5α E. coli cells with 1 μL suspension.

We expected to obtain some self-ligated products that resulted from in vivo nonhomologous end-joining activity in budding yeast; however, under the conditions that we used, we did not obtain any plasmids that lacked an insert (Table 1). When the background is high, self-ligation products can be reduced by phosphatase treatment of a digested vector.

We have used the three-fragment homologous recombination method to successfully generate eight constructs, each of which encoded a different point mutation. Sequencing of all of the plasmids rescued from yeast transformants (19 independent colonies) confirmed the presence of the desired mutations and the absence of PCR-introduced errors; however, two mutations were found within the oligonucleotide sequences of the PCR primers (Table 1). Because the frequency of PCR errors depends on the fidelity of the Taq DNA polymerase and the size of the product, and because a subpopulation of oligonucleotide primers may have a certain undesired deletion or mutation, the amplified region should be sequenced. However, the efficiency by which the desired mutation is introduced should not be affected by these factors. Thus, the use of better reagents (e.g., a Taq DNA polymerase with higher fidelity or a pool of oligonucleotide primers that is more pure) should improve this three-fragment method.

If the template DNA is within a yeast vector, then any remaining template DNA in the PCR is a key source of false positives. Instead of purifying PCR products, we digested the remaining plasmids with DpnI (New England Biolabs) to reduce false positives. DpnI can cut the plasmids, but not the PCR products, because the enzyme cuts only methylated target sites, and the plasmids that we used were amplified in E. coli strains DH5α or DH10B, which express Dam methylase. Alternatively, false positives can be reduced by using a template that does not contain yeast vector DNA or by using a vector that carries a yeast amino acid marker gene that is different from that of the template vector DNA.

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Benchmarks

Table 1. Accuracy of Targeted Changes

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Targeted Mutations</th>
<th>Vector Used</th>
<th>Size of the Amplified Region (kb)</th>
<th>No. Correctly Targeted Clones</th>
<th>No. Clones without Additional Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKP1</td>
<td>A241G, A242C (N81A)</td>
<td>pOBD2</td>
<td>0.6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>YMR111C</td>
<td>A841G, A842C (N281A)</td>
<td>pOBD2</td>
<td>1.4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>SGT1</td>
<td>T92C (L31P)</td>
<td>pOBD2</td>
<td>1.2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>T295C (F99L)</td>
<td>pOBD2</td>
<td>1.2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>A638T (N213I)</td>
<td>pOBD2</td>
<td>1.2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>T511G (S171A)</td>
<td>pRS314</td>
<td>2.0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>T511G, C512A, A513T (S171D)</td>
<td>pRS314</td>
<td>2.0</td>
<td>2</td>
<td>2</td>
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<tr>
<td></td>
<td>T544G (S182A)</td>
<td>pRS314</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>C-terminal</td>
<td>pRS414</td>
<td>2.0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

aNumbers in parentheses indicate amino acid positions.
bMutations were found within the oligonucleotide sequence of the primer region.

Figure 2. An epitope tagging method. (A) Oligonucleotides with a complementary tag sequence (green bars) that is longer than 30 bases. (B) The primers (red and orange arrows) amplify the gene in the opposite direction from the tag position. (C) Crossing lines represent homologous recombination within homologous DNA sequences. (D) The rescued plasmid with a tag at the desired position.

We have used a similar strategy to insert a tag sequence (i.e., a sequence encoding a hemagglutinin epitope) into a specific position within a yeast plasmid (Figure 2). Primers were designed as indicated in Figure 2A. Two complementary primers that had a single hemagglutinin tag sequence were used in two independent PCRs. Each tag sequence primer was paired in PCR with a primer that hybridized to a region located 30 bp away from the multiple cloning site where the vector was digested (Figure 2B). Because high-quality synthesis of oligonucleotides is generally limited to approximately 90 bases, this method may be useful in adding only a few tags such as hemagglutinin, Myc, FLAG, 6xHis, or a Cre-loxP site. The subsequent steps of this method are the same as those described in the procedure for site-directed mutagenesis (Figure 2, C and D).

The homologous recombination system in yeast has been used for gene disruption, plasmid construction, and mutagenesis (3,4,6,7,12,13). We found that a three-fragment homologous recombination system in yeast cells can efficiently and specifically generate mutated genes in yeast plasmids with very few errors (Table 1). This site-directed mutagenesis system has several features that the QuickChange has and some that are better: (i) no reliance on the presence or absence of a restriction site in the target sequence; (ii) no tricky PCR is needed, (i.e., double PCR or long PCR for amplification of large vectors); (iii) no worry about potential mutations in the vector region; (iv) no need to construct the template plasmid before the experiment, because any yeast vectors can be used; and (v) the PCR-amplified region can be minimized if the target plasmid has a unique restriction enzyme site close to the target site.

In summary, this high-yield system of obtaining correct recombinant plasmids from three PCR fragments by using homologous recombination will be applicable to a variety of methods of modifying plasmids that do not have unique restriction enzyme sites (e.g., mouse knockout or knock-in constructs). Although PCR-based, site-directed mutagenesis requires extensive sequencing of thousands of nucleotides, this process is no longer an excessive drain on laboratory resources (financial or manpower). The fidelity of recently developed commercial Taq DNA polymerases is high enough to prevent PCR amplification errors within a product that is several kilobases in length. In fact, we did not detect any PCR errors in several 1- to 2-kb fragments when we used Pfx, a high-fidelity Taq DNA polymerase, under the conditions described. Therefore, this method may be applicable to constructs that require amplification of a region that is several kilobases long. In addition, this method may also be applicable to other organisms or cell lines in which homologous recombination is efficient such as E. coli (ET recombination) (15) and chicken DT40 cells (14).

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Winding, P. and M.W. Berchtold. 2001. The chicken B cell line DT40: a novel tool for semisynthesized, or directly cloned libraries that are usually inserted into the 5′-end of the gene III sequence in the phagemid. A phagemid containing an ORF sequence will produce protein III as a fusion and thus function (9). Protein-3 (pIII, a minor protein) and protein-8 (pVIII, the major protein) are two bacteriophage coat proteins that are generally used to display polypeptides as fusion proteins because their amino terminal regions are exposed on the phage particle surface and tolerate foreign polypeptide insertions. Such fusion proteins are usually expressed by phagemid constructs in the host cell and packed into the phage particle by the helper phage.

Most phage display studies involve libraries that are usually synthesized, semi-synthesized, or directly cloned from cDNA libraries. Unexpectedly, after panning against target proteins, a large percentage of isolated clones contain disrupted open reading frame (ORF), including in-frame stop codons, deletions, or frame-shift mutations (3.5, 6). Although the expression of non-ORF clones is much less efficient than that of ORF clones, as suggested by some studies (8), these non-ORF clones are usually enriched after several rounds of phage panning. One reason for the enrichment of non-ORF clones relates to the inhibitory role of pIII in phage superinfection. The infection titer of pIII-expressing bacterial cells by the helper phage is 2-3 orders of magnitude lower than that of non-pIII parental cells (4). In phage display, the library sequences are usually inserted at the 5′-end of the gene III sequence in the phagemid. A phagemid containing an ORF sequence will produce protein III as a fusion and thus function (9).

Deoxycholate-Based Method to Screen Phage Display Clones for Uninterrupted Open Reading Frames


Phage display is a powerful technique broadly used in the expression of polypeptides with proper folding and thus function (9). Protein-3 (pIII, a minor protein) and protein-8 (pVIII, the major protein) are two bacteriophage coat proteins that are generally used to display polypeptides as fusion proteins because their amino terminal regions are exposed on the phage particle surface and tolerate foreign polypeptide insertions. Such fusion proteins are usually expressed by phagemid constructs in the host cell and packed into the phage particle by the helper phage. Most phage display studies involve libraries that are usually synthesized, semi-synthesized, or directly cloned from cDNA libraries. Unexpectedly, after panning against target proteins, a large percentage of isolated clones contain disrupted open reading frame (ORF), including in-frame stop codons, deletions, or frame-shift mutations (3.5, 6). Although the expression of non-ORF clones is much less efficient than that of ORF clones, as suggested by some studies (8), these non-ORF clones are usually enriched after several rounds of phage panning. One reason for the enrichment of non-ORF clones relates to the inhibitory role of pIII in phage superinfection. The infection titer of pIII-expressing bacterial cells by the helper phage is 2-3 orders of magnitude lower than that of non-pIII parental cells (4). In phage display, the library sequences are usually inserted at the 5′-end of the gene III sequence in the phagemid. A phagemid containing an ORF sequence will produce protein III as a fusion and thus function (9).

To identify phage clones containing interrupted ORF, we describe here a simple method based on the sensitivity of the host cell to theionic detergent deoxycholate. Expression of pIII has been shown to change the property of the bacterial outer membrane. As a consequence, the host bacteria appear to be sensitive to deoxycholate and more tolerant to certain colicins. In addition, leakage of periplasmic proteins such as β-lactamase can be detected (2). We were interested in determining if the deoxycholate sensitivity of pIII to host bacteria could be applied to the pIII-fusion expressed by phagemids. We expressed the AHNP-Y peptide (YCDGFYACYMDV) (1) by phage display in the fusion to the 5′-end of the streptavidin core sequence (clone H21). Using H21 as a template, we introduced random sequences to replace AHNP-Y by PCR strategy. Briefly, a 5′ random primer (Figure 1) and a 3′ streptavidin-specific primer were used to amplify the template, producing cDNAs of about 450 bp, which was subsequently cloned back into the phage vector pHage (Maxim Biotech, San Francisco, CA, USA). Several clones containing non-amber stop codons in the random peptide region are chosen with the original clone H21 and the empty vector pHage to test the deoxycholate sensitivity of the host strain TG1 (Table 1 and Figure 2). In the TG1 strain, H21 and pHage express the full-length pIII as a fusion protein, while others express a short peptide when the translation is stopped by the non-amber stop codons. The deoxycholate [1.5% (w/v)] plates were made by mixing 2YT-agar