Targeted deletions created in yeast vectors by recombinational excision

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ABSTRACT

We have developed a simple method for creating defined deletions in yeast vectors by utilizing the ability of Saccharomyces cerevisiae to perform homologous recombination. Two complementary single-stranded oligonucleotides are designed so that the 5′ and 3′ halves of the resulting double-stranded oligonucleotide are homologous to the 5′ and 3′ side of a desired deletion junction, respectively. The sequence to be deleted is cleaved by restriction endonuclease digestion, followed by co-transformation of the linearized plasmid and the oligonucleotide into yeast. By homologous recombination in vivo, a subset of the plasmids will recircularize and simultaneously acquire the deletion as defined by the oligonucleotide.

INTRODUCTION

The budding yeast Saccharomyces cerevisiae has proven to be a powerful model organism for the study of basic cellular functions. A variety of frequently used approaches rely on the ability of plasmid-borne genes to complement a defect in the genomic copy, and often extensive deletion analysis of the plasmid-borne sequences is required to define and locate functional domains (1). Also, truncation analysis of proteins engaged in specific protein–protein interactions has become a widely used procedure to delimit the protein domains involved in such interactions (2,3). We have taken advantage of the high efficiency of homologous recombination activity in yeast to introduce precise deletions into a plasmid-borne sequence by an oligonucleotide-based, PCR-free strategy. This method expands the range of recombination-based techniques developed for manipulating DNA in yeast, which includes the creation of chromosomal null alleles by integration into a gene of interest, the rescue of chromosomal segments into a plasmid by gap-repair (4) and recombinational insertion of a PCR product into a linearized target plasmid (5,6). Contrary to these techniques, the strategy described here utilizes the recombination potential of S. cerevisiae to delete a defined sequence rather than to insert a fragment into an episome. From a mechanistic point of view, the method can be seen as a transfer of a deletion carried by an oligonucleotide into a yeast vector by gap-repair.

MATERIALS AND METHODS

Deletion oligonucleotide

Equal amounts of the two complementary single-stranded oligonucleotides were mixed, heated to 100°C for 3 min in a water bath, and subsequently allowed to anneal by slowly cooling the water bath to room temperature. Correct deletion junctions were verified by DNA sequencing using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems). All other DNA manipulations were performed according to standard protocols (7).

Yeast manipulation

All yeast transformations were carried out as described elsewhere (8) and were performed in a W303 strain background (9). Usually, 200 ng of the linearized vector were co-transformed with ~25, 125 and 250 pmol deletion oligonucleotide, respectively. After 2–3 days of growth at 30°C, single colonies were subjected to a colony PCR reaction (10), diagnostic for the oligonucleotide-directed deletion event.

RESULTS

Two complementary single-stranded oligonucleotides are synthesized so that the 5′ half of the annealed double-stranded oligonucleotide is homologous to the 5′ side of the deletion junction, whereas the 3′ half of the oligonucleotide matches the 3′ side of the deletion junction (Fig. 1). This oligonucleotide, containing the desired deletion junction, is then co-transformed into yeast together with the plasmid linearized by a restriction cut anywhere in the segment to be deleted. By homologous recombination, a fraction of the plasmids recircularizes and acquires the deletion. These recombination events have been shown to occur even with regions of homology as small as 15 bp (11).

To illustrate the potential of the strategy, the following will describe the creation of two deletion constructs. In the first example, a 297 bp fragment was deleted using a 54 bp deletion oligonucleotide. Approximately 25 pmol of the two complementary single-stranded deletion oligonucleotides were annealed and co-transformed (8) with 200 ng of the target plasmid that had been linearized by a restriction cut in the 297 bp domain destined to be deleted. After 2 days of growth on selective medium the resulting transformants were screened directly by colony PCR (10) using

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primers flanking the deletion junction. These primers amplify a 693 bp fragment in the absence of a deletion event, whereas a 396 bp fragment is amplified if the deletion has occurred. As indicated in Figure 2A, ~10% of the transformants had acquired the deletion. Two of the deletion constructs were isolated and a correct deletion junction was confirmed by DNA sequence analysis. The method can also be utilized to change restriction sites or to introduce site-specific mutations near restriction sites, either alone or in combination with a deletion event. This is shown in the second example where a 54 bp deletion oligonucleotide was designed so that it facilitates the removal of a 51 bp fragment and simultaneously introduces a restriction site located exactly at the deletion junction. Again, 200 ng of the vector linearized within the 51 bp sequence were co-transformed with 25 pmol of the deletion oligonucleotide. A colony PCR reaction was performed with primers flanking the deletion site and the resulting PCR products were subsequently digested with the restriction enzyme specific for the introduced restriction site. In this experiment, ~20% of the recombinants had acquired the restriction site and therefore also the deletion (Fig. 2B). Correct deletion junctions were confirmed by sequencing two isolated plasmid constructs.

**DISCUSSION**

We have routinely used this oligonucleotide-based strategy for generating deletions in yeast plasmids. In each case, subsequent PCR analysis and DNA sequencing of the junction have demonstrated that between 5 and 20% of the recombinants acquired the deletion, where small domains are generally deleted more effectively than larger domains. These efficiencies might seem low; however, screening for correct deletion constructs by colony PCR of the transformants (10) is a simple and straightforward process, making it easy to screen fairly large numbers of transformants in a relatively short time. Therefore, even deletion efficiencies of ~5% are easily scored. In this study, the method was used to create deletions in *S. cerevisiae* sequences. Thus, the observed background is presumably a result of either a recombination event between the linearized plasmid and the chromosomal locus or, alternatively, due to repair of the gapped vector by an end-joining reaction (12). The sequence in which the deletion is desired does not have to be of yeast origin and the method could therefore be very useful for characterizing various interactions using one of the ‘*n*‘-hybrid systems (3). Control transformation using 200 ng of the linearized plasmid alone did not reduce the amount of observed recombinants. Likewise, dephosphorylation of the linearized vector did not markedly reduce the background. The frequency of correct deletions was not significantly altered using 250 pmol deletion oligonucleotide, but the overall transformation efficiency was noticeably reduced. A higher deletion efficiency could presumably be obtained by using longer deletion oligonucleotides; however, synthesis of long single-stranded oligonucleotides is accompanied by an elevated risk of the incorporation of the wrong nucleotides.

The method described here has several advantages over other deletion strategies. It evades the risk of introducing deleterious mutations inherent to PCR-based procedures, which eliminates the need for extensive sequencing of PCR products (13, 14). Also, no time-consuming subcloning in *Escherichia coli* is required as the deletion constructs can be created directly in the yeast strain of choice. The only requirement is a restriction site in the sequence that is to be deleted. Optimally, this restriction site should be unique, but we have successfully applied this technique by linearizing the plasmid with a partial restriction enzyme

![Figure 1](image1.png)

**Figure 1.** Schematic representation of the oligonucleotide-based deletion method. The white box represents the sequence to be deleted, whereas the striped and gray boxes represent areas of homology between the oligonucleotide designed to create the deletion and the sequences flanking the deletion junction.

![Figure 2](image2.png)

**Figure 2.** (A) PCR analysis showing the recombinational excision of a 297 bp fragment in our favorite gene. A set of PCR primers flanking the deletion site generates a 693 bp fragment in the absence of recombination between the oligonucleotide and the plasmid, whereas a 396 bp fragment is produced if a deletion event has occurred (lanes 5 and 10). (B) A 54 bp deletion oligonucleotide was designed in order to delete a 51 bp fragment and simultaneously to introduce a restriction site not present in the wild-type gene. A PCR reaction was performed with primers flanking the deletion, which creates a 1257 or 1186 bp fragment. The PCR products were digested with the restriction enzyme specific for the introduced site. Based on the sizes of the digestion products we conclude that recombinants in lane 3, 14, 15 and 19 have acquired the deletion. Correct deletion junctions were confirmed by sequencing the constructs from #3 and 14.
digestion. As demonstrated, this method of recombination between a designed oligonucleotide and a linearized plasmid can also be utilized to change restriction sites or to introduce site-specific mutations. Thus, this oligonucleotide-directed manipulation of yeast plasmids \textit{in vivo} provides a convenient and reliable method with a variety of applications.

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\textbf{REFERENCES}