

Using the FailSafe™ PCR System to Construct Molecular Tools for the Functional Characterization of a *Saccharomyces cerevisiae* Gene

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Introduction

The functional characterization of a new gene requires the construction of several molecular tools which very often includes PCR amplification steps. Thus, having an efficient, easy handling and faithful PCR system represents a real gain of time and can greatly facilitate the first steps toward the elucidation of the *in vivo* function of a gene.

We are interested in the transcriptional control of ribosome biogenesis in *Saccharomyces cerevisiae*, and we started the study of a new gene (Our Favourite Gene, *OFG*) potentially involved in this process.^{1,2} First, we wanted to determine the cellular location of the *OFG* protein product and to assess the impact of a chromosomal deletion of *OFG* on the cell physiology. To do so, we needed to tag the protein with an influenza hemagglutinin (HA) epitope and create an *ofg::kanMX4* disruption cassette. We successfully generated these two DNA constructs using the FailSafe™ PCR System.

Materials and Methods

Construction of a plasmid-borne HA-epitope tagged *OFG* allele by fusion PCR

In order to immunodetect the protein product of *OFG*, we introduced by fusion PCR an *in-frame* HA-epitope tag encoding sequence just after the start codon of a plasmid-borne *OFG* allele (Figure 1). The modified *OFG* allele is controlled by its own promoter so that expression

is driven in a physiological manner. A portion of the promoter region of *OFG* (PCR 1) was amplified from plasmid YCplac33-*OFG* using primers OFG-C (5' **GTC ATA GGG ATA GCC CGC ATA GTC AGG AAC ATC GTA TGG GTA TGC CAT CAA AGG CGT CGG TAT TG** 3') and the M13/pUC sequencing primer (-20, BioLabs). The N-terminal region of *OFG* (PCR 2) was amplified from the same template using primers OFG-B (5' **GCG GGC TAT CCC TAT GAC GTC CCG GAC TAT GCA TTC ATC AAA CAG TCT GAA AAA** 3') and OFG-A (5' TTC GAC GGA ATC ATG AGA T 3'). Bolded nucleotides encode the HA-epitope; overlapping bases between OFG-C and OFG-B are underlined.

PCR reactions were performed using the FailSafe PCR Enzyme Mix and a set of 12 FailSafe PCR PreMixes (A-L). The PreMixes contain a buffered salt solution with all four deoxyribonucleotides, and various amounts of MgCl₂ and the FailSafe PCR Enhancer (with betaine). Each 50 µl reaction contained 200 ng of the DNA template, 0.5 µM of each primer, less than 1.25 U of the FailSafe PCR Enzyme Mix, and 25 µl of a FailSafe PCR 2X PreMix. The cycling profile for PCR 1 and PCR 2 was one cycle at 94°C for 2 minutes followed by 30 cycles of 94°C for 45 seconds, 52°C for 45 seconds, 72°C for 1.2 minutes, and a final extension at 72°C for 5 minutes. Five microliters of each PCR reaction was further analysed by electrophoresis on a 1% agarose gel, the expected size for PCR 1 and 2 products is 0.9 kb and 1 kb, respectively

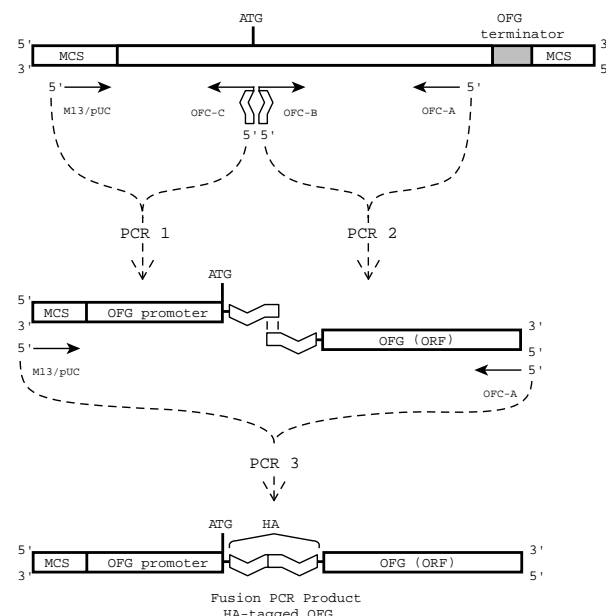


Figure 1. Scheme of the fusion PCR procedure. The 5' region of primer OFG-C, used in the amplification of PCR product 1, is complementary to a segment of PCR product 2 (i.e., the 5' region of primer OFG-B). The HA sequence is therefore created when this overlap is extended and co-ordinately amplified with the inclusion of outside primers M13/pUC and OFG-A.

(Figure 2). A third PCR corresponding to the fusion PCR was performed using PCR products 1 and 2 as DNA template and the two oligonucleotides OFG-A and M13/pUC. A 50 μ l reaction contained ~300 ng of gel-purified PCR 1 product (buffer D) and ~300 ng of gel-purified PCR 2 product (buffer D), 0.5 μ M of each primer, 1.25 U of FailSafe PCR Enzyme Mix, and 25 μ l of FailSafe PCR PreMix D. The cycling profile was one cycle at 94°C for 2 minutes followed by 30 cycles of 94°C for 45 seconds, 51°C for 45 seconds, 72°C for 2.5 minutes, and a final extension at 72°C for 5 minutes. Two microliters of the PCR reaction (Figure 3) were further analysed by electrophoresis on a 1% agarose gel, the expected size of the fusion PCR product is 1.9 kb. After ethanol precipitation, the fusion PCR product was digested with *EcoR* I, gel purified and further subcloned into a *EcoR* I-restricted YCplac33-*OFG* plasmid. The final plasmids, YCplac33-HA-*OFG*, fully complemented an *ofg*-disrupted strain.

Generation of an *ofg::kanMX4* disruption cassette

To perform the chromosomal disruption of *OFG*, a *kanMX4* cassette harbouring *OFG* short flanking homology regions was generated using the FailSafe PCR System with the plasmid pFA6a-*kanMX4* as the DNA template and OFG-P1 (5' GGC TTT TGT TCT TTG ATG TTA ATT CGG CAA TAC CGA CGC CTT TGC GTA CGC TGC AGG TCG AC 3') and OFG-P2 (5' TGT TAT GAA GCT ATA TGG TAA AGA ATA CAT GGT GTC ATA TAG ATT CGA TGA ATT CGA GCT CG 3') as primers. PCR reactions were set up with the 12 PCR 2X PreMixes as described above for PCR 1 and 2 (data not shown). The cycling profile for the PCR reaction was one cycle at 94°C for 2 minutes followed by 30 cycles of 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 1.5 minutes, and a final extension at 72°C for 5 minutes. After concentration by ethanol precipitation, the *ofg::kanMX4* product (1.5kb) was used to transform the yeast strain W303.

Transformants were selected on YPD plates containing 200 mg/ml G418.³

Results and Discussion

With each PCR reaction performed with the FailSafe PCR System, we succeeded on the first try, recovering the expected product in sufficient amounts for the following steps and in most cases sufficiently pure to avoid any further gel-purification steps. A clear advantage of the FailSafe PCR System lies in the 12 PreMixes which allow a direct, easy, and quick determination of the best amplification conditions for each primer pair and DNA template. For the construction of an HA epitope-tagged *OFG* allele, PCR 1 and 2 employed primer pairs highly heterogeneous in size (17 and 65 bases; 19 and 54 bases) with, in both cases, the longest primer having a long 3' non-hybridizing tail. As shown in Figure 2, large amounts of the expected products were obtained with several of the PreMixes but not with all of them, emphasizing the importance of the buffer conditions in the PCR reaction. The amplifications were highly specific in all cases since no significant aberrant products were generated.

Similarly, the PCR reaction yielding the 1.5 kb *ofg::kanMX4* disruption cassette employed a pair of long primers (> 60 bases) with a long non-hybridizing tail (~40 bases). We also obtained high amounts of a highly specific product with 10 of the 12 PreMixes tested (data not shown). As in the previous PCR series, the poorest results were obtained with PreMixes I, J and L.

In the case of the fusion PCR, potential difficulty resides in the nature of the DNA template which consists of two DNA molecules of ~1kb (PCR products 1 and 2) having only a very short overlapping region (18 bp) (Figure 1). When the assay was performed with DNA template that was not purified and two different PreMixes, three products (0.9, 1.0 and 1.9 kb) were amplified in

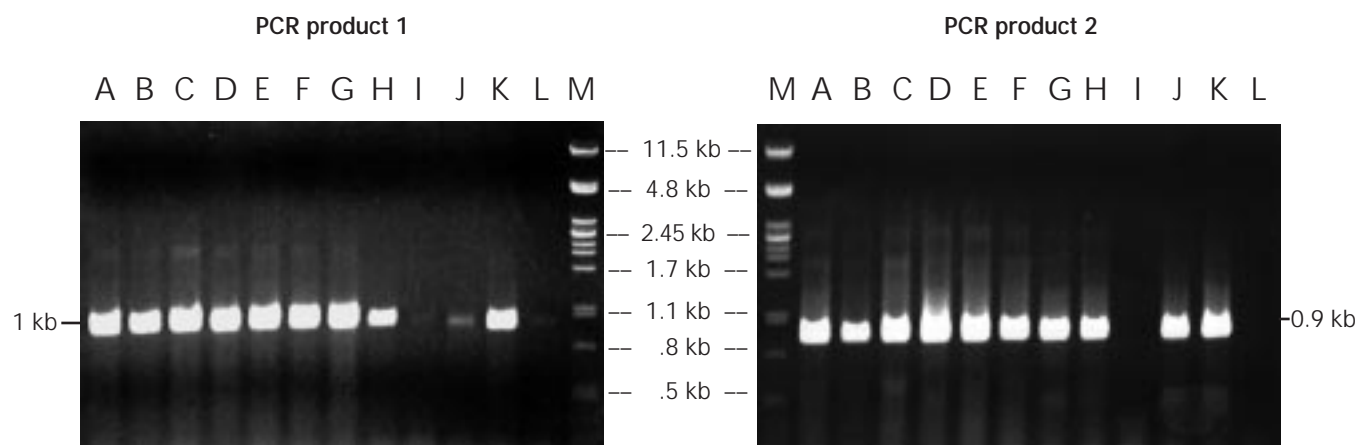


Figure 2. FailSafe PCR amplification of the promoter (PCR1) and N-terminal (PCR2) regions of *OFG*. Lanes A-L show the amplification products resulting from PCR using the 12 FailSafe PCR PreMixes. M, molecular weight marker.

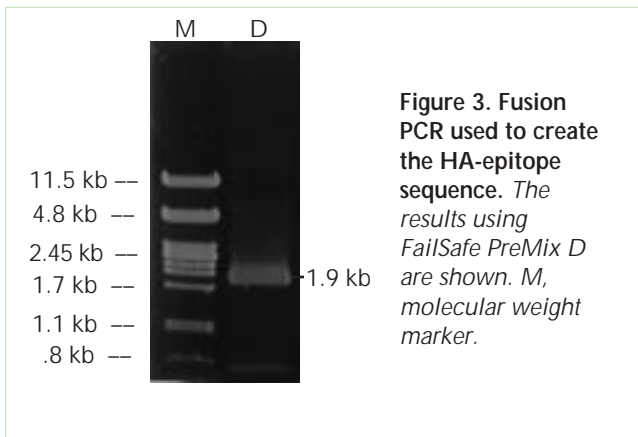


Figure 3. Fusion PCR used to create the HA-epitope sequence. The results using *FailSafe PreMix D* are shown. *M*, molecular weight marker.

comparable amounts (data not shown). The unexpected amplification of the PCR 1 and 2 products was due to traces of primers OFG-A and OFG-B. This was easily overcome by using gel-purified PCR 1 and 2 products as DNA template (Figure 3). We did not sequence the plasmid-borne HA-*OFG* alleles recovered after subcloning of the fusion PCR product, but the fact that they fully complemented the *ofg*-disrupted strain argues in favor of the high fidelity of the *FailSafe* PCR Enzyme Mix.

References

1. Warner, J. R. (1999) *Trends Biochem. Sci.* **24** (11), 437.
2. Mizuta, K. et al. (1997) *Gene* **187** (2), 171.
3. Gietz, D. et al. (1992) *Nucleic Acids Res.* **20**, 1425.

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Figure. Sequencing through a GC-rich trinucleotide repeat. Supercoiled plasmid template containing (CGG)₂₃ was sequenced using the *SequiTherm EXCEL II* isothermal sequencing protocol.