Vector for Positive Selection of In-Frame Genetic Sequences

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Polymerase chain reaction (PCR)-based approaches are becoming increasingly important for the identification of members of extended multigene families as well as homologous gene structures present in phylogenetically divergent species (2,5,10,11). Many of these approaches rely on the use of highly degenerate primers and/or reduced priming stringencies that can generate a broad range of products, including significant numbers of amplification products that contain frameshift(s) and/or termination codon(s). Recently, we introduced the use of short, minimally degenerate primers complementing conserved structural motifs for PCR amplification of homologs of antigen-binding receptor genes in phylogenetically diverse species (6,8–10). This approach is also associated with the generation of amplification artifacts that require DNA sequencing to be distinguished from products that warrant further study. To facilitate identification of amplification products containing open reading frames, we have engineered a vector, pGFPfs, that affords positive selection of recombinants based on the continuity of coding sequence within a *lacZ*:insert:GFP (green fluorescent protein) fusion construct that is expressed in *E. coli*.

pGFPfs was derived from pGFPuvTM (CLONTECH Laboratories, Palo Alto, CA, USA) by ligating a linker (formed by annealing the partially complementary oligonucleotides: 5'-GATCGAT-ATCTCGAGT-3' and 5'-CTAGACTC-GAGATATCGATCTGCA-3') into the multiple cloning site (MCS) of *Pst*I/*Xba*I-digested pGFPuv. Incorporation of this linker, which was confirmed by DNA sequencing, disrupts the GFPuv reading frame and introduces additional unique restriction sites (Figure 1A). The GFP variant in these vectors, GFPuv, differs from wild-type GFP (7) in that it is optimized for bacterial expression, solubility and fluorescence of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *lacZ*:GFPuv

fusion protein (3). Colonies producing GFPuv fusion proteins are identified by viewing under longwave UV light. Positive selection of recombinants containing in-frame inserts is based on correction of a frameshift that has been engineered within the 5' MCS of pGFPfs.

PCR products were generated using methods that have been described previously by our laboratory (8–10) and were ligated into *Hind*III/*Xba*I-digested pGFPfs. Ligation mixtures were used to transfect *E. coli* DH5 α F'TM (Life Technologies, Gaithersburg, MD, USA). Cells were plated on LB plates containing 10 mM IPTG and 100 μ g/mL ampicillin, and colonies were grown at 30°C for 48 h.

Plasmid DNA was isolated for sequencing using a QIAprep[®] Spin Miniprep Kit (Qiagen, Chatsworth, CA, USA) in accordance with the manufacturer's recommended protocol. Sequencing was performed with a 4000L Automated Sequencer (LI-COR, Lincoln, NE, USA) using a SequiThermTM Long-ReadTM Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA). Plates were viewed using a UVL 56 Blak-Ray[®] (366 nm) longwave ultraviolet lamp (UVP, Upland, CA, USA).

IPTG induction of pGFPfs generates a 17-amino acid protein that is encoded by a fragment of *lacZ*, the contiguous cloning sites and a 3' termination codon (Figure 1A); GFPuv is not expressed. Because cloning sites are 5' of the GFPuv coding sequence, expression of GFPuv requires frame continuity within an insert. Therefore, directional cloning of PCR-derived products that are devoid of stop codons can be used to correct the reading frame to that of the GFPuv coding sequence (Figure 1B). Primers incorporating restriction sites can be designed to either remove the frameshift (e.g., *Hind*III/*Kpn*I cloning) or introduce a second frameshift within the 3' primer (e.g., *Hind*III/*Pst*I cloning) to reestablish the correct reading frame and allow expression of GFPuv. The capacity of pGFPfs to discriminate between coding and noncoding amplification products is dependent on noncoding products having an internal stop codon(s) or shifted reading frame; i.e., ± 1 base. It is apparent that 66% of noncoding products will not have the

Benchmarks

Table 1. pGFPfs Cloning

Sequencing Events*	green fluorescent colonies	37
Desirable Products	in-frame (ca. 200 bp)	31
Undesirable Products	in-frame artifacts (45–129 bp)	5
	out-of-frame (stop codon)	1
Efficiency	(31/37) 84%	

*As controls, 15 GFPuv-nonexpressing (white) colonies were recovered and determined to be out-of-frame artifacts or vector.

correct number of nucleotides needed to restore the GFP coding sequence. However, the variable prevalence of stop codons in PCR-derived products provides an additional basis for exclusion

of noncoding recombinants. Validation studies of the pGFPfs construct were carried out initially by (re)amplification of previously characterized in-frame and out-of-frame

single-strand M13 templates, derived in the course of earlier studies (10). Sequencing of templates recovered from isolated nonfluorescent colonies transfected with pGFPfs or recombinant pGFPfs containing out-of-frame inserts as well as from fluorescent colonies transfected with recombinant pGFPfs-containing inserts with continuous reading frames confirmed the correlation (100%) of fluorescence with in-frame sequences. Cloning of products derived by amplification with short, minimally degenerate primers using total mRNA-derived cDNA as template yields GFP-expressing recombinants, nonexpressing recombinants and uncut vector. False-positive colonies have been observed and typically are the result of the integration of short inserts lacking termination codons that could not be eliminated by size selection. The generation of such interfering amplifications correlates directly with decreased primer stringency and/or template heterogeneity. In experiments using total mRNA-derived cDNA as template, undesired in-frame recombinants represent less than 15% of positives. In our experience with conventional M13 cloning and sequencing of short primer amplification products (in excess of 1000 analyses), less than 15% of recombinants represent in-frame sequences (8–10). The use of pGFPfs cloning from total mRNA-derived cDNA resulted in a 5–6-fold overall improvement in the efficiency of identification of both known and novel in-frame sequences (Table 1). In-frame inserts of >800 bp have been shown to produce detectable GFPuv fusion proteins.

The vector can be utilized in searches for previously unrecognized members of extended multigene families. In addition, pGFPfs cloning can be used to identify out-of-frame sequences, such as those arising from disease-causing mutations. For such an application, which could serve as a primary diagnostic tool, amplifications would be carried out under highly stringent conditions using primers that are designed to require a frameshift (associated with disease) immediately upstream of the 3' primer to rescue GFP expression; amplification of the corresponding normal sequence would not rescue GFP expression. In a general sense, pGFPfs is

Figure 1. (A) pGFPfs vector diagram and 5' MCS. The vector sequence shows the oligonucleotide linker (boxed), termination codon (reverse image) and the out-of-frame GFPuv coding sequence as well as restriction enzyme sites (labeled directly above the corresponding nucleotides). Other than the addition of the oligonucleotide linker, the vector is identical to pGFPuv. The 17-amino acid pGFPfs translation product is also shown above its coding sequence (stippled box). The circular vector diagram shows the out-of-frame GFPuv sequence (white). Shown to the right of the circular vector diagram are partial sequences of primer pairs used to correct the GFPuv reading frame; note the incorporation of nucleotides (N) used to maintain (5') and correct (3') product frame. (B) The lacZ:insert:GFPuv fusion protein is shown above its coding sequence; various stippling is used to indicate contributions from lacZ, insert and GFPuv and corresponds to the circular vector diagram. The nucleotides (N) that are introduced in this specific example to maintain continuous frame are shown (reverse text) within the insert-derived sequence (boxed).

a convenient, highly efficient tool for distinguishing in-frame from out-of-frame and/or termination codon-containing sequences and has potential application for distinguishing wild-type and certain mutant sequences.

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