Benchmarks

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Generation of Epitope-Tagged Proteins by Inverse PCR Mutagenesis

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The polymerase chain reaction (PCR) has been extensively used for the mutation of DNA sequences (1-5,7,8). Many variations of the protocol have been implemented, but most involve a first step in which PCR is used to modify the molecule, and a second step in which the new sequence is subcloned into a vector. A new method, named inverse PCR mutagenesis (IPCRM) has been shown to be an efficient and rapid way to introduce point mutations into DNA sequences cloned in plasmid vectors without the requirement of a subcloning step (2,3,7,8). The technique consists of a PCR amplification of the vector using a set of two adjacent nonoverlapping oligonucleotides, one of which encodes the desired mutation. Since the PCR product can be longer than 10 kb, the use of *Pfu* DNA polymerase is recommended, not just because of its high fidelity but also because it generates blunt-ended PCR products, eliminating the need for end polishing. The PCR product is then ligated, treated with *DpnI* to digest methylated contaminating parental plasmid

Table 1. Modified IPCRM Protocol

1. Both primers (50 pmol/ μ L each) were phosphorylated in the same tube for 30 min at 37°C using T4 Polynucleotide Kinase (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
2. The PCR mixture (50 μ L) was prepared with 50 pmol/ μ L of each phosphorylated primer, 200 mM of each dNTP, 1.25 U of *Pfu* DNA Polymerase, 20 ng of template plasmid and 5 μ L of *Pfu* Reaction Buffer (Stratagene, La Jolla, CA, USA).
3. Following an initial 94°C denaturing step for 4 min, the reaction was cycled 16 $\times$ through 94°C for 30 s, 60°C for 30 s and 72°C for 20 min (2.5 min/kb of template). The reaction was terminated by a final extension step at 72°C for 25 min.
4. The amplified product was confirmed by running 5 μ L of the reaction in 1% agarose gel.
5. The remaining sample was purified by GENECLAN® Kit (BIO 101, Vista, CA, USA), resuspended in 7.5 μ L of 10 mM Tris-HCl, pH 8.5, and mixed with 1 μ L ligation buffer, 1 μ L 50 mM dithiothreitol (DTT) and 2 U T4 Ligase from a PRIME EFFICIENCY™ DNA Ligation Kit (Eppendorf Five Prime, Boulder, CO, USA). The ligation reaction was run for 40 min at room temperature, followed by heat-inactivation for 10 min at 65°C.
6. After ligation, 8 μ L of water and 2 μ L of *DpnI* (New England Biolabs) were added to the sample and incubated for 4 h at 37°C.
7. One microliter of the final reaction was used to transform 50 μ L of Epicurian coli® XL2-Blue cells (Stratagene) according to the company protocol.
8. After overnight incubation, ten colonies were selected and grown for plasmid minipreps.

and subsequently introduced into competent cells through transformation. IPCRM is a suitable method to produce substitution and deletion mutations in sequences already cloned into a specific vector (3).

We have extended the IPCRM method to permit insertion of epitope tags into a target protein of interest. Routinely, tagged proteins are generated by cloning the coding sequence into a multiple cloning site of a vector that already contains the epitope sequence (6). Changing the epitope or even its location with respect to the target protein requires either the use of new vectors or extensive cloning procedures. Using IPCRM, it is possible to insert short peptide sequences anywhere within the vector, requiring only a pair of primers.

To illustrate the procedure, we have fused the FLAG® epitope sequence

Figure 1. Overview of Modified IPCRM Protocol.

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(DYKDDDDK) in-frame with the carboxyl terminus of calcium sensing receptor (CaR) DNA, which was already subcloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). For the insertion of the epitope, we designed a pair of adjacent primers, each containing half of the FLAG sequence (Figure 1). The upstream primer consisted of 20 nucleotides complementary to the end of the CaR DNA without the stop codon plus 12 in-frame nucleotides corresponding to the first half of the FLAG DNA sequence (5'-GTCCTTGTAAGTCTGAATCACTACGTTTTCTG-3'). The downstream primer contained the other half of the FLAG DNA sequence plus 20 nucleotides complementary to the vector (5'-GATGACGACAAGTAATCGAGTCTAGAGGGCCCGTT-3').

The procedure was done according to Fisher and Pei (3) with modifications

as illustrated in Figure 1 and the protocol in Table 1.

Before sequencing the constructs, we compared the isolated plasmids with the wild type by submitting them to *Bgl*II (New England Biolabs, Beverly, MA, USA) digestion. Nine out of ten samples analyzed showed the correct bands. To be certain that the FLAG epitope sequence was correct and in-frame, the samples were transfected into HEK-293 cells previously plated in 8-well Lab-Tek® Chamber Slides (Nalge Nunc International, Rochester, NY, USA). After 24 h, the cells were fixed with acetone and analyzed by immunofluorescence with anti-FLAG antibody (Sigma, St. Louis, MO, USA). Seven out of nine samples displayed positive fluorescence in the membranes, implying that CaR and the FLAG epitope were properly fused. Positive results were confirmed by automated sequencing (Model 377 DNA Sequencer; PE Biosystems, Foster City, CA, USA) in the region encompassing the epitope tag. Constructs chosen for use in further studies were sequenced over the entire coding region to confirm the absence of PCR-induced errors. The two non-fluorescent mutants were also sequenced and displayed the deletion of a single nucleotide in the ligation region, which could have occurred either during the PCR cycles or the ligation step.

The method described here was also used to create additional recombinant proteins by inserting other peptides [including c-myc (EQKLISEEDL), an enterokinase site (DDDDK) or His₆] into CaR or G protein-gated inward rectifier potassium channel (GIRK). The inserted peptides varied from 5–10 amino acids and were introduced into different regions of the target proteins. The new constructs were generated and confirmed in less than five days, and purchase of new plasmids was not necessary. Thus, IPCRM offers a rapid, reliable and cost-effective method for generating fused proteins by permitting the insertion of short sequences into a given DNA without the need for engineering of new restriction sites or acquisition of new vectors.

We describe a novel way to tag proteins by the insertion of epitope sequences using a modification of the enzymatic IPCRM technique. No re-

striction enzyme site is required, and the epitope can be fused anywhere within the molecule without subcloning it into a new vector. The method proved to be an efficient alternative for generation of recombinant fusion proteins.

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