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Production of PCR Mimics for Any Semi-quantitative PCR Application

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Semiquantitative polymerase chain reaction (PCR) is a powerful tool for determining DNA and RNA copy numbers in biological samples (4,5,8). Essentially, the technique involves amplification of the target with specific primers and comparison of the signal intensity with that of a standard that was amplified within the same tube after separation of the products by gel electrophoresis. Signal intensity is proportional to target copy number in semiquantitative PCR. The standard can either be an endogenous sequence (e.g. housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*]; see References 16 and 17) or be added prior to amplification at a specific copy number (14).

An optimal competitor sequence should be amplified at a similar efficiency as the target gene. Therefore, it should use the same primers as the tar-

get DNA and have similar size, nucleotide composition and secondary structure. Hence, optimal competitor 'mimics' tend to be constructed from the cloned target gene. Several strategies exist to do this. Restriction enzymes can be used to create a deletion within the target gene or permit insertion of a stuffer fragment (9). However, in the absence of suitable restriction sites, this approach is not feasible. A recent publication describes the application of inverse polymerase chain reaction (IPCR) to generate PCR mimics without the requirement of restriction enzymes (18). This method is rapid and simple, but does have one major drawback. If the plasmid containing the target gene is large, then amplification of the entire plasmid can be difficult unless the target gene is first subcloned into a smaller vector.

We present an application of PCR-ligation-PCR (PLP) mutagenesis (2,3) for the production of an ideal mimic sequence for any semiquantitative PCR application. Like the IPCR method, this method is rapid and does not require the use of restriction sites at the point of the deletion. However, unlike IPCR, there is no requirement for amplifica-

Figure 1. Construction of a PCR mimic using PLP mutagenesis. (A) Sequences from the transferrin gene contained within a baculovirus expression vector (1) were amplified by PCR using the two primer sets A1/A2 and B1/B2. The PCRs were performed in 100- μ L volumes containing 1 ng of template, 200 μ M each dNTP, 1 μ M primers, 4 U of Vent[®] DNA Polymerase and 1 $\times$ Vent reaction buffer (both from New England Biolabs, Schwalbach/Taunus, Germany). The amplifications were performed over 25 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s and then primer extension at 72°C for either 30 s (upstream gene fragment, 196 bp) or 45 s (downstream gene fragment, 639 bp). An aliquot of 5 μ L of each PCR product (A and B) was phosphorylated at 37°C for 30 min in 50- μ L reactions comprising 1 mM ATP, 50 U T4 polynucleotide kinase and 1 $\times$ T4 polynucleotide kinase reaction buffer (both from New England Biolabs). The reactants were heated for 5 min before addition of enzyme. Ligation was performed by combining 5 μ L of each phosphorylated PCR fragment with 400 U of T4 DNA Ligase (New England Biolabs) for 15 min at room temperature. The 835-bp fusion product was PCR-amplified from 1 μ L of the ligation mixture using the primer set A1/B2 as described above, but using a primer extension time of 1 min. The resulting product was then subcloned back into the transferrin baculovirus expression vector. (B) 5- μ L aliquots of the phosphorylated PCR products (A and B) and 5 μ L of the fusion gene product (AB) electrophoresed on ethidium bromide-stained agarose gels.

tion of the whole plasmid; therefore the procedure can be applied for generating PCR mimics from any plasmid vector.

To illustrate this application of PLP mutagenesis, we have created a deletion within the transferrin gene contained in a baculovirus transfer vector (Figure 1). The transferrin gene is approximately 2.1 kb in length, but for semiquantitative PCR purposes, we amplify a smaller fragment of 957 bp. An ideal PCR mimic should be approximately 10%–20% smaller. The first step to create the deletion was PCR amplification of two adjacent transferrin gene fragments separated by a distance of 122 nucleotides. After a phosphorylation reaction (30 min), the two PCR products were ligated in the same buffer (15 min), and the fusion gene was amplified from an aliquot of the ligation reaction mixture by PCR using the 5' primer of the upstream gene fragment and the 3' primer of the downstream gene fragment. This PCR product was then subcloned back into the transfer vector, thereby creating a mimic that contained a deletion of 122 bp within the transferrin gene. Following large-scale DNA preparation and purification using a cesium chloride gradient and phenol-chloroform extraction,

the DNA was quantitated by measuring its absorbance at 260 nm (12).

To test the performance of the mimic in a semiquantitative PCR application, we co-amplified a fixed quantity of the target (parental transferrin baculovirus transfer vector) and dilutions of the mimic in single reactions (Figure 2A). As the number of copies of the mimic decreases, so does the intensity of the amplified mimic band

(835 bp). Amplification of the target by PCR yielded a fragment of 957 bp. In semiquantitative PCR, the concentration of target is determined from the point at which the intensity of the competitor and mimic bands are equivalent. In this experiment, this occurs in the reaction analyzed in lane 4, indicating that 10^5 copies of the target amplicon were present in the PCR samples. The actual quantity of the target added was

Figure 2. Characterization of semiquantitative PCR mimic generated by PLP mutagenesis. (A) In single reactions, 1×10^5 copies of target amplicon (wild-type baculovirus transferrin expression vector) together with a titration of mimic ($1-10^8$ copies) were subjected to PCR using primers A1 and B2, as before (Figure 1) with the exception that the reactions were performed in 50- μ L volumes containing 0.5 U *Taq* DNA Polymerase (Promega, Madison, WI, USA). Aliquots of 5 μ L were electrophoresed on ethidium bromide-stained gels. The mimic and wild-type bands are 835 and 957 bp, respectively. Lane 11 is the control PCR containing no template. (B) To verify that the target amplicon and mimic were amplified at the same efficiency, the two templates were co-amplified by PCR as described above over the same titration range.

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1×10^5 copies. To confirm that both products were amplified with similar efficiency, co-amplification of a titration of both the target and the mimic at the same concentration yields bands of similar intensity over the entire range tested ($1-10^8$ copies; Figure 2B). We have applied this method for titrating recombinant baculoviral stocks, and this data will be published separately.

The production of PCR mimics using PLP mutagenesis could be simplified even further. The fusion gene, amplified in the second mutagenic PCR, can itself be used as a PCR mimic after removal of primers and precise quantitation. As such, a single PCR could generate enough mimic for several thousand semiquantitative PCRs. That PCR fragments can function well as PCR mimics was demonstrated recently (13,15) using PCR mimics that were larger than the target amplicon. To generate these larger mimics, extra nucleotides were added to termini of the target cDNA sequence using PCR. It should be noted, however, that the extra sequences were carefully selected to create minimum disruption of nucleotide composition and to avoid generating stable secondary structure. In practice, effects of additional nucleotides on the amplification efficiency of the mimic, whether added terminally or as a stuffer fragment, are difficult to predict.

As a standard method, we prefer PLP mutagenesis to other mutagenesis technologies such as splice overlap extension PCR (6,7) and ligase chain reaction PCR (10,11) because of its simplicity and reproducibility. We found it to be a rapid, inexpensive method to reliably generate mutations. In addition, use of PLP mutagenesis as a universal method for creating ideal PCR mimics is just one of the many applications that we have identified for this technique.

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