Exonuclease III-Generated Series of Homologous Competitor DNA Fragments for Competitive PCR

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Competitive PCR is currently the most sensitive and accurate assay available for quantitation of the expression level of any gene in cells or tissues (18,25). However, heteroduplex formation between the target gene and competitor DNA, which bears sequence homologous to the target gene, hampers accurate quantitation of the target gene (10,17). To circumvent this problem, several approaches involve the inhibition of heteroduplex formation (3,10,12,14,22), correction of heteroduplex formation (1,11) or use of the heteroduplex signal for quantitation of target sequences (9,17,23).

To avoid biased quantification results and correct heteroduplex formation for accurate quantitation of target sequences, separation of heteroduplex fractions from the homoduplex bands for target gene and competitor DNAs are necessary (1,11,17). It is preferable to generate a competitor DNA of an appropriate size relative to the target gene (11,17). This requires preparation of multiple competitors of different sizes because the rate of migration of heteroduplex DNA during agarose gel electrophoresis is usually unpredictable and has to be determined empirically (12). However, the procedures for generating competitor sequences usually require either a significant amount of cloning or the use of multiple, specifically synthesized oligonucleotides. This is especially true when multiple competitors are required. Here, we describe a simple and efficient technique for the generation of a series of multiple competitor DNA fragments, based on a wild-type gene, by making use of exonuclease III (6,21). This technique only requires the same two oligonucleotides to be used in the competitive PCR assay (18). The procedure was initially applied to the human interferon α/β receptor gene (IFNAR2) (15).

Human peripheral blood mononuclear cells were purified from 10 mL heparinized whole blood from a consenting healthy volunteer using Ficoll-Paque® (Amersham Pharmacia Biotech, Uppsala, Sweden) density centrifugation. Total RNA was isolated using Ultraspec™ RNA (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer's instructions. The quality and integrity of the RNA were tested using spectrophotometry and denaturing agarose gel electrophoresis. cDNA was then synthesized using a random hexamer (Takara Shuzo, Otsu, Japan) and M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA). Two PCR oligonucleotide primers for IFNAR2 (IFNRBS1: 5'-ACAAGTGGCGGTGGCTATAC-3'; and IFNRBLAS1: 5'-TGGGAAAG-GTTGGCTGTGTC-3') were synthesized. PCR was carried out on cDNA corresponding to 50 ng total RNA in 30 μ L volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, 1 μ M each primer and 1 U of AmpliTaq Gold® DNA polymerase (Roche Molecular Systems, Summitville, NJ, USA). The amplified IFNAR2 cDNA fragment was obtained following initial denaturation for 9 min at 95°C and 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, followed by a final extension of 10 min at 72°C in a DNA thermal cycler model PJ2000 (PE Biosystems, Foster City, CA, USA). The cDNA was directly cloned into the T-vector, pCR2.1, using the TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A plasmid was purified from 3 mL LB culture medium including 50 mg/mL kanamycin (Sigma, St. Louis, MO, USA) using the QIAprep® spin miniprep kit (Qiagen, Valencia, CA, USA) and sequenced by the fluorescent-based Taq dye deoxy terminator cycle sequencing system (PE Biosystems), using M13 universal primers according to the manufacturer's instructions.

The purified plasmid (2 μ g) was digested using Bsu36I (New England Biolabs, Beverly, MA, USA), which is unique for the cloned cDNA fragment of IFNAR2 but not for the vector pCR2.1. In the next step, the mixture was extracted once with phenol/chloro-

form and once with chloroform, followed by ethanol precipitation together with 1/10 volume 3 M sodium acetate, pH 5.2. The pellet DNA was resuspended with 10 μ L sterile water and added to a 60- μ L volume containing 66 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 100 mM NaCl and 100 U exonuclease III (New England BioLabs) (6,21). A bidirectional deletion reaction was then carried out at 25°C, and 3 μ L reaction mixture were sampled every 30 s for 5 min, followed by sampling every minute.

To produce blunt ends of dsDNA, each timed-sample was immediately mixed with 3 μ L 60 mM sodium acetate, pH 4.6, 200 mM NaCl, 2 mM ZnSO₄ and 1.5 U S1 nuclease (Takara Shuzo) (20) and incubated at 37°C for 30 min. Then, 1 μ L 0.1 M EDTA, pH 8.0, was added and the mixture was heat inactivated at 65°C for 10 min. Ligation reaction of the deleted plasmid was performed using 3 μ L each timed sample in a 20- μ L volume containing 50 U T4 DNA ligase (New England BioLabs) at 16°C for 2 h. Then, 1 μ L each ligation mixture was subjected to PCR as a 20- μ L volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 200 μ M each dNTP, 1 μ M each primer and 1 U of AmpliTaq Gold DNA polymerase as described earlier. This was followed by electrophoresis through a 1.4% NuSieve® 3:1 agarose (BMA, Rockland, ME, USA) gel containing 1 μ g/mL ethidium bromide in TAE (Figure 1A) (21).

Appropriate ligation mixtures were selected according to DNA size on the agarose gel, and 2 μ L each mixture was used to transform 50 μ L competent TOP10F' *E. coli* (Invitrogen). The transformed bacteria were grown on an LB agar plate containing 50 mg/mL kanamycin. Isolated bacterial colonies were directly screened for appropriate plasmid size using colony PCR (7,16). The PCR products were analyzed along with the product amplified from a wild-type cDNA by electrophoresis through a 3% NuSieve 3:1 agarose gel as described (Figure 1B). Equimolar amounts (40 amol) of wild-type cDNA (target gene) and each competitor of various sizes were further competitively amplified in 30 μ L PCR mixture and analyzed by electrophoresis through a 3% NuSieve 3:1 agarose gel as

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described earlier (Figure 1C). Separation of the heteroduplex band from homoduplex bands could not be achieved with some competitors despite successful separation of homoduplex bands for wild-type cDNA and competitor DNAs (Figure 1C, lanes 3–6).

However, successful separation of all three bands was achieved with other competitors (Figure 1C, lanes 7–10), which allowed the selection of an appropriate competitor. Substantial differences in the ratio of the upper wild-type cDNA containing bands to the lower competitor bands with and without providing clear heteroduplex band were noted (Figure 1C, lanes 3–6 and 7–10), again emphasizing the importance of separating heteroduplex from homoduplex bands (1,11,17). Subsequently, appropriate clones were selected, grown

in LB medium containing kanamycin, purified and sequenced as described to serve as DNA competitors in subsequent competitive PCR. Alternatively, amplified products can be directly used as competitive DNA after purification and estimation, following agarose gel electrophoresis.

There are several published procedures for the generation of competitor sequences (25). While some of these procedures are simple, the generated competitors, heterogeneous to the target gene sequences (2,24), usually require verification that the amplification efficiencies of the heterologous competitor fragment and target molecule are equal (17,19). The use of homologous competitors in competitive PCR seems to be preferable in terms of similar amplification efficiencies compared

with the target sequences. However, heteroduplex formation between the amplified products of target and homologous competitor sequences sometimes hampers an accurate estimation of each product via agarose gel electrophoresis (10,17). For accurate and absolute quantitative measurement of gene expression by competitive PCR, detection and inclusion of heteroduplexes in the quantification is necessary (11).

In this study, we have presented an efficient procedure to overcome this problem. We constructed a series of deletion mutants in a single deletion reaction, with no need for multiple oligonucleotides, depending on a cloned wild-type gene using exonuclease III. In the procedure, only a single appropriate restriction endonuclease site is required for the cloned gene. There are several reported approaches to overcome the problem of heteroduplex formation in competitive PCR by quantitating the heteroduplex DNA (4,8,9,23). Some of these methods are sophisticated but cumbersome because they require either HPLC, PAGE or an additional ELISA procedure for the detection and quantitation of each amplified product including heteroduplex DNA. By comparison, the competitor DNA fragments generat-

Figure 1. Analysis by PCR and agarose gel electrophoresis of ligation mixtures and plasmid clones for serial deletion mutants. (A) Serial deletion mutant genes amplified by PCR. Lanes 1 and 16: 100-bp DNA size marker (New England BioLabs). Lanes 2 and 15: 454-bp fragment (arrowhead) amplified by PCR from cloned wild-type IFNAR2 cDNA. Lanes 3–14: serial deletion mutant genes amplified by PCR from ligation mixture derived from timed-sample (shown in min at the bottom of the figure). (B) Example of colony screening for mutant clones by PCR. Plasmid DNA was directly amplified by PCR. Lanes 1 and 13: 100-bp DNA size marker. Lanes 2 and 12: 454-bp fragment as in Figure 1A. Lanes 3–11: amplified products by PCR of the colonies derived from each timed-sample of 1, 3 and 5 min for lanes 3 to 5, lanes 6–8 and lanes 9–11, respectively. (C) Analysis of heteroduplex formation between wild-type cDNA and various sizes of competitors and resolution of homo- and heteroduplex bands by electrophoresis through a 3% NuSieve 3:1 agarose gel. Lanes 1 and 13: 100-bp DNA size marker. Lanes 2–10: amplified products by competitive PCR of wild-type cDNA and various competitors as described in the text. Competitors used in lanes 2–10 correspond respectively to those in lanes 3–11 of Figure 1B. Heteroduplex bands were separated as distinct bands between wild-type cDNA and competitor bands in lanes 7–10 (arrowhead).

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ed in our method require only agarose gel electrophoresis containing ethidium bromide for the quantitation of amplified DNA, thus promoting its practical application (1, 11).

Based on our results, it appears that the shorter the deleted competitors, the more readily separable are the heteroduplex and homoduplex bands (Figure 1C). However, it should be noted that shortened homologous competitors exhibited both heteroduplex formation and significantly altered amplification efficiency as reported by McCulloch et al. (17). Thus, we recommend the selection of competitors of a size larger and closer to that of the target gene, as long as the formed heteroduplex can be visualized as a distinct band.

The deletion reaction with exonuclease III, representing the most critical reaction for the size of competitors, is affected by temperature, salt concentration, DNA sequence and the amounts of enzyme and plasmid DNA (6,13). Therefore, appropriate reaction conditions must be determined empirically. In our hands, the average reaction was approximately 25 bases/min using the reaction condition described.

Under these conditions, we were able to generate an appropriate mutant DNA clone size within three days, starting from a cloned wild-type gene. However, separation of heteroduplexes alone is not sufficient for reliable quantitative PCR analysis. Note that every quantitative PCR system requires careful validation according to items such as amplification efficiency, linear range and sensitivity (5).

We have used the same approach as described here to generate other homologous competitor DNA fragments for several receptors and cytokines with similar results. We believe the procedure described here will facilitate the analysis of biological material using competitive PCR.

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