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# A Simple Method for Generation of Homologous Internal Standards for Competitive PCR

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**Abstract:** In competitive PCR, which is used to quantify target DNA, an internal standard is needed. Here we present a simple method to construct homologous competitive standards. The method, which is based upon deletion of a portion of the target DNA, does not require any additional primers. This is the simplest method developed thus far to construct a competitive standard. The whole procedure, from construction of a competitive standard to quantitation by PCR, can be completed within a single day.
**Key words:** competitive standard, PCR.

The application of quantitative polymerase chain reaction (PCR) in association with cDNA synthesis (reverse transcription PCR or RT-PCR) is currently the method of choice for quantifying mRNA sequences. In particular, RT-PCR is applicable where only small amounts of RNA are available, or where cellular expression of the sequence of interest is relatively low. The accurate quantitation of PCR products relies on the use of standards to compensate for the high number of uncalculable factors affecting the yield of PCR products.

In competitive PCR (for a review, see Clementi *et al.*, 1994), in which the DNA sequence to be quantified competes for the same primers as a standard DNA fragment, most of these uncalculable effects can be circumvented. Both DNA fragments undergo the same procedures before PCR products are evaluated. Recently, rapid methods which employ an adaptor PCR primer (Föter, 1994), an additional set of primers (Borriello and Lederer, 1995; Zarlenga *et al.*, 1995), or a heterologous spacer gene (Heuvel *et al.*, 1993) to construct such internal standards have been reported. In this note, we present a much simpler method to construct homologous competititve standards which maintain the size disparity from the target DNA for easy differentiation. This new method does not require any extra primers or additional cloning steps. The presented protocol was used to generate a competitive standard for lipocortin-1 cDNA (Wallner *et al.*, 1986) which was successfully used to characterize the differential expression of lipocortin-1 mRNAs in various cells.

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## Materials and Methods

Cellular RNA was prepared from $2\times10^6$ of cultured U937 cells by the guanidium thiocyanate method (Chomczynski and Sacchi, 1987). One μg of RNA was reverse transcribed in 20 μl of buffer (50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1 mM oligo (dT), 0.1 mg/ml BSA, 1 mM dNTP) using SuperScript™II Rnase H⁻ reverse transcriptase (GIBCO BRL, NY, USA). PCR amplification was performed using 1 μl of the prepared cDNA. Forward primer (primer F: 5′-CAGAGACATTAACAGGGTCTACAG-3′) and reverse primer (primer R: 5′-CATGTCATGCTTACTGTACTTGGTG-3′), determined from data published elsewhere, amplify a 298 bp fragment from human lipocortin-1 cDNA (Wallner *et al.*, 1986). PCR was performed in 50 μl of standard buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dNTPs), and 1 μM each of primers R and F. After heating the solution to 94℃ for 5 min, 1.25 U of AmpliTaq$^R$ DNA polymerase (Perkin-Elmer, Norwalk, USA) was added, and the reaction was cycled 30 times through 93℃ for 10 s denaturation, 60℃ for 30 s annealing, and 72℃ for 30 s polymerization in a Perkin-Elmer DNA Thermal Cycler 9600.

Competitive PCR was performed in 20 μl of the standard buffer containing 1 μM each of primers F and R, and a constant amount of the competitive standard. A series of 2-fold dilutions of the U937 cell cDNA were made and added separately to each of the seven

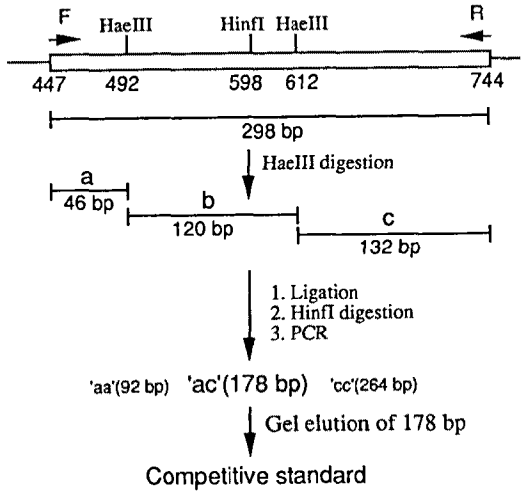


**Fig. 1.** A schematic representation of the construction of a competitive standard. A portion of lipocortin-1 cDNA containing *Hae*III and *Hin*fI restriction sites is as indicated. The number below the cDNA represent the base number from the first codon. Three *Hae*III-digested fragments (46 bp fragment "a", 120 bp fragment "b", and 132 bp fragment "c") are also shown. Primers F and R are forward and reverse primers, common to both the lipocortin-1 cDNA and the competitive standard, and are defined within the text.

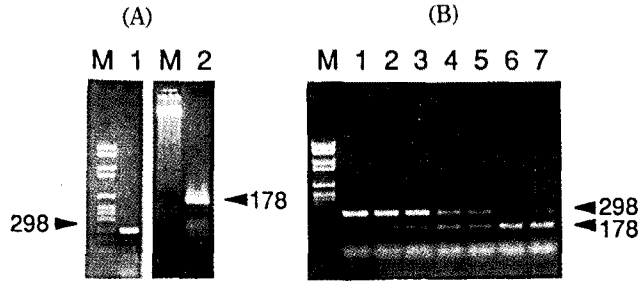


**Fig. 2.** Analysis of PCR products by 2% agarose gel electrophoresis. (A) PCR products from the scheme shown in Fig. 1: Lane M, Molecular marker. cDNA from U937 cells was amplified with primers F and R(lane 1), *Hae*III digested, ligated, *Hin*fI digested, then amplified with primers F and R (lane 2). The 178 bp DNA in lane 2(arrow) was eluted and used as standard. (B) Competitive PCR using the 178-bp standard. A series of 2-fold dilutions of cDNA synthesized from U937 cells was supplemented with a constant amount of competitor ($10^3$ copies) prior to PCR amplification, as defined in the text. Lane M, molecular weight standards. Lanes 1-7, two-fold serial dilutions of original synthesized cDNA. The arrows indicate 298 bp and 178 bp DNA.

PCR tubes. The tubes were heated to 94℃ for 5 min prior to the addition of 0.5 U of AmpliTaq$^R$ DNA polymerase. The mixture was cycled 30 times through 93℃ for 10 s, 60℃ for 30 s, and 72℃ for 30 s.

## Results and Discussion

The method to obtain competitor molecules is schematically shown in Fig. 1, in which a shorter template fragment containing an internal deletion is generated by exploiting a pair of restriction sites. The 298 bp PCR product was verified on 2% agarose gel (Fig. 2A, lane 1), followed by phenol:chloroform-extraction, ethanol-precipitation, then it was restriction enzyme-digested with *Hae*III generating three fragments ("a", "b", and "c" in Fig. 1). The mixture was ligated without further purification, and restriction enzyme-digested for a HinfI site of the fragment "b" to preclude this fragment from being amplified in the subsequent PCR amplification. The reaction mixture was subjected directly to PCR amplification using the same pair of primers. Among others, fragments "ac", "aa", and "cc", which exist in a 2:1:1 ratio in theory, are major species amplifiable by these primers. As expected, the 178 bp fragment "ac" was the main product from the PCR amplification (Fig. 2A, lane 2). This 178 bp PCR fragment has identical 5′ and 3′ ends with the 298 bp fragment and the same DNA sequences, except for the missing 120 bp fragment "b". The PCR solution was run on a 2% agarose gel, the 178 bp band was cut out, and DNA was then eluted. This was used as a competitive standard.

Competetive PCR was performed as described in Methods and the PCR products from each tube were separated on 2% agarose gel, then stained with ethidium bromide. As shown in Fig. 2B, a titration effect was observed in which the 178 bp lower band, resulting from amplification of the competitive standard, increased in intensity because of progressively less competition for the common primers F and R from the sample cDNA. On the other hand, the 298 bp higher band, derived from the sample lipocortin-1 cDNA, decreased in intensity because of relatively more competition from the competitive standard. In this way the intensity of the ethidium bromide-stained competitor molecule of known concentration was equated with the product resulting from cDNA amplification of similar staining intensity.

There are two distinct advantages to this method over similar methods previously described (Heuvel *et al.*, 1993; Föter, 1994; Borriello and Lederer, 1995; Zarlenga *et al.*, 1995). First, this is the simplest method developed thus far to construct a competitive standard. This method does not require any cloned cDNA as starting material. Instead, the sample to be quantified is used as starting material. Therefore, the speed with which this method can be accomplished is very fast. Whole experiments can be routinely conducted ranging from the construction of a competitive standard to actual quantitation of an unknown concentration of cognate cDNA molecules in less than a single day. The second advantage, noteworthy for economy, this meth-

od does not require any additional primers to generate a competitive standard.

An availability of suitable restriction sites is essential for this method. The example given above with lipocortin-1 cDNA presents an ideal situation in which two identical restriction sites, and an internal unique restriction site, encompass the deleted fragment. With a large selection of restriction enzymes, which are available from major suppliers, finding suitable restriction enzymes is generally not difficult.

Using this lipocortin-1 competitive standard, copy numbers of lipocortin-1 mRNA were determined from various samples, such as peripheral blood lymphocytes, tonsillar lymphocytes, and U937 cells under different culture conditions (data not shown). Similarly, copy numbers of *Mycobacterium leprae* DNA from leprosy patients were determined (Choi *et al.*, 1995).

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