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Genome Res. 1994 3: 252-255

Rapid Construction of Deleted DNA Fragments for Use as Internal Standards in Competitive PCR

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Reverse transcriptase polymerase chain reaction (RT–PCR) can be used to amplify mRNA to determine expression of rare messages from small numbers of cells. Quantitative analysis of these messages can be achieved by a modification known as the competitive polymerase chain reaction (cPCR),[1–5] in which an internal standard is coamplified with the sample and the relative amount of each product is determined. Because the efficiency of amplification of the standard is identical to that of the sample template, cPCR circumvents the problems associated with tube-to-tube or sample-to-sample variability of the PCR reaction,[1] thereby providing a means to estimate the level of mRNA accurately. Different types of internal standards may be created for cPCR; these include templates different in length from the target sequence,[1,6–8] templates mutated to either create or delete a restriction site,[3,4] and templates derived from other genes that fortuitously use the same primers but whose internal sequence remains unknown.[2] Although each of these techniques provides certain advantages, the steps of mutagenesis and cloning needed to construct these internal standards are often time-consuming. We describe here a rapid and simple method that uses PCR to generate deletion constructs that can be used as internal standards to measure the level of a specific mRNA by cPCR. The basis of this method is PCR amplification of part of the target gene sequence with the same 3′ primer but a recombinant 5′ primer to produce a shortened template that can be amplified by the original primer pair and is then used as an internal standard for cPCR. We demonstrate the use of this method to produce a competitive internal standard for the ciliary neurotrophic factor (CNTF) gene that is 100 bp shorter than the native sequence, and the use of this standard to quantitate the amount of CNTF mRNA in rat sciatic nerve.

MATERIALS AND METHODS

Generation of cPCR Fragments

All oligomers were synthesized on an Applied Biosystems Automated DNA Synthesizer by the University of Michigan DNA Synthesis Core Facility. Clone pCrC3 (the gift from Dr. Georger Yancopoulos, Regeneron Pharmaceuticals, Inc., Tarrytown, NY), a 3.5-kb rat cDNA clone that contains the entire rat CNTF coding-region sequence (600 bp) or cDNA of CNTF prepared by the RT–PCR of total RNA from rat sciatic nerve was used as a template.

The wild-type cDNA was amplified using a 21-bp 5′ primer (primer w) and a 22-bp 3′ primer (primer d), which correspond to bases 82–102 and are complementary to bases 558–579 of CNTF sequence, respectively (GenBank) and which produce a 498-bp (wild-type) PCR product. A 38-bp primer for producing a deleted mutant (primer m) was constructed to include the sequence of primer w appended to the 5′ end of a sequence corresponding to bases 210–226 of the CNTF gene (Table 1). PCR amplification using primer m as the 5′ primer and primer d as the 3′ primer amplifies 369 bp of the CNTF sequence (210–579), which with the appended 21 bp of primer w sequence results in a 391-bp DNA fragment containing the binding sequence for primer w at its 5′ end and the binding sequence for primer d at its 3′ end.

PCR was performed as described previously.[9] Reactions were carried out in a final volume of 100 μl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl at pH 8.3), 3 mM $MgCl_2$, 0.2 mM each deoxyribonucleoside triphosphate, 100 pmoles each of primer m and primer d, either 10 pg of plasmid pCrC3 or CNTF produced by RT–PCR of rat sciatic nerve RNA, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer). The reactions were carried out in a DNA Thermal Cycler 480 (Perkin-Elmer) using 10 cycles of 1 min denaturing at 94°C, 1 min at 48°C, 50 sec at 72°C; followed by 30 cycles of 1 min at 94°C, 50 sec at 57°C, and 50 sec at 72°C; and, finally, 6 min of extension at 72°C. The PCR products were separated by electrophoresis in 2% Synergel agarose gel (Diversified Biotech). The product of 391 bp was excised from the gel, purified with a QIAEX DNA gel extraction kit (QIAGEN), and stored in aliquots at −20°C.

RT–PCR

Total RNA was prepared from rat sciatic nerve with Tri-Reagent (Molecular Research) according to the manufacturer's protocol and stored at −70°C. Reverse transcription of RNA was performed in a final volume of 20μl containing PCR buffer, 5 mM $MgCl_2$, 1 mM each deoxyri-

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TABLE 1 The Primers Employed

Recombinant primer (primer m): 5′ primer for mutation
| ⟵ primer w sequence ⟶ | ← binding sequence→ |
5′-CTTTCGCAGAGCAAACACCTC.AATAAAAATATCAACCT-3′
Amplification primer (primer d): 3′ primer for mutation and amplification of the target
5′-ACTGTGAGAGCTCTTGAAGGAC-3′
Amplification primer (primer w): 5′ primer for amplification of the target
5′-CTTTCGCAGAGCAAACACCTC-3′

bonucleoside triphosphate, 1 unit of RNase inhibitor, 50 units of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer), 1.25 μM oligo$(dT)_{16}$, and 0.5 μg of total RNA. The samples were incubated at 45°C for 15 min, and the reverse transcriptase was inactivated by heating to 99°C for 5 min and cooled at 5°C for 5 min. The internal standard was then added into the cDNA samples, along with a PCR master mix to bring the final volume to 100 μl containing PCR buffer, 3 mM $MgCl_2$, 2.5 units of *Taq* DNA polymerase, 100 pmoles each of target mRNA 5′ and 3′ primer (primer w, primer d), and 0.5 μl (50 μCi) of [α-^{32}P]dCTP (~110 TBq/mmole, ~3000 Ci/mmole) (Amersham). The reaction mixtures were heated for 2 min at 94°C; followed by 30 cycles of 1 min at 94°C, 50 sec at 57°C, and 50 sec at 72°C; followed by 6 min of final extension at 72°C.

Quantitation

The PCR products were separated by electrophoresis through a 5% polyacrylamide gel. The gel was fixed in 6% acetic acid for 10 min, washed with water for 10 min, and dried. The amount of radioactivity in individual bands was determined directly using a radioanalytic imaging detector, AMBIS 4000, and photographic copies were made by exposing the gel to Kodak X-OMAT AR film for 10–30 min.

The net counts per minute of the 498-bp bands from samples and the 390-bp bands of internal standards were plotted against the amount of added internal standard. The point at which the sample and standard lines intersect represents the point at which the two PCR products are present at equal masses; that is the point at which the amount of sample initially present in the reaction was equal to the known amount of added internal standard.

RESULTS

The procedure used to construct a mutant template is outlined in Figure 1. A deleted DNA fragment was produced as described by utilizing primer m and primer d. A 391-bp PCR product was obtained by PCR amplification of the mutant templates (created either from CNTF clone pCrC3 or from total RNA of rat sciatic nerve) with primer w and primer d (Fig. 2, lanes 1,2), and a 498-bp band was produced using the same primers (primer w and primer d) with the wild-type template (Fig. 2, lane 3). When both internal standard and wild-type template were included in the reaction mixture, two bands were obtained (Fig. 2, lane 4).

A serial dilution of internal standards (0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 pg) was added to 500 ng of total nerve RNA and amplified by RT–PCR. An inverse linear relationship of counts per minute in the sample band to the amount of added standard was observed (Fig. 3). The two lines cross at ~2 pg of internal standard added, indicating that ~2 pg of CNTF cDNA could be detected after reverse transcription of 500 ng total RNA. A more slowly migrating band ap-

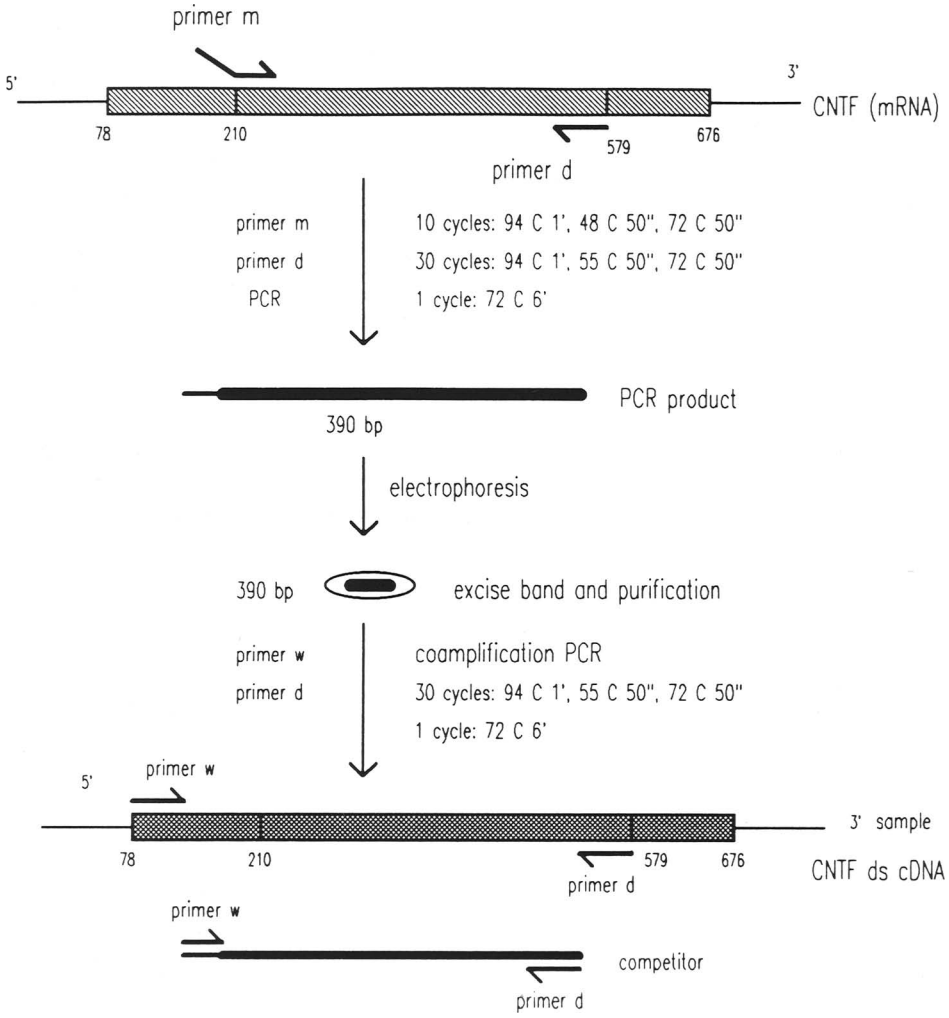

FIGURE 1 Scheme for the synthesis of DNA deletion internal standards. The process is illustrated using the rat CNTF template as an example. The individual steps are described in the text.

pears in some of the lanes. The origin of this artifactual band is not known, but it did not affect the counts obtained from the gel.

DISCUSSION

The method reported here is a rapid and simple means for producing deletion construct templates for quantitative PCR. The internal standard may be produced from cloned plasmids or directly from the sample DNA. Only one extra primer, the recombinant 5′ primer, is required to generate the deleted DNA template. The production of an internal standard that is similar in length and percent of GC to the target sequence, and that is amplified with identical primers as the target sequence, serves to ensure equivalent amplification efficiency of the target and standard templates.

The major considerations in the design of primer m were the character of the oligonucleotide sequence, that is, minimum loop, dimer, or other secondary structure, appropriate annealing temperature, and the length of the sequence to be deleted. The GCG program package (Genetics Computer Group, University of Wisconsin, Madison) was used to select primer sequences matching these characteristics.

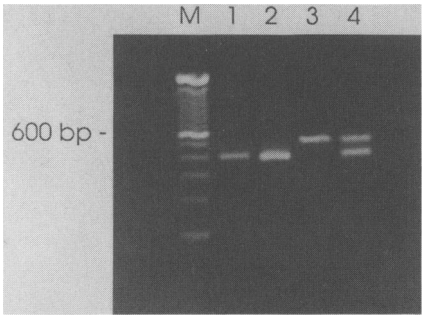

FIGURE 2 Agarose gel analysis of CNTF deletion construct. Lane *M* is 100 bp of DNA ladder. (Lane *1*) A 391-bp product from total RNA of rat sciatic nerve by RT–PCR using primer m and primer d. (Lane *2*) The 391-bp PCR product from plasmid (pCrC3) containing CNTF sequence by using the same pair of primers. (Lane *3*) A 498-bp PCR product from plasmid (pCrC3) containing CNTF sequence by using primer w and primer d. Fifty picograms of 391-bp competitor was coamplified with ~0.5 ng of plasmid pCrC3, of which 0.69 ng (13.8%) corresponds to the 498-bp fragment being amplified (lane *4*). All lanes were loaded with 10 μl of PCR products.

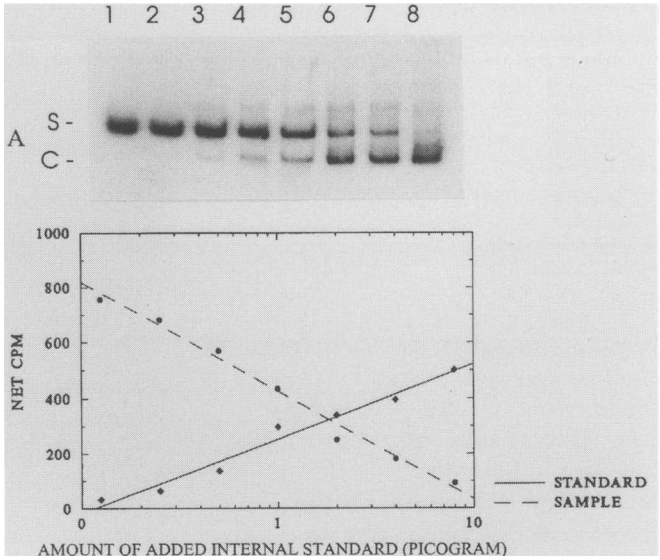

FIGURE 3 Competitive PCR analysis of CNTF RNA. cDNA was synthesized from 0.5 μg of total cytoplasmic RNA using oligo(dT)$_{16}$. Those samples were amplified for 30 cycles in the presence of different amounts of the 391-bp mutation fragment, and the products, 10 μl of a 100-μl reaction mixture, were separated through a 5% polyacrylamide gel (*A*). The amount of internal standard added was 0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 pg (lanes *1–8*). The gel was scanned and analyzed using a radioanalytic imaging detector, and the net counts per minute of both amplified products were determined. This analysis is representative of the results obtained in several different experiments.

Two different thermal cycles were used in the PCR generation of the competitive fragments to avoid the production of undesired bands that may be produced by a low annealing temperature. The first 10 cycles were carried out with an annealing temperature of 48°C, which was calculated according to the binding sequence (17 bp) of the recombinant primer. The subsequent 30 cycles, where the template for the reaction was the product of the previous 10 cycles, were carried out with an annealing temperature of 57°C, which was computed using the entire (38 bp) sequence of the recombinant primer.

The use of an internal DNA standard allows effective control of tube-to-tube and sample-to-sample variability in PCR. To control for variability in the reverse transcriptase reaction, it may be desirable in some cases to use a mutated internal RNA standard. The method reported here can be adapted to the production of deleted RNA standards either by appending the T7 or SP$_6$ RNA polymerase promoter sequence to the 5′ end of the recombinant 5′ primer (reverse transcription with hexmer) or by creating two recombinant primers, one with the RNA polymerase sequence and the other containing a poly(T) sequence [reverse transcriptase with oligo(dT)]. The practical application of including the T7 promoter sequence in PCR primers to produce RNA from PCR-generated templates has already been demonstrated.[10,11]

In summary, the method presented is easily adapted for any target gene sequence to produce an internal standard to be used for quantitative competitive PCR. As quantitative PCR becomes more common, the ability to produce internal standards easily and rapidly should prove extremely valuable.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health and the Veteran's Affairs Medical Center. We thank Ms. Marcia Bertrand for secretarial assistance and Mr. Daniel Cutler for assistance in photography.

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Received July 12, 1993; accepted in revised form November 12, 1993.