BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Joining of DNA Fragments by Repeated Cycles of Denaturation, Annealing and Extension

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The linkage and subcloning of DNA fragments can be a time-consuming technical problem. In many instances, partial cDNA clones isolated from libraries or generated by polymerase chain reaction (PCR) may not contain overlapping restriction sites for common 6-base cutters. In these situations, the ligation to achieve the formation of full-length functional cDNA molecules can be difficult and relatively time-consuming. Certain ligations may also be difficult for reasons that are not overtly obvious. To overcome such situations, we have developed a simple reproducible approach for generating full-length cDNA clones from partial clones containing compatible overhangs. This method is a ligation-independent chain reaction that uses repetitive cycles of denaturation, annealing and extension to facilitate the formation of full-length cDNA.

Leukotriene (LT) B_4 omega hydroxylase is the cytochrome P-450 that catalyzes the inactivation of the chemotactic lipid LTB $_4$ (3–6). Within hematopoietic cells, it is expressed exclusively within polymorphonuclear leukocytes (PMN) (3–5). It is coded for by a cDNA of 2073 bp with an open reading frame of 1560 bp coding for 520 amino acids. Our goal was to obtain a cDNA coding for the complete open reading frame in order to perform structural studies. To prepare RNA from peripheral blood PMN, the cells were first isolated from peripheral venous blood by dextran sedimentation followed by Hypaque-Ficoll® centrifugation (Ficoll-Paque®; Pharmacia Biotech, Piscataway, NJ, USA) as described by Boyum (1). Undegraded total RNA was prepared by guanidium isothiocyanate extraction followed by cesium chloride centrifugation (2). Two fragments coding for base pairs 1–789 and 710–1899, respectively, were generated by reverse transcription (RT)-PCR and cloned into the TA Cloning® Vector (Invitrogen,

San Diego, CA, USA) and sequenced to confirm their identity. Though these two fragments contained a *Sac*I site in the overlapping region, initial attempts to join them at this site were unsuccessful. We therefore developed a different strategy. The 5' fragment was amplified with a sense primer corresponding to bp 1–27 and antisense primers corresponding to bp 754–789. The large fragment was amplified with a sense primer corresponding to bp 710–730 and an antisense primer corresponding to bp 1878–1899. The PCR products were identified on 1% agarose gels and extracted and purified with QIAX™ (Qiagen, Chatsworth, CA, USA).

The approach that was developed to join these fragments is shown in Figure 1A. In step 1, the fragments are repetitively denatured, annealed and extended using proofreading polymerase. In step 2, the 1.9-kb cDNA is gel-purified and either ligated directly into a chosen vector or amplified using appropriate primers. For the actual ligation, 120 ng of the 1190-bp partial cDNA and 80 ng of the 789-bp cDNA were mixed with 1 μ L (20 pmol) of dNTPs, 1 U of ULTma™ DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 3 μ L of 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.8, 100 mM KCl; Perkin-Elmer) and 3 μ L of a 25 mM MgCl $_2$ stock solution (Perkin-Elmer) in a total volume of 30 μ L. One drop of mineral oil was placed on the surface of the mixture. The mixture was cycled (94°C denaturation for 1 min, 64°C annealing for 2 min and 72°C extension for 3 min) in a Perkin-Elmer GeneAmp™ PCR Instrument System (DNA Thermal Cycler) for different numbers of cycles as indicated. Ten microliters of each reaction were analyzed by 1% agarose gel (Figure 1B). Increasing the number of cycles from one (lane 2) to 40 (lane 6) progressively increased the yield of the 1.9-kb cDNA. From this reaction, an aliquot was amplified using primers that bracketed the coding region and allowed directional cloning into the desired expression vector (lane 7). Amplification buffer was the same, with the template being 3 μ L from the initial reaction added to a final volume of 50 μ L. Cycling conditions were altered to 94°C for 1 min denaturing, 55°C for 1 min annealing, 72°C for 1.5 min exten-

Figure 1. Enrichment for the extended full-length cDNA encoding the leukotriene B₄ omega-hydroxylase gene. (A) Strategy for ligation-independent joining of DNA fragments with an overlapping region. (B) Agarose gel electrophoresis of DNA products with various cycles of denaturation, annealing and extension. Lane M contains a DNA marker (pGEM[®] DNA molecular weight marker; Promega, Madison, WI, USA). Two DNA fragments encoding N terminal region (789 bp, amino acids 1–250) and C terminal region (1190 bp, amino acids 226–520) are shown in lane 1. Products of increasing numbers of chain reaction cycles are shown in lanes 2–6. These are 1, 10, 20, 30 and 40 cycles, respectively. Amplification of 1.9-kb cDNA was performed using an aliquot of 30-cycle reaction mixture as a template (lane 7). Lane 8 shows results when primers that bracket 1.9-kb cDNA are added at beginning of the ligation-independent chain reaction.

sion or 30 cycles followed by a final extension of 10 min at 72°C. One subclone of the product from lane 7 was sequenced in its entirety and found to be identical to the published sequence (3). The addition of primers at the beginning of the reaction process was not effective in generating 1.9-kb cDNA. This may be because nucleotides are used in the reactions extending the annealed partial cDNAs after denaturation and reannealing.

This method offers several advantages in the joining of double-stranded DNA fragments. (i) Cloning from cDNA libraries frequently yields partial cDNAs, which may have no suitable common restriction site; (ii) ligation is not required; and (iii) a minimal number of cycles can be used to generate large amounts of full-length cDNA.

REFERENCES

1. Boyum, A. 1968. Isolation of lymphocytes, granulocytes, and macrophages. *Scand. J. Lab. Invest.* 21:77-89 (Supplement 97).
2. Chirgwin, J.J., A.E. Przybyla, R.J. MacDonald and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources rich in ribonuclease. *Biochemistry* 18:5294-5299.
3. Kikiuta, Y., E. Kusonose, K. Endo, S. Yamamoto, K. Sogawa, Y. Fuji-Kuriyama and M. Kusonose. 1993. A novel form of cytochrome P-450 family in human polymorphonuclear leukocytes. *J. Biol. Chem.* 268:9376-9380.
4. Powell, W.S. 1984. Properties of leukotriene B₄ 20-hydroxylase from polymorphonuclear leukocytes. *J. Biol. Chem.* 259:3082-3089.
5. Shak, S. and I.M. Goldstein. 1985. Leukotriene B₄ omega hydroxylase in human polymorphonuclear leukocytes. Partial purification and identification as a cytochrome P-450. *J. Clin. Invest.* 76:1218-1228.
6. Soberman, R.J., T.W. Harper, R.C. Murphy and K.F. Austen. 1985. Identification and functional characterization of leukotriene B₄ 20-hydroxylase in human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. USA* 82:2292-2295.

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**Jaehong Kim, Mark Puder
and Roy J. Soberman**
*Massachusetts General Hospital
Charlestown, MA, USA*