Benchmarks

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Improvement of GC-Rich Template Amplification by Inverse PCR

Inverse polymerase chain reaction (iPCR) is a very attractive method for performing site-directed mutagenesis, since it requires a single pair of primers. On the other hand, the method involves the amplification of the whole recombinant plasmid, a specially difficult step when a large insert of high G+C content is concerned. Inverse PCR also proves to be useful in cloning missing parts of genes by using self-ligated genomic DNA fragments as templates. However, the method is difficult when applied to high G+C-containing DNAs.

In order to improve PCR on these arduous high G+C-containing templates, several DNA polymerases have been tested both with and without the addition of different organic solvents.

A pUC18-derived 4.8-kb plasmid containing a 1980-bp insert encoding the *Actinomadura* R39 DD-carboxypeptidase [74% G+C rich (7)] served as template.

The synthesis of a pair of primers was designed in inverted tail-to-tail directions: 5′-GCCCTCGGCGGCGGTACCGCAT-3′ (T_m = 70°C) annealing perfectly with the target sequence and 5′-GTGGTCGAGGCCCACACCGGGACGATG-3′ (T_m = 61.6°C) introducing two non-contiguous mismatches in view of changing a triplet in the coding sequence. The T_m values were calculated according to the Primer Analysis Software OLIGO version 3.4 distributed by National Biosciences (Hamel, MN, USA) (4,6), and three mismatches were considered for the second primer.

Different DNA polymerases were tested: AmpliTaq™ Polymerase (from *Thermus aquaticus*; Perkin-Elmer, Norwalk, CT, USA), *Tth* DNA Polymerase (from *Thermus thermophilus*; Pharmacia Biotech, Brussels, Belgium), EuroTaq™ Polymerase (from *Thermus aquaticus*; Eurogentec, Seraing, Belgium), DynaZyme™ Polymerase (from *Thermus brockianus;* Finnzymes Oy, Espoo, Finland) and Vent™ DNA Polymerase (from

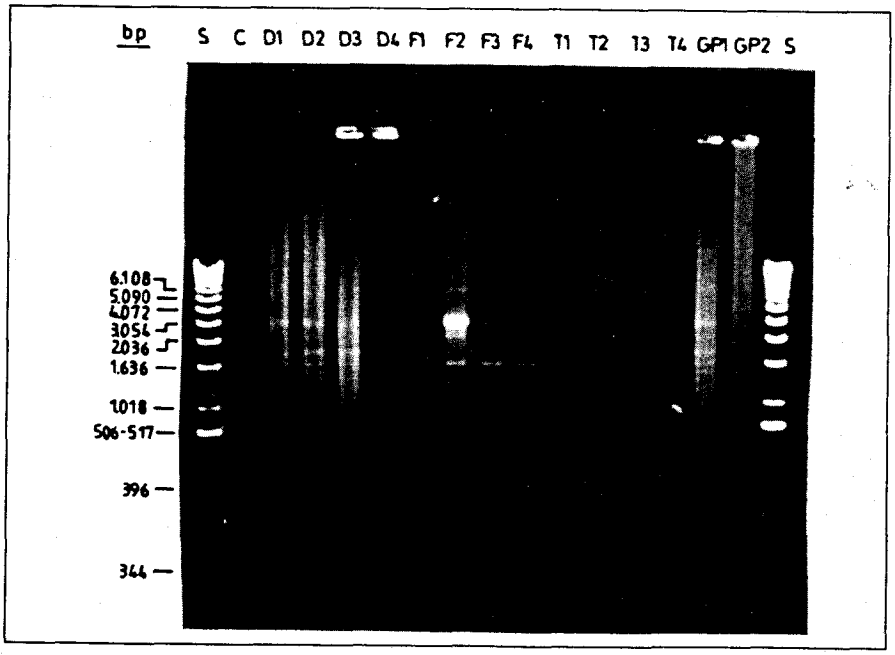

Figure 1. Amplification by iPCR of a 4.8-kb plasmid, containing a GC-rich (74%) 1980-bp insert. 10 µL of each sample were loaded onto a 0.7% agarose gel. Lane S, 1-kb ladder size standard; lane C, control iPCR under standard conditions without organic solvent; lanes D1, D2, D3 and D4, iPCR in presence of 8%, 10%, 15% and 20% DMSO, respectively; lanes F1, F2, F3 and F4, iPCR in presence of 5%, 10%, 15% and 20% formamide, respectively; lanes T1, T2, T3 and T4, iPCR in presence of 10^{-2} M, 10^{-3} M, 10^{-4} M and 10^{-5} M TEMACl, respectively; lane GP1, iPCR in presence of 8% DMSO with 3 µg of T4 gene 32 protein; lane GP2, iPCR under standard conditions with 3 µg of T4 gene 32 protein.

Thermococcus litoralis; New England Biolabs, Beverly, MA, USA).

A standard three-step cycling protocol (1 min of denaturation at 94°C, 2 min of annealing at 55°C [T_m: -6°C] and 5 min [1 min/kb of expected product] of elongation at 72°C) was applied to 100-µL samples, containing the supercoiled plasmid (ca. 25 ng), the primers (2 µM each), the deoxyribonucleoside triphosphates (dNTPs) (200 µM each), one of the above-mentioned DNA polymerases (2 units) and 10 µL of 10× DNA polymerase buffer (the composition of which varies according to the supplier).

No fragment of the correct size was amplified with any DNA polymerase tested.

With the purpose of abolishing stable secondary structures, either dimethylsulfoxide (DMSO) (from 8% to 20%) (3,16), formamide (from 5% to 20% (3,14,15) or tetramethylammonium chloride (TEMACl) (8) (from 10^{-5} to 10^{-2} M) were added to the described mixture.

Analysis of the PCR products in the presence of these organic solvents revealed that only Vent DNA Polymerase succeeded in amplifying the 4.8-kb plasmid and that a 10% concentration of formamide was the most effective additive (Figure 1). DMSO at 8% or 10% was also useful, but the addition of less than 8% completely failed to amplify the plasmid. The T4 gene 32 protein, a single-stranded DNA-binding protein used to overcome secondary structures (1,2), did not appear really helpful, and the addition of TEMACl was not effective at all.

The lack of amplification by most of the DNA polymerases tested suggested that they could be highly sensitive to the added organic solvents.

Vent™ (exo⁻) and Deep Vent™ DNA Polymerases (New England Biolabs) were not considered in our study because they have been modified for specific applications other than iPCR (12,13). *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA), an enzyme very interesting in terms of thermostability and fidelity (10), but more expensive on a unit basis was also not considered.

iPCR was repeated with different pairs of primers, and in each case the whole 4.8-kb plasmid was successfully amplified by Vent DNA Polymerase in the presence of 10% formamide, in the above-described conditions.

The Vent DNA Polymerase (5,9,11) with its excellent level of fidelity (5-fold to 15-fold higher than that of *Taq* DNA Polymerase)—due to a strong 3′–5′ proofreading exonuclease, its high thermostability (half-life of 6–7 h at 95°C), its production of blunt-ended DNA fragments and its tolerance to organic solvents—thus appears to be the enzyme of choice for iPCR on GC-rich templates.

The combination of Vent DNA Polymerase and 10% formamide is now used as a routine protocol to amplify very GC-rich templates up to 5 kb with only minimal further adjustment.

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This work was supported in part by the Belgian programme on Interuniversity Poles of attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming (PAI No. 19), the Fonds de la Recherche Scientifique Médicale (Contract No. 3,4531,92), the Fonds de la Recherche Fondamentale Collective (Convention No. 2,4525,92) and Convention tripartite between the Région wallonne, SmithKline Beecham, UK, and the University of Liège. C.D. and J.D. are Chercheurs qualifiés of the Fonds National de la Recherche Scientifique, Brussels. Address correspondence to J. Dusart, Centre d'Ingénierie des Protéines, Institut de Chimie, B6, Université de Liège, Sart Tilman, B-4000 Liège, Belgium.

Received 12 July 1993; accepted 8 March 1994.

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