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Fast Amplification of the Low Density Lipoprotein Receptor Gene and Detection of a Large Deletion by Means of Long Polymerase Chain Reaction

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Summary: To demonstrate the usefulness of Long PCR in analyzing gene structures and large deletions we have developed a method to amplify the entire LDL receptor gene, including the promoter region and intron 1. This opens new ways for studies of the gene and allows the detection of certain LDL receptor-specific deletions.

For the amplification of the LDL receptor gene, spanning approximately 45.5×10^3 bases and divided into 18 exons and 17 introns, we have designed overlapping PCR products (ranging from 4 to 16×10^3 bases in length), which can be amplified simultaneously overnight for fast results.

It was possible to positively identify two samples from heterozygote carriers of the "5 kB French-Canadian" deletion using this method. As a side result the length of intron 1 of the LDL receptor gene could be established to be approximately 9.5×10^3 bases. The method is sensitive enough to detect deletions in 1 : 10 mixes of positive control with wildtype DNA.

Introduction

In recent years there have been several reports about Long PCR describing the successful amplification of DNA sequences longer than the 3 to 4×10^3 bases that seemed to be the upper limit of PCR until a few years ago (1–3). Most of them described how specific target sequences were amplified by modifying the components of the reaction mixture, for example by applying specially adjusted reaction buffers and/or other polymerases, as well as by using special reaction protocols. In the meantime, commercial kits specifically designed for use with Long PCR protocols have appeared on the market (4, 5). Still, however, amplification of longer DNA fragments is not routinely performed.

The most obvious clinical importance of Long PCR lies in its potential to significantly simplify methods determined to analyze gene structures (through amplificates covering several exons/introns) and to detect extensive structural aberrations, like large deletions and insertions (e. g. familial hypercholesterolaemia or muscular dystrophy *Becker/Duchenne*) or long repetitive regions (e. g. fragile X syndrome, myotonic dystrophy or *Huntington*'s disease) which so far required laborious procedures (like *Southern* blot analysis) to be revealed.

This study has been performed to show the practicability of Long PCR using the example of the LDL receptor gene. The LDL receptor gene, featuring numerous mutations (6, 7), of which a substantial number are large deletions resulting in a defective LDL-receptor protein and thus leading to familial hypercholesterolaemia, was our target of choice to demonstrate this method. During the development of this method the LDL receptor gene was amplified from the DNA of 36 normal test persons by means of 6 overlapping PCR products using the Expand™ Long Template PCR System (Boehringer Mannheim, Germany). The fragments to be amplified were selected to possibly cover target sequences of interest as well as to allow the use of primers with similar and possibly high annealing temperatures. In addition, 2 fragments were amplified, one covering exons 1 to 4 (first half) and designed to detect the "5 kB French-Danadian" LDL receptor deletion (8) and another fragment starting at the promoter region of the gene and ranging up to exon 4 (first half) as well. All of the respective PCR products can amplified at the same time using the same reaction protocol allowing to check the entire gene for deletions within a short period of time.

Materials and Methods

Expand™ Long Template PCR System

The Expand™ Long Template PCR System was purchased from Boehringer Mannheim (Mannheim, Germany). The kit contains a mixture of *Taq* and *Pwo* DNA polymerases[1]) and comes with three

[1]) Enzymes
Expand Long Template PCR System, DNA deoxynucleotidyltransferase, EC 2.7.7.7

Tab. 1 LDL receptor fragments amplified and primers used

PCR product	Start/End		Primers used			Fragment (10^3 bases)
LP1–3	forward	5' end of exon 1	SP90	5'-CATTGAAATGCTGTAAATGACGTGG-3'	(11)	~12.4
	reverse	3' end of exon 3	SP60	5'-AATAGCAAAGGCAGGGCCACACTTA-3'	(11)	
LP1–4RB	forward	5' end of exon 1	SP90	5'-CATTGAAATGCTGTAAATGACGTGG-3'	(11)	~15.1
	reverse	first half of exon 4	4RB	5'-ACTTAGGCAGTGGAACTCGAAGGCC-3'		
LPP1–4RB	forward	5' end of promoter	P1	5'-GGATCCCACAAAACAAAAAATATTTTTTTGG-3'		~15.8
	reverse	first half of exon 4	4RB	5'-ACTTAGGCAGTGGAACTCGAAGGCC-3'		
LP3–7	forward	5' end of exon 3	SP59	5'-TGACAGTTCAATCCTGTCTCTTCTG-3'	(11)	~7,8
	reverse	3' end of exon 7	SP67	5'-AGGGCTCAGTCCACCGGGGAATCAC-3'	(11)	
LP4–8	forward	5' end of exon 4	SP61	5'-TGGTCTCGGCCATCCATCCCTGCAG-3'	(11)	~6.0
	reverse	3' end of exon 8	SP69	5'-CCACCCGCCGCCTTCCCGTGCTCAC-3'	(11)	
LP8–11	forward	5' end of exon 8	SP68	5'-CCAAGCCTCTTTCTCTCTCTTCCAG-3'	(11)	~5.0
	reverse	3' end of exon 11	SP75	5'-TGGCTGGGACGGCTGTCCTGCGAAC-3'	(11)	
LP11–16	forward	5' end of exon 11	SP74	5'-CAGCTATTCTCTGTCCTCCCACCAG-3'	(11)	~12.0
	reverse	3' end of exon 16	SP85	5'-CGCTGGGGGACCGGCCCGCGCTTAC-3'	(11)	
LP16–18	forward	5' end of exon 16	SP84	5'-CCTCACTCTTGCTTCTCTCCTGCAG-3'	(11)	~3.8
	reverse	3' end of exon 18	SP11	5'-GCTTTGGTCTTCTCTGTCTTTGAAT-3'	(11)	

PCR buffers (differing in $MgCl_2$ concentration and detergent content).

Template DNA

Human genomic DNA, ranging from 156 to 652 mg/l in concentrations was used as template DNA. The samples from the 36 test persons were prepared from peripheral blood using the salting-out procedure described by *Miller* et al. (9). Two DNA's from patients heterozygous for the "5 kB French Canadian" mutation[2]) (labelled FC1 and FC2) served as positive controls.

Additionally, DNA from these two carriers was mixed 2 : 3, 1 : 2, 1 : 5, and 1 : 10 (by volume) with wildtype DNA (adjusted in concentration to match the concentration of the carrier sample) to test if this method was suited for pool screening.

Primers

With the exceptions of the forward primer, labelled P1, for the promoter sequence – as published by *Südhof* et al. (10) – and the reverse primer for the first half of exon 4, labelled 4RB, we have used the primers as published by *Leitersdorf* et al. (11). Detailed information about the primers and PCR products is given in table 1. It should be noted that sparse information about the sequence of the intron/exon boundary regions of the LDL receptor gene and technical reasons relating to the possible sequencing of the long fragment have kept the authors from modifying *Leitersdorf*'s primers to closer fit the common annealing temperature. PCR product sizes were calculated based on the data published by *Südhof* et al. (10) as well as based on the lengths of the PCR products covering intron 1 (LP1–3, LP1–4RB and LPP1–4RB, as compared with the molecular mass markers used).

Reaction mix

The reaction mix was prepared as specified by the manufacturers instructions, with slight modifications as to buffer usage, DNA and primer concentration. Two mixes were prepared, a lower mix containing dNTP's and both primers in a total volume of 25 µl and an upper mix containing the reaction buffer, DNA and the enzyme in a total volume of 25 µl. Both mixes were combined to a 50 µl mix immediately before placing the tubes in the PCR cycler. The respective reaction mixes are specified in table 2.

Since all of the fragments are amplified simultaneously primer concentrations have to be adapted to achieve sufficiently high concentration of each PCR product as well as specificity of each product at the preset common (and thus suboptimal) annealing temperature. Usually this leads to relatively high primer concentrations for some of the reactions and relatively low ones for others. Fragment LP16–18 required unusually high amounts of primer (determined empirically), both when amplified alone at its optimal annealing temperature as well as when amplified along with the other products.

Polymerase chain reaction protocol

The PCR reactions were performed in Perkin-Elmer GeneAmp PCR Systems 2400 or 9600, respectively. The amplification was designed as a 3-step PCR with the following conditions: an initial denaturation for 2 minutes at 94 °C, followed by 10 cycles of 10 s denaturation at 94 °C, 30 s annealing at 63 °C and 15 minutes extension at 68 °C, then followed by 20 identical cycles, where the extension time was automatically prolonged by 20 s per cycle, finally followed by a 7 minute incubation at 68 °C.

Polymerase chain reaction product analysis

Five µl of PCR product were mixed with 3 µl of xylene cyanol stopper (1 g/l bromphenol blue, 1 g/l xylene cyanol, 20 mmol/l EDTA) and loaded onto a standard 5 g/l agarose gel stained with ethidium bromide running in Tris-borate/EDTA buffer (0.089 mol/l Tris-borate, 0.025 mol/l EDTA, pH 8.3) at 100 V for 3 hours. A "1 kB" DNA ladder (Gibco BRL) and a "Lambda Mix" DNA ladder (MBI Fermentas, Vilnius, Lithuania) were used for length determination. To achieve higher resolutions it is possible to use special agaroses adapted to give greater resolution with longer PCR products (e.g. SeaKem GTG®, FMC BioProducts, Rockland, Maine, designed for separation of fragments $> 1 \times 10^3$ bases).

As most of the products are longer than $2-4 \times 10^3$ bases in size it is usually recommended to use longer gels allowing for a greater resolution of bands in this range. We have found gels measuring 16 cm in length to be sufficient.

Results

It was possible to successfully amplify the LDL receptor genes of all 36 test DNA's using the method described

[2]) Kindly provided by Dr. *Anne Minnich*, Clinical Research Institute of Montreal, Quebec, Canada

Tab. 2 Reaction mixes for the respective fragments

	Lower mix								Upper mix	
Exons:	P1–4RB	1–4RB	1–3	3–7	4–8	8–11	11–16	16–18		
Each dNTP	70 nmol	70 nmol	70 nmol	70 nmol	70 nmol	70 nmol	70 nmol	70 nmol	Buffer 3[a]	5.00 µl
5'-Primer	20 pmol	20 pmol	20 pmol	4 pmol	2 pmol	2 pmol	2.5 pmol	60 pmol	DNA	500 ng
3'-Primer	20 pmol	20 pmol	20 pmol	4 pmol	1 pmol	2 pmol	2.5 pmol	60 pmol	Enzyme	0.75 µl
H_2O	to 25 µl	to 25 µl	to 25 µl	to 25 µl	to 25 µl	to 25 µl	to 25 µl	to 25 µl	H_2O	to 25 µl

[a] Supplied "Buffer 3": 500 mmol/l Tris-HCl, pH 9.2 (25 °C), 160 mmol/l $(NH_4)_2SO_4$, 22.5 mmol/l $MgCl_2$, dimethylsulphoxide, volume fraction 0.2; Tween® 20, volume fraction 0.01.

(fig. 1). To prove that the PCR fragments produced actually do cover the expected number of exons they were tested by using them as templates for reamplification. Each single exon could be reamplified in its expected length from the respective PCR product(s) the exon was covered by (data not shown).

Additionally, 2 fragments of interest were created, one covering the gene from the promoter to the first half of exon 4 and another one ranging from exon 1 to the first half of exon 4 (fig. 2). The latter fragment was amplified from control DNA and from the two DNA samples heterozygous for the "5 kB French-Canadian" deletion (FC1 and FC2). While the control DNA lane showed the expected 15.1 × 10^3 bases band, the products from samples FC1 and FC2 displayed a shorter, intense band

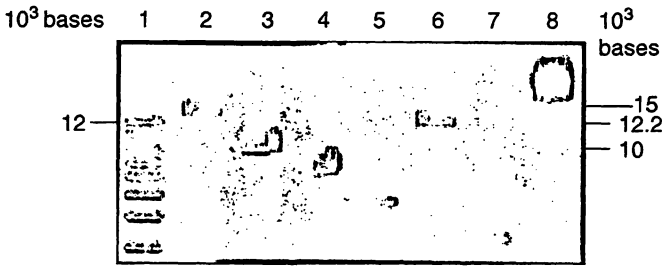

Fig. 1 Six PCR products covering the LDL receptor gene from exon 1 to exon 18, one of the 36 test individuals shown as example.
Lane 1: 1 × 10^3 bases ladder (visible from the 3 × 10^3 to the 12 × 10^3 bases band), lane 2: fragment LP1–3, lane 3: fragment LP3–7, lane 4: fragment LP4–8, lane 5: fragment LP8–11, lane 6: fragment LP11–16, lane 7: fragment LP16–18, lane 8: Lambda Mix DNA ladder.

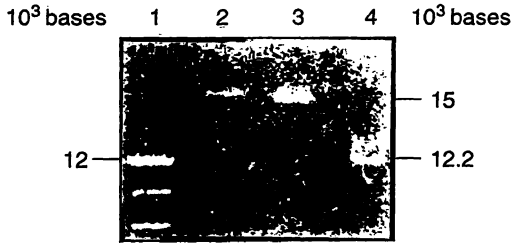

Fig. 2 Two PCR products covering intron 1 of the LDL receptor gene.
Lane 1: 1 × 10^3 bases ladder (visible from the 5 × 10^3 to the 12 × 10^3 bases band), lane 2: fragment LPP1–4RB (15.8 × 10^3 bases), lane 3: fragment LP1–4RB (15.1 × 10^3 bases), lane 4: Lambda Mix DNA ladder.

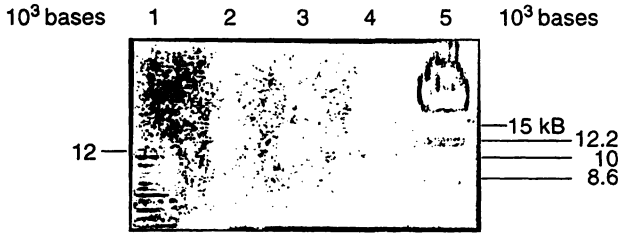

Fig. 3 Two samples heterozygous for the "5 kB French Canadian" deletion.
Lane 1: 1 × 10^3 bases ladder (visible from the 6 × 10^3 to the 12 × 10^3 bases band), lane 2: control sample (fragment LP4–4RB, 15.1 × 10^3 bases), lanes 3 and 4: samples FC1 and FC2 both heterozygote for the "5 kB French Canadian" deletion (upper band 15.1 × 10^3 bases, lower band 10.5 × 10^3 bases) lane 5: Lambda Mix DNA ladder.

at 10.5 × 10^3 bases, corresponding to the allele affected by the deletion, and a second, much less intense band at 15.1 × 10^3 bases, representing the normal allele (fig. 3). This difference in intensity supports the detection of affected alleles and is most likely based on the fact that the shorter (by 5 × 10^3 bases) fragment is more efficiently amplified. In extreme cases the longer, normal band might be almost undetectable thus bearing the risk of interpreting the sample as homozygote for the deletion. To the best of the author's knowledge no homozygote for familial hypercholesterolaemia "5 kB French-Canadian" has been identified so far.

The amplification of this fragment using mixes of DNA from the patients carrying the deletion with wildtype DNA allowed a positive identification of their heterozygosity in mixes up to the 1 : 10 dilution (fig. 4).

Based on the lengths of the 3 PCR products covering intron 1 (LP1–3, LPP1–4RB, LP1–4RB) the length of intron 1 could be calculated as 9.5 × 10^3 bases.

Discussion

Recently there has been a report about the application of Long PCR to study the structure of the LDL receptor gene (12). It describes a method to amplify two fragments from the gene, 16.1 and 20.0 × 10^3 bases long, whereas exon 1 and intron 1 have not been covered.

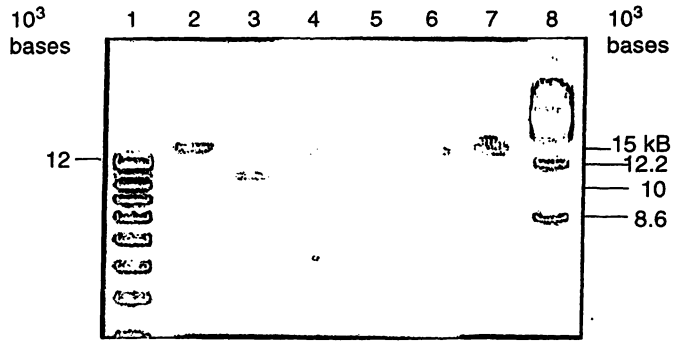

Fig. 4 Mixes of sample FC1 (heterozygous for the "5 kB French-Canadian" mutation) with wildtype DNA.
Lane 1: 1×10^3 bases ladder (visible from the 4×10^3 to the 12×10^3 bases band), lane 2: wildtype control (fragment 1–4RB, 15.1×10^3 bases), lane 3: sample FC1 ($15.1 \times 10^3/10.5 \times 10^3$ bases), lanes 4–7: mixes of sample FC1 with wildtype control in steps 2 : 3, 1 : 2, 1 : 5 and 1 : 10, lane 8: Lambda Mix DNA ladder.

Our results show that it is indeed possible to amplify the entire LDL receptor gene by using overlapping Long PCR fragments, including the so far recalcitrant sequences of exon 1 and intron 1 of the gene. Since PCR products of greater size than the approximately 10×10^3 bases necessary to span intron 1 can be created without similar problems in other regions of the gene as well as in other human genes (13) it is likely that the unknown structure of intron 1 was contributing to the earlier difficulties with its amplification. It can be confirmed that Long PCR is a suitable method to screen for large deletions and that this method can be modified to allow effective pool-based screening.

Although ready-to-use kits are a great help in performing Long PCR it has to be noted that there are additional useful steps for achieving reproducible results with long target sequences. Long PCR kits provide a mix of polymerases with and without 5' → 3' exonuclease activity (e.g. r*Tth/Vent* or *Taq/Pwo* mixes) overcoming nucleotide misincorporations and thus surpassing the length limitations of standard *Taq* polymerase ($3-4 \times 10^3$ bases) and are supplied with optimized buffer systems.

Nevertheless, other factors influencing the Long PCR reaction should be adapted as well. Only high-quality template will lead to reliable and reproducible results, and even then it might be necessary to substantially increase the initial amount of DNA. This is probably because Long PCR requires a sufficient "starting" amount of undamaged target template.

In regard to primers our experience was that primer lengths between 20–25 nucleotides, and therefore primers often already existing in primer libraries, in most cases are sufficient to ensure successful amplification. It is to be expected though, that designing longer primers (30 to 40 nucleotides) will help to facilitate the amplification of recalcitrant sequences or improve results where non-specific products appear, unless the primer sequence itself (or part of it) is the reason for the unsatisfying amplification. It is generally advised that primers with a sufficiently high content of G and C bases, and therefore with a rather high melting temperature, be selected. This again allows the use of higher annealing temperature thus reducing the likeliness of non-specific secondary products to occur, which, because shorter, tend to be more efficiently amplified than the target sequence itself (2).

Furthermore, combining forward and reverse primers with similar melting temperatures (± 2 °C) will often result in a more successful amplification. If there are several DNA fragments of a gene to be amplified at the same time, effort should be directed at fitting the melting temperatures of all primers into a limited frame. If this can be achieved only partially, or not at all, (e.g. due to limitations in primer design due to knowledge about the sequence or due to its nature) then PCR product intensity can be controlled via the amount of primers in the reaction mix.

There are publications recommending the use of hot-start protocols to increase product specificity (1, 13). This is usually achieved by adding one decisive reaction component after the reaction mixture has reached a temperature of about 70–80 °C or by separating the reaction components via a wax layer (e.g. AmpliWax® Gems, Perkin-Elmer). While we have tested both manual hot-start protocols, as well as semi-automated ones, there was no large improvement to be seen in our application. Obviously the use of a lower and an upper reaction, combined immediately before placing the final mix in the thermal cycler was sufficient to minimize the risk of generating non-specific products.

Several aspects need to be addressed to optimize the reaction protocol for the amplification of long DNA fragments. To achieve complete strand denaturation, an initial step at 94 °C, lasting 2–5 minutes, is advised (1). However, further denaturation steps should be kept short to ensure only minimal damage to the template (strand breaks, depurination). For this reason it is recommended to minimize the denaturation time in successive cycles as far as possible. Experience has shown that denaturation times as short as 10 s are still sufficient. The extension time basically is calculated depending on the length of the template fragments to be amplified. One minute of extension time per 1×10^3 bases of target template is usually sufficient. Prolongation of the extension times via the cycler's auto-extension feature will compensate the decrease in enzyme amount and activity (1, 13).

When running different Long PCR reactions at once (as is required to amplify the entire gene in one step) the optimization of the annealing phase has an immense importance for successful amplification. While annealing time needs not be longer than 30 s, the best annealing

temperature in most cases has to be determined empirically. Provided that primer combinations have been chosen which all have a similar melting temperature, a standard 3-step or 2-step PCR protocol can be used. Should it be the case that the calculated melting temperatures of the respective primer combinations are more widely spread, then touch-down techniques have given better results. However, we have observed that such a touch-down program often is not able to compensate the loss of yield as well as of specificity resulting from inadequately selected primer combinations.

It can be concluded that Long PCR as a relatively easy method has the potential to replace *Southern* blot analysis in many experiments aimed the detection of larger structural aberrations (deletions rather than very large insertions which might not be amplified under the conditions optimized for a specific Long PCR product) and studies to reveal gene structures.

References

1. Foord OS, Rose EA. Long-distance PCR. PCR Methods and applications 1994; Vol 3, No 6, S149–S61.
2. Cheng S, Chang S-Y, Gravitt P, Respess R. Long PCR. Nature 1994; 369:684–5.
3. Barnes WM. PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. Proc Natl Acad Sci USA 1994; 91:2216–20.
4. Boehringer Mannheim. High-yield, high-fidelity amplification with the Expand Long Template PCR System. Biochemica 1995; 1:11–2.
5. Nielson K, Scott B, Bauer JC, Kretz K. (Stratagene Cloning Systems). TaqPlus™ DNA polymerase for more robust PCR. Strateg Mol Biol 1995; 7:64–5.
6. Hobbs HH, Brown MS, Russell DW, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. Annu Rev Genet 1990; 24:133–70.
7. Soutar AK. Familial hypercholesterolemia and LDL receptor mutations. N Engl J Med 1992; 317:734–7.
8. Ma Y, Betard C, Roy M, Davignon J, Kessling AM. Identification of a second "French Canadian" LDL receptor gene deletion and development of a rapid method to detect both deletions. Clin Genet 1989; 36:219–28.
9. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucl Acids Res 1988; 3:1215.
10. Südhof TC, Goldstein JL, Brown MS, Russell DW. The LDL receptor gene: a mosaic of exons shared with different proteins. Science 1985; 228:815–22.
11. Leitersdorf E, Tobin EJ, Davignon J, Hobbs HH. Common low-density lipoprotein receptor mutations in the French Canadian population. J Clin Invest 1990; 85:1014–23.
12. Rodningen OK, Leren TP. Application of long polymerase chain reaction in the study of the LDL receptor gene. Scand J Clin Lab Invest 1996; 56:93–6.
13. Cheng S. Longer PCR amplifications. PCR Strategies 1995; 313–24.

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