Benchmarks

Long-Distance Genome Walking Using the Long and Accurate Polymerase Chain Reaction

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Many methods using polymerase chain reaction (PCR) have been developed and modified to isolate an unknown DNA sequence adjacent to a known sequence; these are collectively called gene-walking methods (1,4-9). However, their usage has been restricted because most previous methods had practical problems, including low sensitivity, low efficiency (small probability of success) and/or are laborious with multiple steps. Furthermore, the extended nucleotide stretch is usually short in length. Except for laborious inversion PCR (up to 2-kb extension; Reference 8), most methods produce PCR products only 200-800 nucleotides in length.

To increase the length and the accuracy of walking products, we have applied the long and accurate PCR (LA-PCR) technique (2,3) in the new method. Using our new method, called long-distance genome-walking PCR (LDGW-PCR), extended fragments up to 4.0 kb have been obtained. In addition, the reliability of this technique is high.

Most of our work has been on the hexamerin gene of the mosquito (*Anopheles gambiae*), which will be described in detail; we also studied the *hairy* gene in *Drosophila*. Genomic DNA was extracted from a single mosquito or *Drosophila* using Protocol 1 of the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual.

Three primers are needed for gene walking: two primers specific to the 3' end of the known sequence (SP1: 5'-TCA TCA TCA CGC CGT ACA CGG C-3'; SP2: 5'-CGC TTC TAC GAC AGC CTG CCG TTC-3') and an adapter primer [AP; *EcoRI*-(G)₁₅: 5'-CGA GGA ATT CGG GGG GGG GGG GGG G-3'], which contains an *EcoRI* site near its 5' end. For internal sequence probe, two additional specific primers were used (PKM122 + 21: 5'-

GGA TAT CCG TTC GAT CGC TCA-3'; PKM122 - 300: 5'-CAC TAG AAG CGA CCG AGA GAG-3'). PKM122 + 21 is located 10 bases downstream from SP2. PKM122 - 300 is located 300 bases downstream from the 3' terminus of the hexamerin gene (3'-flanking region).

The new method has three steps (Figure 1). The extension reaction was performed to extend single-stranded (ss)DNA of target sequence using one primer (SP1). The reaction mixture contains 1× LA-PCR buffer [40 mM Tricine-KOH (pH 9.2; Catalog No. 8417-1; CLONTECH Laboratories, Palo Alto, CA, USA), 3.5 mM magnesium acetate, 15 mM potassium acetate, 75 µg/mL bovine serum albumin (BSA)], 0.2 mM of each dNTP, 50 pmol of SP1, 50 ng mosquito genomic DNA and 2.5 U KlenTaq polymerase (CLONTECH) in a final reaction volume of 50 µL. Thermal cycling was performed using a PCR System 9600 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) with the following conditions: 96°C for 10 s, 64°C for 10 s

and 68°C for 4 min for 35 cycles.

The primer extension products were purified using GENECLEAN® III (BIO 101, Vista, CA, USA) and adjusted to a volume of 10 µL. The purified amplification product was incubated for 5 min at 95°C in a water bath and chilled immediately on ice for 2 or 3 min. For tailing, 4 µL of 5× tailing buffer (1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/mL BSA, pH 6.6), 3 µL of 5 mM CoCl₂, 1 µL of 100 pmol dCTP and 50 U of terminal deoxyribonucleotidyl transferase (TdT; Boehringer Mannheim, Indianapolis, IN, USA) were added, and the mixture was incubated for 20 min at 37°C followed by heating for 10 min at 70°C. The reaction mixture was diluted to 100 µL in distilled water, and 1-5-µL aliquots were used for PCR.

PCR was performed using two primers (SP2 and AP) with the following conditions: 96°C for 30 s, 65°C for 10 s and 68°C for 5 min for 40 cycles followed by a 5-min incubation at 68°C. Longer denaturation time was applied to the second reaction than to

Figure 1. Schematic representation of LDGW-PCR. The reaction is performed to extend and enrich ssDNAs using only one primer (SP1) specific to the known sequence at the 3' end. The amplification products are purified, and 3' ends are tailed with poly(dC) using TdT. The reaction product is diluted five-fold with distilled water and directly used as PCR template. The second reaction is performed using two primers (SP2 and AP) to produce extended PCR products. All PCRs were performed under high-stringency conditions.

Table 1. Results of Six Consecutive Attempts at Gene Walking Using LDGW-PCR

Species	Gene	Direction	Time (min)	Length (kb)
<i>A. gambiae</i>	Hexamerin	3'→5'	4	3.8
<i>A. gambiae</i>	Hexamerin	3'→5'	4	4.0
<i>A. gambiae</i>	Hexamerin	5'→3'	2	2.5
<i>A. gambiae</i>	Hexamerin	5'→3'	2	1.5
<i>D. nebulosa</i>	<i>hairy</i>	3'→5'	4	4.2
<i>D. willistoni</i>	<i>hairy</i>	3'→5'	4	3.5

The hexamerin gene was studied in the mosquito *A. gambiae*, and the *hairy* gene was studied in two species of *Drosophila*. "Time" is the polymerization time of the first reaction. "Length" is the approximate size of the largest discrete band detected on an agarose gel that was subsequently sequenced and confirmed to be an accurate extension of the known sequence.

the first to denature the AP primer [including poly(dG) tail] completely. Also, twice the amount of KlenTaq polymerase (5 U for each tube) was used in the PCR, and an additional aliquot of polymerase (1–2 U for each reaction tube) was added during the denaturation step of the 30th cycle.

To confirm that we produced extended fragments, Southern blot analysis and sequencing were performed. DNA molecular weight marker (1-kb ladder) and PCR products were loaded onto a 1.0% agarose gel containing ethidium bromide. DNA fragments were trans-

ferred to a HybondTM-N membrane (Amersham International plc, Little Chalfont, Bucks, England, UK). Digoxigenin-labeled internal nucleotide sequence (Random-Primed DNA Labeling method; GeniusTM 1 Kit; Boehringer Mannheim) was used as a probe. Hybridization was performed overnight at 48°C in hybridization solution: 50% formamide, 5× standard saline citrate (SSC), 2% (wt/vol) blocking reagent (Boehringer Mannheim), 0.2% sodium dodecyl sulfate (SDS) and 0.1% *N*-lauroylsarcosine. The membrane was washed twice for 15 min at 68°C in 0.5× SSC with 0.1% SDS.

The two largest PCR products (about 4.0 kb) were isolated from a 1.2% low-melting agarose gel and purified by GENECLEAN III. Both purified fragments were sequenced using the cycle sequencing protocol with dye-labeled dideoxyribonucleotides on a Model 373A DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). The quality of the product was sufficient to produce 400 bp or more of accurate sequence per sequencing reaction. The purified fragments were also cloned into the TA Cloning[®] vector using the TA Cloning Kit (Invitrogen). Cloned plasmids were purified by the QIAGEN[®] Plasmid Mini Kit (Qiagen, Chatsworth, CA, USA) and sequenced.

Using this technique, numerous bands were obtained per reaction, and nearly all the bands between 2 and 4 kb appeared to be specific gene sequences from Southern blot analysis (Figure 2). However, two small bands, 0.5–2.0 kb, appeared in the agarose gel but were not detected in Southern blot analysis.

Figure 2. Genome walking products from LDGW-PCR. Amplification products were obtained using primers SP2 and AP. (A) Agarose gel stained with ethidium bromide. Lane M: DNA molecular weight marker (1-kb ladder). Lanes 1 and 2: PCR products obtained from two replicates of the initial extension reaction. (B) Southern blot analysis using internal sequence probe of the same gel.

Therefore this gene-walking technique accurately amplifies longer fragments (3–4 kb) while some of the shorter ones (<2 kb, Figure 2) might be artifacts. Furthermore, larger bands (5–7 kb) could also be detected by both Southern blot and agarose analyses, although the bands were too weak to detect easily.

Because the SP2 primer was about 350 bp from the 3' end of the known sequence, this provides an internal control for artifacts. In sequencing both the 3.5- and 4.0-kb products, we confirmed that these fragments are true extensions of the known region because the first 350 bp of the 5' ends of both are identical to the known 3' ends.

The reliability of the technique is high. Once having worked out the initial conditions and procedures, we have not had a failure in six attempts at walking for both the mosquito hexamerin gene and the *Drosophila hairy* gene (Table 1). Walking in both the 3' and 5' directions is possible by simply using alternative strands. Another aspect of the length of the walk suggested by Table 1 is that the length of the product is dependent on the polymerization time during the thermal cycling. One somewhat unexpected result is that there is one predominant discrete band (Figure 2). This is probably not due to any secondary structures because, as can be seen in Table 1, the size of the predominant product is a function of time of polymerization. Furthermore, we have sequenced multiple clones from the discrete band that vary in size by 100–200 bp, so while the band appears discrete, it contains molecules that vary in size.

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ADDENDUM

The following is an addendum to a June 1995 *BioTechniques* article; Shapiro, M., 1995, DNAdraw: A Program for Preparing DNA and Protein Data for Publication, 18: 1064-1067.

An update of the DNAdraw software described is now available at the DNAdraw Web site (<http://www.cvn.net/~dnadraw>). The new Version 3.0 represents a major upgrade, with additional features including direct printing, color output, optional page breaks and lots more. It can be downloaded from the Web site.

Relative Amplification Efficiency of Differently Sized Templates by Long-Distance PCR

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A common concern with polymerase chain reaction (PCR) is the variation of amplification efficiency with template size. Length-dependence problems can arise when amplifying differently sized alleles (2), pseudogenes (6) or heterogeneous genomes involving deletions (4,5). In conventional PCR, smaller fragments are more efficiently amplified than significantly larger templates. However, accounts of long-distance (LD)-PCR amplifying smaller fragments preferentially, albeit to a lesser extent than conventional PCR (4,5), remain controversial (6). This work evaluates the quantitative relationships between template size and LD-PCR amplification efficiency.

Defined quantities of templates of different sizes were co-amplified with a constant amount of a reference template. PCR targets were complete or deleted human immunodeficiency virus type 1 (HIV-1) genomes cloned into pUC and *Aat*II-linearized (5). HIV-1 genome sizes were 9709 bp (pNL4-3; Reference 1), 4313 bp (p Δ nipdj; Reference 5), 2693 bp (p Δ focep; Reference 5) and 1948 bp (p Δ cesmo; Reference 5). Two distinct sets of primers were used for amplification: primers LTR U5/LTR R (Figure 1) yielded products of 9.1, 3.6, 2 and 1.3 kb with targets pNL4-3, p Δ nipdj, p Δ focep and p Δ cesmo, respectively, while primers CL-NCS and CL-NEF (5'-ATCTCTCGACGACGAGCTCGGCTTGCTGAA-GCGCGCA-3' and 5'-CCATGTTTTTCTAGGTCTCGAGAT-ACTGCTCC-3', respectively) yielded products of 8.2, 2.8, 1.2 and 0.4 kb. Templates were subjected to LD-PCR with 1.75 U of Expand™ Long-Template PCR enzyme (Boehringer Mannheim, Meylan, France) in a 50- μ L reaction mixture containing 50 mM Tris-HCl, pH 9.2, 14 mM (NH₄)₂SO₄, 1.75 mM MgCl₂, 350 μ M each dNTP, 7.5 pmol of each primer and 100 ng of human genomic DNA carrier. Templates were amplified with 25 cycles at 94°C