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Ligation-mediated PCR for quantitative in vivo footprinting

Shu-Mei Dai¹, Hsiu-Hua Chen¹, Cheng Chang¹, Arthur D. Riggs¹, and Steven D. Flanagan^{2*}

Divisions of ¹Biology and ²Neurosciences, Beckman Research Institute of the City of Hope, Duarte, CA 91010-3011. *Corresponding author (sflan@coh.org).

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Ligation-mediated polymerase chain reaction (LM-PCR) is a genomic analysis technique for determination of (1) primary DNA nucleotide sequences (2) cytosine methylation patterns (3) DNA lesion formation and repair, and (4) in vivo protein–DNA footprints^{1–4}. However, LM-PCR can be limited by the multiple steps required and the relatively short stretch of sequence (usually <200 bp) that can be analyzed per reaction. We report here a simplified, one-day LM-PCR protocol in which all pipetting steps can be performed by a robotic workstation and which, moreover, provides longer reads (>350 bp) and enhanced signal quality by use of non-radioactive detection and a LI-COR DNA sequencing instrument. Sensitivity comparable to radiolabeling is achieved using oligonucleotide primers that are 5'-end labeled with infrared fluorochromes. We showed that the technique could be used for sensitive and reproducible in vivo photofootprinting of the human phosphoglycerate kinase 1 (PGK1) promoter, as well as providing good Maxam–Gilbert sequence information. The methods described here should allow high-throughput, high-resolution analysis of transcription factor binding and chromatin structure, and also may be useful for sequencing gaps that are refractory to cloning.

To achieve exponential amplification, polymerase chain reactions (PCR) require primer sequences flanking both sides of the targeted DNA region⁵. Ligation-mediated PCR¹, however, requires sequence information from only a single flank, because one primer hybridizes to a universal sequence that has been ligated onto the DNA fragments. As illustrated in Figure 1, LM-PCR consists of four stages. First, single-strand breaks, such as created by Maxam–Gilbert chemical sequencing reactions or derived from UV photodimers, are converted to blunt-ended duplex DNA fragments by primer extension. Second, a linker-primer oligonucleotide is ligated to the blunt

ends. Third, exponential PCR amplification is performed, followed by an optional exonuclease treatment to remove primers from previous steps. Fourth, the distribution of products is detected either by Southern blotting and hybridization with a radiolabeled probe², or by a final round of primer extension using a third gene-specific labeled oligonucleotide¹. For work reported here, the third primer (IRD-P3 in Fig. 1B) is labeled at the 5' end with a dye that fluoresces in the infrared⁶.

The LM-PCR protocol (Fig. 1B) presents an assortment of technical challenges for automation. Although the first, third, and

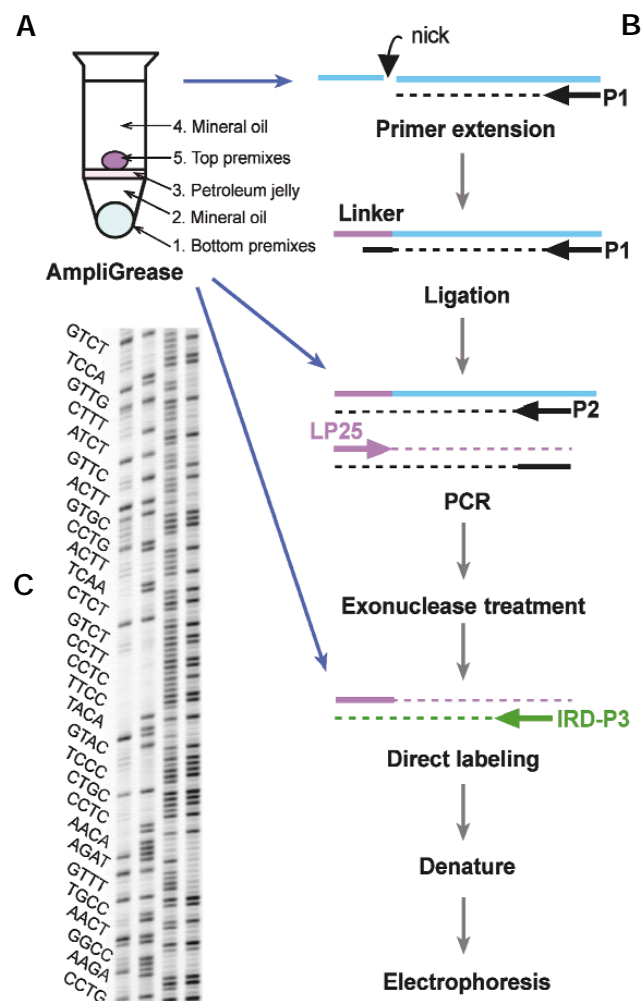


Figure 1. Flowchart of automated LM-PCR. (A) Automated AmpliGrease hot-start LM-PCR is a modification of the manual protocol for the use of petroleum jelly as a barrier separating two aqueous components until heated¹⁰. The components are numbered in the order of addition to the tube using a liquid-handling robot. The first component is a premix containing PCR buffers, deoxynucleotides, oligonucleotide primers, and template, which can be added separately. Mineral oil is pipetted into the tube at two stages, first as a 7.5 μ l component (2) to protect the bottom premix from splashing and to absorb heat from the molten petroleum jelly (3), and then as a larger aliquot (142.5 μ l) to allow the petroleum jelly to form a single phase on heating. Finally, a second aqueous aliquot is pipetted into the mineral oil containing polymerase. Equalization of buffer and Mg^{2+} concentrations in both aqueous phases was found to improve reliability. (B) LM-PCR (ref. 24) was automated by diluting products from each stage into the next utilizing AmpliGrease in three stages as indicated by the blue arrows. (C) Example of sequence of human TP53 exon-5 along upper stand. Lanes (from left to right) are G, G+A, C+T, and C products of Maxam–Gilbert²⁵ sequencing reactions performed using HeLa cell DNA. The sequence displayed lies 137–251 bp from the primer sequence (P53R7028).

fourth stages are polymerase catalyzed, and therefore have the same salt and substrate concentration requirements, three different nested primers are utilized. The second stage, the ligation step, requires higher Mg^{2+} and NaCl concentrations than the first, third, and fourth polymerase-mediated stages. Either the salt and buffer conditions for polymerase activity must be broad, or the components from previous stages must be replaced. Furthermore, the primers and adapter oligonucleotides included in the previous stages may cause mispriming in succeeding stages. Because of these considerations, a variety of DNA polymerases, buffer conditions, and purification techniques have been extensively explored to streamline and to optimize LM-PCR protocols^{1,3,7}, with less than optimal success. Hot-start protocols, which have proved so useful for optimizing PCR^{8,9}, have remained largely unexplored for use with LM-PCR. One such method uses Ampligrease, which is petroleum jelly suspended in mineral oil, to separate two aqueous premix components until heated, whereupon the jelly becomes miscible with the oil, allowing the top premix to combine with the bottom¹⁰. In this report, we demonstrate that combining this hot-start method with the single-tube approach of Cairns and Murray¹¹, as modified by us³, yields excellent results for Maxam–Gilbert sequencing (Fig. 1C). The automated LM-PCR also produces superior in vivo UV photofootprints (Fig. 2), as well as dimethylsulfate in vivo photofootprints (data not shown). A variety of gene targets have been successfully analyzed using the automated procedure, including all five human tumor protein p53 (TP53) exons (Fig. 1C), human TWIST promoter, and human PGK1 (Fig. 2). It should be noted that human PGK1 is very G+C-rich, and not an easy target even for standard sequencing methods.

Using the procedure reported here, all the pipetting steps in LM-PCR can be performed on a Biomek 2000 liquid handling robotic workstation. After thermocycling is completed in the first reaction, the product is diluted into the next stage for ligation. After a 2 h ligation reaction coupling the linker-primer to the primer extension product, one-half of the ligation mixture is used for an exponential PCR reaction, again using Ampligrease (see Fig. 1A). Before direct labeling with IRD-tagged primer 3 (IRD-P3), residual P1 and P2 primers may be removed by exonuclease treatment. (Note that this step is optional and only required when primers are designed without appropriate gaps in T_m , i.e., 60°C, 64°C, 66°C for P1, P2, and P3, respectively.) Electrophoresis with simultaneous detection is performed on the LI-COR DNA sequencer. Automated LM-PCR, combined with the use of infrared fluorochrome (IRD)-labeled primers and detection using the LI-COR DNA sequencer, routinely provides

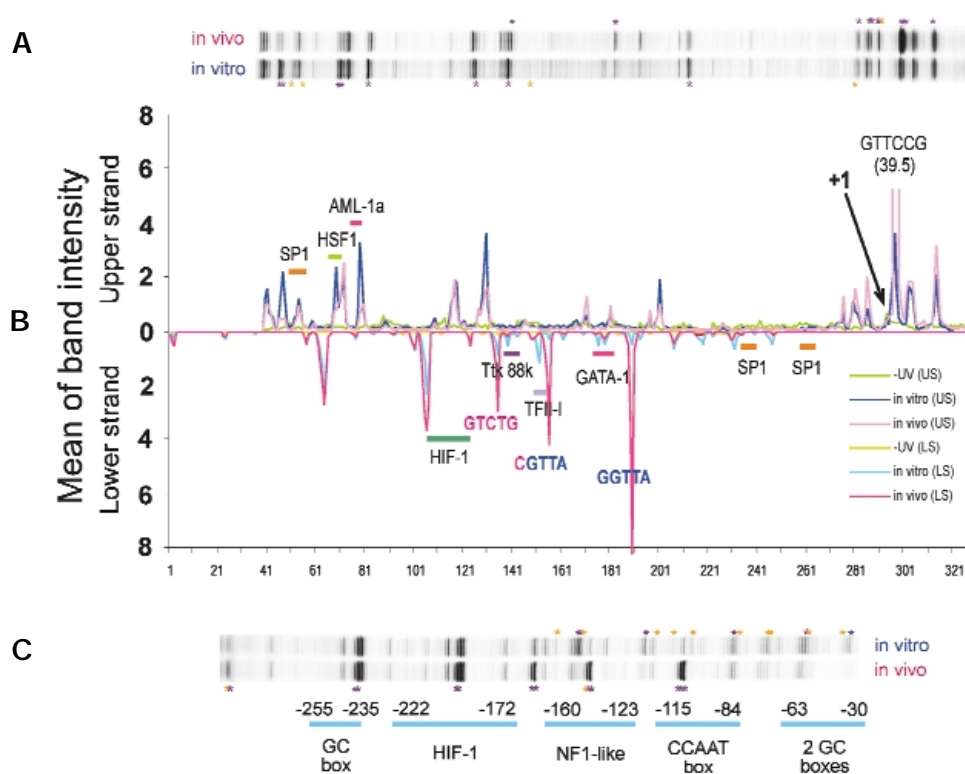


Figure 2. Quantitative analysis of UV photofootprinting. (A) UV footprinting of the human PGK1 promoter was analyzed in intact cells (in vivo) and for isolated DNA (in vitro) along the upper strand. The digitized fluorescence data (TIFF file) from the LI-COR Model 4200 sequencer were displayed using Adobe PhotoShop. Asterisks (*) designate bands that reach a statistically significant difference ($P < 0.01$) in t -test analysis of in vivo versus in vitro conditions in four replicates of the LM-PCR protocol on a pair of DNA samples. Bands of intensity < 0.5 are indicated by orange asterisks and ≥ 0.5 , by purple, with the asterisks positioned adjacent to the gel image displaying enhanced intensity. (B) The band intensities from four LM-PCR reactions were analyzed using the RFLPscan software and normalized using the sum of the bands for each LM-PCR reaction. The mean values of the normalized band intensities were plotted. The upper and lower panels illustrate results from the upper and lower strands, respectively. On a single LI-COR sequencing gel, five promoter transcription binding sites were prominently revealed. Along the lower strand between positions -160 and -30 relative to the transcription start site, three enhancements and several suppression sites were observed by comparison of in vivo to in vitro results. This region contains an NF1-like consensus site, a CCAAT box, and two GC boxes. On the upper strand, footprints revealed by suppression of UV dipyrimidine formation are evident within the GC box and HIF-1 binding site¹⁵ (bp -255 to -172) and an enhancement (17-fold) near the transcription initiation site. (C) Gel image of the lower strand is displayed as for (A).

more than 350 bp of sequence. In some experiments where longer fragments are analyzed, 700–1,000 bp of sequence can be resolved and useful information obtained over the entire range.

The promoter of the human PGK1 gene has been well characterized by earlier LM-PCR experiments^{2,12–14}. These earlier studies included UV photofootprinting, and thus provided a good standard by which to evaluate automated LM-PCR results. As shown in Figure 2, an excellent UV-induced footprint is revealed by comparing the in vivo footprint (red lines) with that obtained by the in vitro UV treatment of purified DNA (blue lines). For easy comparison, both the lower and upper strands are plotted in the same graph, but inverted relative to one another (Fig. 2B). The digitized data obtained by use of the LI-COR, combined with a low background and constant spacing between bands, allow superior quantitative information to be obtained, with changes less than twofold being quite meaningful if the results from independent repeat reactions are averaged (see below).

The role of consensus binding sites in PGK1 transcriptional control has been investigated previously^{14,15}. In addition to the footprints previously noted, we also observed footprints between GGTTA and CGTTA (half of a CCAAT consensus core¹⁶). A consen-

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sus recognition site for GATA-1 (5'-NNGATNNNN), which interacts synergistically with adjacent CCAAT binding proteins¹⁷, was observed to produce an 8.5-fold suppression (calculated using data displayed in Fig. 2B) on the lower strand and detectable enhancement on the upper strand. Two suppression footprints were observed within the pyrimidine-rich sequence of the NF1 binding site, including the indicated consensus recognition sites for the Ttk 88K (ref. 18) and TFII-I (ref. 19) transcription factors. A possible binding site (TGT/CGGT) for runt-factor AML-1 (ref. 20) and RGAANRTTCN for HSF1 (ref. 21) are also observed between the GC box and the HIF binding site along the upper strand.

It is generally accepted that LM-PCR can reliably detect twofold changes in footprint intensity^{2,12}, but we report here the first quantitative, statistical study of band variation. To assess the statistical variance of the automated LM-PCR process, we replicated the automated LM-PCR protocol four times for a single set of UV-treated DNA samples under both in vivo and in vitro conditions. The digitized data obtained from the LI-COR instrument were used for semiautomated lane and band identification using RFLPscan; integrated intensities were obtained for 300 bands by Gaussian curve fitting. When LM-PCR was performed in quadruplicate on a pair of in vitro and in vivo UV-treated samples, the coefficient of variation values obtained were close to 10% for bands with an intensity of >0.5. Individual bands were subjected to two-tailed *t*-tests assuming unequal variance. Bands meeting a *P* < 0.01 criteria are indicated by asterisks in Figure 2. The binding sequences along the lower strands for Ttk 88K, TFII-I, GATA-1, and CCAAT binding proteins were all observed to produce more than a fivefold change in footprinting intensities (Fig. 2). In UV photofootprinting, a DNA-binding protein produces a "signature" by modulating the quantum yield for cross-linking adjacent pyrimidine bases, presumably by rotating the bases away or toward a preferred orientation. In the PGK promoter region one sees as much as a 17-fold enhancement of reactivity (CCAAT box) and up to a 16-fold suppression (TFII-I site) of UV reactivity. As the human and other genome projects near completion, a major challenge will be to use this sequence information to learn more about chromatin structure and the regulation of genes. In vivo footprinting by LM-PCR is one of the few ways to obtain information about true native chromatin structure. High-throughput automated or partially automated LM-PCR has the potential to use the sequence data to gain high-resolution information on in vivo chromatin structure.

The protocol presented here represents a variety of modifications to existing protocols. The use of infrared dyes on the LI-COR sequencer simplified data analysis because the background signal was consistently low and the bands were evenly spaced. The automated hot-start technique proved crucial for setting up reactions at room temperature; furthermore, hot-start protocols consistently provided better results than achieved using manual protocols where samples were cooled on ice during pipetting steps. *Pfu* Turbo provided significant improvements when compared with results using a mixture of native Vent and Vent (exo)-polymerases. The effect of exonuclease treatment before direct labeling with the IRD-P3 primer generally provided a stronger signal with the effects dependent upon the spacing of *T_m* values for a given primer set.

Experimental protocol

Cell lines, UV irradiation, and DNA purification. Chinese hamster hybrid cells (Y162-11C) containing an active human X chromosome² were used for UV in vivo footprinting as described¹⁴. Cells were lysed and DNA was purified and treated with T4 endonuclease V and *Escherichia coli* photolyase as described earlier²².

Nested PGK1 oligonucleotide primers for LM-PCR. The primer design program, ospX (ref. 23), was utilized to construct two primer sets with pro-

gressively increasing *T_m* and overlapping sequence. The primer set (labeled P1, P2, and P3, respectively, in Fig. 1B) used to map the lower strand includes an extension primer (HP102, *T_m* = 59.6°C), 5'-CCTGGGTCTCGCACATTC; a PCR primer (HP112, *T_m* = 63.7°C), 5'-GCACATTCTTCACGTCCGTTCCCA; and an IRD-labeled primer (HP122, *T_m* = 67.5°C), 5'-CACGTC-CGTTCCGACGTCACC). The corresponding primer set for the upper strand consisted of (HP298R1, *T_m* = 59.7°C), 5'-GGGAGAGAGTCCGGT-GATT; (HP310R2, *T_m* = 63.8°C), 5'-GGTGATTCCGTCAACGAGGGAG; and (HP318R3, *T_m* = 65.8°C), 5'-GGTCAACGAGGGAGCCGACTG.

TP53 primers. The corresponding primer set for exon-5 consisted of (P53R7015, *T_m* = 59.2°C), 5'-TGGGGACCTGGGCAA; (P53R7027, *T_m* = 64.0°C), 5'-GCAACCAGCCCTGTCTCTC; and (P53R7028, *T_m* = 65.7°C), 5'-CAACCAGCCCTGTCTCTCTC.

Hot-start primer extension. All pipetting steps for LM-PCR were performed on a Biomek 2000 liquid handling robotic workstation (Beckman Instruments, Inc., Fullerton, CA). The bottom premix (6.5 µl) contained 1 µl (1 µg/µl) DNA, 0.65 µl 10× cloned *Pfu* buffer (Stratagene, La Jolla, CA); 0.2 µl each of dATP, dCTP, dGTP, and dTTP (2.5 mM), 0.6 µl of 2 µM primer 1, and 3.45 µl distilled H₂O. First, 5.5 µl bottom premix was transferred to the tube by the Biomek 2000 robotic workstation, and 1 µl of DNA was added (manually or automatically). Next, 7.5 µl of mineral oil and about 20 µl of warm AmpliGrease were added to the top of the bottom premix. (The warm AmpliGrease was set up by incubating petroleum jelly in a VWR heat block and transferred with a hairdryer—preheated Biomek P250 pipette tip.) Finally, 142 µl of mineral oil were pipetted on top of the petroleum jelly, followed by 3.5 µl of top premixes (0.2 µl of 2.5 U/µl *Pfu* Turbo, 0.35 µl of 10× cloned *Pfu* buffer, 1.0 µl of 70% sucrose and 1.95 µl H₂O). The hot-start primer extension is then performed in the thermocycler by a denaturing step at 95°C for 5 min, an annealing step at the oligonucleotide *T_m* (calculated by ospX) of primer 1 for 30 min, and a primer extension at 76°C for 10 min.

Ligation. After the primer extension step, about 95% of the suspension of AmpliGrease in mineral oil was removed. Then, 6.4 µl of ligation premix were transferred to the tube with the primer extension products and mixed well. The ligation premix contains 0.4624 µl of 1 M Tris-HCl (pH 7.5), 0.1024 µl of 1 M MgCl₂, 0.32 µl of 1 M dithiothreitol (DTT), 0.1568 µl of 100 mM adenosine triphosphate (ATP), 0.0784 µl of 10 mg/ml bovine serum albumin (BSA), 1.6 µl of 20 µM linker, 0.32 µl of 20 U/µl T4 DNA ligase (Promega, Madison, WI), and 3.36 µl of H₂O. The ligation was carried out at 17°C for 2 h.

Hot-start PCR. One-half of the ligation product was removed, and warm AmpliGrease was added directly to the top of the ligation product and the pre-existing mixture of mineral oil and AmpliGrease. After adding an additional 142 µl of mineral oil, 3.5 µl of PCR premix was added to the top. The PCR premix consisted of 1.0 µl 70% sucrose, 0.67 µl of 10× *Pfu* cloned buffer, 0.4 µl of 25 mM dNTP, 0.2 µl of 20 µM primer 2, 0.2 µl of 20 µM LP25, 0.4 µl of 2.5 U/µl *Pfu* Turbo, and 0.63 µl H₂O. The conditions for PCR were: one cycle of 95°C for 2 min, *T_m* of primer 2 for 2 min, and 76°C for 3 min; 18 cycles of 95°C for 45 s, *T_m* of primer 2 for 2 min, and 76°C for 3 min; and one cycle of 95°C for 45 s, *T_m* of primer 2 for 2 min, and 76°C for 10 min.

Exonuclease treatment (optional). About 95% of the mixture of AmpliGrease and mineral oil was removed. One microliter of 1 U/µl *E. coli* Exonuclease I (10 U/µl from US Biochemicals (USB), Cleveland, OH, diluted to 1 U/µl with 1× PCR buffer) was added to the PCR products. The reaction tube(s) were put in the thermocycler at 37°C for 30 min, and then at 72°C for 15 min to inactivate the exonuclease.

Hot-start direct labeling. After exonuclease treatment, 20 µl of warm AmpliGrease, 142 µl mineral oil, and 3.5 µl direct labeling premix were added, in order, to the top of the exonuclease-treated samples. The direct labeling premix contained 1.15 µl distilled H₂O, 1.0 µl 70% sucrose, 0.35 µl 10× *Pfu* cloned buffer, and 1 µl of 1 µM IRD-labeled primer. This step was performed under yellow light to protect the IRD-labeled primer, which is kept as dark and cold as possible between steps. The direct labeling was carried out in the thermocycler with conditions of 95°C for 2 min, five cycles of 95°C for 45 s, *T_m* of primer 3 for 3 min, 76°C for 2 min, and 76°C for 10 min.

Electrophoresis. Electrophoresis and scanning were performed in a LI-COR DNA sequencer model 4200 following manufacturer's instructions (LI-COR, Inc., Lincoln, NB). The collected TIFF image can be visualized either by PhotoShop (Adobe Systems, Inc., San Jose, CA) or lane analysis program such as RFLPscan (Scanalytics, Inc., Fairfax, VA). RFLPscan was also used to quantitate band intensity for further variation analysis.

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