Mutation detection using ligase chain reaction in passivated silicon-glass microchips and microchip capillary electrophoresis

Xing Jian Lou¹, Nicholas J. Panaro¹, Peter Wilding¹, Paolo Fortina², and Larry J. Kricka¹
¹University of Pennsylvania School of Medicine and ²Thomas Jefferson University
Philadelphia, PA, USA

BioTechniques 37:392-398 (September 2004)

The ligase chain reaction (LCR) following PCR is one of the most sensitive and specific methods for detecting mutations, especially single nucleotide polymorphisms (SNPs). Performing LCR in microchips remains a challenge because of the inhibitory effect of the internal surfaces of silicon-glass microchips. We have tested a dynamic polymer-based surface passivation method for LCR conducted in oxide-coated silicon-glass microchips. The combination of polyvinylpyrrolidone 40 (PVP-40) at 0.75% (w/v) with an excess of the ligase produced successful LCR in the silicon-glass microchips, with yields of ligated primers comparable to reactions performed in conventional reaction tubes. Ligated primers were detected and quantified simply and conveniently using microchip capillary electrophoresis.

INTRODUCTION

In two randomly selected human genomes, 99.9% of the DNA sequence is identical. The remaining 0.1% of DNA contains sequence variations. Among these variations, the most common type is a single nucleotide polymorphism (SNP), and this accounts for more than 90% of all differences between individuals (1,2). SNPs are distributed throughout the genome and have been shown to be associated with susceptibility to diseases such as cystic fibrosis (3), high blood pressure (4), diabetes (5), and certain cancers (6,7). SNPs also form the basis of pharmacogenomics, the tailoring of medicines to suit an individual’s genome, and thus the detection of SNPs is expected to be of increasing importance in the management of patients (8).

Many methods have been developed to either screen for unknown SNPs or detect known SNPs. Methods for screening unknown SNPs include DNA sequencing (9,10), microarray or variant detector arrays (11–15), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (16). Methods for detecting known SNPs include PCR-based mutation detection systems (for review, see Reference 17), ligase chain reaction (LCR) (18), and denaturing high-performance liquid chromatography (DHPLC) (19). Some of the above methods are good for both screening unknown SNPs and detecting known SNPs. Among these methods, LCR is emerging as the most sensitive method with high specificity for known SNP detection (20). LCR was first developed by Barany (18), who used thermostable DNA ligase to discriminate between normal and mutant DNA and to amplify the allele-specific product. A mismatch at the 3' end of the discriminating primer prevents the DNA ligase from joining the two fragments together. By using both strands of genomic DNA as targets for oligonucleotide hybridization, the products generated from two sets of adjacent oligonucleotide primers, complementary to each target strand in one round of ligation, can become the targets for the next round. The amount of the products can thus be increased exponentially by repeated thermal cycling (18).

Microchip technology has been effectively applied in molecular biology research and disease detection (21,22). Our laboratory has previously reported the use of silicon-glass microchips for mutation detection using LCR. However, the amount of LCR products in such microchips was significantly decreased as compared with the same reaction performed in GeneAmp® reaction tubes (Applied Biosystems, Foster City, CA, USA) (23). The reduced yield was most likely caused by the binding of a significant amount of critical LCR components to the internal surface of the microchip. Because the surface area/volume ratio is high in microchips, any low-level binding activity can cause a significant binding of LCR components such as metal ions, oligonucleotide primers, and enzymes. We have recently reported that dynamic surface passivation using polymers developed by Giordano et al. (24,25) can significantly increase the efficiency of PCR in silicon-glass microchips (26). In particular, a comparison of the surface passivation effects of polyethylene glycol 8000 (PEG 8000) and polyvinylpyrrolidone 40 (PVP-40) showed that the latter had a more prominent effect on PCR efficiency (26). Although PCR and LCR have different analytical principles, they have in common the use of enzymes and oligonucleotides as reagents. Therefore, in this study, we investigated the effect of PVP-40 dynamic surface passivation of oxide-coated silicon-glass microchips in an LCR-based mutation detection assay.

MATERIALS AND METHODS

Silicon-Glass Chips

Silicon-glass microchips (反应体积，大约10 μL；表面面积，大约200 mm²) were microfabricated at Cornell University Nanotechnology Center (Ithaca, NY, USA) using standard photolithographic procedures as previously described (26). All the silicon components of silicon-glass microchips used in this study were statically passivated by coating with a 2000 Å thick layer of SiO₂ (27,28).

Genomic DNA Isolation

Blood from healthy volunteers was drawn into tubes precoated with EDTA. Genomic DNA was isolated using a QIAamp® DNA Blood Mini Kit purchased from Qiagen (Valencia, CA, USA).
Isolation of Human Lymphocytes

Blood drawn from healthy volunteers was equilibrated to room temperature in tubes precoated with EDTA with rotation. Lymphocytes were then isolated using Red Blood Cell Lysis Buffer from Roche Applied Science (Indianapolis, IN, USA) as previously described (26).

PCR Amplification

PCR amplifications for the fragment flanking SNP 908 within the NOD2/CARD15 gene involved in inflammatory bowel disease were performed in GeneAmp reaction tubes using a conventional thermal cycler. The final reaction volume was 50 μL, and reagents include GeneAmp 1× Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM MgCl₂, 1.5 Unit AmpliTaq Gold® DNA polymerase (Applied Biosystems), 0.2 μM for each primer (forward primer: 5′-GATGGAGGCAGGTCCACCTT3′; reverse primer: 5′-CACCTGATCTCCAAAGAAA-3′), 0.5 mM dNTPs (Applied Biosystems), and 50 ng of the above isolated genomic DNA. The reaction mixture was initially heated for 10 min at 95°C for denaturation, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and completed by extension at 72°C for 10 min. When PCR was performed directly from isolated lymphocytes (200 cells/μL), the number of PCR cycles was increased from 35 to 45. The PCR product was 303 bp in length and was purified using QIAquick® PCR Purification Kit (Qiagen) for subsequent sequencing reaction or treated with alkaline phosphatase (USB, Cleveland, CA, USA) and Exonuclease I (USB) for further LCR analysis. Purified or treated PCR products were quantitated using capillary electrophoresis. The amplified PCR products were then sequenced at the DNA Sequence Facility of University of Pennsylvania (Philadelphia, PA, USA) using ABI Prism® 3100 capillary sequencers with BigDye™ Taq FS Terminator V 3.1 chemistry (Applied Biosystems).

Mutation Detection Using LCR

Each reaction was performed in 10 μL containing 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 10 mM MgCl₂, 0.1% (v/v) Nonidet® P40 (NP40), 0.01 mM rATP, 1 mM dithiothreitol (DTT), 50 ng enzyme-treated PCR product, and allele-specific primers for SNP 908. Final concentrations from 0.025 to 0.2 U/μL Pfu DNA ligase (Stratagene, La Jolla, CA, USA) and 0.75% to 3% (w/v) polyvinylpyrrolidone (PVP-40; Sigma, St. Louis, MO, USA) were added to the mixture accordingly. Sequences of the set of allele-specific primers for SNP 908 wild-type and a G to C mutation are shown in Figure 1A. Reactions were initially denatured at 94°C for 4 min and ligated at 60°C for 3 min, followed by 25 cycles of 94°C for 30 s and 60°C for 30 s. The microchip cycler runs two microchips simultaneously. Identical LCR controls were also run.

Figure 1. Ligase chain reaction (LCR) primer sequences and schematic for the PCR and LCR processes. (A) Sequence of LCR primers for genotype determination. Eight primers (four for wild-type and four for mutant) were required in the LCR mixture to determine whether the target DNA was wild-type or a C to G mutant. However, among these eight primers, two primers, primer A and primer D, phosphorylated at the 5′ end, were common to both wild-type and mutant detection. Primer BW and primer CW were wild-type-specific primers, and primer BM and primer CM were mutant-specific. (B) PCR and LCR processes. A DNA fragment containing single nucleotide polymorphism (SNP) 908 is first amplified by PCR from wild-type human genomic DNA (isolated or from lymphocytes without any preprocessing). Four oligonucleotide primers complementary to the wild-type target then hybridize to the target DNA fragment, and Pfu thermostable ligase only ligates adjacent primers that are perfectly complementary to the target (left). Ligated LCR primers from one round of ligation become targets for the next round, thus the amount of ligated LCR primers increases exponentially. Primers containing a single-base mismatch at the junction do not ligate, therefore no ligated LCR primers are formed (right). CE, capillary electrophoresis.
in parallel in GeneAmp reaction tubes under the same cycling condition using an ABI PRISM 2400 thermal cycler (Applied Biosystems).

**Capillary Electrophoresis**

The PCR and LCR products were detected and quantified using either a DNA 1000 or DNA 500 LabChip® Kit (Caliper Technologies, Mountain View, CA, USA) and the Agilent 2001 Analyzer (Agilent Technologies, Palo Alto, CA, USA) (29).

**RESULTS AND DISCUSSION**

Figure 1B illustrates the PCR and LCR process for mutation detection of SNP 908 in the NOD2/CARD15 gene. In brief, _Pfu_ DNA ligase links two adjacent oligonucleotide LCR primers when hybridized at 60°C to the complementary target (generated by PCR) only when the nucleotides are perfectly base-paired at the junction. The ligated primers are exponentially amplified by thermal cycling of the ligation reaction in the presence of a second set of adjacent LCR primers, complementary to the first set and the target.

LCR was first optimized in GeneAmp reaction tubes using a conventional thermal cycler. From these experiments, we found that 0.1 U/μL ligase in the reaction mixture (half of the amount suggested by the manu-

![Figure 2. Effect of the concentration of ligase on ligase chain reaction (LCR) in tubes. Each experiment was performed in triplicate. The x-axis indicates ligase concentration. The amount of ligated LCR primers in 0.1, 0.05, and 0.025 U/μL was normalized to that in 0.2 U/μL (100%). The relative amount on the y-axis represents an average of the replicates, and the error bar shows the standard deviation.](image)

![Figure 3. Effect of polyvinylpyrrolidone 40 (PVP-40) on ligase chain reaction (LCR) in microchips. Capillary electrophoresis was used to detect and quantify the ligated LCR primers. (A) The x-axis represents polymer concentration, which was 0% or 0.75%. Each experiment was performed in duplicate. The amounts of ligated LCR primers were normalized to that produced by the same reaction mixture in tubes (100%). The y-axis represents an average of the duplicates, and the error bar shows the standard deviation. (B) Representative capillary electrophoresis traces. Peak 1 and peak 5 are internal markers of DNA, and the rest of conditions were the same as those in row e. Peak 2 is unligated primers. Peak 3 is ligated LCR primers. Peak 4 is PCR-amplified DNA fragments containing single nucleotide polymorphism (SNP) 908. (a) Ligase (0.1 U/μL) in the absence of PVP; (b) ligase (0.1 U/μL) in the presence of 0.75% PVP; (c) ligase (0.2 U/μL) in the absence of PVP-40; (d) ligase (0.2 U/μL) in the presence of 0.75% PVP-40; (e) ligase (0.1 U/μL) in the absence of PVP in tubes as a control. In rows a through e, primers were complementary to wild-type target DNA. (f) Primers matched the mutant target DNA but did not match the wild-type target DNA, and the rest of conditions were the same as those in row e.](image)
facturer) was sufficient for LCR using the SNP 908 primers (Figure 2). However, 0.05 U/μL ligase significantly decreased the efficiency of the LCR, indicating an insufficient amount of enzyme. We therefore chose to use 0.1 U/μL ligase as a starting point to test the dynamic surface passivation effect of PVP-40 for LCR assays in thermal oxide-coated chips. At this lower level of enzyme, any detrimental effect due to the chip would be more readily apparent. A reaction volume of 10 μL (equivalent to the volume in the chip) and 20 μL (reaction volume suggested by the manufacturer) using 0.1 U/μL ligase were also tested in tubes, and no difference was observed (data not shown), thus confirming that the low reaction volume in the microchip was not detrimental to LCR. When LCR was performed in tubes using PCR fragments directly amplified from isolated human lymphocytes (no genomic DNA isolation was performed), a similar amount of ligated LCR primers was observed, as compared to performing LCR using PCR fragments amplified from genomic DNA (data not shown). This result suggested that our PCR-LCR approach can detect SNPs associated with disease using human blood cells as well as genomic DNA.

When we ran the LCR in thermal oxide-coated silicon-glass chips, only a very small amount of ligated LCR primers was detected by capillary electrophoresis, while the same LCR mixture run in tubes generated a large amount of ligated LCR primers (3–4 ng/μL; Figure 3A). The amount of ligated LCR primers formed in the chips was too small to be quantified by the capillary electrophoresis system (Figure 3B). Based on our previous experience with dynamic surface passivation for microchip-based PCR (26), and because it is known that polymers can reduce adsorption of biomolecules to surfaces (30,31), we tested the passivation effect of the polymer PVP-40 on the surface of microchips used for LCR. The presence of 0.75% (w/v) PVP-40 significantly increased the amount of ligated LCR primers in microchips using 0.1 U/μL ligase (the optimized amount in tubes), but the total amount of ligated LCR primers was only 47% of that in the tubes (Figure 3). When LCR was run using 0.2 U/μL rather than 0.1 U/μL ligase in the absence of 0.75% (w/v) PVP-40 in microchips, there was only a very small increase in the amount of ligated LCR primers (Figure 3). However, when we increased the ligase concentration to 0.2 U/μL, the presence of 0.75% (w/v) PVP-40 generated a LCR signal that was identical to that obtained in tubes (Figure 3). Detailed examination of the
capillary electrophoresis traces (Figure 3B) shows a small peak corresponding to unligated primers still present in the microchip reaction containing 0.2 μL ligase and 0.75% PVP-40 (Figure 3B, trace d) compared to the tube reaction containing 0.1 μL ligase (Figure 3B, trace e), but this residual peak was not consistently detected, and it is clear that both reactions are close to completion.

To exclude the possibility that a detergent property of PVP-40 acted to facilitate LCR, we tested the effect of PVP-40 on LCR in tubes. No significant increase in LCR signals was detected in the presence of PVP-40 at three different ligase concentrations (Figure 4). Nonspecific signals remained at zero in reactions containing various concentrations of PVP-40 with mutant LCR primers (Figure 4). Thus, the presence of PVP-40 did not decrease the specificity of the LCR assay.

In this study, we have shown that the combination of dynamic coating with polymers and static passivation with thermal oxide made LCR in silicon-glass microchips as efficient as LCR performed in conventional reaction tubes. The high specificity of the LCR in the chip-based assay was retained. Our protocol provides an effective means of transferring LCR assays into microchip format and will extend the scope of microchip-based assays.

ACKNOWLEDGMENTS

This work was supported by grant no. NCI RO1-CA 78848-04 from the National Cancer Institute (to P.W. and L.J.K.).

COMPETING INTERESTS

STATEMENT

The authors declare that they have no competing interests.

REFERENCES


Received 22 April 2004; accepted 21 May 2004.

Address correspondence to Larry J. Kricka, Department of Pathology and Laboratory Medicine, University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, USA. e-mail: kricka@mail.med.upenn.edu