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Analysis of ligase chain reaction products amplified in a silicon–glass chip using capillary electrophoresis

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Abstract

Ligase chain reaction (LCR) is a useful molecular technique for detecting known point mutations. We report the first example of the use of a disposable silicon–glass micro-chip for LCR and the first application of capillary electrophoresis (CE) to analyze samples amplified by LCR in a chip. Silicon–glass chips were manufactured using conventional photolithography and anodic bonding. The chips provide three distinct advantages for LCR: excellent thermal conductivity, a micro reaction volume (<10 μl), and reproducible, low-cost manufacturing. Investigation and quantitation of amplification efficiency of LCR in a chip or in a tube requires an analytical technique that is faster and more convenient than the conventional slab gel methods. Slab gel electrophoresis uses relatively large amounts of sample and is labor-intensive and time-consuming, and thus is unsuitable for the separation and detection of LCR products. In contrast CE requires sample volumes (original LCR products) of less than 1 μl and is therefore well-suited to analysis of the micro-volume reaction mixture from chips. We combined CE with a sensitive laser induced fluorescence (LIF) detection system for the rapid separation and quantitative detection of LCR products amplified from the *lacI* gene in a silicon–glass chip. Comparative studies were made with LCR between tubes and silicon–glass chips. CE–LIF analysis is ideally suited to examination of micro-LCR amplification with high thoughput. The technologies may find medical uses in disease diagnosis and research.

Keywords: Ligase chain reaction products; Silicon–glass chip; DNA; Oligonucleotides

1. Introduction

There are many methods that have been developed for detecting mutations in both RNA and DNA. These methods can be classified into two broad categories: (a) the screening for unknown mutations; and (b) the detection of known mutations. Techniques for the screening of unknown mutations include denaturing gradient gel electrophoresis (DGGE) [1], heteroduplex polymorphism assay (HPA) [2], enzymatic and chemical cleavage [3,4], single strand conformation polymorphism analysis (SSCP) [5] and meta-stable single strand conformation polymorphism analysis (mSSCP) [6]. Methods used in the detection of known mutations include the amplification refractory mutation system-PCR (ARMS-PCR) [7], the ligase chain reaction (LCR) [8], PCR restriction fragment length polymorphism

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(PCR-RFLP) [9] and mini-sequencing or primer extension [10].

The ligase chain reaction involves a cyclical accumulation of the ligation products of two pairs of complementary oligonucleotide probes. The sequence of the probes is identical to the target sequence, fragmented as two double stranded segments. The amplification reaction is triggered by the presence of the proper target sequence. If the probe sequences perfectly match the target sequences, the probes will then hybridize in juxtaposition on each of the target segments and become ligated. LCR does not amplify copies of any genomic sequence other than that represented by the probes added, thus the product is entirely composed of ligated copies of the input probes. The entire process can be regarded as a detection reaction followed by signal amplification. The LCR principle is illustrated in Fig. 1.

Micro-fabrication technology has been applied to a range of analytical problems in recent years [11–17] and has found several applications in molecular biology. Various types of DNA analyses are possible using silicon chip-based devices and DNA amplification in silicon–glass chips has been reported [18–20]. Several groups are performing genome mapping and DNA sequencing by oligonucleotide array hybridization on either glass chips or silicon chips on which synthesized oligonucleotides have been immobilized [21,22]. In addition, antisense DNA oligonucleotides and restricted DNA fragments were separated and detected by planar chip capillary electrophoresis [23,24]. In this paper we report the first description of the LCR reaction of known point mutations in a micro-fabricated silicon–glass chip.

Capillary electrophoresis has been established as an important analytical tool due to its high resolving power, speed, automation and reproducibility. CE has been combined with various mutation detection methods such as HPA [25], constant denaturant capillary electrophoresis (CDCE) [26], ARMS-PCR [27], PCR-RFLP [28], and SSCP [29,30]. We also report here on the application of CE for the analysis of the LCR products amplified in silicon–glass chips compared to amplification in MicroAmp reaction tube. Further, we have evaluated the amplification efficiency in these macro and micro reaction vessels.

Our results suggest that a combination of the beneficial properties of chip LCR and CE, namely low sample volume (<1 μl) and high throughput, will be advantageous where the screening of a large number of DNA samples for mutations is required, such as in medical diagnostics and medical research. The miniaturization of the LCR reaction system is the initial step towards a fully integrated, chip-based, highly efficient, portable DNA mutation detection instrument where a chip CE will be used for detection.

2. Experimental

2.1. Silicon–glass chips

The silicon–glass chips were manufactured by Alberta Microelectronic Center (Edmonton, Canada) with standard photolithographic procedures (Fig. 2). The silicon–glass chips were anodically bonded as described previously [18].

2.2. LCR amplification reaction in silicon–glass chips

Separate LCR reactions were performed using the LCR kit from Stratagene (La Jolla, CA, USA). Individual silicon–glass chips were filled with 10 μl of the LCR mixture. The operational protocol for filling and emptying the chips was the same as that described previously [18]. Briefly, the LCR reaction mixture was introduced into the chip by pipetting it into the entry port on the chip and then applying a slight negative pressure to the exit port. During cycling, the two ports were sealed with small rubber washers mounted on the edge of the chip holder. The prototype thermal cycling device was designed in our laboratory and built by Faulkner Instruments (Pitman, NJ, USA). It incorporated a heater–cooler (9500/071/040; ITI Ferrotec, Chelmsford, MA, USA) centrally located under an oxygen-free copper block (40×40 mm) containing a 10-kΩ thermistor (YSI 44016; Yellow Springs Instruments, OH, USA) (Fig. 3). The heater–cooler and thermistor were connected to a modular laser diode controller (LDC-3900; ILX Lightwave, Bozeman, MT, USA), which was controlled remotely through an RS-232 interface by a virtual instrument built on LabVIEW for Windows (National Instruments, Austin, TX, USA).

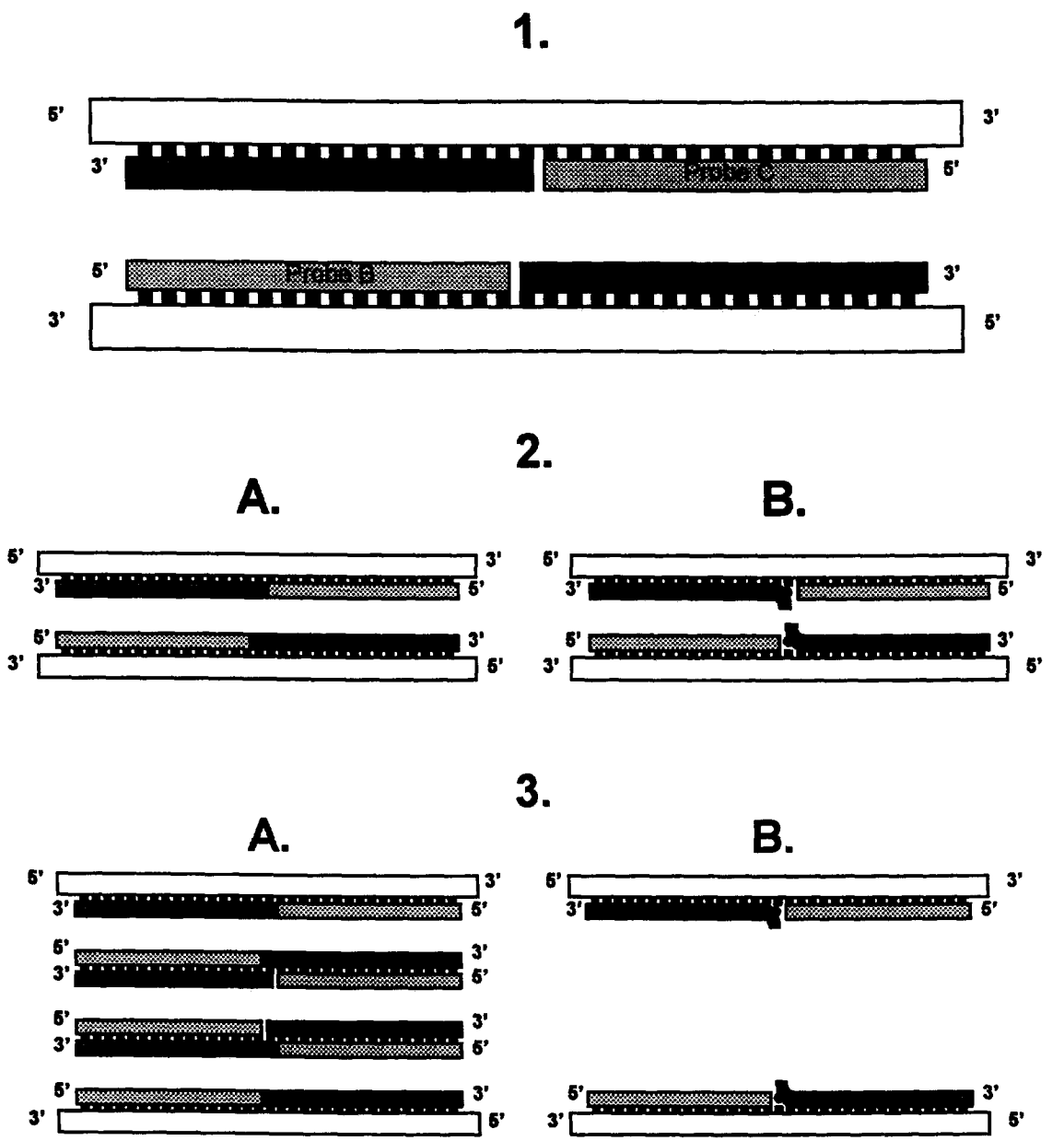

Fig. 1. Mechanisms of the ligase chain reaction. Stage 1: denaturation of double stranded DNA template at 94°C and probe annealing at 65°C. Stage 2: ligation of the probes with the thermal stable ligase at 65°C. A, ligation taking place between 3′ and 5′ termini of probes annealed with the wild type template DNA. B, no ligation occurs with mutant template DNA because annealed primer termini do not abut. Stage 3: products resulting from 20 to 30 repetitions of the thermal cycle.

The virtual instrument automated the thermal cycling. One complete cycle took 113 s. It was necessary to keep a constant air flow under the thermal cycling device in order to dissipate heat. The air flow was monitored by a flowmeter (Gilmont F-400; Gilmont Instruments, Barrington, IL, USA) and kept constant at approximate 40 $l\,min^{-1}$ to ensure consistent cooling.

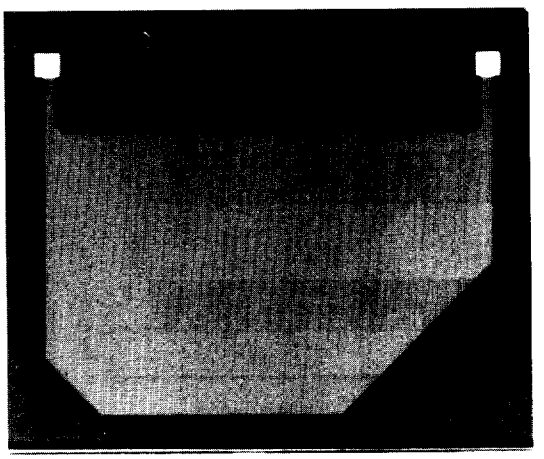

Fig. 2. The silicon–glass chip used for LCR reaction. The reaction chamber was etched to a depth of 80 μm with a total volume of about 10 μl. The linear dimensions of the chip are 17×15 mm.

The reaction mixture contained 1 μl Pfu LCR buffer, 4 ng of each of the four oligonucleotide probes (probe A: 5′-TTGTGCCACGCGGTTGGG-AATGTA-3′; probe B: 5′-AGCAACGACTGTTT-GCCCGCCAGTTG-3′; probe C: 5′-TACATT-CCCAACCGC-GTGGCACAAC-3′ and probe D: 5′-AACTGGCGGGCAA ACAGTCGTTGCT-3′) and 1.8 units of Pfu DNA ligase enzyme. The wild-type DNA template used as a positive control (120 pg) was a plasmid construct containing the pBluescript II phagemid and a normal *lacI* sequence. A separate mutant template (120 pg), containing a C to T transition mutation within the insert, was also run as a negative reaction control. A third reaction mixture, used as a second negative control, was created containing only salmon sperm DNA (200 ng) with zero annealing information. The amplification processes anticipated for the positive template and the mutant DNA template are shown in Fig. 1 (steps 2A and 3A and steps 2B and 3B, respectively). The thermal conditions used for LCR in chips were: one cycle 92°C/4 min and 60°C/3 min followed by 30 cycles of 92°C/20 s and 60°C/20 s.

2.3. LCR amplification reaction in MicroAmp reaction tubes

LCR reactions were performed using the LCR kit from Stratagene in a total mixture of 10 μl in individual MicroAmp reaction tubes (Perkin-Elmer, Norwalk, CT, USA). The reaction mixture contains 1 μl Pfu LCR buffer, 4 ng of each of the four

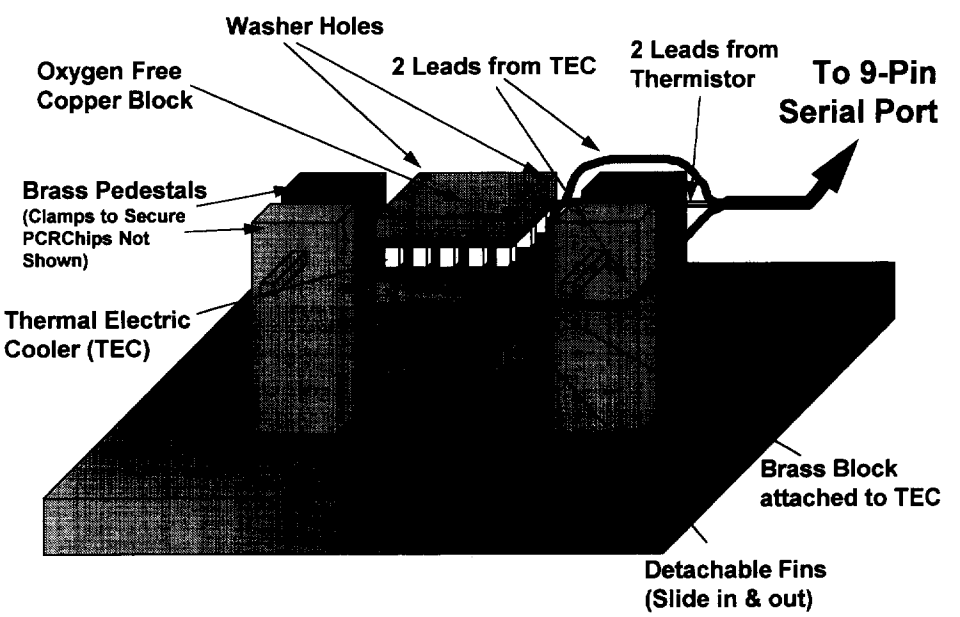

Fig. 3. An illustration of the custom-made chip thermal cycler. The Peltier is abbreviated by TEC (thermal electric cooler).

oligonucleotide probes (the sequence of the four probes were the same as that described above) and 1.8 units of Pfu DNA ligase enzyme. Wild-type template DNA (120 pg) was added to the above reaction mixture as sample with positive information. Mutant plasmid template (120 pg) was added to a second reaction mixture as negative control. The LCR was performed in a GeneAmp PCR System 9600 (Perkin-Elmer) with the thermal conditions used as follows: one cycle 92°C/4 min and 60°C/3 min followed by 30 cycles of 92°C/20 s and 60°C/20 s with the transition rate at maximum.

2.4. Capillary electrophoresis

Capillary electrophoresis was performed on the P/ACE system 5500 with a laser-induced fluorescence (LIF) detector (Beckman, Fullerton, CA, USA) in the reversed polarity mode (negative potential at the injection end of the capillary column). The excitation wavelength on the CE was fixed at 488 nm and the emission wavelength was 520 nm (both wavelengths were fixed by the manufacturer). The external temperature of the capillary column was set at 22°C. Before the analysis, 1 μl of each LCR sample was removed from the chip and diluted with 5 μl of 0.2 μM TOTO-1 (maximum excitation wavelength and emission wavelength was 513 nm and 533 nm) (Molecular Probes, Eugene, OR, USA) in 0.1× TE (10 mM Tris-base, 1 mM EDTA, pH 7.5) buffer. The samples were then placed in a 4°C refrigerator for 30 min to avoid the photodecomposition of TOTO-1 and also to provide enough time to allow the dye to intercalate into the DNA molecules. Sample injections were conducted at 5 kV for 25 s and the separation was performed at a field strength of 228 V/cm for 20 min. Post-run analysis of the data was performed using the Gold chromatography data system (Version 8.0). The buffer system used for separation consisted of 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA, pH 8.4, to which 0.5% (w/v) (hydroxypropyl)methyl-cellulose (HPMC) was added. The viscosity of a 2% aqueous solution for this cellulose derivative (H-7509, Sigma) was 4000 cP at 25°C. HPMC was dissolved in the buffer using the method recommended by Ulfelder et al. [28]. Afterwards, glycerol (5.0%, v/v) and fresh 0.2 μM TOTO-1 fluorescent dye was added to the buffer. The buffer was filtered using a 2.0-μm filter and then degassed for 15 min by sonication before use. Samples were analyzed on a surface modified fused-silica capillary DB-1 (J&W Scientific, Folsom, CA, USA). The capillary column (47 cm×100 μm I.D.) was conditioned with five volumes of distilled water followed by five volumes of separation buffer, and then subjected to voltage equilibration for 30 min until a stable base line was achieved. After each run the column was washed with acetonitrile for 30 s, with deionised water for 1 min, and then with separation buffer for 3 min.

3. Results and discussion

Analysis of LCR products from a positive reaction performed in the conventional tube format and in chips are shown in Fig. 4A and 4B. It can be seen that the chip LCR is efficient, and discrete ligation products identical in size to the tube LCR products are generated. It can also be seen that the amplified LCR products were separated from the redundant probes by CE and detected by LIF. The first two peaks represent the two pairs of reannealed double-stranded probes (ds-probes) (each approximately 25 bp in length), and the third peak reflects the double-stranded DNA fragment amplified by LCR (50 bp in length). The results were highly reproducible as the reaction was repeated four times in both tubes and chips and each sample was analyzed twice on the CE machine. Fig. 4C and Fig. 4D shows results of the CE analysis of micro-chip LCR reaction mixtures obtained from the mutant template DNA and the negative (non template) control with salmon sperm DNA, respectively. In both reactions only the two pairs of annealed ds-probes could be detected, indicating that chip LCR can demonstrate the same level of reaction specificity as the conventional tube LCR. The yields of the amplified products from the chips were lower than those obtained using a conventional tube LCR (compare Fig. 4A with B). The yield of LCR product from the chip (Fig. 4B) was approximately 44% of that from the tube based on the integrated peak areas. This lower yield may be attributable to the non-specific binding of both the template DNA and the ligase enzyme onto the chip

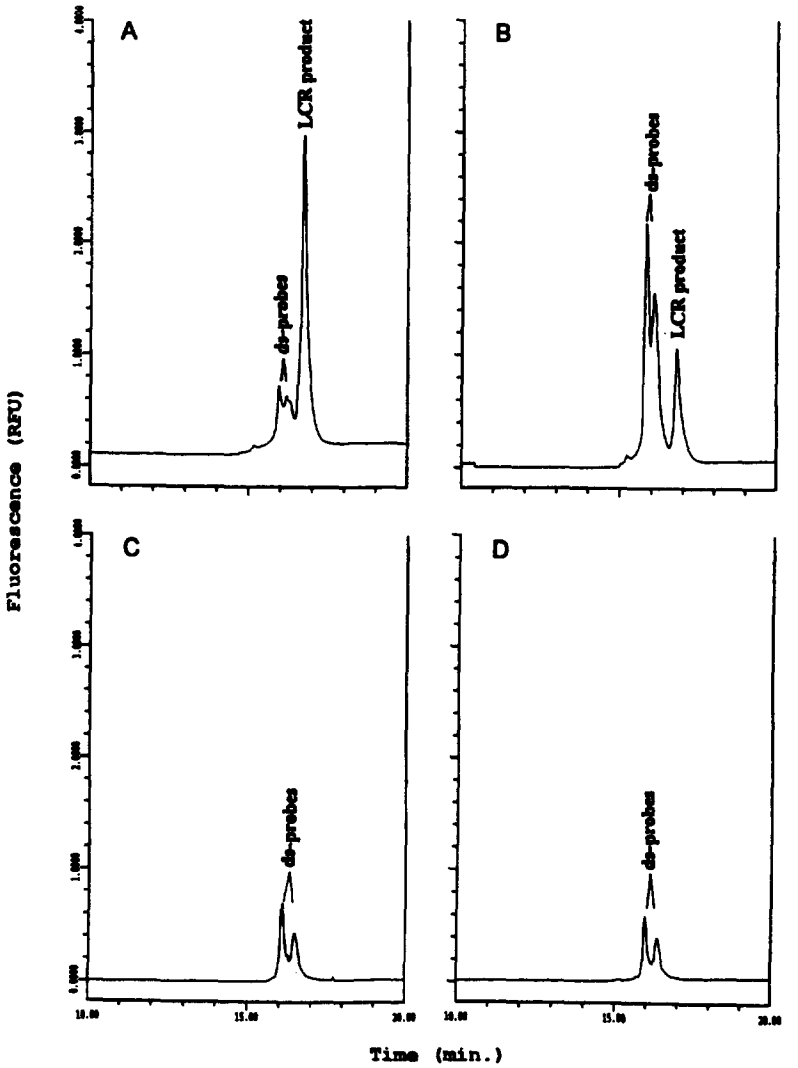

Fig. 4. Electropherograms of the amplification samples from LCR reaction. The LCR products were separated by CE in an inverted mode and detected using LIF. (A) Positive amplification sample from wild-type DNA where the reaction took place inside the MicroAmp reaction tube. (B) Positive amplification sample from wild-type DNA template from the silicon–glass chip. (C) Negative amplification product from the mutant DNA template from the silicon–glass chip. (D) Negative control where there is no template DNA present from the silicon–glass chip. Samples were electrokinetically injected at 5 kV for 25 s. The separation was completed in a DB-1 coated capillary column.

surface and further studies are under going to improve the amplification efficiency.

LCR amplification products were separated using an CE system similar to that developed previously for DNA mutation analysis [25,30]. The mechanism for the improved separation by the CE buffer is that the glycerol additive increases the buffer viscosity, and may help to reduce the pore size of the entangled network by covalently reacting with HPMC [31]. Separation of LCR products by CE provides an efficient means for detecting the amplification products. An analysis of an LCR sample can be completed in less than 20 min, whereas an analysis using conventional gel electrophoresis may take several hours. Moreover, band diffusion is always a problem for separating short LCR fragments with slab gel

electrophoresis mainly because of insufficient dissipation of Joule heating. In contrast, the Joule heat is effectively dissipated through the thin wall of the capillary in CE, and band diffusion is reduced to a minimum, as can be seen in Fig. 4.

Capillary electrophoresis combined with LIF detection does not require large amounts of sample for analysis. Usually, less than 1 μl of the amplified LCR reaction mixture is sufficient, compared to the 10 μl of sample needed for slab gel electrophoresis. The geometry of the chip (see Fig. 2) was a modification of the previous PCR chip [18]. The new design increases the ease of filling and empting the chip and reduces the entrapment of air bubbles within the chip. The volume of the silicon–glass chip reaction chamber can be further reduced to between 1 μl and 4 μl permitting an even higher throughput in sample processing. These smaller volumes of LCR product could be readily detected and analyzed by the CE–LIF system.

4. Conclusion

The product yield of the LCR reaction achieved in the chip and in the MicroAmp reaction tube are comparable, bringing close the possibility of miniaturization of reactions for point mutation detection. CE has the advantage of automation (20 to 50 samples each batch), fast analysis (20 min compared to the 2-4 h slab gel methods), minimal sample band diffusion, and low consumption of both sample (a few nanoliters) and chemical reagents (a few milliliters) compared to the conventional gel electrophoresis methods. It eliminates the use of radioisotopes and labor-intensive procedures. Further investigation is under way to integrate the LCR chip and the CE chip for the development of a portable DNA mutation detection device.

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References

[1] E.S. Abrams, S.E. Murdaugh and L. Lerman, Genomics, 7 (1990) 463.
[2] M.B. White, M. Carvalho, S. O'Brien, D. Derse and M. Dean, Genomics, 12 (1992) 301.
[3] R.M. Myers, Z. Larin and T. Maniatis, Science, 230 (1985) 1242.
[4] R.G.H. Cotton, N.R. Rodrigues and D.R. Campbell, Proc. Natl. Acad. Sci. USA, 85 (1988) 4397.
[5] M. Orita, Y. Suzuki, T. Sekiya and K. Hayashi, Genomics, 5 (1989) 874.
[6] T. Kasuga, J. Cheng and K.R. Mitchelson, PCR Methods Appl., 4 (1995) 227.
[7] C.R. Newton, A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith and A.F. Markham, Nucleic Acids Res., 17 (1989) 2503.
[8] F. Barany, Proc. Natl. Acad. Sci. USA, 88 (1991) 189.
[9] U. Landegren, Genet. Anal. Tech. Appl., 9 (1992) 3.
[10] L. Ugozzoli, J.M. Wahlqvist, A. Ehsani, B.E. Kaplan and R.B. Wallace, Genet. Anal. Tech. Appl., 9 (1992) 107.
[11] R.F. Service, Science, 268 (1995) 26.
[12] A. Manz, D.J. Harrison, E. Verpoorte and H.M. Widmer, in P.R. Brown and E. Grushka (Editors), Progress in Chromatography, Marcel Dekker, New York, 1993, p. 1.
[13] P. Wilding, D. Nozak and L.J. Kricka, J. Int. Fed. Clin. Chem., 6 (1994) 54.
[14] L.J. Kricka and P. Wilding, in C.P. Price and D.J. Newman (Editors), Principles and Practice of Immunoassay, 2nd ed., 1996, Macmillan Press Reference Books, London, in press.
[15] L.J. Kricka, X. Ji, O. Nozaki and P. Wilding, J. Biolumin. Chemilumin., 9 (1994) 135.
[16] L.J. Kricka, I. Faro, S. Heyner, W.T. Garside, G. Fitzpatrick and P. Wilding, Clin. Chem., 41 (1995) 1358.
[17] A. Manz, Y. Miyahara, J. Miura, Y. Watanabe, H. Miyagi and K. Sato, Sensors Actuators, B1 (1990) 249.
[18] P. Wilding, M.A. Shoffner and L.J. Kricka, Clin. Chem., 40 (1994) 1815.
[19] P. Wilding, M.A. Shoffner, J. Cheng, G. Hvichia and L.J. Kricka, Clin. Chem., 41 (1995) 1367.
[20] M.A. Northrup, C. Gonzalez, S. Lehew and R. Hills, in A. van den Berg and P. Bergveld (editors), Micro Total Analysis Systems, Kluwer Academic Publishers, Dordrecht, 1994, p. 139.
[21] R. Drmanac, S. Drmanac, Z. Strezoska, T. Paunesku, I. Labat, M. Zeremski, J. Snoddy, W.K. Funkhouser, B. Koop, L. Hood and R. Crkvenjakov, Science, 260 (1993) 1649.
[22] K.L. Beattie, W.G. Beattie, L. Meng, S.L. Turner, R. Coral-Vazquez, D.D. Smith, P.M. McIntyre and D.D. Dao, Clin. Chem., 41 (1995) 700.

[23] C.S. Effenhauser, A. Paulus, A. Manz and H.M. Widmer, Anal. Chem., 66 (1994) 2949.
[24] A.T. Woolley and R.A. Mathies, Proc. Natl. Acad. Sci. USA, 91 (1994) 11348.
[25] J. Cheng, T. Kasuga and K.R. Mitchelson, E.R.T. Lightly, N.D. Watson, W.J. Martin and D. Atkinson, J. Chromatogr. A, 677 (1994) 169.
[26] O. Khrapko, J.S. Hanekamp, W.G. Thilly, A. Belenkii, F. Foret and B.L. Karger, Nucleic Acids Res., 22 (1994) 364.
[27] H. Arakawa, K. Uetanaka, M. Maeda and A. Tsuji, J. Chromatogr. A, 664 (1994) 89.
[28] K.J. Ulfelder, H.E. Schwartz, J.M. Hall and F.J. Sunzeri, Anal. Biochem., 200 (1992) 260.
[29] A.W.H.M. Kuypers, P.M.W. Willems, M.J. van der Schans, P.C.M. Linssen, H.M.C. Wessels, C.H.M.M. de Bruijn, F.M. Everaerts and E.J.B.M. Mensink, J. Chromatogr., 621 (1993) 149.
[30] J. Cheng, T. Kasuga, N.D. Watson and K.R. Mitchelson, J. Cap. Elec., 2 (1995) 24.
[31] J. Cheng and K.R. Mitchelson, Anal. Chem., 66 (1994) 4210.