RECENT DEVELOPMENTS IN MINIATURIZED PCR-MICROCHIPS, MICROARRAYS AND MICRODROPLETS

Larry J Kricka¹, Eleanor S Pollak¹, Paolo Fortina²
¹Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
²Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107

Corresponding Author:
Larry J Kricka
Department of Pathology and Laboratory Medicine
University of Pennsylvania School of Medicine Philadelphia
PA 19104, USA
Tel.: 215 662 6575
Fax: 215 662 7529
e-mail kricka@mail.med.upenn.edu

ABSTRACT
Microminiaturization of assays and lab-on-a-chip devices hold considerable promise for the future of analysis, especially in point-of-care testing. This article focuses on developments that have occurred during the last five years in the specific area of microchip PCR and miniaturized PCR in arrays of reaction vessels and droplets. Although, this area continues to be an active focus of research and development and the variety and ingenuity of microchip PCR and integrated microchip PCR devices continue to increase, commercialization lags behind the progress being made in digital PCR and arrays for real-time PCR.

Key-words: PCR, RT-PCR, digital PCR, microchip, lab-on-a-chip, genetic testing, miniaturization

INTRODUCTION
PCR performed at a miniaturized scale in µL-nL volumes continue to attract the attention of the research community; a diverse range of “chip”, “card” and “array” devices have been created. This article focuses on developments that have occurred in this field during the last five years. A number of recent reviews have surveyed different aspects of miniaturized PCR and PCR microchips (1-4). More extensive coverage of the earlier literature on these types of analytical devices is presented elsewhere (5-7).

PCR MICROCHIPS
Chip fabrication materials and design - The first PCR microchips were fabricated from silicon and glass (8), but subsequently other types of material, especially polymers have been evaluated. This has been driven in part by issues concerning both ease of fabrication and production cost. In this context polyester has been utilized for PCR microchip fabrication by laser-printing of toner onto polyester films (9). This technique produces microfluidic devices with channel depths on the order of tens of micrometers. Deeper channels (e.g., 270 µm) can be produced using a CO2 laser to cut microchannels in a uniform layer of printed toner coated onto polyester sheets. The final chip is constructed by sandwiching the channel layer between uncoated cover sheets of polyester in which access holes have been precut. This type of chip has been used to perform solid phase extraction of DNA from blood (>65% recovery) and amplify a 520 bp fragment of lambda-phage DNA. The low thermal conductivity of glass or polymers used to fabricate microchips limits both heat transfer rates and the speed of thermal cycling. Reducing the volume of a PCR reaction chamber can improve the speed of thermal cycling, but this strategy is not suited to analyzing samples with low analyte concentrations. Faster thermal transitions can be achieved by providing a heat
sink. In the context of glass chips, a heat sink component has been shown to substantially reduce thermal resistance opposing heat dissipation into the ambient environment, and eliminates the parasitic thermal capacitance of other microchip regions that do not need to be heated (10).

On-chip components and integration - The scope of on-chip integration continues to expand and includes chips that combine solid phase extraction and PCR (~500 nL reaction chamber) (11); template purification, polymerase chain reaction (250 nL), post-PCR cleanup and inline injection, and capillary electrophoresis (CE) (12); cell lysis, DNA binding, washing, elution and PCR in the same reaction chamber (13); DNA extraction and PCR (14-16); and cell pre-concentration (via immunomagnetic beads), purification, PCR (100 nL), and capillary electrophoretic analysis (17,18).

Cell isolation is often required as part of an integrated PCR microchip assay and different strategies have been implemented (19) such as bead capture (20) and electrokinetic capture (21). Another option is to use on-chip electrokinetic capture (22). This has been accomplished on a chip by fabricating two interconnected chambers each containing electrodes. The first set of electrodes inside a larger chamber (0.6 µL) diverts bacterial cells from a flowing stream into a smaller chamber (0.4 nL) that contains interdigitated electrodes that actively trap and concentrate the bacterial cells using dielectrophoresis.

One strategy for microchip PCR has been to have separate zones for each of the steps in a PCR reaction (melting, annealing, extension) and move the reaction mixture between zones. However, this ultimately becomes a design challenge. A closed-loop ferrofluid-driven PCR chip provides an elegant solution to fluid movement in this type of multi-zone chip (23). In this prototype, the PCR reaction mixture was contained in a circular closed microchannel in a polymethyl methacrylate chip. Flow of the reaction mixture was driven by the magnetic force generated by an external magnet through a small oil-based ferrofluid plug and amplification of genetically modified soy or maize was achieved in <13 minutes.

Another aspect of integration is to store reagents on a chip. This has been achieved in a PCR chip by means of just-in-time release reagents, paraffin-passivated, dry reagents (24). The paraffin protects the stored reagents during storage in the chip and during the sample preparation phase of the assay. In the final analytic phase, the PCR chamber is filled with target. Heating the PCR chamber to the denaturation temperature melts the paraffin and releases the reagents that then rehydrate in the target DNA solution and subsequently initiate PCR. A benefit of this strategy is that it reduces the number of analytical operations and simplifies the flow control on the chip.

Nanotechnology

Nanotechnology and the applications of nanofabricated objects have assumed considerable importance in many branches of science (25). Nanotechnology has also found application in PCR microchips (26-31).

Poly(quaternary ammonium)-modified gold nanoparticles have been used for efficient on-chip cell lysis prior to microchip PCR for the rapid detection of bacteria (3). The nanoparticles remain in the PCR solution thus facilitating integration of cell lysis and PCR as both processes are accomplished in the same reaction chamber; however, one problem was PCR inhibition caused by the gold nanoparticles which was eventually overcome by treating the PCR chamber with 1-10% BSA and increasing the annealing temperature. Gold nanorods can also be used to lyse cells for one step DNA extraction and real-time PCR of pathogens in a single chamber (32). The longitudinal resonance of gold nanorods transforms near infrared energy into thermal energy within a chip, and the heat generated causes cell lysis.

Another application of nanotechnology in the context of PCR microchips has been the development of a magnetic nanoparticle-based microheater embedded in polydimethylsiloxane (PDMS) microchips (29). Heat generated by the microheater could be controlled by the number of embedded particles and by the intensity of the applied AC magnetic field. This new type of chip with an embedded magnetic nanoparticle-based heater was shown to amplify a 732 bp target DNA with good efficiency (>90%) compared to a conventional PCR thermocycler.

Types of amplification reaction

Real time PCR - One target of microchip research has been the development of small portable or handheld instruments for point-of-care DNA analysis that could be used for medical diagnosis or environmental monitoring. To this end an electrochemical real-time PCR has been implemented on a silicon-glass microchip for simultaneous DNA amplification and detection (33). The onset thermal cycle at which the analytical signal became distinguishable from the background, was found to be much lower for the electrochemical real-time PCR compared to a fluorescence-based counterpart (template DNA 3 x 106 copies/µL).

A two-step ultra-rapid real-time (URRT) PCR has been described using a commercial silicon microchip system, the GenSpector TMC-1000 (34). Rapid detection of Enterohemorrhagic E. coli was accomplished in a chip with a 6 µL total reaction volume using 1 sec denaturation and 3 sec combined annealing/extension steps. The STX2 gene target of the Enterohemorrhagic E. coli was detected in ~7 minutes including melting point analysis (detection limit ~3 cfu/PCR) (35).

Reverse transcription PCR (RT-PCR) - RT-PCR in an integrated microdevice has also been developed for single-cell gene expression analysis. The microchip comprises integrated nanoliter metering pumps, a 200-nL RT-PCR reactor with a pad for single-cell capture, and an affinity capture matrix for the purification and concentration of products. The latter is coupled to a microfabricated capillary electrophoresis separation channel for analysis of the product. This microchip was successfully used to measure siRNA knockdown of the GAPDH gene in single Jurkat cells (26).
Point-of-care testing (POCT) detection of human immunodeficiency virus (HIV) has also motivated development of microchips for RT-PCR and an associated analyzer (36). This was achieved using a polymer lab-on-a-chip device coupled with a portable analyzer that provides non-contact infrared-based temperature control and chemiluminescence detection. Analysis of HIV (primer sets for p24 and gp120) with this system was completed in < 1 hour.

Similarly, development of a low cost (~$1000 in component costs), portable and integrated microfluidic instrument has been the motivation for other microchip-based RT-PCR devices (37). Reactions were performed in a tri-layered glass-PDMS microchip that consists of integrated pneumatically-actuated valves and pumps, a thin-film resistive combined heater and temperature sensor, and channels for capillary electrophoresis. The chip fits onto a platform that houses a laser diode and a charged coupled device (CCD) camera, circuitry for thermal control, and mini-pumps to operate the on-chip pumps and valves.

### MINIATURIZED PCR ON ARRAYS AND IN DROPLETS

One route to real-time PCR miniaturization has been to design cards that contain arrays of microwells linked to sample application reservoirs (e.g., TaqMan® Array Card, 384 wells, Applied Biosystems, Foster City, CA) (38). The Fluidigm Digital Array (Fluidigm Corp, South San Francisco, CA) integrated fluidic circuit is another example of a device that partitions a PCR reaction mix into hundreds of individual PCR reactions in order to perform “digital PCR” (39-41). These devices have an on-chip network of microfluidic channels, chambers, and valves that automatically assemble individual PCR reactions ranging from 2304 to 39,960 reactions per device in volumes ranging from 0.85 - 10 nL. Partitioning of PCR reaction mixtures localizes individual nucleic acid molecules in separate regions so that each location will contain either zero molecules or one molecule, i.e., a negative or positive reaction. Quantification of target nucleic acid is then simply achieved by counting the positive regions (42).

Droplets of emulsified PCR reaction mixtures have become a popular choice for miniaturizing PCR to perform digital PCR. For example, the BioRad QX100 Droplet Digital PCR system (Bio-Rad Laboratories Inc, Hercules, CA) produces ~20,000 monodisperse droplets from a 20 µL sample of PCR reaction mixture (43). The RainDance system (RainDance Technologies, Inc., Lexington, MA) produces up to 80 million partitions per run with a volume of only 5 pl per partition (44). Another approach is the BEAMing (Beads, Emulsions, Amplification, and Magnetics) Technology (Inostics Inc., Baltimore, MD) (http://www.inostics.com) (45, 46). This performs single-molecule PCRs on magnetic beads in an emulsion droplet (average diameter of emulsion compartment 5 µm).

### CONCLUSIONS

Miniaturization of PCR reactions, especially in emulsion droplets, has progressed rapidly in order to reap the benefits of digital PCR and several commercial systems are available. However, commercial development of PCR lab-on-a-chip devices has not kept pace with research and development activities in this area and the full potential of lab-on-a-chip devices for PCR-based analysis remains in the future. Thus, the question remains not if but when this future will materialize.

### References

1. Arora A, Simone G, Salieb-Beugelaar GB, Kim JT, Manz A. Latest developments in micro total analysis systems. Anal Chem 2010; 82(12): 4830-47
2. Njoroge SK, Chen HW, Witek MA, Soper SA. (2011) Integrated microfluidic systems for DNA analysis. Top Curr Chem 2011; 304:203-60
3. Wan W, Yeow JT. Integration of nanoparticle cell lysis and microchip PCR for one-step rapid detection of bacteria. Biomed Microdevices 2012; 14(2): 337-46
4. Neuži P, Gieselbrecht S, Länge K, Huang TJ, Manz A. Revisiting lab-on-a-chip technology for drug discovery. Nat Rev Drug Discov 2012; 11(8): 620-32
5. Rios A, Zougagh M, Avila M. Miniaturization through lab-on-a-chip: Utopia or reality for routine laboratories? A review. Anal Chim Acta 2012; 740: 1-11
6. Kricka LJ, Wilding P. Microchip PCR Anal Bioanal Chem 2003; 377(5): 820-5
7. Anonymous. Lab on Chip PCR - LOC PCR. www.gene-quantification.de/lab-on-chip.html (Accessed August 2012).
8. Zhang C, Xing D, Li Y. Micropumps, microvalves, and micromixers within PCR microfluidic chips: Advances and trends. Biotechnol Adv 2007; 25(5): 483-514 (Review).
9. Duarte GR, Price CW, Augustine BH, Carrilho E, Landers JP. Dynamic solid phase DNA extraction and PCR amplification in polyester-toner based microchip. Anal Chem 2011; 83(13): 5182-9
10. Hoang VN, Kaigala GV, Atrashov A, Pilarski LM, Backhouse CJ. Strategies for enhancing the speed and integration of microchip genetic amplification. Electrophoresis 2008; 29(23): 4684-94
11. Hagan KA, Reedy CR, Bienvenue JM, Dewald AH, Landers JP. A valveless microfluidic device for integrated solid phase extraction and polymerase chain reaction for short tandem repeat (STR) analysis. Analyst 2011; 136(9): 1928-37
12. Liu P, Li X, Greenspoon SA, Scherer JR, Mathies RA. Integrated DNA purification, PCR, sample cleanup, and capillary electrophoresis microchip for forensic human identification. Lab chip 2011; 11(6): 1041-8
13. Zhang Y, Park S, Yang S, Wang TH. An all-in-one microfluidic device for parallel DNA extraction and gene analysis. Biomed Microdevices 2010; 12(6): 1043-9.

14. Bienveneu JM, Legendre LA, Ferrance JP, Landers JP. An integrated microfluidic device for DNA purification and PCR amplification of STR fragments. Forensic Sci Int Genet 2010; 4(3): 178-86.

15. Pan X, Jiang L, Liu K, Lin B, Qin J. A microfluidic device integrated with multichamber polymerase chain reaction and multichannel separation for genetic analysis. Anal Chim Acta 2010; 674(1): 110-5.

16. Reedy CR, Hagan KA, Marchiarullo DJ, Dewald AH, Barron A, Bienveneu JM, Landers JP. A modular microfluidic system for deoxyribonucleic acid identification by short tandem repeat analysis. Anal Chim Acta 2011; 687(2): 150-8.

17. Beyor N, Yi L, Seo TS, Mathies RA. Integrated capture, concentration, polymerase chain reaction, and capillary electrophoretic analysis of pathogens on a chip. Anal Chem 2009; 81(9): 3523-8.

18. Thaitrong N, Toriello NM, Del Bueno N, Mathies RA. Polymerase chain reaction- capillary electrophoresis genetic analysis microdevice with in-line affinity capture sample injection. Anal Chem 2009; 81(4): 1371-7.

19. Lindström S, Andersson-Svahn H. Overview of single-cell analyses: microdevices and applications. Lab Chip 2010; 10(24): 3363-72 (Review).

20. Karle M, Miwa J, Czilwik G, Auwärter V, Roth G, Zengerle R, von Stetten F. Continuous microfluidic DNA extraction using phase-transfer magnetophoresis. Lab Chip 2010; 10(23): 3284-90.

21. Bhattacharya S, Salamat S, Morissette D, Banada P, Akin D, Liu YS, Bhunia AK, Ladisch M, Bashir R. PCR-based detection in a micro-fabricated platform. Lab chip 2008; 8(7): 1130-6.

22. Dharmasiri U, Njoroge SK, Wetke MA, Adebiyi MG, Kamande JW, Hupert ML, Barany F, Soper SA. High-throughput selection, enumeration, electrokinetic manipulation, and molecular profiling of low-abundance circulating tumor cells using a microfluidic system. Anal Chem 2011; 83(6): 2301-9.

23. Sun Y, Kwok YC, Foo-Peng Lee P, Nguyen NT. Rapid amplification of genetically modified organisms using a circular ferrofluid-driven PCR microchip. Anal Bioanal Chem 2009; 394(5): 1505-8.

24. Kim J, Byun D, Mauk MG, Bau HH. A disposable, self-contained PCR chip. Lab chip 2009; 9(4): 606-12.

25. Grodzinski P, Ward W, Liu R, Scott K, Sutura S, Fortina P. The use of microelectronic-based techniques in molecular diagnostic assays. In: “Molecular Diagnostic Techniques” Ansorge W, Patrinos GP, eds., chapter 2, pp. 349-363, 2nd edition. Academic Press, Elsevier Science, New York, NY, 2009.

26. Toriello NM, Douglas ES, Thaitrong N, Hsiao SC, Francis MB, Bertozzi CR, Mathies RA. Integrated microfluidic bioprocessor for single-cell gene expression analysis. Proc Natl Acad Sci USA 2008; 105(51): 20173-8.

27. Jain KK. Personalized clinical laboratory diagnostics. Adv Clin Chem 2009; 47: 95-119.

28. Fournier-Wirth C, Coste J. Nanotechnologies for pathogen detection: Future alternatives? Biologicals 2010; 38(1): 9-13.

29. Kim JA, Lee SH, Park H, Kim JH, Park TH. Microheater based on magnetic nanoparticle embedded PDMS. Nanotechnology 2010; 21(16): 165102.

30. Chen P, Pan D, Fan C, Chen J, Huang K, Wang D, Zhang H, Li Y, Feng G, Liang P, He L, Shi Y. Gold nanoparticles for high-throughput genotyping of long-range haplotypes. Nat Nanotechnol 2011; 6(10): 639-44.

31. Sun Y, Dhumpa R, Bang DD, Høgberg J, Handberg K, Wolff A. A lab-on-a-chip device for rapid identification of avian influenza viral RNA by solid-phase PCR. Lab Chip 2011; 11(8): 1457-63.

32. Cheong KH, Yi DK, Lee JG, Park JM, Kim MJ, Edel JB, Ko C. Gold nanoparticles for one step DNA extraction and real-time PCR of pathogens in a single chamber. Lab chip 2008; 8(5): 810-3.

33. Yeung SS, Lee TM, Hsing JM. Electrochemistry-based real-time PCR on a microchip. Anal Chem 2008; 80(23): 8975-81.

34. Goodgene. Genespector TMC-100. http://www.goodgene.co.kr/eng/html/products/medicine_genespector.php (Accessed August 2012)

35. Dinhson BJ, Koppes NM, Kwan SH, Cho SH, Yoo BS, Han SH, Yoon BS. Rapid detection of virulence STX2 gene of Enterohemorrhagic Escherichia coli using two-step ultra-rapid real-time PCR. Biotechnol Lett 2010; 32(5): 681-8.

36. Lee SH, Kim SW, Kang JY, Ahn CH. A polymer lab-on-a-chip for reverse transcription (RT)-PCR based point-of-care clinical diagnostics. Lab chip 2008;8(12): 2121-7.

37. Kaigala GV, Hoang VN, Sticker A, Lauzon J, Manage D, Pilarski LM, Backhouse CJ. An inexpensive and portable microchip-based platform for integrated RT-PCR and capillary electrophoresis. Analyst 2008; 133(3): 331-8.

38. Rachwal PA, Rose HL, Cox V, Lukaszewski RA, Murch AL, Weller SA. The potential of TaqMan Array Cards for detection of multiple biological agents by real-time PCR. PLoS One 2012;7(4): e35971.

39. Fluidigm. Copy Number Variation. http://www.fluidigm.com/copy-number-variation.html (Accessed August 2012).

40. Barley AJ, Mohr S, Day PJ. High-throughput droplet PCR. Methods 2010; 50(4):277-281.

41. Baker M. Digital PCR hits it stride. Nature Methods 2012; 9(6): 541-4.

42. Blow N. PCR’s next frontier. Nat Methods 2007; 4(10): 869-875.

43. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, Kitano TK, Hodel MR, Petersen JF, Wyatt PW, Stenblock ER, Shah PH, Bousie LJ, Troup CB, Mellen JC, Wittmann DK, Erndt NG, Cauley TH, Koehler RT, So AP, Dubre S, Rose KA, Montesclaros L, Wang S, Stumpe DP, Hodges SP, Romine S, Milanovich FP, White HE, Regan JF, Karlin-Neumann GA, Hindson CM, Saxovon S, Colston BW. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 2011; 83(22): 8604-10.

44. Kiss MM, Ortolona-Donnelly L, Beer NR, Warner J, Bailey CG, Colston BW, Rothberg JM, Link DR, Leamon JH. High-throughput quantitative polymerase chain reaction in picoliter droplets. Anal Chem 2008; 80(23): 8975-81.

45. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, Diaz LA Jr, Goodman SN, David KA, Juhi H, Kinzler KW, Vogelstein B. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci USA 2005; 102(45): 16368-73.

46. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods 2006;3(7): 551-9.