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Miniaturised nucleic acid analysis

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The application of micro total analysis systems has grown exponentially over the past few years, particularly diversifying in disciplines related to bioassays. The primary focus of this review is to detail recent new approaches to sample preparation, nucleic acid amplification and detection within microfluidic devices or at the microscale level. We also introduce some applications that have as yet to be explored in a miniaturised environment, but should benefit from improvements in analytical efficiency and functionality when transferred to planar-chip formats. The studies described in this review were published in commonly available journals as well as in the proceedings of three major conferences relevant to microfluidics (Micro Total Analysis Systems, Transducers and The Nanotechnology Conference and Trade Show). Although an emphasis has been placed on papers published since 2002, pertinent articles preceding this publication year have also been included.

Introduction

The polymerase chain reaction (PCR) has succeeded in revolutionising the analysis of nucleic acids. Disciplines both directly and indirectly related to the life sciences can readily exploit PCR, and with various genome study initiatives increasingly making available sequence data, this trend is set to continue into the future. Most striking has been the synonymous advent of real-time PCR and delivery of a robust quantitative gene measuring tool. However, the post-genome era brings with it new problems and exposes old fissures that have undermined the application of PCR. The scaling-up of pre-existing procedures for higher throughput would be inappropriate for continuous environmental monitoring and population studies. Indeed, when attempted this approach reveals the necessary requirement to integrate gene analysis into a process that serves best to eliminate both sampling and experimental error. Miniaturisation of PCR integrated bioassays potentially possesses all of the desired qualities and comes with additional benefits relating to sample tracking, contamination, and savings in finances and time. Long ignored issues relating to the meaningfulness of data are starting to be questioned. Biological matter is comprised from the unit of life, the cell, and gene analysis is portrayed in terms of gene abundance, presence, or absence, within a particular biological sample. Tissues are heterogeneous, in that many types of different cells co-exist within biological samples. However, most PCR experimental testing to date does not clearly relate the gene content measured to the contribution made by all specific cell types. Increasingly the need to analyse from the single cell is gaining in popularity, and once again the packaging and process offered by integrated miniaturised PCR offers great promise. Indeed, when considered alongside the potential to dissect the PCR process itself to understand more how to increase and maintain PCR efficiency, the miniaturisation of PCR and future PCR applications seem to become permanently entwined. The majority of developments in miniaturisation have stemmed from institutes with strong analytical, engineering, fabrication and optical specialisations. The influx of interdisciplinary interest offers distinct and critical benefits to the application driven life sciences. Therefore, to allow researchers from engineering and life science backgrounds to reflect on the recent progress and to help traverse interdisciplinary boundaries, we have compiled a concise review of papers based around nucleic acid assay miniaturisation and microfluidic PCR, and set these against a framework of biological applications. The reader is also directed to other reviews covering either a wide range of applications,[1,2] or specific fields such as DNA and protein analysis,[3] genomics and proteomics analysis[4] or molecular diagnostics.[5]

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Pierre-Alain Auroux.

Theoretical aspects

Description of the polymerase chain reaction (PCR)

The PCR is a three-step amplification process first introduced by Saiki and co-workers in 1985.[6] This in-vitro enzyme-mediated method facilitates the generation of nucleic acid sequences based on the choice of specific primers. During the first step—called denaturation—the hydrogen bonding stabilising the double stranded DNA template is broken to form two complementary single strands (see Fig. 1). In order to provide the energy necessary to break the bonding, this step is commonly performed at temperatures between 94 °C and 96 °C. The

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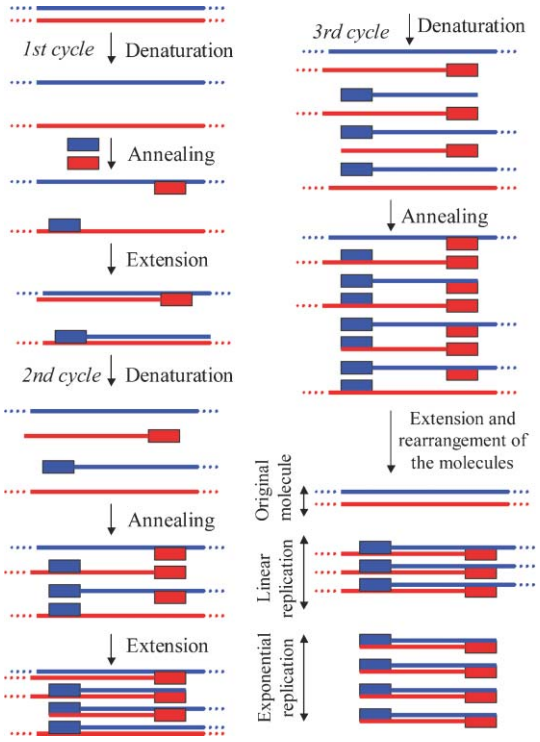

Fig. 1 Schematic description of the PCR process. A DNA strand is amplified during cycles composed of three steps: denaturation (80 °C–95 °C), annealing (50 °C to 65 °C) and extension (close to 72 °C). It is only after the 2nd cycle that a primer-specific molecule is generated.

temperature is then lowered for the annealing step: primers (synthetic sequences of single stranded DNA—normally 17–25 nucleotides long—which flank the target region to be amplified) specifically bind to the complementary sequences of the DNA template. At this stage, the temperature is primer-dependant and usually varies between 50 °C and 65 °C. Finally the temperature is raised to allow extension: the template is typically replicated by a thermostable DNA polymerase. This temperature is often close to 72 °C, which is related to the temperature optimum of the DNA polymerase in conjunction with retaining hybridisation specificity of primers. The denaturation-annealing-extension cycle is repeated, typically between 25 and 40 times. Although these three steps are performed at three different temperatures, it is not uncommon to combine the annealing and the extension into one operation. Interestingly, at the end of the 1st cycle, the product obtained is defined only at one end, as the replication is a function of time at the extension temperature. It is only at the end of the 2nd cycle that the specific product is first produced. Another interesting point is that two types of replication occur at the same time: semi-specific products such as those from cycle 1 lead to linear replication, whereas truly specific products or amplicons that are defined at both ends lead to exponential replication. Although the linear replication process is negligible in terms of amounts of DNA produced, it is a crucial intermediary step in PCR as it is through linear replication that a specific amplicon is obtained, thus rendering exponential amplification possible.

Since 1985, different parameters for the PCR have been extensively studied to optimise the reaction and maximise amplification yield throughout a number of constraints imposed by different bioassays. The extraction of a thermostable enzyme, *Taq*, from the bacterium *Thermus aquaticus* was the first breakthrough.[7–9] Prior to this discovery, the enzyme was heat labile and lost its activity after each denaturation step. Consequently, the polymerase had to be replenished after each cycle which was arduous, but importantly increased the risks of contamination during PCR as the sample vial had to be re-opened, and made optimisation almost impossible to achieve. The introduction of a thermostable enzyme obviated this cumbersome procedure and also enabled the automation of the process. Ensuing *Taq* polymerase engineering succeeded in enhancing processivity (which is defined for polymerases as the number of nucleotides added per unit to a strand during one sequence of binding and dissociation from the primer template) and fidelity (capability of a polymerase to faithfully replicate a DNA strand without expansion or deletion in the number of base pairs, nor substitution of the bases (AT ↔ CG)).[10] In this study, a recombinant *Taq* polymerase-thioredoxin displayed an increase in processivity when compared to the wild-type *Taq* polymerase (from 50–80 nt to >300 nt). Other enzymes such as *Pfu*,[11] KlenTaq,[12] *Vent*[13] or *Tfi*[14] have been applied to PCR. Significantly, some enzymes are nowadays modified to block their activity until they are heat activated. This is for example the case of PfuUltra™ Hotstart DNA polymerase, which is commercialised by Stratagene. In this instance, heat labile antibodies neutralise the enzyme activity until they are denaturated during the initial PCR denaturation step. Such a procedure limits the formation of non-specific products, as it impairs the enzyme activity until thermocycling temperatures are reached. Priming methods comparing poly(A) tails and random hexamers for cDNA synthesis were studied by Resuehr and co-workers,[15] while Halford *et al.* studied the effect of primer-dimer formation over the PCR yield.[16] Protocols to optimise PCR reactions are nowadays readily available.[17,18] PCR has also been studied from a more theoretical approach and statistical models describing the reaction are often based on the branching theory.[19] Based on this theory, the number of molecules generated by a PCR reaction starting with a single molecule after n cycles is: $(1 + p)^n$, where p is the probability for a molecule to duplicate. Such an equation leads to two consequences. Firstly, the value p is positively linked to the PCR yield. Secondly, for large values of p, the distribution of the DNA population becomes multinodal, presenting multiple probability peaks. This is due to the amplification during the initial cycle becoming critical and a failure for the original molecule to replicate having a lasting impact. It is consequently essential to optimise the PCR parameters in order to obtain the most favourable conditions and enhance the PCR yield.

Strand-displacement based amplification

Although PCR is the most popular DNA amplification technique, other methods also exist[20] and are potentially suitable for microfluidic-based devices. For example, S*ingle Strand Displacement Amplification* (SSDA) is an isothermal technique first introduced by Walker *et al.* in 1992.[21] Comparably to PCR, this method allows exponential amplification of double-stranded DNA. The *Multiple Strand Displacement Amplification* (MSDA) technique is similar to SSDA, with the only difference being that during extension of the complementary strand, new annealing sites are generated for the first primer. Although MSDA can be applied with specific primers, it has also been used with random hexamers, leading to the amplification of entire genomes.[22] Such an amplification process produces tree-like molecules leading to amplified molecules of different lengths. Indeed the generated strands are not cleaved from the strand molecule from which the amplification originated. A few products using this technique are commercially available, such as GenomiPhi™ DNA amplification offered by Amersham Biosciences, and REPLI-g™ from Molecular Staging. Notomi *et al.* developed another method for DNA amplification: *Loop-mediated isothermal AMPlification* (LAMP).[23] This technique is based on MSDA but instead of using a set of two specific primers, or random hexamers, it involves 3 sets of specific primers.

Other DNA amplification techniques

Lizardi and co-workers used circularisable oligonucleotide priming to investigate amplification of circular DNA templates.[24] This method, called *Rolling-Circle Amplification* (RCA), produces a single-stranded product initialised by only one primer. Although this technique might seem limited as it only applies to circular templates with an appropriate promoter, a wider variety of applications are technically possible as circular templates include viruses and plasmids. RCA has been coupled to other techniques such as MSDA,[24] or RCA itself (*Circle-to-Circle Amplification*).[25]

The *Ligase Chain reaction* was initially introduced by Landefren *et al.* in 1988 and is based on the ability of two oligonucleotides to hybridise next to each other given an appropriate complementary DNA target molecule.[26] Although this method was introduced as a single-base-modification detection method, it has since been used for the sole purpose of DNA replication.[27]

The merging of PCR with gene micro-array applications has been demonstrated by *Solid Phase DNA Amplification* (SPA).[28,29] In this method, the primers are surface-bound and thus do not freely diffuse in solution. A Monte Carlo Lattice model was proposed by Mercier and co-workers to discribe solid phase amplification.[19] As the amount of DNA generated is directly dependent on the number of primers immobilised at the surface, SPA would highly benefit from on-chip applications where area-to-volume ratios are greatly enhanced. The application of PCR to define specific sequences for subsequent use as micro-array probes has also recently been described by Greiner *et al.*[30]

Substrates used and surface treatments to minimise bio-molecule adsorption

Substrates

Although microfluidic devices were originally structured in silicon or glass, polymers such as poly(dimethylsiloxane) (PDMS), Poly(methylmethacrylate) (PMMA) and Polycarbonate (PC) have increasingly been used as alternative substrate materials. The search for new substrate materials is still a burgeoning area, and many recent microfluidic devices for PCR have been fabricated using SU-8, PEEK, PTFE, Cyclic-Olefin-Copolymer (COC) or Zeonor (polymer commonly used for compact discs and DVDs). For a more detailed overview of some of the materials used, the reader should refer to references in Table 1.

Surface treatment

The efficiency of biological reactions is often limited by interactions between the microchannel surfaces and the bio-molecules present in solution. This issue is even more exacerbated by the wide variety of possible substrate material (see Table 1) in conjunction with the vast range of biological matter used as analyte; consequently various surface treatments have been investigated. One of the most widely used processes to prevent on-chip adsorption of molecules consists of silanisation, a method first introduced by Hjertén in 1985.[58] For example, El-Ali *et al.* performed a gas phase silanisation of a SU-8 chamber surface in order to enhance its PCR compatibility.[44] Similarly, Shin and co-workers studied different silane compositions to decrease protein adsorption at glass surfaces.[59] Noteworthy is that even though silanisation has been successfully applied to microfluidic devices, criticisms regarding the reproducibility of such coatings have been argued.[60] To circumvent this issue, several methods have been investigated, and for instance, different strategies were implemented to prevent protein adsorption in PDMS microfluidic devices. Choi *et al.* favoured a mixture of poly(ethylene glycol) and poly(acrylic),[61] while Xiao and co-workers used a polyacrylamide gel.[49] Alternatively, Shin and co-workers coated PDMS micro-channels with parylene in order to prevent adsorption on the chip surface.[62] An additional approach was introduced when Kirby *et al.* studied the effect of polarity and surface charge on cell adsorption by controllably modifying the zeta potential at the surface of their device.[39]

Table 1 Examples of materials used for micro-chip fabrication

Material	References
Glass	31–33
Borofloat-glass	34–36
Fused silica	37, 38
Silica	39
Soda-lime glass	40
Silicon	41, 42
SU-8	43–45
Acrylic (PSA)	46
PDMS	47–49
PEEK	50
COC	51
PMMA	51
Polyimide	46
PTFE	52
Fluorocarbon film	53
PC	54
Diamond coated chip	55
Zeonor	56, 57

Sample preparation

The following section addresses recent studies in the areas of cell culturing, cell identification and cell trapping, cell lysis and DNA extraction within microfluidic formats.

Cell culture

Much attention has focussed on the transfer of cell culturing techniques to planar chip formats. For example, Grodrian *et al.* applied the principle of segmented flow on chip for parallel cell culture by separating culture media plugs with immiscible fluid.[63] Alternatively, Tan and co-workers recreated a tissue-like structure in a micro-fluidic environment using cell-matrix assemblies and biopolymers,[64] while Schaak *et al.* have performed cell culture in droplets.[65] To assess the viability of the cultured cells Goto and co-workers implemented a protocol to detect nitric oxide released by macrophages.[66]

Cell identification and cell trapping

Biological samples are most often present as a complex matrix. It is therefore of paramount importance to be able to differentiate cells or targets of interest from other biological material. Different approaches have been implemented on chip, where for example, Lin *et al.* introduced an in-vitro impedance measurement of cells to distinguish between abnormal cells and normal cells under different frequency domains.[67] Alternatively, Emmelkamp and co-workers chose an optical detection technique for sorting cells based on their intrinsic fluorescence signal within a microfluidic structure.[36]

After having detected the cell of interest, the next step is usually some form of isolation and concentration. To this extent, different techniques have been implemented on chip, such as dielectrophoresis (DEP). Fuchs and co-workers have used DEP to trap individual particles among a few thousand cells suspended in a liquid on a microelectronic chip.[68] Similarly, during a study of blood components and their inhibitory effects on PCR, Wolff and co-workers captured cells on electrodes by DEP while inhibitors were eluted by

washing.[69] An alternative to conventional DEP has been proposed by Lapizco-Encinas *et al.* and involved electrodeless DEP for cell trapping.[70] Capillary electrophoresis was also used by Park *et al.* to demonstrate the capture and subsequent release of DNA fragments within PEEK tubing.[50] Although DEP and electrophoresis are widely used techniques for cell trapping, other methods have also been investigated. For example, Denoual *et al.* presented a microfluidic device in which cells could be directed to cell traps by the fluid flow.[71] Lettieri and co-workers have also introduced a microfluidic device for performing controlled micro re-circulating flow capable of DNA trapping.[72] Two flows (an electroosmotic flow and a pressure-driven flow) were opposed and molecules were trapped by adjusting the different velocities. From another perspective Goubault *et al.* captured rare cells using magnetic filaments and adjusted the specific/non-specific discrimination ratio by optimising flow rates,[73] while Lu and co-workers presented a rapid organelle separation by free flow isoelectric focusing.[74] Electrophoresis has also been used to increase sample concentration, where Halle *et al.* introduced a microfluidic free-flow zone electrophoresis device to concentrate biological particles in buffer.[75]

Cell lysis and DNA extraction/manipulation

In order to extract the different nucleic acid analytes from within a cell, controlled lysis is usually performed. Although regularly achieved by heating the cell,[54,76,77] other lysis methods have been developed. For example, strong ultrasonic fields were applied by Tachibana *et al.*,[78] whereas Marmottant and co-workers presented an experiment during which gentle bubble oscillations were successfully applied to rupture lipid membranes.[79] Lu and co-workers also presented a miniaturised device for controlled cell lysis in order to release sub-cellular materials.[80] Chemical lysis was performed by both Schilling *et al.*,[81] and Hong and co-workers.[82]

Once cell lysis has been performed, the issue of DNA extraction can be addressed. For example, Reddy and co-workers presented an organic-aqueous liquid extraction on a micro-device in order to purify DNA obtained directly from cells.[83] On the other hand, Bienvenue *et al.* implemented a sol-gel technique to extract DNA.[34] Other groups have favoured more targeted approaches to isolate DNA from its medium. For example, Washizu and co-workers used electrostatic fields for direct DNA manipulations[84] and Mohanty *et al.* applied dielectrophoresis and electrical impedance spectroscopy for single cell manipulation within microfluidic systems.[85] Finally, Sundararjan and co-workers demonstrated immobilisation and programmable release of single DNA molecules by electrical heating.[86]

Microfluidic DNA amplification

Since its introduction PCR sustains a tremendous impact in several fields, including clinical diagnostics, medical sciences and forensics. However this technique presents several drawbacks. For example, the costs associated with each reaction are still quite high (mostly due to the price of the enzyme), even if current parallel processing has attenuated costs. It is also well documented that PCR is highly prone to contamination due to its efficiency and high sensitivity.[87,88] Additionally, a common advantage of miniaturised systems over macro apparatus is their reduced thermal mass: as they present less inertia to temperature changes ramping rates for heating or cooling are drastically increased, and this gives rise to more rapid gene amplification, and may present benefit for some of the conformation fluorescence resonance energy transfer (FRET) probes used in real-time PCR. Whilst several of the more successful and reproducible applications of PCR are manifested in qualitative analyses, increasingly the requirements of PCR testing are placing a burden on elucidating a robust means to accurate and precise gene quantitation.[89] For example, the definition of therapeutically significant disease thresholds could be used to more effectively treat infections or other diseases. Therefore the cumbersome processes that endure sample losses and sample degradation, ultimately compromise gene quantitation can be circumvented by employing closed μTAS.

Well-based amplification

A microfluidic approach to DNA amplification would present several advantages. Not only could system features easily be integrated to ease complex sample handling and tracking, but also possible risks of contamination would be reduced. Indeed several operations (such as sample preparation, PCR and DNA detection) could ultimately be integrated into one device. By rendering the system quasi-autonomous, interactions with the sample would be minimised and the number of possible sources of contamination would be greatly lessened. These attractive features explain why the miniaturisation of the PCR apparatus has been of a growing interest in the scientific community. The first on-chip PCR devices were presented by Northrup and co-workers in 1993[90] and Wilding *et al.* in 1994.[91] Both devices consisted of a silicon chip with a micro-well in which the sample was loaded (see Fig. 2). The entire chip was heated and cooled to provide the adequate thermocycling conditions.

Since its introduction, this model has been replicated by numerous groups, sometimes with major amendments. These include integration of electrophoretic product sizing on a monolithic device,[92] as well as optimisation of conditions to provide faster PCR (under 7 minutes),[93] single-molecule detection,[94] real-time applications[95] and multiplexing.[96,97] The use of micro-arrays[96,98,99] and drop-metering[100] provided these micro-devices with high throughput (more than 10,000 parallel reactions have been reported[101–104]) and better fluid-handling capabilities, whereas thermocycling conditions, such as temperature uniformity[105] and heat transfer optimisation,[106] were closely studied. A high versatility in terms of thermocycling conditions was shown by Kajiyama and co-workers when they presented independently-addressable wells.[107] In addition, a variety of applications were successfully demonstrated, such as ligase chain reaction[108] and isothermal nucleic acids amplification (NASBA).[109] There have only been few devices based on a well approach that differed significantly from the original design presented by Northrup. For example, Oda *et al.* addressed the invasive characteristics of heating elements conventionally used by replacing the heaters with a transparent contact-free heating technique based on infra-red wavelengths.[110] This device has since been developed further.[35,111] Pal and co-workers performed PCR using induction heating, another non-contact heating method.[112] In this paper the chips were mounted onto a ferrous ring, while the inductor was made of a copper wired wound around a ferrite core. By alternating the current going through the inductor, magnetic fields were produced. They then induced Eddy currents in the ferrous ring, which generated localised heat without any physical contact. In 2003, Krishnan *et al.*

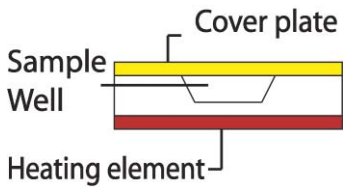

Fig. 2 Micro-well PCR. The sample is introduced into a sample well. The chip is then heated and cooled to provide thermocycling conditions.

presented an original device relying on convection to provide thermocycling conditions.[113] Two heaters sandwiched the Rayleigh-Bénard cell and were maintained at constant temperatures, namely 97 °C at the bottom and 61 °C at the top. Due to the difference in temperature, a convection flow developed in the cell, with an upward flux in the centre and downward fluxes on the sides. As the sample moved up and down in the cell, PCR conditions were provided and DNA was amplified. This device was later modified by Wheeler and co-workers.[114] It is to be noted that these devices are fairly flexible in terms of design: the well geometry can be tailored to a specific application, or arrays of wells are easily manufactured. However, they still do not take full advantage of lithographic techniques. For example, it would be difficult to implement efficient mixing structures. In addition, because the temperature of the entire chip needs to be altered for thermocycling, they display unnecessary inertia.

Continuous flow-through based amplification

A dynamic, moving-sample system particularly addresses previously described issues relating to full process integration. In the case of continuous-flow chips for example, transition times to change temperature depends only on the sample pumping rate and the time the sample needs to reach temperature equilibrium. Also heat inertia is reduced to a minimum because only the sample thermal mass needs to be taken into consideration rather than the chip plus sample as in the case of microwell PCR, with a direct consequence of enhanced rates of heating and cooling.

One approach to continuous flow-through based amplification was introduced in 1994 by Nakano and co-workers when they used capillary-based systems. Their concept consisted of a capillary going through three different oil baths in a loop.[115] The length of capillary in each bath determined the duration of the annealing, extension and denaturation steps. The sample was continuously pumped through the capillary unidirectionally and DNA amplification occurred as the sample was going through each temperature cycle. Based on a comparable concept, Curcio and co-workers performed a high-throughput DNA analysis system in a Teflon tube by separating different sample plugs with immiscible organic plugs.[116] A similar experiment was performed in a capillary by Park *et al.*[117] Friedman and co-workers proposed a static sample approach to nucleic acid amplification in 1998.[118] They enclosed the PCR sample inside a capillary, the ends of which were blocked by rubber pads. Each capillary was coated with a transparent Indium Tin Oxide (ITO) layer, ensuring that thermal cycling conditions could be independently controlled. Finally Chiou *et al.* presented a bi-directional sample pumping system.[119,120] In this device, the capillary was rested on three heating blocks and the sample was pumped back and forth to provide thermal cycling. An optical system stopped the pumping mechanism when the sample reached the desired temperature zone.

The first continuous-flow PCR chip presented in 1998 by Kopp and co-workers provided the basis for subsequent developments.[121] It consisted of a serpentine channel that passed through three heating zones maintained at constant temperature by copper blocks (see Fig. 3). Remarkably, specifically amplified products were detected after 20 cycles in 1.5 minutes.

Device improvements have included optimisation of the PCR conditions to prevent any cross-contamination during injection series,[122] modification of the design to combine RNA Reverse-Transcription and PCR[123] as well as a sensitivity study.[124] Applications such as high throughput and single molecule amplification[125] and infectious biological agent monitoring[126] have also been reported. Different approaches have been used to simulate the temperature distribution in continuous-flow PCR chips, such as finite element analysis,[127]

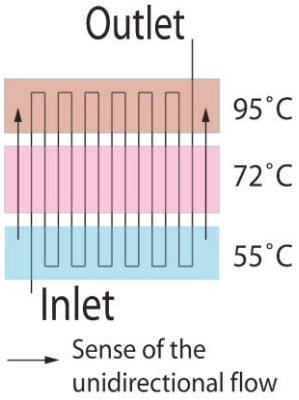

Fig. 3 Continuous-flow PCR. The sample is introduced at the inlet and pumped unidirectionally towards the outlet. Three heaters are maintained at constant temperature to provide adequate thermocycling conditions.

CFD-ACE+ (computational fluid dynamics and multi-physics software)[128,129] and Lattice Boltzmann simulations.[130] Although the continuous-flow approach allows for fast heating and cooling rates (thus improving product specificity) parallelisation is not easily applicable as it would significantly complicate the design and most likely increase the footprint of the PCR chip. In addition, this type of device is restrictive in terms of number of cycles: the number of meanders is decided during the design procedure and cannot be modified to increase the number of cycles once the chip is manufactured. However, Liu *et al.* circumvented this drawback by manufacturing a rotary device,[131] where a12nL-sample was introduced in the chip and subsequently pumped at a rate to enable 2–3 revolutions per minute. Integrated heaters provided the temperature zones necessary for thermocycling. Although the number of cycles could easily be adjusted (by changing the number of revolutions) the manufacturing process required additional and complex steps.

Novel approaches to DNA amplification

Although the majority of microfluidic devices for PCR utilise batch or continuous-flow strategies, new approaches for DNA amplification have started to emerge. For example, an on-chip sample-shunting technique has been introduced by Auroux *et al.*[43,132] This approach, based on shunting the sample back and forth over heating zones in a straight channel by applying alternate pressure, combines the cycling flexibility of the well-chips with the quick temperature transition of the continuous-flow PCR micro-structure (see Fig. 4).

A theoretical evaluation of a system based on this concept was provided by Bu and co-workers.[133] A sample-shunting device using ferrofluidics for actuators has also been introduced by Hardt *et al.*[134] From another perspective, Pollack and co-workers used an electrowetting process to control droplets containing PCR reagents and demonstrated that such fluid handling did not inhibit DNA amplification.[135]

A few approaches have also been developed on macro-scale apparatus and could also be of interest for microfluidic PCR. For example, high-speed PCR could be achieved on chip by using a microwave-assisted technique first demonstrated by Fermér *et al.*[136] DNA amplification in a water-in-oil emulsion, presented by Nakano *et al.*,[137] could be pushed to further limits thanks to the better fluid handling provided by micro-devices. In addition, since cells can be manipulated in a microfluidic environment (see section 'Sample Preparation'), another application could be *in situ* (meaning inside a cell) DNA amplification. This technique, first introduced in 1990 by Haase *et al.*,[138] has since been optimised[139] and has been combined to reverse transcription[140] and to LAMP

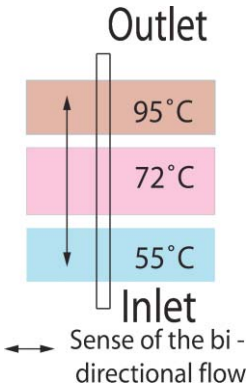

Fig. 4 Sample-shunting PCR. The sample is introduced in the inlet and pumped back and forth in a straight channel. Three heaters provide the necessary temperature zones.

amplification,[141] offering enhanced flexibility in terms of possible applications.

Inhibitors and enhancers

One of the main concerns when performing on-chip PCR is the loss in efficiency. Although this problem can be sometimes traced to inhibitory materials used during micro-device fabrication (*e.g.*, residual chromium from chrome masks in glass chip fabrication[142] or thermocouples[110]), the most predominant problem is the elevated adsorption of the PCR components onto the chip surface due to the increase of the surface-to-volume ratio in a micrometer-scale environment. For example, Erill and co-workers pinpointed that the component the most prone to adsorption was the *Taq* polymerase enzyme.[143] Although surface coating strategies such as silanisation are possible solutions (see section 'Surface treatment'), their effectiveness is sometimes questionable due to a lack of reproducibility.[60] A second approach, called *dynamic passivation*, includes passivating reagents directly in the PCR mixture. The most common additives are polymers such as polyethylene glycol (PEG)[144] and polyvinylpyrrolidone (PVP)[144] or proteins such as bovine serum albumin (BSA).[143] Interestingly, Robinson *et al.* studied the influence of the molecular weight of PVP over the coating efficiency, as well as the effect of analytes in solution over BSA adsorption.[145] Additional dynamic coatings have also been studied by Giordano *et al.* and include hydroxyethylcellulose (HEC), epoxy (poly)-dimethylacrylamide (EPDMA), and diaminobutane (DAB).[146] Cui and co-workers investigated the effect of single-walled carbon nanotubes on the PCR yield.[147] Results showed that the nanotubes increased the amount of DNA amplified when they were introduced in concentrations below 3 μg/μL. Finally, Yoon and co-workers examined the influence of pH, potential and electrode polarity in an electrowetting–based microdevice in order to prevent bio-molecule adsorption.[148]

DNA detection methods

Sequence independent detection methods

Due to cost-effectiveness, the use of intercalators (molecules that upon binding to double-stranded DNA exhibit significant enhancements in their fluorescence quantum efficiencies) in combination with gel electrophoresis is probably the most widely used method in biological laboratories for the detection of post-PCR products. With this technique, the DNA molecules are effectively labelled with an intercalator dye and subsequently separated according to their sizes. Popular intercalating dyes include ethidium bromide and SYBR® Green, but other intercalators have also been studied such as BEBO,[149] YOYO[150] and TO-PRO-3.[151] The use of intercalators to detect DNA molecules presents two main advantages: it enables real-time detection[152,153] and it is highly versatile, as the dyes have been selected to bind selectively to dsDNA. However, indiscriminate binding is also a major drawback: both specific and non-specific products will generate the same type of signal, making it difficult to differentiate between them. Integrating a size separation method or denaturing gradient profile often assists the identification of authentic amplicons.

One of the most widely used on-chip detection techniques for DNA molecules is capillary electrophoresis (CE). CE was first demonstrated on chip by Manz *et al.*[154] and has since then been applied in many fields,[155] including the analysis of PCR products.[156] Recent applications include the detection of short tandem repeats by Mitnik and co-workers,[157] while Tseng *et al.* studied the effect of bubble cells on the resolution and the sensitivity of DNA separation during CE.[158] Efforts to develop matrices for on-chip CE with lower viscosity comprised a novel sieving buffer based on hydroxypropylmethylcellulose presented by Han *et al.*,[159] as well as a study of a series of poly(ethylene oxide) matrices by Xu and co-workers.[160] A high-throughput device capable of genotyping simultaneously 384 samples in less than 325s was presented by Emrich *et al.*[161]

Other sequence-independent detection methods have also been investigated. For example, Messina *et al.* introduced a high-frequency sensor that enabled the differentiation between pre-amplification and post-PCR products.[162] Zangmeister and co-workers developed a novel fluorescence detection method based on the displacement of a fluorescent-tagged oligomer by the untagged target molecule.[163]

Sequence-specific detection methods

Real-time probes

It is the authors' belief that, although real-time probes are not yet widely used for on-chip detection due to complex detection system requirements, they will soon be routinely implemented for on-chip quantitative and qualitative real-time PCR (RQ-PCR). In this section the reader will find a brief overview of some of the commercially available real-time probes.

Hydrolysis probes. *TaqMan® probes.* The selectivity of real-time PCR was greatly improved with the introduction of fluorogenic probes (oligonucleotides whose sequences are complementary to part of the target molecules),[164] such as the TaqMan® or hydrolysis probes. The detection principle is based on the quenching phenomenon known as Fluorescence Resonance Energy Transfer (FRET). FRET corresponds to the transfer of energy from a fluorescent reporter molecule (R) to a quencher molecule (Q), leading to light emission by Q at a longer wavelength than the donor. After hybridisation (and during annealing) the probe is cleaved due to the 5′ nuclease activity of the enzyme. As FRET is impaired, the reporter's emission can be detected. RQ-PCR optimisation using TaqMan® probes has been performed and include the study of the best possible combinations between commonly used fluorophores and quenchers by Marras *et al.*,[165] as well as quantitative co-amplification of multiple targets (multiplexing) by Wang and co-workers.[166] Based on a similar approach, Gilliland *et al.* introduced PCR quantitation by competitive PCR.[167]

Cycling probe technology. Duck *et al.* developed in 1990 the Cycling Probe Technology (CPT).[168] This detection method is based on a chimeric DNA-RNA-DNA probe labelled at one end with a reporter and at the other end with a quencher.[169] Upon hybridisation with the target sequence, the enzyme RNaseH specifically cleaves the RNA region of the probe,

resulting in fluorescence emission. This method has been implemented on chip with success in Professor Harrisson's group.[170–172]

DNAzyme. A detection method similar to the CPB and also based on a hybridisation probe was recently released by BD Biosciences: the BD Qzyme™ Assay.[173] The innovative component is the DNAzyme, a catalytically active oligonucleotide capable of cleaving nucleic acid substrates at specific locations. The DNAzyme operates in coordination with a DNAzyme-specific fluorogenic target that is cleaved during the PCR process, and consequently the fluorescent signal enables the real-time monitoring of DNA amplification. Since the introduction of the DNAzyme probes, several applications have been reported in the literature such as quantitation of nucleic acid sequences in real-time,[174] study of the probe fluorescence signal[175] and elaboration of a circular probe.[176]

MGB™ technology. A new probe approach based on Minor Groove Binders (MGB) has recently been introduced by Epoch Biosciences. MGB are flat molecules that can fit in the minor groove and interact with the phosphate-sugar backbone either by hydrogen bonds or by hydrophobic interactions. Such characteristics were proved to stabilise DNA hybridisation, resulting in shorter primers retaining higher melting temperatures[177] and increased probe specificity.[178] Similar MGB technology is now used both by Applied Biosystems for their TaqMan® chemistry and by Epoch Biosciences for their MGB Eclipse Probe Systems. MGBs are used in conjunction with fluorogenic probe technologies and are gaining in popularity with the increasing requirement to place probes over exact regions of target sequences, such as exon-exon boundaries used in splicosomic studies.

Conformation probes. *Molecular Beacons.* Operating on a comparable FRET principle as TaqMan® probes, Molecular Beacons were introduced by Tyagi and co-workers.[179,165,180] They consist of single-stranded oligonucleotides arranged in a stem-loop structure, with a reporter probe attached to one of the stem arms and a quencher to the other. The loop portion is complementary to part of the target sequence, whereas the stem portion flanks the loop and are self complementary and unable to hybridise to any of the target sequence. To simplify, Molecular Meacons exist in two different states. When they are in a loop-stem structure, the fluorescence signal is quenched by FRET or proximity effects. On the contrary, during the annealing step, the loop part hybridises to the target molecule to separate the reporter and quencher dyes and a signal can be detected. Molecular Beacons have been used in a microfluidic environment to detect the breast cancer gene BRCA1 by Culha and co-workers,[181] and also as a mean to study DNA hybridisation kinetics and to perform DNA melting temperature analysis by Dodge *et al.*[182] Based on a similar concept, Nazarenko and co-workers introduced self-quenched primers.[183] These single-fluorophore labelled primers emit fluorescence with different intensities depending on their conformation: a weak emission is noticed when the primers are in a stem-loop configuration, whereas the fluorescence signal is at its maximum when the primers are incorporated in a double-stranded DNA helix.

Scorpions. Scorpion primers are an alternative to Molecular Beacons.[184] A Scorpion primer consists of a three-part molecule based on the following model: (PCR primer) – (blocker molecule) – (probe). During the PCR process, the primer hybridises to the target molecule and is then extended. Upon denaturation, the extended-DNA-strand/target hybrid is dissociated and so are the two arms of the Scorpion. Once the temperature reduced for the annealing/extension phase, the probe-part of the Scorpion anneals to the extended strand, the quenching process is impaired and fluorescence is detected. Upon extension the probe is displaced and recovers its quenched conformation with a resultant drop in the fluorescence signal.

Hybridisation probes. *LC probes.* Roche Molecular Diagnostics have also developed a mutation analysis kit based on hybridisation probes. As with ligase chain detection, the probes are specifically designed to target the amplicon at adjacent sites. However, one probe is labeled at the 3′ end with fluorescein (the "donor" probe), whereas the two other probes are labeled with an acceptor dye on the 5′ end (either LC-Red 640 or LC-Red 705, each dye corresponding to a mutation possibility). The detection principle is based on FRET: the single-base-mutation is detected depending on the wavelength at which the signal is detected, as LC-Red 640 and LC-Red 705 emit at two different wavelengths.

AEGIS platform. The latest developments in DNA diagnostic technologies include the expansion of the DNA alphabet. EraGen Biosciences, Inc. developed the AEGIS (An Extended Genetic Information System) platform that extends the DNA alphabet by up to 8 new bases. Two of the new bases, isoC and isoG, have already been combined to the natural DNA bases to produce the company's diagnostic technology. The GENE-CODE system is a real-time nucleic acid detection system based on the reduction in fluorescent signal during PCR. During the amplification process, a site-specific quencher is inserted opposite a complementary AEGIS base in one of the primers, resulting in signal diminution by FRET phenomenon. This technology is still fairly new but clinical diagnostics assays based on the AEGIS bases isoC and isoG are already commercially available (such as the VERSANT HIV Assay developed by Bayer Diagnostics).

Although real-time PCR methods using hydrolysis probes, conformation probes or hybridisation probes are powerful techniques, they are inherently limited in some cases, as pointed out by Gardner and co-workers regarding TaqMan® probes.[185] These probes are indeed highly specific, rendering the detection of viruses with substantial genetic variation among strains, such as the HIV virus, impractical.

RQ-PCR data analysis strategies. At the same time as the above techniques enabled quantitative PCR, the issue relating to data analysis became a paramount concern. Not only were systematic errors in fluorescence measurements investigated,[186] but different methods of analysis were also introduced. The most widely used procedure of analysis is the threshold method.[187] Based on the background fluorescence signal, a certain level of fluorescence signal (usually around 10 times the value of the average background fluorescence value during early cycles) is imposed as the threshold limit. After plotting the fluorescence signal obtained during PCR against the number of cycles performed, the intersection of this curve with the threshold limit can be determined. It is often called the threshold value, or C_T value, and represents the number of cycles necessary to generate an exact amount of DNA. With this method, C_T values can be compared and DNA template concentrations can be calculated. Although this method is well accepted among the scientific community, new approaches have been proposed. For example, Rutledge and co-workers studied the mathematics of quantitative PCR and offered an alternative analysis tool based on DNA mass at threshold limit,[188] whereas Liu *et al.* favoured the kinetic curves to extrapolate the initial amount of gene transcript.[189] New algorithms have also been developed to promote automatic analysis of real-time PCR data.[190]

Single Nucleotide Polymorphism (SNP)

Whilst quantitative PCR assays present a significant challenge for microfluidic applications, the development of qualitative assays such as the analysis of genetic mutations has attracted much attention. Single Nucleotide Polymorphisms (SNPs) are frequent non-random base substitutions, present at 1 per 1000 base pairs within the genome, and are avidly studied for association with diseases. Several techniques to detect SNPs have been implemented on chip. For example, Kimbal and co-workers applied Temperature Gradient Gel Electrophoresis (TGGE) in a polycarbonate micro-device.[191] Buch *et al.* combined standard gel electrophoresis to TGGE in their 2-D separation device.[192] In the first dimension, molecules were separated by gel electrophoresis according to their sizes, while TGGE allowed a SNP analysis in subsequent perpendicular channels. Using hydroxy-ethylcellulose as a sieving matrix, Liu and co-workers used TGCE and successfully separated a mixture of wild type DNA, mutant type DNA and the corresponding heteroduplexes.[193] Ito *et al.* favoured Affinity Capillary Electrophoresis (ACE) to develop a gene mutation assay.[194] Zhong and co-workers performed SNP genotyping by applying a combination of allele-specific oligonucleotides attached to the chip surface, detector probes and a thermo-stable DNA ligase.[195] Another approach was presented by Russom *et al.*: they analysed SNPs by extending allele-specific fluorescently labelled nucleotides.[196]

Hybridisation based detection

DNA hybridisation is widely exploited to achieve high selectivity and sensitivity. Different approaches have been applied on chip. For example, Lai and co-workers developed an enzyme-linked immunosorbent assay on a compact disk platform,[56] while Cai *et al.* determined by electrochemical impedance spectroscopy that DNA hybridisation is strongly frequency-dependent.[197] Trau and co-workers applied PCR reactors combined to microarrays to genotype Chinese medicine plants.[198] Magnetic beads were also used in microfluidics devices to immobilise by hybridisation alternatively the target template,[54] the capture probe[42] or various sensing agents.[48] A different approach was introduced by McKendry *et al.*:[199] using cantilever assays they were able to determine the sequences of unlabeled DNA molecules present at nanomolar concentrations.

Integrated systems

One of the main challenges in miniaturisation is the integration of functional components to perform several operations without the need for macro apparatus or manual user input. A few groups have started to tackle this issue and a brief overview of the integrated systems available at the moment is given in this section.

A number of instances of integrated on-capillary systems have been recently presented in the literature. For example, Li and co-workers developed a system that could perform cell counting, cell-lysis, PCR, on-column hybridisation and product detection.[200] In addition, an integrated on-capillary arrangement capable of cell-lysis, reverse transcription PCR and product analysis was reported by Matsunaga *et al.*,[77] while a set-up capable of PCR, sequencing and purification in capillaries was introduced by Hashimoto *et al.*[201]

Several examples of on-chip integrated systems have also been published in the literature. One of the devices combining the most operations was presented by Liu *et al.*[54,76] Their device enabled the following steps: cell capture, cell concentration, cell lysis, purification, PCR, SNP assaying and hybridisation. In addition, valves, mixers and pumps were incorporated onto the chip. Another system offering various capabilities—namely cell capture, cell lysis, purification and DNA amplification—was introduced by El-Ali and co-workers.[44] Other structures have also been investigated, and for a more detailed overview of some of the most recent, the reader should refer to Table 2. As can be seen in this table, the pre-PCR steps that are integrated the most include cell capture,[44,54,76,82,202–205] cell lysis[44,54,76,82,202–204,206,207] and sample purification.[35,44,54,76,82,203,205,208] The well-approach PCR[41,44,54,76,208–217] seems to be favored for integrated systems when compared to the continuous-flow process.[35,123,125,206,218–220] This might be due to an increased simplicity in sample handling for the former method. The physical elements the most integrated are heaters[41,44,125,205,206,209–214,216–219] and valves.[41,54,76,82,202,206,209,210,212,216–218]

DNA amplification is mostly performed to increase the detection sensitivity either of a specific molecule among a heterogeneous population or to characterise and/or measure a given section of a target. However, even though the merits of PCR are widely recognised, the process of DNA amplification impairs the speed, the cost-effectiveness and even sometimes

Table 2 Components of several integrated systems

Operation / Article	[206]	[207]	[54, 76]	[82]	[202]	[203]	[204]	[44]	[205]	[35]	[208]	[41, 209, 210]	[221]	[211]	[218]	[212]	[213]	[219]	[214]	[215]	[216, 217]	[123, 220]	[125]
Cell loading							•					•											
Cell capture			•	•	•	•	•	•	•														
Cell concentration / Cell culture			•						•														
Cell analysis					•	•	•																
Cell lysis / Digestion	•	•	•	•		•	•	•															
Purification / Filtering / Extraction			•	•		•		•	•	•	•		•										
Reverse transcription																						•	
PCR – well			•					•			•	•		•		•	•		•	•	•		
PCR – continuous flow	•									•					•			•				•	•
Electrophoresis		•					•				•	•	•			•	•			•	•		
SNP			•												•								•
Hybridisation			•																•				
Integrated components																							
Heaters	•							•	•			•		•	•	•	•	•	•		•		•
Electrodes								•				•									•		
Valves	•		•	•	•							•			•	•					•		
Mixers			•	•	•																		
Pumps	•		•																				

the accuracy and the sensitivity of such detection methods. To resolve this issue, U.S. Genomics has developed a unique method with which single molecules from an un-amplified sample can be detected using the GeneEngine™. This system relies on isolating the target from the sample, fluorescently tagging it and finally injecting it into the test platform where each molecule is subsequently detected by laser illumination. Using DirectMolecular™ Analysis, quantitation, SNP analysis and molecule interaction quantification can be performed by colour coincidence counting. Analysis in less than one minute and femtomolar range sensitivity are claimed to be achieved. Alternatively, DirectLinear™ Analysis enables the sequencing of single linear genomic DNA molecules. Sequence-specific probes are first hybridised to the DNA molecule. The strand is then stretched and the probes are detected linearly by the GeneEngine™. A spatially resolved map of specific sequence locations on the DNA can then be generated, leading to a 'genetic barcode' for each individual molecules. Applications of such barcodes include genetic comparison of samples or populations, SNP detection and genome mapping or microorganisms.

Conclusion

The future of high throughput gene analysis will benefit greatly from the implementation of micro Total Analysis Systems, particularly for the analyses requiring multi-step procedures such as those including sample preparation. Microfluidic devices will serve therefore to provide a level of standardisation not presently achievable with current laboratory standard operating procedures, especially those based upon minute quantities of samples. As the development of microfluidic devices continues, newly devised microstructures need to utilise the better production and micro-engineering procedures. Importantly however, the devices should also innately incorporate the requirements of each bioassay to enable them to be customised to the exact requirements of the specific bioassay and thus permit the optimum co-selection of device and choice of assay chemistry.

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References

1 P.-A. Auroux, D. R. Reyes, D. Iossifidis and A. Manz, Micro Total Analysis System 2. Analytical Standard Operations and Applications, *Anal. Chem.*, 2002, **74**, 2637.
2 T. Vilkner, D. Janasek and A. Manz, Micro total analysis systems. Recent developments, *Anal. Chem.*, 2004, **76**, 3373.
3 J. Khandurina and A. Guttman, Bioanalysis in microfluidic devices, *J. Chromatogr. A*, 2002, **943**, 159.
4 G. H. W. Sanders and A. Manz, Chip-based microsystems for genomic and proteomic analysis, *Trends Anal. Chem.*, 2000, **19**, 364.
5 J. P. Landers, Molecular diagnostics on electrophoretic microchips, *Anal. Chem.*, 2003, **75**, 2919.
6 R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim, Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, *Science*, 1985, **230**, 1350.
7 A. Chien, D. B. Edgar and J. M. Trela, Deoxyribonucleic acid polymerase from the extreme thermophile Thermus Aquaticus, *J. Bacteriol.*, 1976, **127**, 1550.
8 A. S. Kaledin, A. G. Slyusarenko and S. I. Gorodetskii, Isolation and properties of DNA polymerase from extremely thermophilic bacterium Thermuc aquaticus YT1, *Biokhimiya*, 1980, **45**, 644.
9 K. R. Tindall and T. A. Kunkel, Fidelity of DNA synthesis by the Thermus aquaticus DNA Polymerase, *Biochemistry*, 1988, **27**, 6008.
10 J. F. Davidson, R. Fox, D. D. Harris, S. Lyons-Abbott and L. A. Loeb, Insertion of the T3 DNA polymerase thioredoxin binding domain enhances the processivity and fidelity of Taq DNA polymerase, *Nucl. Acids Res.*, 2003, **31**, 4702.
11 K. S. Lundberg, D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge and E. J. Mathur, High-fidelity amplification using a thermostable DNA polymerase isolated from Pyrococcus furiosus, *Gene*, 1991, **108**, 1.
12 W. M. Barnes, The fidelity of Taq polymerase catalyzing PCR is improved by an N-terminal deletion, *Gene*, 1992, **112**, 29.
13 P. Mattila, J. Korpela, T. Tenkanen and K. Pitkanen, Fidelity of DNA-synthesis by the Thermococcus-Litoralis DNA- Polymerase-an extremely heat-stable enzyme with proofreading activity, *Nucl. Acids Res.*, 1991, **19**, 4967.
14 J. J. Choi, S. E. Jung, H.-K. Kim and S.-T. Kwon, Purification and properties of Thermus filiformis DNA polymerase expressed in Escherichia coli, *Biotechnol. Appl. Biochem.*, 1999, **30**, 19.
15 D. Resuehr and A.-N. Spiess, A real-time polymerase chain reaction-based evaluation of cDNA synthesis priming methods, *Anal. Biochem.*, 2003, **322**, 287.
16 W. P. Halford, V. C. Falco, B. M. Gebhardt and D. J. J. Carr, The inherent quantitative capacoty of the reverse transcription-polymerase chain reaction, *Anal. Biochem.*, 1999, **266**, 181.
17 P. Markoulatos, N. Siafakas and M. Moncany, Multiplex polymerase chain reaction: a practical approach, *J. Clin. Lab. Anal.*, 2002, **16**, 47.
18 O. Henegariu, N. A. Heerema, S. R. Dlouhy, G. H. Vance and P. H. Vogt, Multiplex PCR: critical parameters and step-by-step protocol, *Bio Techniques*, 1997, **23**, 504.
19 J.-F. Mercier, G. W. Slater and P. Mayer, Solid phase DNA amplification: a simple Monte Carlo Lattice model, *Biophys. J.*, 2003, **85**, 2075.
20 B. Schweitzer and S. Kingsmore, Combining nucleic acid amplification and detection, *Curr. Opin. Biotechnol.*, 2001, **12**, 21.
21 G. T. Walker, M. C. Little, J. G. Nadeau and D. D. Shank, Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 392.
22 F. B. Dean, S. Hosomo, L. Fang, X. Wu, A. F. Faruqi, P. Bray-Ward, Z. Sun, Q. Zong, Y. Du, J. Du, W. Driscoll, W. Song, S. F. Kingsmore, M. Egholm and R. S. Lasken, Comprehensive human genome amplification using multiple displacement amplification, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 5261.
23 T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase, Loop-mediated isothermal amplification of DNA, *Nucl. Acids Res.*, 2000, **28**, e63.
24 P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, Mutation detection and single-molecule counting using isothermal rolling-circle amplification, *Nat. Genet.*, 1998, **19**, 225.
25 F. Dahl, J. Baner, M. Gullberg, M. Mendel-Hartvig, U. Landegren and M. Nilsson, Circle-to-circle amplification for precise and sensitive DNA anlysis, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 4548.
26 U. Landegren, R. Kaiser, J. Sanders and L. Hood, A ligase-mediated gene detection technique, *Science*, 1988, **241**, 1077.
27 F. Barany, Genetic disease detection and DNA amplification using cloned thermostable ligase, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 189.
28 D. H. Bing, C. Boles, F. N. Rehman, M. Audeh, M. Belmarsh, B. Kelley and C. P. Adams, Bridge amplification: a solid phase PCR system for the amplification and detection of allelic differences in single copy genes, *Genetic Identity Conference Proceedings, 1996.*
29 C. Adessi, G. Matton, G. Ayala, G. Turcatti, J.-J. Mermod, P. Mayer and E. Kawashima, Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms, *Nucl. Acids Res.*, 2000, **28**, e87.
30 O. Greiner and P. J. R. Day, Avoidance of nonspecific hybridization by employing oligonucleotide micro-arrays generated from hydrolysis polymerase chain reaction probe sequences, *Anal. Biochem.*, 2004, **324**, 197.
31 T. S. Hug, D. Parrat, P.-A. Kuenzi, U. Staufer, E. Verpoorte and N. F. de Rooij, Fabrication of Nanochannels with PDMS, silicon and glass walls and spontaneous filling by capillary forces, 29, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
32 Y. Tanaka, K. Sato, M. Yamato, T. Okano and T. Kitamori, Micro liver system for bioreactor and bioconversion, 777, in *Micro total Analysis Systems*, 2003, Squaw Valley, CA, USA.
33 L. E. Rodd, G. Rosengarten, S. T. Huntington, K. Lyytikainen, D. V. Boger and J. J. Cooper-White, The effect of surface character on flows in cylindrical microchannels, 951, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA,USA.
34 J. M. Bienvenue, J. M. Karlinsey, Q. Wu, R. D. McConnell, J. P. Ferrance, P. M. Norris and J. P. Landers, Methods for high-

speed, efficient purification of nucleic acids from diverse clinical and bio-hazardous samples on glass and hybrid PDMS-glass microchips, 789, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
35 J. P. Ferrance, Q. J. Wu, B. C. Giordano, H. Hernandez, Y. Kwok, K. Snow, S. Thibodeau and J. P. Landers, Developments toward a complete micro-total analysis system for Duchenne muscular dystrophy diagnosis, *Anal. Chim. Acta*, 2003, **500**, 223.
36 J. Emmelkamp, R. da Costa, H. Andersson and A. van den Berg, Intrinsic autofluorescence of single living cells for label-free cell sorting in a microfluidic system, 85, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
37 S. Song, A. K. Singh, T. J. Shepodd and B. J. Kirby, Microchip dialysis of proteins using in situ photopatterned nanoporous polymer membranes, *Anal. Chem.*, 2004, **76**, 2367.
38 L. R. Huang, E. C. Cox, R. H. Austin and J. C. Sturm, Tilted brownian ratchet for DNA analysis, *Anal. Chem.*, 2003, **75**, 6963.
39 B. J. Kirby, A. R. Wheeler, R. N. Zare, J. A. Fruetel and T. J. Shepodd, Programmable modification of cell adhesion and zeta potential in silica microchips, *Lab Chip*, 2003, **3**, 5.
40 C. H. Lin, L.-M. Fu, K.-H. Lee, R.-J. Yang and G.-B. Lee, Novel surface modification methods and surface property analysis for separation of DNA bio-molecules using capillary eletrophoresis, 1081, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
41 Z. Zhao, Z. Cui, D. Cui and S. Xia, Monolithically integrated PCR biochip for DNA amplification, *Sens. Actuators A*, 2003, **108**, 162.
42 M. Gabig-Ciminska, A. Holmgren, H. Andresen, K. B. Barken, M. Wümpelmann, J. Albers, R. Hintsche, A. Breitenstein, P. Neubauer, M. Los, A. Czyz, G. Wegrzyn, G. Silfversparre, B. Jürgen, T. Schweder and S.-O. Enfors, Electric chips for rapid detection and quantification of nucleic acids, *Biosens. Bioelectron.*, 2004, **19**, 537.
43 P.-A. Auroux, P. J. R. Day and A. Manz, Sample-shunting based PCR microfluidic device, 67, in *The nanotechnology conference and trade show*, 2004, Boston, MA, USA.
44 J. El-Ali, I. R. Perch-Nielsen, C. R. Poulsen, M. Jensen, P. Telleman and A. Wolff, Microfabricated DNA amplification device monolithically integrated with advanced sample pre-treatment, 214, in *Transducers*, 2003, Boston, MA, USA.
45 M. Schlüter, S. Mammitzsch, M. Martens, S. Gasso and H. J. Lilienhof, Micro fluidic immunoassay chip with integrated liquid handling, 275, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
46 Y. Huang, J. M. Yang, P. J. Hopkins, S. K. Kassegne, M. Tirado, A. H. Forster and H. Reese, Separation of simulants of biological warfare agents from blood by a miniaturized dielectrophoresis device, *Biomed. Microdev.*, 2003, **5**, 217.
47 M. Kanai, D. Uchida, S. Sugiura, Y. Shirasaki, J. S. Go, H. Nakanishi, T. Funatsu and S. Shoji, PDMS microfluidic devices with PTFE passivated channels, 429, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
48 C. A. Marquette and L. J. Blum, Direct immobilization in poly(dimethylsiloxane) for DNA, protein and enzyme fluidic biochips, *Anal. Chim. Acta*, 2004, **506**, 127.
49 D. Xiao, T. V. Le and M. J. Wirth, Surface modification of the channels of poly(dimethylsiloxane) microfluidic chips with polyacrylamide for fast electriphoretic separations of proteins, *Anal. Chem.*, 2004, **76**, 2055.
50 S.-R. Park and H. Swerdlow, Concentration of DNA in a flowing stream for high-sensitivity capillary electrophoresis, *Anal. Chem.*, 2003, **75**, 4467.
51 J. Kai, Y. S. Sohn and C. H. Ahn, Protein microarray on cyclic olefin copolymer (COC) for disposable protein lab-on-chip, 5, in *Micro Total Analysis Systems*, 2003, Squaw Valley, California USA.
52 M. Kanai, H. Abe, T. Munaka, Y. Fujiyama, D. Uchida, A. Yamayoshi, H. Nakanishi, A. Murakami and S. Shoji, Integrated micro chamber for living cell analysis with negligible dead volume sample injector, 288, in *Transducers*, 2003, Boston, MA, USA.
53 S.-H. Lee, C.-S. Lee, B.-G. Kim and Y.-K. Kim, Surface characterization and a lift-off process of a fluorocarbon thin film for micro protein patterning, 1093, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
54 R. H. Liu, J. Yang, R. Lenigk, J. Bonanno and P. Grodzinski, Self-contained, fully integrated biochip for sample preparation, polymerase chain reaction amplification, and DNA microarray detection, *Anal. Chem.*, 2004, **76**, 1824.
55 T. Hanaoka, O. Takai, K. Takahashi and S. Tsugane, Chip ligating human genomic DNA serves as storage material and template for polymerase chain reaction, *Biotechnol. Lett.*, 2003, **25**, 509.
56 S. Lai, S. C. Wang, J. Luo, L. M. Lee, S.-T. Yang and M. J. Madou, Design of a compact disk-like micrfluidic platform for enzyme-linked immunosorbent assay, *Anal. Chem.*, 2004, **76**, 1832.
57 J. Kameoka, H. G. Craighead, H. Zhang and J. Henion, A polymeric microfluidic chip for CE/MS-Determination of small molecules, *Anal. Chem.*, 2001, **73**, 1935.
58 S. Hjertén, High-performance electrophoresis-Elimination of electroendosmosis and solute adsorption, *J. Chromatogr.*, 1985, **347**, 191.
59 D.-S. Shin, C.-K. Kang, J.-K. Kim, W.-J. Chung, K.-H. Jang and Y.-S. Lee, Surface modification technology for bio-mems, 1746, in *Transducers*, 2003, Boston, MA, USA.
60 B. C. Giordano, E. R. Copeland and J. P. Landers, Towards dynamic coating of glass microchip chambers for amplifying DNA via the polymerase chain reaction, *Electrophoresis*, 2001, **22**, 334.
61 H.-G. Choi, Z. Zhang, P. Boccazzi, P. E. Laibinis, A. J. Sinskey and K. F. Jensen, Poly(ethylene glycol) (PEG)-modified Poly-(dimethylsiloxane) (PDMS) for protein-and cell-resistant surfaces in microbioreactor, 1105, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
62 Y. S. Shin, K. Cho, S. H. Lim, S. Chung, S.-J. Park, C. Chung, D.-C. Han and J. K. Chang, PDMS-based micro PCR chip with Parylene coating, *J. Micromech. Microeng.*, 2003, **13**, 768.
63 A. Grodrian, J. Metze, T. Henkel, K. Martin, M. Roth and J. M. Köhler, Segmented flow generation by chip reactors for highly parallelized cell cultivation, *Biosens. Bioelectron.*, 2004, **19**, 1421.
64 W. Tan and T. A. Desai, Layer-by-layer microfluidics for biomimetic three-dimensional structures, *Biomaterials*, 2004, **25**, 1355.
65 B. Schaak, B. Fouqué, S. Porte, S. Combe, A. Hennico, O. Filhol-Cochet, J. Reboud, M. Balakirev and F. Chatelain, Cell culture in microdrops, a new format for cell on chip technology, 669, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
66 M. Goto, K. Sato, M. Tokeshi and T. Kitamori, Development of microchip-based bioassay system using cultured cells, 785, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
67 Y.-C. Lin, M. Li, C.-Y. Wu, W.-C. Hsiao and Y.-C. Chung, Microchips for cell-type identification, 729, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
68 A. Fuchs, N. Manaresi, D. Freida, L. Altomare, C. L. Villiers, G. Medoro, A. Romani, I. Chartier, C. Bory, M. Tartagni, P. N. Marche, F. Chatelain and R. Guerrieri, A microelectronic chip opens new fields in rare cell population analysis and individual cell biology, 911, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
69 A. Wolff, I. R. Perch-Nielsen, C. R. Poulsen, J. El-Ali and D. D. Bang, Removal of PCR inhibitors using dielectrophoresis for sample preparation in a microfabricated system, 1137, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
70 B. H. Lapizco-Encinas, B. A. Simmons, E. B. Cummings and Y. Fintschenko, High-throughput electrodeless dielectrophoresis of viruses in polymeric microdevices, 607, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
71 M. Denoual, K. Aoki, A. Mita-Tixier and H. Fujita, A microfluidic device for long-term study of individual cells, 531, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
72 G.-L. Lettieri, L. Ceriotti, N. F. de Rooij and E. Verpoorte, On-chip DNA trapping and preconcentration employing recirculating flow devices, 737, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
73 C. Goubault, J.-L. Viovy and J. Bibette, Capture of rare cells by magnetic filaments, 239, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
74 H. Lu, S. Gaudet, P. K. Sorger, M. A. Schmidt and K. F. Jensen, Micro isoelectric free flow separation of subcellular materials, 915, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
75 K. J. Halle, J. J. Li, M. S. Munson, J. Monteith, E. Guzman, S. Feather, J. Verba, Q. Porter, V. Kenning, A. E. Kamholz, B. H. Weigl, P. Saltsman, R. Bardell and P. Yager, Capture-and-release concentration of bacteria using free-flow zone electrophoresis, 559, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
76 R. H. Liu, J. Yang, R. Lenigk, J. Bonanno, P. Grodzinski and F. Zenhausern, Self-contained, integrated biochip system for sample-to-answer genetic assays, 1319, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
77 H. Matsunaga, T. Anazawa and E. S. Yeung, Integrated on-capillary instrumentation for gene expression measurement directly from cells, *Electrophoresis*, 2003, **24**, 458.
78 K. Tachibana, T. Uchida, K. Ogawa, N. Yamashita and K. Tamura, Induction of cell-membrane porosity by ultrasound, *Lancet*, 1999, **353**, 1409.

79 P. Marmottant and S. Hilgenfeldt, Controlled vesicle deformation and lysis by single oscillating bubbles, *Nature*, 2003, **423**, 153.
80 H. Lu, S. Gaudet, P. K. Sorger, M. A. Schmidt and K. F. Jensen, Miniaturized electroporating device for controlled cell lysis, 773, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
81 E. A. Schilling, A. E. Kamholz and P. Yager, Cell lysis and protein extraction in a microfluidic device with detection by a fluorogenic enzyme assay, *Anal. Chem.*, 2002, **74**, 1798.
82 J. W. Hong, V. Studer, G. Hang, W. F. Anderson and S. R. Quake, A nanoliter-scale nucleic acid processor with parallel architecture, *Nat. Biotechnol.*, 2004, **22**, 435.
83 V. Reddy, S. Yang and J. D. Zahn, Organic/aqueous two phase microflow for biological sample preparation, 437, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
84 M. Washizu, DNA manipulation in electrostatic fields, 869, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
85 S. K. Mohanty, S. K. Ravula, K. L. Engisch and A. B. Frazier, A micro system using dielectrophoresis and electrical impedance spectroscopy for cell manipulation and analysis, 1055, in *Transducers*, 2003, Boston, MA, USA.
86 N. Sundararajan, X. Su and A. Berlin, Microfluidic immobilization and programmable release of single molecules, 665, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
87 C. E. Corless, M. Guiver, R. Borrow, V. Edward-Jones, E. B. Kaczmarski and A. J. Fox, Contamination and sensitivity issues with a real-time universal 16S rRNA PCR, *J. Clin. Microbiol.*, 2000, **38**, 1747.
88 Committee on DNA Technology in Forensic Science, Chapter 2: DNA typing: Technical considerations, p65, *DNA Technology in Forensic Science*, National Research Council, The National Academies Press, Washington, D.C.
89 F. Ferre, Quantitative or semi-quantitative PCR: reality versus myth, *PCR Methods Appl.*, 1992, **2**, 1.
90 M. A. Northrup, M. T. Ching, R. M. White and R. T. Watson, DNA amplification with a microfabricated reaction chamber, *Transducers '93*, 1993, 924.
91 P. Wilding, M. A. Shoffner and L. J. Kricka, PCR in silicon microstructure, *Clin. Chem.*, 1994, **40**, 1815.
92 A. T. Woolley, D. Hadley, P. Landre, A. J. de Mello, R. A. Mathies and M. A. Northrup, Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device, *Anal. Chem.*, 1996, **68**, 4081.
93 P. Belgrader, W. Benett, D. Hadley, G. Long, R. J. Mariella, F. Milanovich, S. Nasarabadi, W. Nelson, J. Richards and P. Stratton, Infection disease: PCR detection of bacteria in seven minutes, *Science*, 1999, **284**, 449.
94 E. T. Lagally, I. Medintz and R. A. Mathies, Single-molecule DNA amplification and analysis in an integrated microfluidic device, *Anal. Chem.*, 2001, **73**, 565.
95 T. B. Taylor, E. S. Winn-Deen, E. Picozza, T. Woudenberg and M. Albin, Optimization of the performance of the polymerase chain reaction in silicon-based microstructures, *Nucl. Acids Res.*, 1997, **25**, 3164.
96 Q. Zou, Y. Miao, Y. Chen, U. Sridhar, C. S. Chong, T. Chai, Y. Tie, C. H. L. Teh, J. S. Lim and C. K. Heng, Micro-assembled multi-chamber thermal cycler for low-cost reaction chip thermal multiplexing, *Sens. Actuators A*, 2002, **102**, 114.
97 Q. Zou, U. Sridhar, Y. Chen and J. Singh, Miniaturized independently controllable multichamber thermal cycler, *IEEE Sens. J.*, 2003, **3**, 774.
98 X. Yu, D. Y. Zhang, T. Li, L. Hao and X. Li, 3-D microarrays biochip for DNA amplification in polydimethylsiloxane (PDMS) elastomer, *Sens. Actuators A*, 2003, **108**, 103.
99 S. Poser, T. Schulz, U. Dillner, V. Baier, J. M. Köhler, D. Schimkat, G. Mayer and A. Siebert, Chip elements for fast thermocycling, *Sens. Actuators A*, 1997, **62**, 672.
100 M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man, D. Jones, D. Heldsinger, C. H. Mastrangelo and D. T. Burke, An integrated nanoliter DNA analysis device, *Science*, 1998, **282**, 484.
101 J. H. Leamon, W. L. Lee, K. R. Tartaro, J. R. Lanza, G. J. Sarkis, A. D. deWinter, J. Berka and K. L. Lohman, A massively parallel PicoTiterPlate (TM) based platform for discrete picoliter-scale polymerase chain reactions, *Electrophoresis*, 2003, **24**, 3769.
102 H. Nagai, Y. Murakami, Y. Morita, K. Yokoyama and E. Tamiya, Development of a microchamber array for picoliter PCR, *Anal. Chem.*, 2001, **73**, 1043.
103 H. Nagai, Y. Murakami, K. Yokoyama and E. Tamiya, High-throughput PCR in silicon based microchamber array, *Biosens. Bioelectron.*, 2001, **16**, 1015.
104 H. Nagai, Y. Murakami, S. Wakida, E. Niki and E. Tamiya, High throughput single cell PCR on a silicon microchamber array, 268, in *Micro Total Analysis Systems*, 2001.
105 A. M. Chaudhari, T. M. Woudenberg, M. Albin and K. E. Goodson, Transient liquid crystal thermometry of microfabricated PCR vessel arrays, *J. Microelectromech. Syst.*, 1998, **7**, 345.
106 D. S. Yoon, Y.-S. Lee, Y. Lee, H. J. Cho, S. W. Sung, K. W. Oh, J. Cha and G. Lim, Precise temperature control and rapid thermal cycling in a micromachined DNA polymerase chain reaction, *J. Micromech. Microeng.*, 2002, **12**, 813.
107 T. Kajiyama, Y. Miyahara, L. J. Kricka, P. Wilding, D. J. Graves, S. Surrey and P. Fortina, Genotyping on a thermal gradient DNA chip, *Genome Res.*, 2003, **13**, 467.
108 J. Cheng, M. A. Shoffner, K. R. Mitchelson, L. J. Kricka and P. Wilding, Analysis of ligase chain reaction products amplified in a silicon-glass chip using capillary electrophoresis, *J. Chromatogr. A*, 1996, **732**, 151.
109 A. Gulliksen, L. Solli, F. Karlsen, H. Rogne, E. Hovig, T. Nordstrom and R. Sirevag, Real-time nucleic acid sequence-based amplification in nanoliter volumes, *Anal. Chem.*, 2004, **76**, 9.
110 R. P. Oda, M. A. Strausbauch, A. F. R. Huhmer, N. Borson, S. R. Jurrens, J. Craighead, P. J. Wettstein, B. Eckloff, B. Kline and J. P. Landers, Infrared-mediated thermocycling for ultrafast polymerase chain reaction Amplification of DNA, *Anal. Chem.*, 1998, **70**, 4361.
111 B. C. Giordano, J. Ferrance, S. Swedberg, A. F. R. Hühmer and J. P. Landers, Polymerase chain reaction in polymeric microchips: DNA amplification in less than 240 seconds, *Anal. Biochem.*, 2001, **291**, 124.
112 D. Pal and V. Venkataraman, A portable battery-operated chip thermocycler based on induction heating, *Sens. Actuators A*, 2002, **102**, 151.
113 M. Krishnan, V. M. Ugaz and M. A. Burns, PCR in a Rayleigh-Benard convection cell, *Science*, 2003, **298**, 793.
114 E. K. Wheeler, B. Benett, P. Stratton, J. Richards, A. Christian, A. Chen, T. Weisgrader, K. Ness, J. Ortega and F. Milanovich, Convectively driven polymerase chain reaction thermal cycler, 1133, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
115 H. Nakano, K. Matsuda, M. Yohda, T. Nagamune, I. Endo and T. Yamane, High-Speed Polymerase Chain-Reaction in Constant Flow, *Biosci. Biotechnol. Biochem.*, 1994, **58**, 349.
116 M. Curcio and J. Roeraade, Continuous segmented-flow polymerase chain reaction for high-throughput miniaturized DNA amplification, *Anal. Chem.*, 2003, **75**, 1.
117 N. Park, S. Kim and J. H. Hahn, Cylindrical compact thermal-cycling device for continuous-flow polymerase chain reaction, *Anal. Chem.*, 2003, **75**, 6029.
118 N. A. Friedman and D. R. Meldrum, Capillary tube resistive thermal cycling, *Anal. Chem.*, 1998, **70**, 2997.
119 J. Chiou, P. Matsudaira, A. Sonin and D. Ehrlich, A Closed-Cycle Capillary Polymerase Chain Reaction Machine, *Anal. Chem.*, 2001, 2018.
120 J. Chiou, P. Matsudaira, A. Sonin and D. J. Ehrlich, Performance of a closed-cycle capillary polymerase chain reaction, 495, in *Micro Total Analysis System*, 2001.
121 M. U. Kopp, A. J. de Mello and A. Manz, Chemical amplification: continuous-flow PCR on a chip, *Science*, 1998, **280**, 1046.
122 I. Schneegaß, R. Bräutigam and J. M. Köhler, Miniaturized flow-through PCR with different templates types in a silicon chip thermocycler, *Lab Chip*, 2001, **1**, 42.
123 P. J. Obeid, T. K. Christopoulos, H. J. Crabtree and C. J. Backhouse, Microfabricated device for DNA and RNA amplification by continuous-flow polymerase chain reaction and reverse transcription-polymerase chain reaction with cycle number selection, *Anal. Chem.*, 2003, **75**, 288.
124 J. Yang, Y. J. Liu, C. B. Rauch, R. L. Stevens, R. H. Liu, R. Lenigk and P. Grodzinski, High sensitivity PCR assay in plastic micro reactors, *Lab Chip*, 2002, **2**, 179.
125 J. Baker, M. Strachan, K. Swartz, Y. Yurkovetsky, A. Rulison, C. Brooks and A. Kopf-Sill, Single molecule amplification in a continuous flow labchip device, 1335, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
126 P. Belgrader, C. J. Elkin, S. B. Brown, S. N. Nasarabadi, R. G. Langlois, F. P. Milanovich, B. W. J. Colston and G. D. Marshall, A reusable flow-through polymerase chain reaction instrument for continuous monitoring of infectious biological agents, *Anal. Chem.*, 2003, **75**, 3114.
127 Q. Zhang, W. Wang, H. Zhang and Y. Wang, Temperature analysis of continuous-flow micro-PCR based on FEA, *Sens. Actuators B*, 2002, **82**, 75.
128 C.-F. Chou, R. Changrani, P. Roberts, D. Sadler, J. Burdon, F. Zenhausern, S. Lin, A. Mulholland, N. Swami and

R. Terbrueggen, A miniaturized cyclic PCR device-modeling and experiments, *Microelectron. Eng.*, 2002, **61**, 921.
129 D. J. Sadler, R. Changrani and P. Roberts, Thermal management of BioMEMS: temperature control for ceramic-based PCR and DNA detection devices, *IEEE Trans. Compon. Packag. Technol.*, 2003, **26**, 309.
130 T. Mautner, Lattice Boltzmann simulations comparing conventional and a heat conduction based flow-through PCR microdevices, 328, in *The nanotechnology conference and trade show*, 2004, Boston, MA, USA.
131 J. Liu, M. Enzelberger and S. Quake, A nanoliter rotary device for polymerase chain reaction, *Electrophoresis*, 2002, **23**, 1531.
132 P.-A. Auroux, P. J. R. Day, F. Niggli and A. Manz, PCR microvolume device for detection of nucleic acids, 55, in *The nanotechnology conference and trade show*, 2003, San Francisco, CA, USA.
133 M. Bu, T. Melvin, G. Ensell, J. S. Wilkinson and A. G. R. Evans, Design and theoretical evaluation of a novel microfluidic device to be used for PCR, *J. Micromech. Microeng.*, 2003, **13**, S125.
134 S. Hardt, D. Dadic, F. Doffing, K. S. Drese, G. Münchov and O. Sörensen, Development of a slug-flow PCR chip with minimum heating cycle times, 55, in *The nanotechnology conference and trade show*, 2004, Boston, MA, USA.
135 M. G. Pollack, P. Y. Paik, A. D. Shenderov, V. K. Pamula, F. S. Dietrich and R. B. Fair, Investigation of electrowetting-based microfluidic for real-time PCR applications, 619, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
136 C. Fermér, P. Nilsson and M. Larhed, Microwave-assisted high-speed PCR, *Eur. J. Pharm. Sci.*, 2003, **18**, 129.
137 M. Nakano, J. Komatsu, S.-I. Matsuura, K. Takashima, S. Katsura and A. Mizuno, Single-molecule PCR using water-in-oil emulsion, *J. Biotechnol.*, 2003, **102**, 117.
138 A. T. Haase, E. F. Retzel and K. A. Staskus, Amplification and detection of lentiviral DNA inside cells, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 4971.
139 P. Komminoth and M. Werner, Target and signal amplification: approaches to increase the sensitivity of in situ hybridization, *Histochem. Cell Biol.*, 1997, **108**, 325.
140 R. Kher and R. Bacallao, Direct in situ reverse transcriptase-polymerase chain reaction, *Am. J. Physiol.: Cell Physiol.*, 2001, **281**, C726.
141 F. Maruyama, T. Kenzaka, N. Yamaguchi, K. Tani and M. Nasu, Detection of bacteria carrying the stx(2) gene by in situ loop- mediated isothermal amplification, *Appl. Environ. Microbiol.*, 2003, **69**, 5023.
142 T. B. Taylor, S. E. Harvey, M. Albin, L. Lebak, Y. Ning, I. Mowat, T. Schuerlein and E. Principe, Process control for optimal PCR performance in glass microstructures, *J. Biomed. Microdev.*, 1998, **1**, 65.
143 I. Erill, S. Campoy, N. Erill, J. C. Barbé and J. Aguiló, Biochemical analysis and optimization of inhibition and adsorption phenomena in glass-silicon PCR-chips, *Sens. Actuators B*, 2003, **96**, 685.
144 X. J. Lou, N. J. Panaro, P. Wilding, P. Fortina and L. J. Kricka, Increased amplification efficiency of microchip-based PCR by dynamic surface passivation, *Biotechniques*, 2004, **36**, 248.
145 S. Robinson and P. A. Williams, Inhibition of protein adsorption onto silica by polyvinylpyrrolidone, *Langmuir*, 2002, **18**, 8743.
146 B. C. Giordano, M. Muza, A. Trout and J. P. Landers, Dynamically-coated capillaries allow for capillary electrophoretic resolution of transferrin sialoforms via direct analysis of human serum, *J. Chromatogr. B*, 2000, **742**, 79.
147 D. Cui, F. Tian, Y. Kong, I. Titushikin and H. Gao, Effects of single-walled carbon nanotubes on the polyermase chain reaction, *Nanotechnology*, 2004, **15**, 154.
148 J.-Y. Yoon and R. L. Garrell, Preventing biomolecular adsorption in electrowetting-based biofluidic chips, *Anal. Chem.*, 2003, **75**, 5097.
149 M. Bengtsson, H. J. Karlsson, G. Westman and M. Kubista, A new minor groove binding asymmetric cyanine reporter dye for real-time PCR, *Nucl. Acids Res.*, 2003, **31**, e45.
150 C. L. Kuyper, G. P. Brewood and D. T. Chiu, Initiating conformation transitions of individual YOYO- intercalated DNA molecules with optical trapping, *Nano Letters*, 2003, **3**, 1387.
151 A. Guttman, C. Barta, M. Szöke, M. Sasvári-Székely and H. Kalász, Real-time detection of allele-specific polymerase chain reaction products by automated ultra-thin-layer agarose gel electrophoresis, *J. Chromatogr. A*, 1998, **828**, 481.
152 R. Higuchi, G. Dollinger, P. S. Walsh and R. Griffith, Simultaneous amplification and detection of specific DNA-sequences, *BioTechnology*, 1992, **10**, 413.
153 R. Higuchi, C. Fockler, G. Dollinger and R. Watson, Kinetic PCR analysis-Real-time monitoring of DNA amplification reactions, *BioTechnology*, 1993, **11**, 1026.
154 A. Manz, N. Graber and H. M. Widmer, Miniaturized Total Chemical-Analysis Systems-a Novel Concept for Chemical Sensing, *Sens. Actuators B*, 1990, **1**, 244.
155 L. Zhang, F. Dang and Y. Baba, Microchip electrophoresis-based separation of DNA, *J. Pharm. Biomed. Anal.*, 2003, **30**, 1645.
156 S. H. Chen, Microchip electrophoresis and the analysis of polymerase chain reaction products, *LC GC North Am.*, 2002, **20**, 164.
157 L. Mitnik, L. Carey, R. Burger, S. Desmarais, L. Koutny, O. Wernet, P. Matsudaraira and D. Ehrlich, High-speed analysis of multiplexed short tandem repeats with an electrophoretic microdevice, *Electrophoresis*, 2002, **23**, 719.
158 W.-L. Tseng, Y.-W. Lin, K.-C. Chen and H.-T. Chang, DNA analysis on microfabricated electrophoretic devices with bubble cells, *Electrophoresis*, 2002, **23**, 2477.
159 F. Han, B. H. Huynh, Y. Ma and B. Lin, High-efficiency DNA separation by capillary electrophoresis in a polymer solution with ultralow viscosity, *Anal. Chem.*, 2003, **71**, 2385.
160 F. Xu, M. Jabasini and Y. Baba, Fast screening reduced-viscosity mixed polymer solutions using an orthogonal designing approach for microchip separation of specific DNA fragments, 259, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
161 C. A. Emrich, H. Tian, I. L. Medintz and R. A. Mathies, Microfabricated 384-lane capillary array electrophoresis bioanalyzer for ultrahigh-throughput genetic analysis, *Anal. Chem.*, 2002, **74**, 5076.
162 T. C. Messina, L. N. Dunkleberger, G. A. Mensing, A. S. Kalmbach, R. Weiss, D. J. Beebe and L. L. Sohn, A novel high-frequency sensor for biological discrimination, 1223, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
163 R. A. Zangmeister and M. J. Tarlov, Selective DNA sensing elements integrated into microfluidic channels, 1343, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
164 L. G. Lee, C. R. Connell and W. Bloch, Allelic discrimination by nick-translation PCR with fluorogenic probes, *Nucl. Acids Res.*, 1993, **21**, 3761.
165 S. A. E. Marras, F. R. Kramer and S. Tyagi, Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes, *Nucl. Acids Res.*, 2002, **30**, e122.
166 A. M. Wang, M. V. Doyle and D. F. Mark, Quantitation of mRNA by the Polymerase Chain Reaction, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 9717.
167 G. Gilliland, S. Perrin, K. Blanchard and H. F. Bunn, Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 2725.
168 P. Duck, G. Alvarado-Urbina, B. Burdick and B. Collier, Probe amplifier system based on chimeric cycling oligonucleotides, *BioTechniques*, 1990, **9**, 142.
169 M. L. Beggs, M. D. Cave, C. Marlowe, L. Cloney, P. Duck and K. D. Eisenach, Characterization of Mycobacterium tuberculosis complex direct repeat sequence for use in cycling probe reaction, *J. Clin. Microbiol.*, 1996, **34**, 2985.
170 T. Tang, G. Ocvirk and D. J. Harrison, Iso-Thermal DNA Reactions and Assays in Microfabricated Capillary Electrophoresis Systems, *Transducers '97*, 1997, **1**, 523.
171 M. Y. Badal, T. Tang, W. E. Lee, T. Dickinson-Laing, D. E. Bader and D. J. Harrison, An integrated system for gene detection using cycling probe technology, 423, in *Micro Total Analysis Systems 2000, Proceedings*, 2000, Dordrecht.
172 T. Tang, M. Y. Badal, G. Ocvirk, W. E. Lee, D. E. Bader, F. Bekkaoui and D. J. Harrison, Integrated microfluidic electrophoresis system for analysis of genetic materials using signal amplification methods, *Anal. Chem.*, 2002, **74**, 725.
173 B. D. Biosciences, BD QZyme Assays for quantitative PCR, *Clontechniques*, **18**, 2.
174 A. V. Todd, C. J. Fuery, H. L. Impey, T. L. Applegate and M. A. Haughton, DzyNA-PCR: Use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format, *Clin. Chem.*, 2000, **46**, 625.
175 J. W. Liu and Y. Lu, Improving fluorescent DNAzyme biosensors by combining inter- and intramolecular quenchers, *Anal. Chem.*, 2003, **75**, 6666.
176 F. Chen, R. J. Wang, Z. Li, B. Liu, X. P. Wang, Y. H. Sun, D. Y. Hao and J. Zhang, A novel replicating circular DNAzyme, *Nucl. Acids Res.*, 2004, **32**, 2336.
177 I. Afonina, M. Zivarts, I. Kutyavin, E. Lukhtanov, H. Gamper and R. B. Meyer, Efficient priming of PCR with short oligonuleotides conjugated to a minor groove binder, *Nucl. Acids Res.*, 1997, **25**, 2657.
178 I. V. Kutyavin, I. A. Afonina, A. Mills, V. V. Gorn, E. A. Lukhtanov, E. S. Belousov, M. J. Singer, D. K. Walburger, S. G. Lokhov, A. A. Gall, R. Dempcy, M. W. Reed, R. B. Meyer and J. Hedgpeth,

3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures, *Nucl. Acids Res.*, 2000, **28**, 655.
179 S. Tyagi and F. R. Kramer, Molecular beacons: probes that fluoresce upon hybridization, *Nat. Biotechnol.*, 1996, **14**, 303.
180 G. Bonnet, S. Tyagi, A. Libchaber and F. R. Kramer, Thermodynamics basis of the enhanced specificity of structured DNA probes, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 6171.
181 M. Culha, D. L. Stokes, G. D. Griffin and T. Vo-Dinh, Application of a miniature biochip using the molecular beacon probe in breast cancer gene BRCA1 detection, *Biosens. Bioelectron.*, 2004, **19**, 1007.
182 A. Dodge, G. Turcatti, I. Lawrence, N. F. De Rooij and E. Verpoorte, A microfluidic platform using molecular beacon-based temperature calibration for thermal dehybridization of surface-bound DNA, *Anal. Chem.*, 2004, **76**, 1778.
183 I. Nazarenko, B. Lowe, M. Darfler, P. Ikonomi, D. Schuster and A. Rashtchian, Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore, *Nucl. Acids Res.*, 2002, **30**, e37.
184 D. Whitcombe, J. Theaker, S. P. Guy, T. Brown and S. Little, Detection of PCR products using self-probing amplicons and fluorescence, *Nat. Biotechnol.*, 1999, **17**, 804.
185 S. N. Gardner, T. A. Kuczmarski, E. A. Vitalis and T. R. Slezak, Limitations of TaqMan PCR for detecting divergent viral pathogens illustrated by hepatitis A, B, C, and E viruses and human immunodeficiency virus, *J. Clin. Microbiol.*, 2003, **41**, 2417.
186 G. Nishimura and M. Kinjo, Systematic error in fluorescence correlation measurements identified by a simple saturation model of fluorescence, *Anal. Chem.*, 2004, **76**, 1963.
187 Applied Biosystems, Data analysis on the ABI Pprism 7700 sequence detection system: setting baselines and thresholds, http://www.appliedbiosystems.com/support/tutorials/pdf/data_analysis_7700.pdf.
188 R. G. Rutledge and C. Côté, Mathematics of quantitative kinetic PCR and the application of standard curves, *Nucl. Acids Res.*, 2003, **31**, e93.
189 W. Liu and D. A. Saint, A new quantitative method of real time reverse transcritpion polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics, *Anal. Biochem.*, 2002, **302**, 52.
190 J. Wilhelm, A. Pingoud and M. Hahn, Validation of an algorithm for automatic quantification of nucleic acid copy numbers by real-time polymerase chain reaction, *Anal. Biochem.*, 2003, **317**, 218.
191 C. Kimball, J. Buch, C. Lee and D. L. de Voe, Temperature gradient gel electrophoresis in an integrated polycarbonate microsystem, 24, in *Transducers*, 2003, Boston, MA, USA.
192 J. S. Buch, Y. Li, F. Rosenberger, D. L. de Voe and C. S. Lee, Two-dimensional genomic and proteomic separations in a plastic microfluidic network, 477, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
193 P. Liu, W. Xing, D. Liang, G. Huang, Y. Zhou and J. Cheng, Fast screening of single-nucleotide polymorphisms using chip-based temperature gradient capillary electrophoresis, *Anal. Lett.*, 2003, **36**, 2823.
194 T. Ito, A. Inoue, K. Sato, K. Hosokawa and M. Maeda, Detection of single-base mutation by affinity capillary electrophoresis in a PDMS-glass hybrid microdevice, 263, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
195 X.-B. Zhong, R. Reynolds, J. R. Kidd, K. K. Kidd, R. Jenison, R. A. Marlar and D. C. Ward, Single-nucleotide polymorphism genotyping on optical thin-film biosensor chips, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 11559.
196 A. Russom, A. Ahmadian, H. Andersson, P. Nilsson and G. Stemme, Single-nucleotide polymorphism analysis by allele-specific extension of fluorescently labeled nucleotides in a microfluidic flow-through device, *Electrophoresis*, 2003, **24**, 158.
197 W. Cai, J. R. Peck, D. W. van der Weide and R. J. Hamers, Direct electrical detection of hybridization at DNA-modified silicon surfaces, *Biosens. Bioelectron.*, 2004, **19**, 1013.
198 D. Trau, T. M. H. Lee, A. I. K. Lao, R. Lenigk, I.-M. Hsing, N. Y. Ip, M. C. Carles and N. J. Sucher, Genotyping on a complementary metal oxide semiconductor silicon polymerase chain reaction chip with integrated DNA microarray, *Anal. Chem.*, 2002, **74**, 3168.
199 R. McKendry, J. Zhang, Y. Arntz, T. Strunz, M. Hegner, H. P. Lang, M. K. Baller, U. Certa, E. Meyer, H. J. Güntherodt and C. Gerber, Multiple label-free biodetection and quantitative DNA-binding assays on a nanomechanical cantilever array, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 9783.
200 H. Li and E. S. Yeung, Selective genotyping of individual cells by capillary polymerase chain reaction, *Electrophoresis*, 2002, **23**, 3372.
201 M. Hashimoto, Y. He and E. S. Yeung, On-line integration of PCR and cycle sequencing in capillaries: from human genomic DNA directly to called bases, *Nucl. Acids Res.*, 2003, **31**, e41.
202 A. Han, E. Moss, R. D. Rabbitt, K. L. Engisch and A. B. Frazier, A single cell multi-analysis system for electrophysiological studies, 674, in *Transducers*, 2003, Boston, MA, USA.
203 P. R. C. Gascoyne, J. V. Vykoukal, T. Anderson, J. Noshari, F. F. Becker, K. Ratanachoo, K. Kandjanapa, J. Satayavivad and M. Ruchirawat, Programmable dielectrophoretic uTAS sample handling, 919, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
204 J. Gao, X.-F. Yin and Z.-L. Fang, Integrating single cell injection, cell lysis and separation of intracellular constituents on a microfluidic chip, 231, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
205 C. J. Bruckner-Lea, T. Tsukuda, B. Dockendorff, J. C. Follansbee, M. T. Kingsley, C. Ocampo, J. R. Stults and D. P. Chandler, Renewable microcolumns for automated DNA purification and flow-through amplification: from sediment samples through polymerase chain reaction, *Anal. Chim. Acta*, 2002, **469**, 129.
206 T. Fukuba, T. Naganuma and T. Fujii, Microfabricated flow-through PCR device for *in situ* gene analysis in extreme environments, 725, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
207 K. Sakai-Kato, M. Kato and T. Toyo'oka, Creation of an on-chip enzyme reactor by encapsulating trypsin in sol-gel on a plastic microchip, *Anal. Chem.*, 2003, **75**, 388.
208 Z. H. Fan, A. J. Ricco, W. Tan, M.-Q. Zhao and C. G. Koh, Integrating multiplexed PCR with CE for detecting microorganisms, 849, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
209 D.-S. Lee, S. H. Park, H. Yang, T. H. Yoon, S.-J. Kim, H. Kim, Y. B. Shin, K. Kim and Y. T. Kim, Submicroliter-volume PCR chip with fast thermal response and very low power consumption, 187, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
210 J. El-Ali, I. R. Perch-Nielsen, C. R. Poulsen, D. D. Bang, P. Telleman and A. Wolff, Simulation and experimental validation of a SU-8 based PCR thermocycler chip with integrated heaters and temperature sensor, *Sens. Actuators A*, 2004, **110**, 3.
211 S. A. Soper, W. Stryjewski, L. Zhu, Y. Xu, M. Wabuyele, H. Chen, M. Galloway and R. L. McCarley, Polymer-based modular microsystems for DNA sequencing, 717, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
212 E. T. Lagally, J. R. Scherer, R. G. Blazej and R. A. Mathies, Genetic analysis using a portable PCR-CE microsystem, 1283, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
213 I. Rodriguez, M. Lesaicherre, Y. Tie, Q. Zou, C. Yu, J. Singh, L. T. Meng, S. Uppili, S. F. Y. Li, P. Gopalakrishnakone and Z. E. Selvanayagam, Practical integration of polymerase chain reaction amplification and electrophoretic analysis in microfluidic devices for genetic analysis, *Electrophoresis*, 2003, **24**, 172.
214 N. Y. Lee, M. Yamada and M. Seki, Purification of human genomic DNA from a single hair root on a microdevice and direct amplification of its D1S80 locus, 721, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
215 S. Shandrick, Z. Ronai and A. Guttman, Rapid microwell polymerase chain reaction with subsequent ultrathin-layer gel electrophoresis of DNA, *Electrophoresis*, 2002, **23**, 591.
216 C. G. Koh, W. Tan, M.-Q. Zhao, A. J. Ricco and Z. H. Fan, Integrating polymerase chain reaction, valving, and electrophoresis in a plastic device for bacterial detection, *Anal. Chem.*, 2003, **75**, 4591.
217 C. G. Koh, W. Tan, M.-Q. Zhao, A. J. Ricco and Z. H. Fan, Correction-Integrating polymerase chain reaction, valving, and electrophoresis in a plastic device for bacterial detection, *Anal. Chem.*, 2003, **75**, 6379.
218 C.-F. Chou, R. Lenigk, D. Sadler, R. Changrani, S. O'Rourke, R. H. Liu and F. Zenhausern, Rapid genotyping with integrated continuous-flow PCR and bioelectronic detection, 1203, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.
219 K. Sun, A. Yamaguchi, Y. Ishida, S. Matsuo and H. Misawa, A heater-integrated transparent microchannel chip for continuous-flow PCR, *Sens. Actuators B*, 2002, **84**, 283.
220 P. J. Obeid and T. K. Christopoulos, Continuous-flow DNA and RNA amplification chip combined with laser-induced fluorescence detection, *Anal. Chim. Acta*, 2003, **494**, 1.
221 F. Arai, A. Ichikawa, T. Fukuda and T. Katsuragi, Continuous culture and monitoring of selected and isolated microorganisms on a chip by thermal gelation, 757, in *Micro Total Analysis Systems*, 2003, Squaw Valley, CA, USA.