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REVIEW ARTICLE

Extraction, amplification and detection of DNA in microfluidic chip-based assays

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Abstract This review covers three aspects of PCR-based microfluidic chip assays: sample preparation, target amplification, and product detection. We also discuss the challenges related to the miniaturization and integration of each assay and make a comparison between conventional and microfluidic schemes. In order to accomplish these essential assays without human intervention between individual steps, the micro-components for fluid manipulation become critical. We therefore summarize and discuss components such as microvalves (for fluid regulation), pumps (for fluid driving) and mixers (for blending fluids). By combining the above assays and microcomponents, DNA testing of multi-step bio-reactions in microfluidic chips may be achieved with minimal external control. The combination of assay schemes with the use of micro-components also leads to rapid methods for DNA testing via multi-step bioreactions. Contains 259 references.

Keywords DNA · Point-of-care testing · Polymerase chain reaction · Microfluidics · Chip · Microvalve · Micropump · Micromixer

Introduction to microfluidics and DNA

Since the last century, the miniaturization of electronic devices–microelectronics has been regarded as the most significant enabling technology in human history. With integrated circuits and progress in information processing, microelectronics has revolutionized the way we work, live and play. Miniaturization concepts have recently been brought to the forefront of the fluidics since the introduction by Manz et al. at the 5th International conference on Solid-State Sensors and Actuators (Transducers '89): microfluidics, which appeared as the name for the new research discipline dealing with transport phenomena and fluid-based devices at microscopic length scales [1].

One of the most impressive developments of microfluidics in life sciences is Point-of-Care testing (POCT), which is defined as diagnostic testing at or near the site of patient care to make the test convenient and immediate [2]. Patients can receive testing result within minutes utilizing miniaturized and portable devices including hand held instruments (e.g., blood glucose meter) and test kits (e.g., pregnancy test, HIV salivary assay, pregnancy test strip etc.). Such devices can be used in hospitals, at a doctor office or simply by patients by themselves at home without any professional knowledge or particular skill. Additionally, miniaturized devices are likely to impact economy and improve public health significantly in third world countries, bringing cheaper and smaller, but still sophisticated analytical tools to rural areas, and resource-poor regions [3].

The detection of DNA and its variation is critical for many fields, including clinical and veterinary diagnostics, industrial and environmental testing, agricultural researches and forensic science. Disease diagnosis and prognosis are based on effective detection of disease conditions (e.g. cancer), infectious organisms (e.g. HIV) and genetic markers. However, DNA analysis from original specimens is a complex process involving multiple chemical compositions as well as multistep reactions. The concentration of DNA analyte in the test samples is usually not yet high enough for direct detection. Therefore, DNA amplification is a required step to raise the concentration of the target sequence. Since its invention in 1983, polymerase chain reaction (PCR) has been the method of choice for DNA

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amplification for decades [4]. Diseases caused by infectious bacterial and viral DNA can be detected by PCR. PCR's high sensitivity enables virus detection soon after infection and even before the onset of disease.

In the past 20 years, microfluidics has emerged as powerful tools for DNA analysis and the publications in the topic of microfluidic/DNA have increased steadily from 1993 to 2013 (Fig. 1). In this review, we will focus on the development of PCR-based microfluidic chip for DNA analysis. Such kind of device can extract DNA, amplify and detect it–all in one integrated chip or device, and in a very short period of time [5]. An ideal PCR-based microfluidic chip for DNA analysis should be capable of automated operation, with individual assay accomplished by the micro-components. To achieve this "sample-in-result-out" goal, there are three essential on-chip steps: sample preparation, target amplification, and product detection. Consequently, the implementation and the state-of-the-art of these steps as well as the fluidic manipulation are individually summarized and discussed. The emphasis is placed on the role, necessity and criteria of each step or micro-components for fully integrated microfluidic chips.

On-chip sample preparation

Sample preparation is the first while also the most easily overlooked step in the full process of bio-analysis. The problem become more serious for POCT application in which great attention was put on the reaction and detection steps while less on sample preparation for difficulties and negligence [6, 7]. For nucleic acid test in clinical practice, DNA/RNA extraction and purification from a complex bio-sample is usually inevitable. Conventionally this procedure is time consuming, labor intensive and contamination prone, it is not compatible with high throughput or field testing requirement. To integrate the automatic sample pretreatment functions will reduce reagent consuming, assay time and contamination risk. More importantly, it will affect the efficiency and reliability of a test ("garbage in, garbage out") but also determine the feasibility of a final product. It is not an exaggeration to say that the last large obstacle to realize a real practical POCT product is total integration of automatic sample preparation process, at least for the nucleic acid tests system.

In the past decade about 3,000 papers published about on chip DNA tests (See Fig. 1), almost all of them need off-chip sample preparation and reagent handling because. A full function system with sample-in–answer-out capability is still rare [8]. To our best knowledge, the only two fully integrated devices for nucleic acid test in the market are GeneXpert system by Cepheid (Sunnyvale, CA) and Liat Analyzer by IQuum (Allston, MA). Without exception, both of their outstanding lies in the successful integration of sample preparation process smoothly into their system.

In this section, we firstly brief the basic mechanism for conventional DNA extraction method, then discuss they modification to on-chip assay and new technique which only possible by microfluidic.

Conventional sample preparation

In principle, exact DNA extraction methods highly depend on the species of the sample (virus, bacterial, yeast, animal cell or

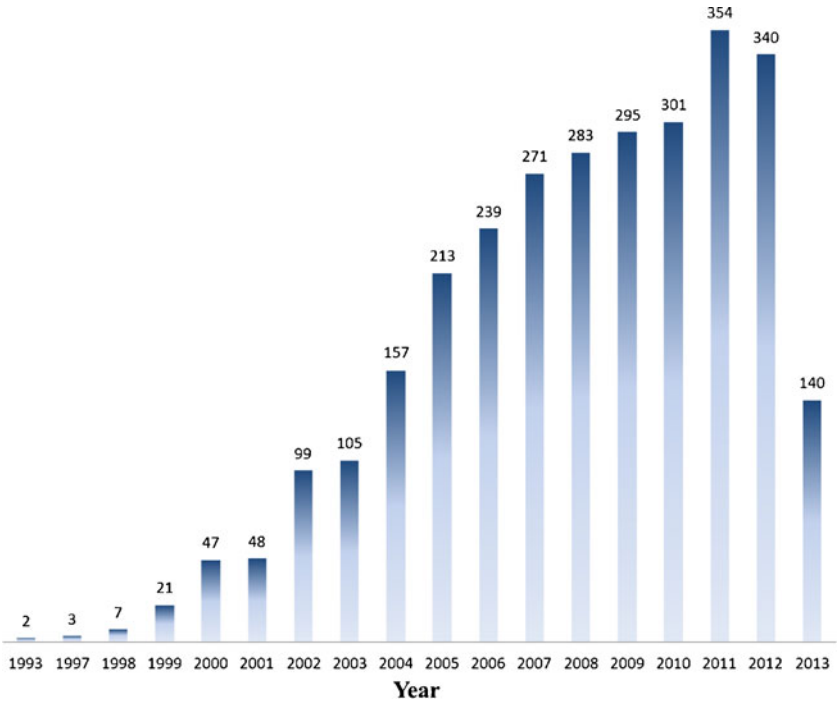

Fig. 1 Publications record per year in the topic of microfluidic/DNA from Web of Science. The publications in 2013 were recorded by July 2013

plant cell) and the raw source of the sample reside in (blood, saliva, urine or tissue section etc.) as well. There is not a universal method for entire diversified samples. For clarity and simplicity, our discussion will be based on a "standard method" and modification of it.

A conventional bench top DNA extraction procedure divided into 3 key steps: (1) Cell isolation and lysis; (2) DNA extraction; (3) DNA purification. Nucleic acid can be extracted out of cells by disrupting the tissue/lysing cells and then exploring the difference in properties between DNA/RNA, protein and other constitutes (lipids, polysaccharides etc.). In disruption process, cell membrane (wall) usually disrupted by mechanical (grinding, heat, sonication, microwave and electric field) or detergents (SDS, Triton-100x enzyme also added when necessary) to release internal genetic materials, in the following separation process, DNA was partitioned into aqueous phase by phase separation (Phenol-Chloroform/water is the mostly used two phase because Phenol-Chloroform can dissolve and denature protein while not dissolve DNA.) or alternatively, by absorbed on a silica-gel surface (or magnetic beads) by affinity method under high-salt buffer. Finally, DNA in aqueous solution (or elute firstly under low-salt buffer if they are adsorbed on solid phase surface) will be precipitated by alcohol (2.5 volumes of ethanol or 1 volume of isopropanol) and then air dried.

For the realization of this function on chip, the challenge is the multi steps handling of heterogeneous biochemical contents and their transfer between multiphase (solid and liquid, organic and inorganic). These contents are usually incompatible with each other harmful to the following PCR amplification process thus must be separated in prior of amplification. Some convenience in DNA template extraction can ease the difficulties are the PCR amplification results are insensitive to quantity of DNA template (from tens to tens of thousand copies), so extraction efficiency is not a big issue here. Another expedience seldom noticed is fierce chemical and physical treatment of DNA template usually did not affect the PCR result (except long distant PCR larger than 10Kb [9] even though long DNA molecule is vulnerable to mechanical shearing, and the PCR efficiency is even greater for smaller-size template DNA. In the following several paragraph, we will discuss cell lysis techniques according to the physical mechanism and DNA extraction and purification according to its solubility and affinity.

On-chip cell lysis

Detergent lysis is the most widely used bench top method in laboratory can be transferred to chip [10–12]. Detergents (like SDS or Triton-X) can disrupt the cell membranes by solubilizing their phospholipids. It is very effective to mammalian cells but bacterial and yeast cells, lysozyme should be added to weaken the cell wall. It also usually be used in combination with other method like mechanical milling, thermal lysis etc.

Thermal lysis use high temperature to rupture cell membrane/wall, animal cells and microbial cell can be disrupt by place in boiling water for 40 s [13]. Maybe this is the easiest method for implementing in a microfluidic chip since thermal treatment can be integrate with PCR thermal cycle [14, 15]. There is plenty of microfluidic system reported use this method [16–19] .

Ultrasound produces tiny gaseous cavitation from dissolved gases or vapors due to drastic pressure variation in the liquid. Imploding of cavitation produce shock waves equivalent to several thousand atmospheres of pressure, which is sufficient to break cells [20, 21]. Ultrasound treatment of mammalian or bacterial cells suspension under 1 $W{\cdot}cm^{-2}$ less than 1 min is enough to release DNA fragment in length of hundreds bp [22]. The advantage of this method is its ubiquitous to cell type and short lysis time, the disadvantage is it hard to be portable because its relative larger profile or external device needed. Nevertheless, it is realized on chip and much progress was made on its miniaturization and efficiency [23, 24]. GeneXpert system by Cepheid (Sunnyvale, CA) also use sonication lysis, it is the first commercialized PCR-based instrument that fully integrates sample preparation, amplification, and detection in a single device [25].

Cell behavior under electrical field was studied for a long time and many properties and parameter was well known [26]. When cell transmembrane potential exceed a critical value (1 Vol), cell membrane will fully breakdown. By optimize electrode design and buffer selection, applying an external field (direct current (DC), alternating current (AC) or Radio frequency (RF) modulated DC) at the order of $KV{\cdot}cm^{-1}$ can compromise cell membranes sufficiently to extract DNA out of cell. This method has a good compatibility with microfluidic chip and so was also widely adopted, though it is not without problems such as bubble generation and relative low efficiency [27–30] .

Another possible lysis mechanism accompanied with electrolysis is electrochemical lysis [31]. It utilizes the hydroxide ions generated by electrolysis of buffer to compromise cell membrane.

On-chip DNA extraction

As mentioned in the beginning, once cell was lysed, DNA need be extracted from the lysate in most situations, otherwise possible reagent mix in the lysate will inhibitor the following PCR reaction. Borrow conventional DNA purification method to on-chip process is the most direct method, like organic-aqueous liquid extraction [32], or affinity column of silica [8, 33–35].

For on-chip DNA separation, the most popular method is using magnetic beads coated with silica or functionalized

groups (carboxyl [36], amine [37], biotin [38], nucleotide probes [39] or etc.) to extract DNA from lysate medium. This method takes the advantage of a compact system for the exquisite manipulation of magnetic beads. This is also the separation method adopted by Liat AnalyzerTM from IQuum Inc, the only few commercialized product in the market.

Some distinctive purification methods which are only reachable on microfluidic platform were also reported. For example, dielectrophoresis trapping [40] or isotachophoresis separation were successfully realized on chip for DNA purification [41]. It is worth to mention that DNA separation/purification is not always necessary if the lysate buffer is specially chose to avoid the interference to PCR reaction.

DNA amplification by PCR

PCR is typical temperature-controlled and enzyme-catalyzed biochemical reaction involving three steps of thermal cycling: denaturation of double strand DNA (dsDNA) into single strand DNA (ssDNA), annealing of primers to the denatured strands and elongation of the primers by a thermostable DNA polymerase enzyme. It is commonly assumed that PCR reaction requires, therefore, three different temperatures: 95 °C for DNA denaturation (denaturing temperature, Td), ~55 °C for annealing (annealing temperatures, Ta), and 72 °C for extension. These cycles are repeated until there are enough copies of DNA to be detected and analyzed. In principle, each PCR cycle can double the number of DNA molecules. Therefore, theoretically more than 1 billion DNA molecules of interest can be generated after 30 cycles. PCR thermal cycling could be carried in either stationary or flowing forms. In the stationary forms, the temperature of PCR solution is switched to repeat the thermal cycling by complicated temperature control device. In the flowing form, PCR solution is flowed or oscillated along two or three constant temperature zones or temperature gradient driven by convection or pump.

Thermal manipulation: temperature control and measurement

Bench thermocyclers are capable of performing automated reactions in plastic tubes at heating/cooling speeds of ~2–3 °C·s^{-1} so the temperature difference of 20 °C can be achieved in ~10 s. Nowadays, in contrast, DNA chips can easily achieve heating/cooling speeds of 10–20 °C·s^{-1} (i.e. a temperature difference of 20 °C can be achieved in less than a second) owing to miniaturization and its attendant enhanced heat-transfer rates [42–44]. Different types of PCR microfluidic technologies have facilitated DNA amplification with much shorter reaction times, owing to a smaller thermal capacity and a higher heat-transfer rate between the PCR sample and the temperature-controlled components. The choice of heating and cooling methods for PCR microfluidics is of importance for achieving high temperature ramping speeds, temperature precision and thermal distribution uniformity. Within current PCR microfluidics, the most commonly used temperature control algorithm is percentage-integrator-differentiator module for triggering either the heating or cooling elements to reach set temperature [45]. Air impingement [46–49] and Peltier-effect-based thermoelectric cooling [50] are two major cooling methods. While for the heating methods, various advanced technologies have been applied and investigated in microfluidics. They could be typically divided into two categories: contact and non-contact heating [44, 45].

Thin film heating elements and Peltier-effect-based thermoelectric heating blocks by are the two main contact heating formats. The thin film (such as Platinum (Pt) [48, 51–54] and polysilicon [55–57]) could be deposited and patterned locally on a substrate using thin film deposition and photolithography techniques, so that it could be further integrated with other components into one chip. Wu and coworkers [48] developed a temperature control system (Fig. 2) using Pt thin film as heater and temperature sensor (Fig. 2b). Rather than integrating more functions into one chip, they designed PCR device consisting of a temperature control system, an optical detection system and an interchangeable (disposable or modular) PCR chip, where the PCR chip is independent from the two functional systems.

The thermoelectric heating blocks are commercially available and have been widely used in PCR microfluidics in spite of larger thermal mass, slower temperature ramping rates and non-transparent [15, 58–61].

For the non-contact heating methods, thermal cycling with a tiny volume sample was performed independently and without physical contact between the heating source and the reaction sample. These methods based on hot air [62, 63], optical absorption (Infrared (IR) [64, 65]) and microwave absorption [46, 66] are capable of very fast temperature control. For example (Fig. 3), Shaw et al. developed a microwave heating system with heating rate up to 65 °C·s^{-1}, minimal over- or under-shoot (±0.1 °C) and high accuracy of temperature(±0.1 °C) [46].

To achieve fast and precise thermal cycling in microfluidics, temperature measurement method should have following criteria: high temperature resolution and acquisition rate. Traditional embedded thermocouple [46, 49, 61, 67] and semiconductor electrical-resistance thermometers [58, 68] are common approaches and easily implemented in microfluidics. The thin film temperature sensors such as Pt could be patterned to μm size and integrated with thin film heater into one chip using photolithography, so that they provides a higher degree of integration and rapid response [48]. The non-contact IR [60, 69] and fluorescent [64, 70] thermometry are easily accessible and full-field techniques which are also capable of mapping temperature over a large area with high acquisition rate and

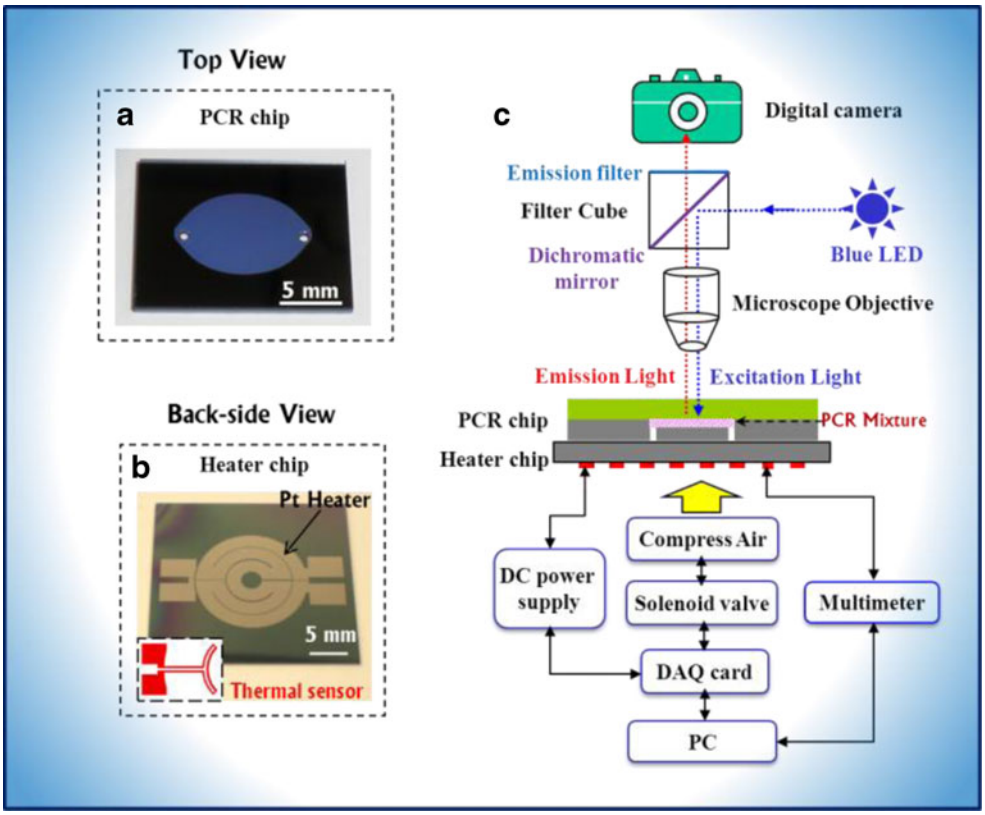

Fig. 2 Interchangeable Pcr chip and temperature control device of Wu et al. [48]. **a** Top view of PCR chip; **b** Back-side view of heater chip, Pt Heater and thermal sensor were integrated in one chip; **c** *Upper panel* is optical detection system. *Central panel* is chip layout of self-sealed. PCR chip was put on the backside of heater chip after PCR mixture was injected into PCR chamber. *Lower panel* is temperature control system connected to heater chip

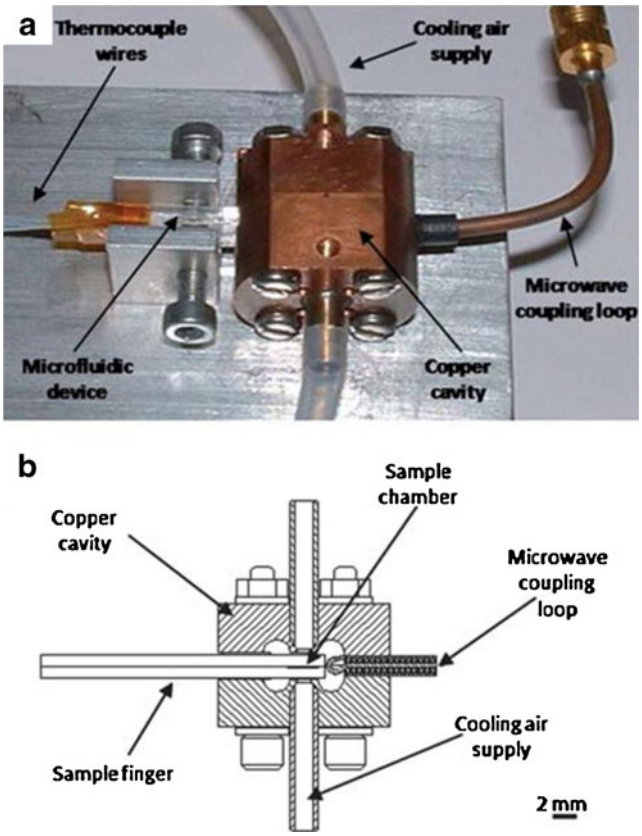

Fig. 3 Photograph (**a**) and schematic cross-section (**b**) of the re-entrant cylindrical microwave cavity from Shaw et al. [46]

spatial resolution up to several μm. Although non-contact methods have disadvantages of lower precision than the contact methods, they could be further coupled with non-contact heating for easy accomplishment of nanoliter droplet PCR [64].

PCR reaction components consideration

DNA amplification is performed by enzymatic reaction, which is composed of various components essential for the reaction to be successful. The PCR reaction components include the DNA template, primers, DNA polymerase enzyme, deoxyribonucleotide (dNTP), a buffer, divalent ions ($MgCl_2$) and KCl. The main engine in this reaction is heat-stable polymerase. Additionally, the right concentration of the reaction components is critical for the success and maximum amplification. The material interaction is much distinct in high surface-area-to-volume ratio in microfluidics. The interaction between the reaction chamber material and the enzymatic reaction components may make some of the components inactive resulting in lower or non-amplification of DNA. Every of the reaction components can be easily tested for possible inhibition within the microfluidic PCR chamber. This is usually done by indirect approach, when the PCR reaction is incubated with the material of interest, and one of the reaction components is omitted. The omitted reaction component is later supplemented to the reaction before running the PCR cycling. If the other reaction components will be

significantly absorbed by the tested material, the reaction will yield only low amplified DNA, or as a result of total inhibition, there will be no DNA amplification [71, 72].

PCR reaction tolerates high concentration of DNA primers and dNTP, which can be added in excess in microfluidics PCR reaction. dNTPs may bind Mg^{2+} ions and thus affect the concentration of free magnesium ions in the reaction. In addition, excessive amounts of dNTPs can increase the error rate of DNA polymerase and even inhibit the reaction. Within limits, increasing primer concentration may improve the outcome of the PCR reaction. Even if some of the oligo primers or the dNTP is absorbed on the material surface, there will be enough free available primers or dNTP to complete the amplification reaction.

DNA template contains the DNA region (target) to be amplified. The amount of DNA template strongly influences the outcome of the reaction. Template DNA to be amplified is a negatively charged polyanionic molecule. DNA is not expected to be absorbed on hydrophobic (such as polymers) or negatively charged surfaces (such as glass or silicon dioxide (SiO_2)). The sample itself, besides DNA may contain some inhibitors that interact with DNA and make it unavailable for the amplification reaction. The sample purity plays a role, as inhibitors like humic acid, collagen or melanin may reduce the final PCR product [73]. Some of the materials used for On-Chip-PCR have been demonstrated to inhibit the PCR reaction through DNA inhibition. This includes yellow shiftwax from Nikka Seiko, Norland Optical Adhesive 61 after exposure to UV light and dried acrylic glue [71].

Salts such as KCl or ammonium sulfate ($(NH_4)_2SO_4$) are important components of PCR buffer, as DNA strand denaturation depends on salt concentration. Especially short DNA molecules are amplified preferentially over long DNA molecules at high ionic strength, as longer DNA molecules denaturate slower than shorter DNA. Additionally buffer provides suitable chemical environment and stable reaction pH for optimum activity and stability for the "reaction engine" polymerase. It is expected pH to be stable at high temperature. The pH of the Tris buffer in the reaction mix will decrease at high temperatures, which may cause depurination of the template DNA, resulting in lower yield of amplicons. A higher pH (such as pH 9.0) is suggested for long DNA templates. While material is not expected to interact with monovalent ions, the influence in pH may be significant. The material influence on pH can be monitored by incubating it in pH indicator diluted solution (such as Cresol Red). Changing color will indicate on material influence on reaction buffer pH. As Cresol Red can be included into PCR mix, it is advisable to use it for easier observation of potential pH changes during PCR [48, 71, 74]. Indeed, we could observe pH changes by incubating PCR reaction mix containing Cresol Red with some of materials.

Magnesium (i.e. $MgCl_2$) is an important component for the PCR, as to work properly DNA polymerase requires free divalent cations, such as magnesium or manganese. The magnesium is easily bound by the dNTP and the DNA, therefore even why small increases in the dNTP concentrations can rapidly inhibit the PCR reaction (magnesium form soluble complexes with dNTPs), whereas increases in magnesium concentration often have positive effects. Magnesium also facilitates the annealing of the oligo primer to the template DNA by stabilizing the primer-template DNA interaction. Mg^{2+} ions stabilize the double strand DNA and raise the melting temperature. Some of the components of the reaction mixture such as template concentration, dNTPs and the presence of chelating agents (Ethylenediaminetetraacetic acid (EDTA)) or some proteins can reduce the amount of free Mg^{2+} present thus reducing the activity of the DNA polymerase. There is s potential to loose Mg^{2+} ions by the absorption by negatively charged PCR chamber walls (such as SiO_2). Therefore it is advisable to include higher end of the Mg^{2+} concentration. Overall, the dNTP concentration should be balanced in such a way that some free Mg^{2+} ions will remain in solution.

Heat-stable DNA polymerase is an engine of PCR reaction, enzymatically assembling a new DNA strand from nucleotides, by using ssDNA as template. The DNA polymerase remains active even after DNA denaturation. Too little enzyme may result in the lack of the amplification products, while too much enzyme, usually because of the high glycerol concentration in the stock solution, may result in an unbalanced amplification and an increase in the background. The optimal amount of polymerase is also dependent on the template size and type.

DNA polymerases have highly conserved three-dimensional structure, with catalytic subunits and domains. Conserved 3D structure is of immense importance for the correct polymerase function. As every protein, DNA polymerase may interact with surface, undergoing conformational changes, losing the 3D structure with the following loss of the amplification ability. Inhibition of DNA polymerase will result in very little or no DNA amplified (PCR product). Protein adsorption itself is a very complex process, which is driven by different protein-surface forces, including hydrophobic, electrostatic and van der Waals forces. Important parameters for adsorption are the properties of DNA polymerase itself, the material surface, ionic strength and the nature of solvent, temperature and pH. Polymerase adsorption is usually only partial reversible because polymerase undergo structural changes due adsorption and they get attached with many amino acid residues to the surface. Increasing ionic strength or changing pH may help desorption of the polymerase from the surface. Studies have shown DNA polymerase inhibition on hydrophobic surfaces (such as hydrophobic Polydimethylsiloxane (PDMS) or the hydrophobic silane surfaces) [75–77]. The interaction between hydrophobic parts of the protein with hydrophobic surface is much larger than to hydrophilic surfaces. Another main force

governing the adsorption is electrostatical interaction between charged DNA polymerase residues (depending on the solution pH) and the charged solid material surface. It has been reported that often a maximum in the absorbed amount could be found around the isoelectric point of the protein [78]. The ionic strength is also influencing protein-protein, surface-protein and intramolecular interactions. Polymerase adsorption, which is controlled by electrostatic attraction, will decrease in the presence of salt because electrostatical affinity between sorbent and protein will decrease [78–80]. Numerous studies have demonstrated DNA polymerase inhibition on various materials, such as silicon, silicon nitride (Si_3N_4), untreated PDMS and so on.

Passivation techniques to avoid reaction inhibition

Already in 1996, Shoffner et al. pointed to the surface interaction in PCR reaction [81]. Bovine serum albumin (BSA) can be added as a carrier-protein to the PCR mix, to compete with the enzyme (such as DNA polymerase) for adsorption at the microfluidic chip walls, thus improving the PCR yield [82]. BSA also facilitates primer annealing, as an osmoprotectant stabilizes both the DNA and polymerase, also acts as a DNA polymerase competitor in inhibitor chelation [83]. The adsorption mechanism both of BSA protein [84, 85] and DNA polymerase [86] has been extensively studied. The adsorption of the DNA polymerase was systematically studied on 13 materials [86]. It was shown that BSA molecules can be adsorbed to both hydrophilic and hydrophobic surfaces [84] with differing adsorption mechanisms and rates [85]. The adsorption of DNA polymerase and DNA was studied on 23 various materials using actual PCR conditions [71].

To avoid inhibitory effects of certain materials' in enzymatic reactions (such as PCR), the material surface can be passivated [45, 87]. The material surface can be passivated by active coating ("dynamic passivation") or by passive coating (or "static passivation"). The dynamic passivation is well known for PCR, as in this case the additives are included in the PCR reaction mix. For the static passivation, biological or chemical molecules are applied to the microfluidic channels prior to the PCR reaction. Passivation techniques are well described in reviews of Zhang [45, 87].

A simple passive coating or static passivation strategy is to deposit silicon oxide onto the silicon surface. Other such depositions have employed PDMS, Polytetrafluoroethylene (PTFE), Polypropylene (PP), Parylene-C or epoxy polydimethylacrylamide (EDPMA). For the purposes of passive coating, surfaces have been treated by silanizing agents such as Hexamethyldisilazane (HMDS), Dichlorodimethylsilane (DCDMS), Bis(trimethylsilyl)trifluoroacetamide (BTMSTFA), Chlorodimethyloctylsilane (CTMS), Trimethoxymethylsilane, Dimethylformamide (DMF) or a commercial hydrophobic surface coating agent (e.g. Aquapel, PPG Industries).

There have been reports on the use of SigmaCote™ or SurfaSil™ silanization for glass. Still other chip-surface-treatment materials have been reported, including Polyvinylpyrrolidone (PVP) for PDMS, Tween-20, Polyethylene glycol (PEG) 8000, carbon nanotubes, Bacteriophage T4 gene 32 protein (gp32) and BSA (Table 1).

In addition to the essential PCR components, a number of adjuvants (additives or co-solvents) are used to enhance product yields. In active coating or dynamic passivation, besides those described in the reviews of Zhang [45, 87], the following adjuvants can be included in the PCR mix: gelatin, Dimethyl sulfoxide (DMSO),, formamide, glycerol, betaine, PEG, PVP, proteins such as BSA, Single-strand binding protein (Ssb), gp32, polyamines such as spermine or spermidine, potassium glutamate, and non-ionic detergents (Nonident P-40, Triton X-100, Tween-20) (Table 2).

PCR reaction optimization through the right selection of the right surface material is of the upmost importance, as it enables to increase the yield of enzymatic reactions in microfluidics.

Fluid manipulation: flow control by micro- valve, pump and mixer

The fluid manipulation for reagents and samples is the key function in realization of a fully integrated microfluidic chip.

Table 1 Passive coating (static passivation) by deposition or treatment of various materials onto the surface

Material	References
SiO_2	[52, 81, 88, 89]
PDMS	[90]
PTFE	[91]
PP	[23, 68, 92–95]
Parylene-C	[96, 97]
EDPMA	[98]
HMDS	[52, 99, 100]
DCDMS	[101–105]
BTMSTFA	[106, 107]
CTMS	[108]
Trimethoxymethylsilane	[109]
DMF	[110]
Aquapel	[111, 112]
SigmaCote™ & SurfaSil™	[59, 81]
PVP	[49, 61, 98, 102]
Tween-20	[100, 102, 104]
PEG 8000	[49, 113–115]
Carbon nanotubes	[98, 116, 117]
gp32	[63]
BSA	[15, 108, 109, 118–122]

Table 2 Active coating (dynamic passivation) by including adjuvants in the PCR mix

Adjuvants	References
Gelatin	[123]
DMSO	[124–126]
Tetramethylammonium chloride	[127–129]
Formamide	[130–133]
Glycerol	[134, 135]
Betaine	[136–141]
PEG	[49, 98, 115, 142]
PVP	[61, 98, 143–146]
BSA	[83, 147, 148]
Ssb	[149–152]
gp32	[83, 130, 152–160]
Spermine & spermidine	[161–163]
Potassium glutamate	[164]
Non-ionic detergents	[134, 165–169]

Flow control includes regulating, driving and mixing, and the corresponding microfluidic component is micro- valve, pump and mixer. The desired characteristics of these micro-components include stability and reproducibility, easy integration, independent operation, compact size, disposability and materials compatibility with samples, reagents and PCR (as discussion in the section of PCR reaction components consideration and Passivation techniques to avoid reaction inhibition). Researchers are striking to develop higher level of micro-PCR integrated systems to assemble all these functions into one chip with capabilities of multiple samples analysis and cross-contamination prevention.

Microvalves for fluid regulation

The function of the micro-valve is to regulate or switch the flow of fluids inside micro- channel or chamber. Kwang and Chong [170] reviewed comprehensive active or passive microvalves in microfluidics based on different actuation mechanisms. For DNA chip, the microvalve is critical to: (1) successful PCR for bubbles and evaporation prevention, and (2) high throughput for avoiding cross-contamination between different samples. Particularly, the valve should be able to withhold the pressure throughout the whole process in order to control the fluid direction or seal PCR sample fluids. Particularly at elevated temperature (denaturation, at about 95 °C) with degassing and air as well as fluid expansion, the valve should be capable of confining the PCR solution inside PCR chamber. Most conventional "on-off" microvavles involve a diaphragm including a thin PDMS layers [53, 171–176], thin latex sheets [177–179], and so on. These diaphragms are deflected to open or close the channel or chamber by a pneumatic pressure [174, 180, 181], a stimuli-sensitive actuator [182–185] and servomotor-controller [186].

Figure 4a–b shows the in-line pneumatic microvalve fabricated by soft lithography in Quake's group [181]. It consisted of two PDMS layers with the upper channel ("control channel") and the lower channel ("flow channel"). Two layers were bonded together in crossed-channel architecture. The lower layer was sealed onto the top of a glass or elastomer substrate. When pressurized gas was applied to the upper pneumatic control channels, the elastomer membrane between the two channels deflected at the intersection and closed the lower layer (Fig. 4b, rounded channel). Liu et al. were further built up large-scale integration (LSI) of microfluidic system (Fig. 4c), with 2,860 in-line microvalves displayed horizontally or vertically that was controlled by only two independent pneumatic pressure supplies [180]. The large valves or the small valves were selectively actuated because they had different thresholds of hydraulic pressure necessary for actuation. A 20×20 microfluidic channel matrix requiring merely 41 pipetting steps for 400 distinct PCR reactions was demonstrated in this LSI microfluidic chip.

Niu et al. designed a membrane valve based on electrorheological (ER) fluid [183, 184]. ER fluid is a type of colloidal suspension whose rheological properties can be varied through the application of an external electric field [187]. Under an applied field on the order of 1~2 $kV{\cdot}mm^{-1}$, the ER fluids exhibit solid like behavior, e.g., ability to transmit shear stress. The transformation from liquid-like to solid-like behavior is relatively quick, on the order of 1~10 ms, and reversible. Figure 5a shows the design and fabrication of micro-ER valve. The ER fluid flows in layer 1, replacing the air-valve channel in Quake's design, and parallel Carbon-PDMS electrodes are tightly integrated on the two sidewalls of the ER fluid channel, forming upstream and downstream control valves. The controlled liquid flows in layer 2. As indicated in the intersectional scheme (Fig. 5a), a 35 μm-thick flexible diaphragm lies between the two layers, separating the cross-channels. ER fluid is continuously pushed at a constant pressure into the chip by a syringe pump (Fig. 5c). With an adequate DC electric field applied alternately to two electrode pairs, the pressure in the ER fluid channel between the two valves can be modulated as the two valves are alternately opened and closed. Such a pressure change within the ER fluid channel will eventually result in the deformation of the flexible diaphragm with a vertical pull-and-push movement. In this way, the liquid flow in flow channel layer 2 is controlled by the pressure changes in the ER fluid channel. The ER fluid effect is able to provide high-pressure changes in ER fluid control channel so as to fully close and open an associated flow channel. The fast response time of the ER fluid and the push-and-pull valve design adopted assure fast switching time of the valve less than 10 ms and sound reliability. This ER-fluid-based microvalve has other advantages

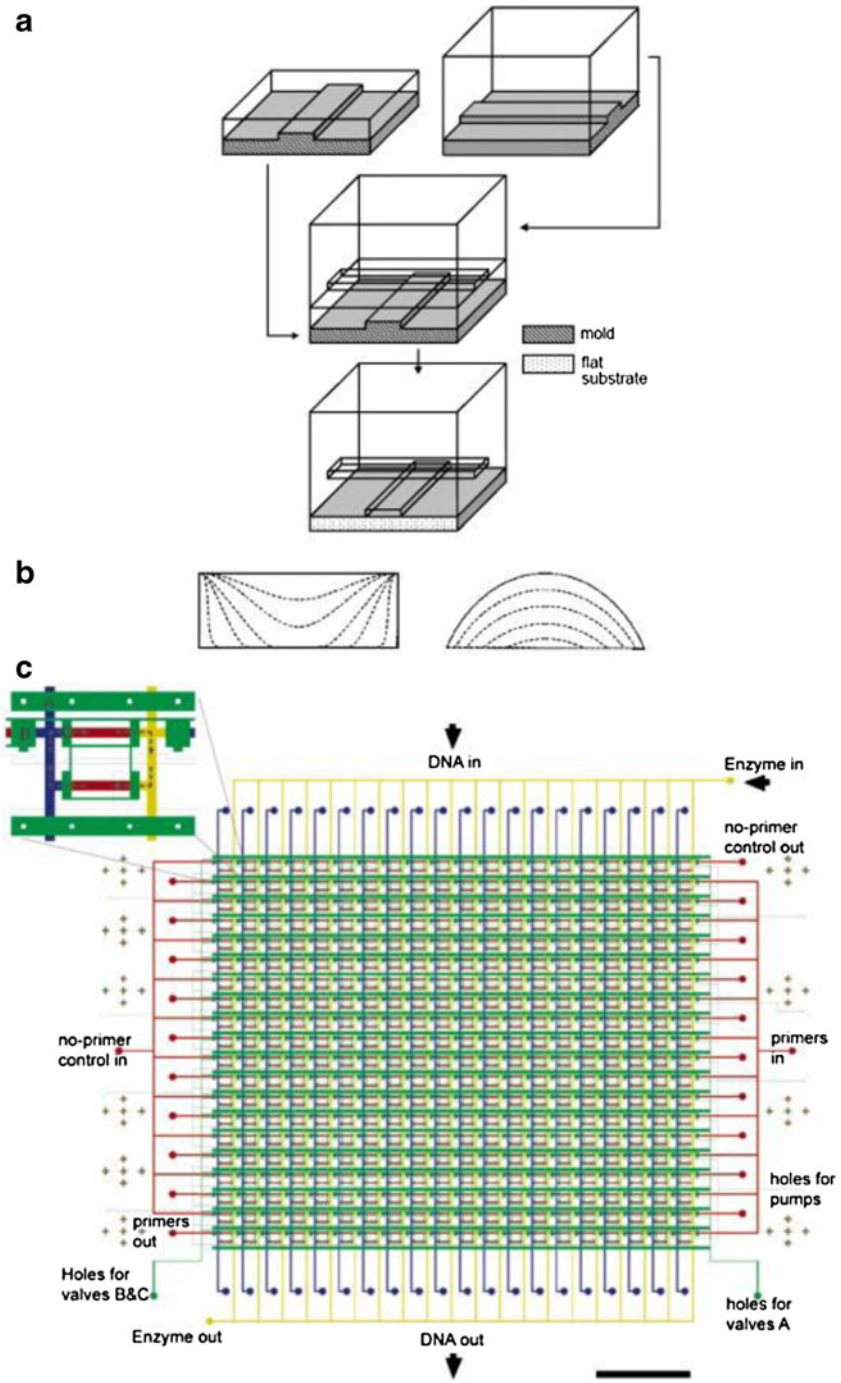

Fig. 4 **a** Process flow for constructing multilayer soft lithography. **b** Schematic of microvalve closing for square and rounded channels. The *dotted lines* indicated the contour of the top of the channel for rectangular (*left*) and rounded (*right*) channels as pressure was increased. Adapted from [181]. **c** Schematic diagram of the $N=20$ matrix chip to perform 400 independent PCR reactions. Adapted from [180]

of easy fabrication and biocompatibility and is suitable for most microfluidic applications.

The membrane-based valves provide fast response time, large resistible pressure and reliable performance but the fabrication of such microvalves involves multilayer complicated construction and they are too expensive to be used for single-use biochip devices. Recently, the phase change effects are employed as new actuation technique for microvalves in microfluidics. The phase change materials used in these microvalves include sol–gel [188], paraffin [15, 189–191], ice [192, 193], and smart hydrogels [10, 194]. Sol–gel, paraffin and ice can change from solid state to liquid state at elevated temperature. The physical or mechanical property (such as volume) of the smart hydrogels can be changed reversibly, reproducibly and significantly under very small environmental stimuli including pH, glucose, temperature, electric and magnetic field, light and antigen. The hydrogel microvalve structure and working mechanism were illustrated in Fig. 6 [194]. It is reported that the closed valve did not exhibit any visible leakage up to a pressure of 200 kPa. The

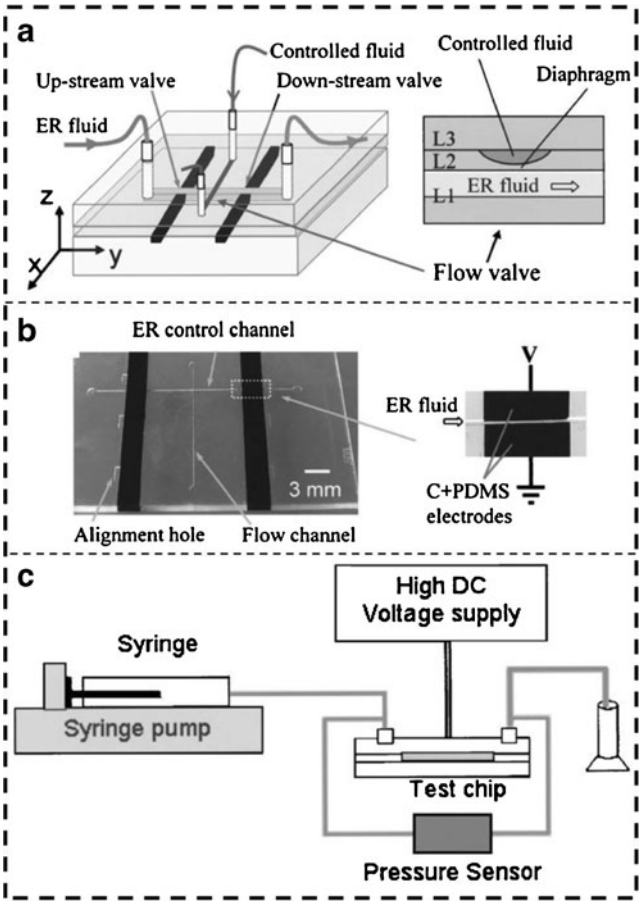

Fig. 5 Design and fabrication of micro-ER valve. Adapted from [184]. **a** Design of four-port valve chip. Right: cross-section of the different layers forming the flow valve; L1 is the ER fluid channel layer, L2 the controlled flow channel layer, and L3 the cover layer. **b** Optical photograph of fabricated ER valve chip. Right: top-view image of the planner electrodes and the ER fluid channel. **c** Experimental setup for microvalve testing

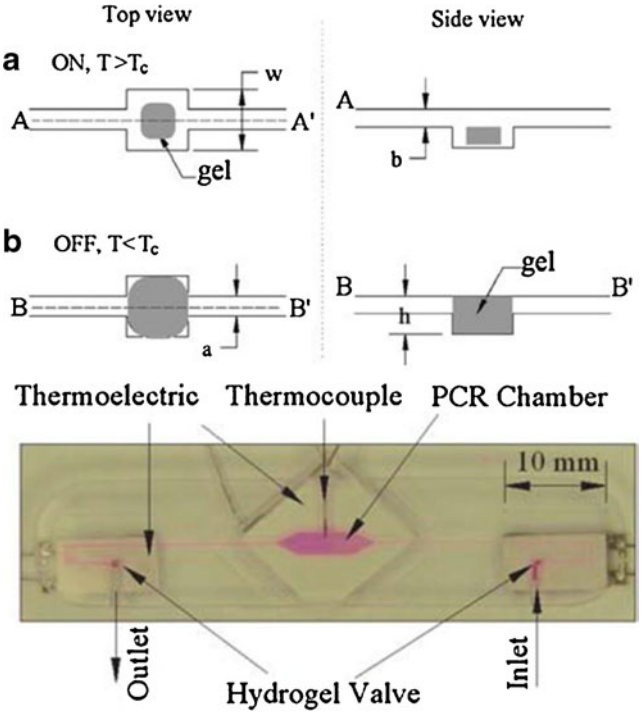

Fig. 6 **a** Illustration of hydrogel volume changes as the gel's temperature varies. The top (*a*) and bottom (*b*) rows correspond, respectively, to temperature above and below the critical temperature. The left and right columns provide, respectively, top and side views. When the hydrogel was dry or when its temperature was increased above the critical temperature (32 °C), the hydrogel remained in the de-swelling (unexpanded) state and the conduit was open to flow. When the temperature decreased below the critical temperature in the presence of aqueous solution, the hydrogel swelled and blocked the flow. **b** A PCR chamber equipped with two hydrogel valves. Adapted from [194]

hydrogel microvalves provide advantages of perfect sealing, the ability to withstand relatively high pressure and simple fabrication and operation. Moreover, in some cases, the microvalve can operate in a self-actuated, open-loop control mode, eliminating the need for a sensor to control the appropriate actuation time [194].

Micropumps for fluid driving

The micropumps are used to drive the samples or reagents into different reaction chambers from inlets or pre-reaction chambers. In the flowing PCR, PCR solution is flowed or oscillated along two or three constant temperature zones or temperature gradient by external pump (such as syringe and peristaltic pump) or internal micropump. Micropumps promise compact device size to achieve all-in-one chip and will be summarized and discussed in the following section.

Up to date, micropumps can be roughly divided into two groups: mechanical micropumps based on diaphragm or peristaltic movement mechanisms and non-mechanical micropumps based on electric or magnetic force generated by applied electric or magnetic field. Various kinds of mechanical micropumps have been adapted in the PCR chips, including pneumatical (including thermopneumatic) [174–176, 180, 195–197], electric-servomotor-driven [186, 198, 199] piezoelectric micropumps [51] and ferrofluid driven [200].

Figure 7 shows the pneumatical pump based on diaphragm (A) and peristaltic (B) movement mechanisms. As shown in Fig. 7a, the diaphragm micropump comprises of a pump chamber and two passive check valves—one at the inlet (or suction side) and one at the outlet (or discharge side) [201]. The performance of inlet/outlet microvalves is critical to the operation of diaphragm micropump. Micropumps with multiple chambers in series and no valves, or micropumps with multiple active microvalves operated in a series of on/off actuation sequences are sometimes referred to as peristaltic micropumps [170, 202, 203]. In the pneumatic peristaltic micropumps (Fig. 7b), three separate microvalves are usually used to peristaltically pump the working fluid [181]. Figure 8 presents ER fluid peristaltic micropoumps reported by Liu et al. [183, 204]. The ER fluid micropump has the same actuation scheme (Fig. 8b) with pneumatic peristaltic

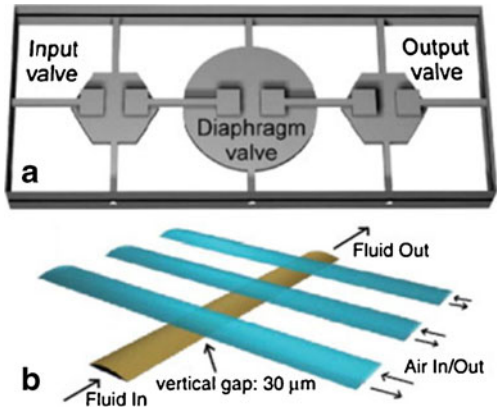

Fig. 7 **a** an oblique view of the three-layer pneumatic diaphragm micropump. Adapted from [201]. **b** A 3D scale diagram of a pneumatic peristaltic micropump. Adapted from [181]

micropump. However the ER fluid micropump exhibits good performance at high pumping frequencies and uniform liquid flow characteristics. In addition, it can be easily integrated with other ER fluid microfluidic components and the programmable digital control also gives the device flexibility in its operations.

Comparing with diaphragm micropumps, the peristaltic micropumps are preferred for PCR chips due to its simplicity in design and operation. Furthermore, the peristaltic micropumps permit bidirectional or unidirectional sample fluid transport by simply modifying the active microvalve actuation scheme. Nevertheless, performance improvements realized with a multi-chamber or multi-active-valve design in the peristaltic micropumps must be balanced against increases in fabrication complexity and overall device size [202].

Mechanical micropumps can provide high volume flow rate and reliable performance, however they always need extern control device and further miniaturization will become a problem. Non-mechanical micropumps have been emerging as a new direction for fully integrated PCR chip, including electrokinetic [16, 205–207], capillary [208, 209], magneto hydro dynamic (MHD) [210], acoustic-wave [211], electrochemical [15] and ferrofluidic magnetic micropumps [212].

Chen et al. developed a novel electrkinetically driven synchronized PCR chip. This chip allowed DNA amplification to be carried out in a continuous flow mode using synchronized electrokinetic pumping, as schematically shown in Fig. 9. The electrokinetic flow can be controlled easily by switching on/off voltages and positive/negative directions. Electrokinetic micropump offer advantages of low cost, simply system design, easy control scheme. However, electrokinetic pump still

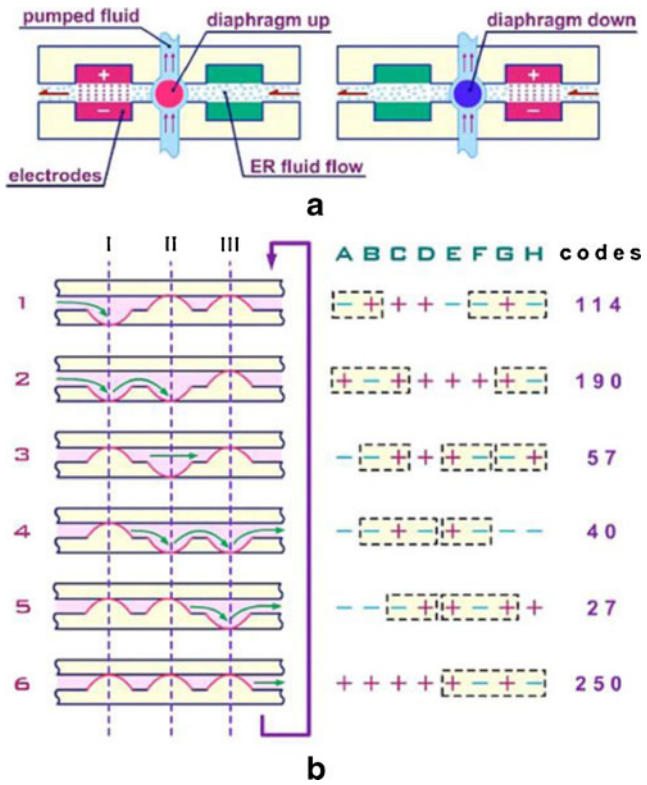

Fig. 8 **a** A the principle of ER valve's operation is illustrated schematically by showing the deformation of a single diaphragm via pumped ER fluid. The diaphragms' pumping sequences and their corresponding signals are shown in (**b**). Adapted from [204]

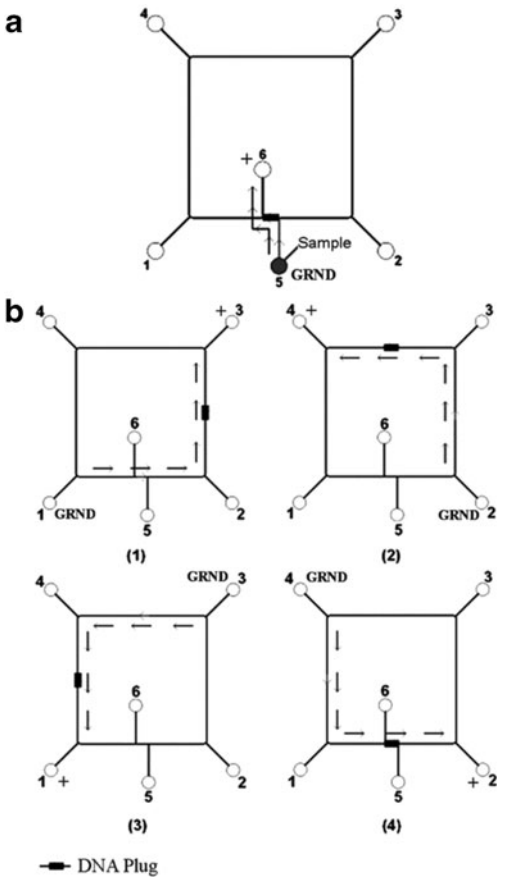

Fig. 9 Diagrammatic principle of electrokinetic synchronized cyclic continuous flow PCR process. **a** Sample injection; **b** Sample cycling. Adapted from [205]

suffer from electrolysis by-products which lead to bubble formation and buffer change in chemical composition, evaporation of solvent due to Joule heating and demixing due to electrophoretic separation.

Micromixers for fluid blending

DNA extraction and PCR are multi-component biochemical reactions. Therefore, the sample and reagents should be effectively mixed in order to achieve complete reaction. In bench experiments, such mixing operations are manually accomplished by a conventional vortexing in a microfuge tube, which certainly can't meet the requirement of LOC platform. Moreover, fluid flow is dominated by laminar flow due to low Reynolds number in microfluidics. Consequently, two or more microfluid streams contacted with each other cannot be mixed within a short path or a reasonable time. Up to date, lots of passive and active micromixers have been developed for non-manually blending the samples and reagents in microfluidic chip [213].

Passive micromixers mainly rely on micro-structures [212, 214–216] or internal flow field in droplet [209, 217, 218] to create chaotic advection. Y/T-shaped micromixers have widely used in accomplishing effective mixing of PCR solution in PCR chip [212, 214–216]. For example, Fig. 10 shows an integrated device is capable of measuring aqueous reagent and DNA-containing solutions, mixing the solutions together in Y-shaped micromixer, amplifying or digesting the DNA to form discrete products, and separating and detecting those products by gel electrophoresis.

Active mixing can be achieved by time-pulsing perturbation [213] owing to a periodical change of electrical fields [16], acoustic fluid shaking [15], electrowetting-based droplet shaking [209] and pumping pressure [176, 180, 183].

Niu et al. developed an active ER fluid mixer (Fig. 11) employing dynamic control to help achieve chaotic mixing [220]. Later Liu et al. developed an all-in-one chip (Fig. 11b) with ER fluid micropump and micromixer as well as microheater [183]. This integrated chip accomplished multi-component and operation PCR process, providing the advantages of small size with a high degree of integration, high polymerase chain reaction efficiency, and digital control.

DNA detection: qualitative and quantitative analysis

Nowadays, DNA detection methods are varied based on different qualitative or quantitative analysis techniques. The amplified DNA could be detected during PCR (real-time PCR) or at the end of PCR (end-point PCR). One of the major difficulties confronted with the development of a truly miniaturized DNA amplification and detection device is to meet the physical constraints required by a portable detection module for DNA detection and quantitation.

The most conventional DNA non-specific detection technique in PCR microfluidic chip is optical approach in combination with

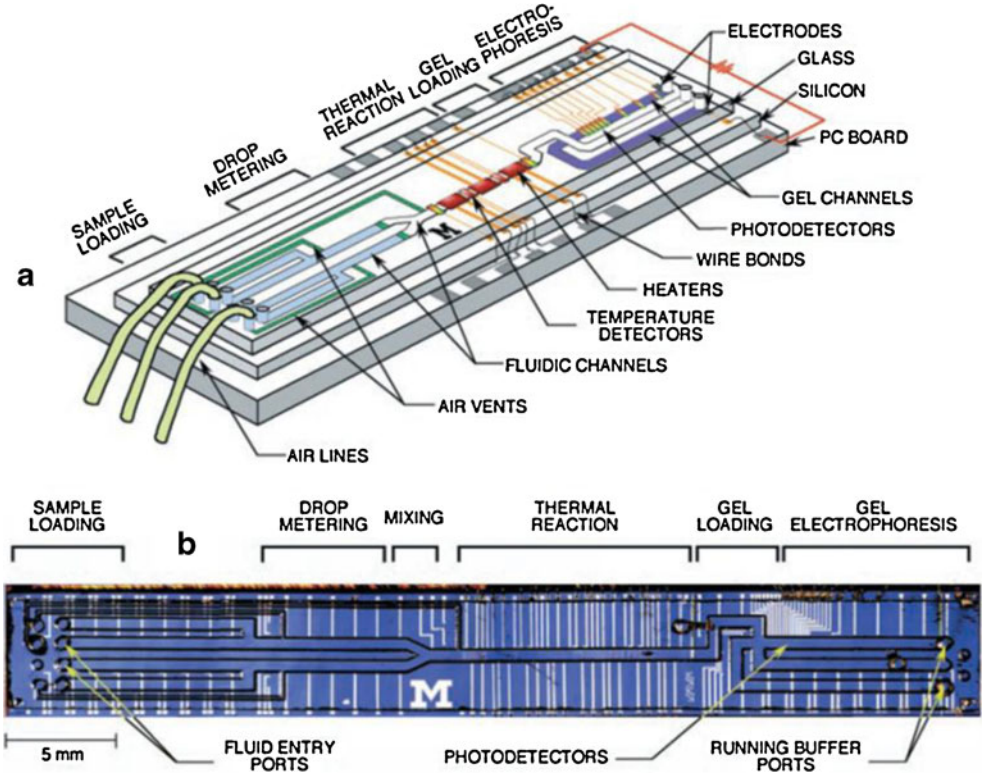

Fig. 10 **a** Schematic of integrated device with two liquid samples and electrophoresis gel present. **b** Optical micrograph of the device from above. Adapted from [214]

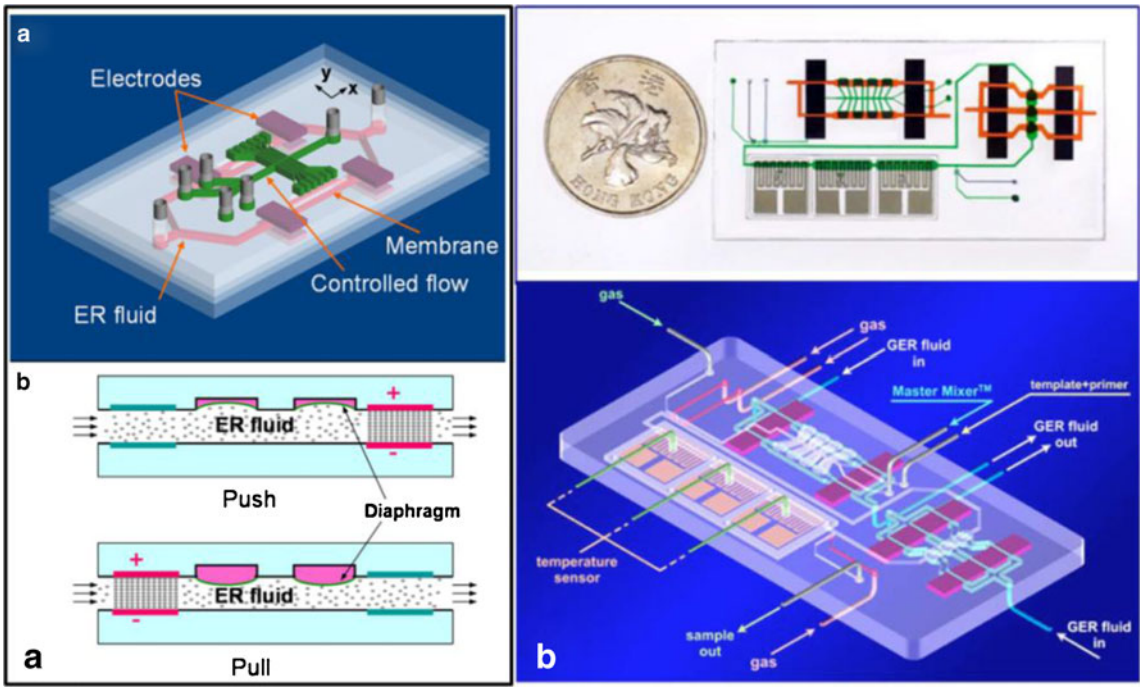

Fig. 11 **a** Scheme of mixer construction. Adapted from [219]. (*a*) Schematic picture showing the PDMS active mixer design and construction. The overall chip size is 3 cm by 1.5 cm. (*b*) Schematic illustration showing the working principle for the push and pull ER valve, for one cross sectional area of ER fluid channel and control bars. **b** The upper panel shows an image of the fabricated device, placed beside a one dollar Hong Kong coin. The lower panel shows a 3D schematic illustration of the integrated chip. Adapted from [183]

capillary electrophoresis [1, 36]. The optical approach is based on the fluorescence signals from binding interaction between the dye or probe and the increasing amount of double-stranded DNA molecules [36, 180, 221]. DNA molecules are separated according to their sizes by capillary electrophoresis. The DNA template concentration could be obtained by fluorescent intensity difference before and after PCR or calculated from threshold value in real-time PCR. Although this technique is sensitive and widely used in PCR, fluorescence-based methods are not amenable for miniaturization and integration onto a single-chip dimension, due to the general requirement of power-intensive laser light sources and an optical detection system of a considerable size [222].

Sequence-specific electrochemical DNA sensors are well suited for hand-held instruments. Because such detection methods (Fig. 12) deliver an electronic signal directly and there is no need for expensive signal transduction equipment (such as

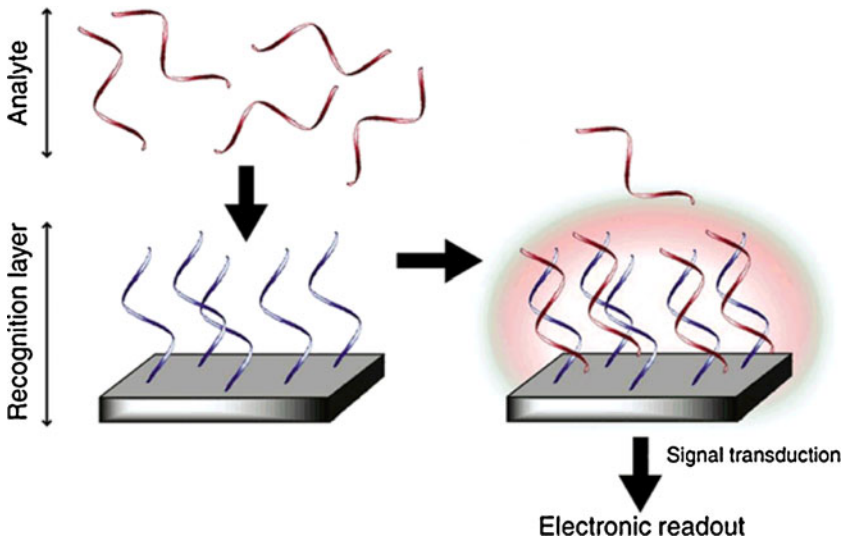

Fig. 12 Target DNA is captured at the recognition layer and the resulting hybridization signal is transduced into a usable electronic signal for display and analysis. In the case of electronic and electrochemical biosensors, signal transduction is greatly simplified, because the incoming signal is already electronic in origin [223]

charge-coupled device for fluorescence-based methods) [223]. The sensing protocol basically involves the immobilization of an oligonucleotide onto a transducer surface, and upon the hybridization of complementary target sequence, the binding event is detected by electrochemical methods [224]. The sensitivity and selectivity of the electronic detection methods are comparable with that of the fluorescence-based detection and would be accommodated in future miniaturization trends [224].

Conditional methods described above are sometimes restricted by the throughput, because they normally could detect only single target. In order to detect or sequence multiple targets in a single device, without employing complex instrumentation, researchers could perform reactions in individual micro- array, sphere or wells. Microarray DNA hybridization technique is multiple nucleic acid detection method based on the specific binding between nucleic acid probe array and their complementary target nucleic acid sequences. It has been used widely for single-nucleotide polymorphism detection and nucleic acid diagnostic application [225]. The microfluidic platform advances DNA microarray hybridization techniques with less sample usage, reduced incubation time and enhanced sensitivity [226].

Microsphere-based suspension array technologies explore a new platform for high-throughput nucleic acid detection. For example, the Luminex® xMAP™ system (Fig. 13) incorporates 5.6 μm polystyrene microspheres that are internally dyed with two spectrally distinct fluorochromes [227, 228]. Each microsphere carries precise amounts of each of these fluorochromes for specific spectral address and possesses a different reactant on its surface (i.e. oligonucleotide capture probe for DNA detection). A third fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction (i.e. DNA hybridization for DNA detection) that has occurred at the microsphere surface. The Luminex® xMAP™ system is capable of analyzing and reporting up to 100 different analytes in a single reaction vessel. Two assay chemistries including direct and competitive DNA hybridization have been used for post-PCR nucleic acid detection on the xMAP system. The speed, efficiency and utility of xMAP technology for simultaneous, rapid, sensitive and specific nucleic acid detection are demonstrated in published literature [228–230] and its capability is potentially further coupled with microfluidics for the automatic multiple nucleic acid detection [231].

Four hundred fifty-four technology perform pyrosequencing in high-density picolitre micro-wells and is able to sequence 25 million bases, at 99 % or better accuracy, in one 4-h run [232–234]. As shown in Fig. 13, after the PCR was performed in emulsion, the amplicons were transferred into the micro-wells in a chip (Figs. 13b and 14).

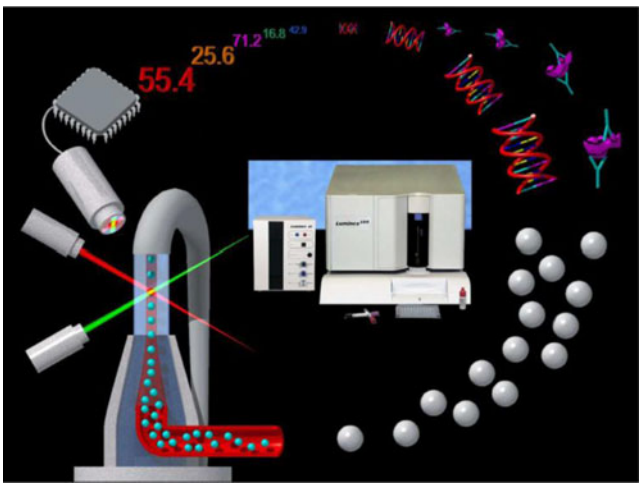

Fig. 13 Luminex xMAP system and components illustration adapted from [228]. The four main components of the xMAP system are shown, clockwise from top-right: biomolecular reactants; fluorescently color-coded microspheres; fluidics and optics; and high-speed digital signal processing. The Luminex 100 analyzer with XY platform and sheath delivery system is pictured in the center

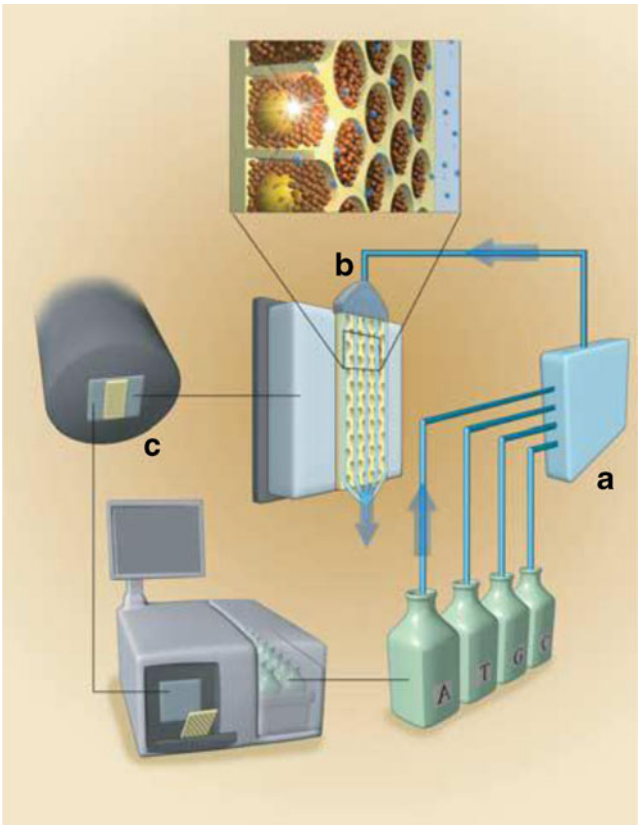

Fig. 14 Four hundred fifty-four technology sequencing instrument illustration adapted from [234]. The sequencing instrument consists of the following major subsystems: a fluidic assembly (**a**), a flow chamber that includes the well-containing fibre-optic slide (**b**), a CCD camera-based imaging assembly (**c**), and a computer that provides the necessary user interface and instrument control

Beside the above DNA concentration or sequencing measurement methods, micro/nanotechnology rendered and developed many novel DNA analysis techniques including: (1) DNA counting based on digital (or droplet) PCR [235–237]; (2) DNA sizing based on DNA stretching length in nanochannels [238–240]; (3) DNA weighting based on differential deflection signals of micro/nano-cantilever [241–243].

Conclusion

Different types of micro/nano-fluidic technologies have facilitated DNA purification, amplification and detection to be integrated into one chip which combine the advantages of small sizes, much shorter reaction times, less manual operation and reduced cost [15, 201, 244]. Successful DNA amplification and detection on chip depends on the optimization of several parameters, which is a cumbersome task due to many variables (conditions and components) typically involved and requires comprehensive knowledge of multi-subject intersecting molecular biology, chemistry, physics, mechanics and micro/nano-fabrication technologies. The choice of an appropriate technology for realizing miniaturization is not a trivial task since the simplicity and integration of the design has to be balanced with performance requirements.

Nevertheless, despite a lot of papers have been published and significant progresses have been made in PCR-based DNA amplification and detection microfluidic chip, a truly miniaturized chip-scale device without external components remains elusive neither in laboratory nor industry. The complexity of PCR microfluidic chip, primarily determined by the fundamental mechanisms of the PCR reaction, might be simplified by raw sample direct amplification [48, 245], solid-phase amplification [246, 247] and isothermal amplifications such as Strand displacement amplification (SDA), Nucleic acid sequence-based amplification (NASBA), Rolling cycle amplification (RCA) and Loop-mediated isothermal amplification (LAMP) [248]. For example, LAMP is not only isothermal, fast, and specific but also highly sensitive and robust so that it does not require the extracted DNA to be highly purified or denaturized [249–251]. Meanwhile, to develop such a low-cost and disposable biochip, there is a need to establish standardization [252–254] for microfluidic interconnection, chip dimension and vocabulary, and to find cheaper material to fabricate the chip. At present there is a wide choice to solve these problems. The ease and cost of manufacture as well as reliability of micro-components (microvalves, micropumps and micromixers) could be further improved by the well-established MEMS [255]. Moreover, various low cost plastics are suitable for PCR chip and we may expect additional materials, such as paper-based chips [256–259].

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