

# Current State of Intellectual Property in Microfluidic Nucleic Acid Analysis

Lidija Malic<sup>1,3,#</sup>, Marc Herrmann<sup>1,3,#</sup>, Xuyen D. Hoa<sup>1,3,#</sup>, and Maryam Tabrizian<sup>1,2,3,4+</sup>

<sup>1</sup>Department of Biomedical Engineering, McGill University, <sup>2</sup>Faculty of Dentistry, <sup>3</sup>Centre for Biorecognition and Biosensors, <sup>4</sup>McGill Institute for Advanced Materials, Montréal, QC, H3A 2A4, Canada

Received: October 2, 2006; Accepted: November 10, 2006; Revised: November 10, 2006

**Abstract:** The development of novel fabrication methods, materials and surface chemistries to implement nucleic acid analysis brings reduced cost, reduced reagent consumption, increased analysis efficiency, portability, ease of use and reliability to today's genomic approach. This trend, as evident by the exponential growth in the number of patent applications, granted patents and commercialized systems, is motivated by the promise for significant breakthroughs and benefits of nucleic acid analysis to drug discovery and point-of-care diagnosis. This review paper aims at identifying the enabling technologies and key patents in microfluidics for nucleic acid analysis. In particular, it seeks to identify granted and pending patents for cell sorting and lysis, nucleic acid extraction and purification, followed by nucleic acid amplification, separation and detection. Additionally, it presents an overview of the current intellectual property environment and seeks to identify trends for the future development. Much of this development is geared increasingly toward fully integrated systems. The convergence of technology and interdisciplinary interests is expected to foster further breakthroughs and commercialization.

**Keywords:** Microfluidic nucleic acid analysis, cell sorting, cell lysis, nucleic acid extraction, nucleic acid separation, polymerase chain reaction, electrophoresis, integrated systems, Lab-on-a-Chip.

## INTRODUCTION

In the near future, one can imagine a device no larger than a credit card able to detect infections and diseases directly from a single drop of blood within minutes. Such is the promise of lab-on-a-chip and microfluidic technologies.

With microfluidics, the precise control and manipulation of microliter or nanoliter volumes of fluids poses significant technological challenges, and yet offers considerable benefits: rapid analysis, minimal reagent and sample consumption. The integration of functional components for sample processing and detection onto a single platform as to create a *lab on a chip*, provides additional advantages: portability, reliability and reduced cost. For point-of-care diagnosis, these advantages must be leveraged to provide a viable and effective tool [1,2]. Generally, in molecular biology or nucleic acid (NA) analysis, these advantages are sought for new applications in drug discovery, cancer research, food and environmental safety.

Over the past decade, much progress has been achieved in the development of novel materials and fabrication processes to overcome the limitations of early microfluidic systems. These devices were the initial attempts to adapt materials, silicon and glass, and microfabrication processes from the semiconductor industry to a novel application. However, the high cost associated with the clean-room processes (photolithography, etching, and bonding) limited the development of disposable devices. Polymer-based chips with alternative low cost manufacturing processes were

introduced to address these issues [3,4]. Increasingly, microfluidic devices are fabricated from rigid transparent polymers such as acrylics or flexible polymers like silicone rubber using the cost-effective techniques of injection molding, embossing and replica molding [5]. Additionally, the development of novel chemistries were introduced to enhance the surface properties of these materials [6-8]. Growing access of these technologies to both research groups and companies has led to a commensurate increase of innovations in the development of NA on-chip analysis [9,10].

In this paper, inventions describing microfluidic technologies for the implementation of on-chip NA analysis systems are presented. In contrast to existent literature reviews, which provide excellent overview of most recent developments, this review focuses on the state of intellectual property as to identify the trends and contributors from which future commercial development must build upon. Given the protracted process of a successful patent prosecution reaching sometimes over 3 years, this review also covers patent pending inventions (published patent applications) as to provide a timely overview of recent trends. The approach is three-fold. In the first tier, key inventions and methods that implement the individual functional components of NA analysis are catalogued. For clarity, a short description of the manipulation steps of NA analysis is provided. A wide variety of materials and physical and chemical approaches are described for the handling of fluids and processing of the genomic samples. The second tier addresses strategies and challenges of the integration of these components into a functional, application-oriented microfluidic NA analysis platform. Examples of patents, granted and pending, of integrated systems are identified. In the final tier of this review, the

<sup>+</sup>Address correspondence to this author at the Department of Biomedical Engineering, McGill University, 3775 University Street, Montréal, QC, H3A 2A4, Canada; Tel: (514)398-8129; Fax: (514)398-7461; E-mail: maryam.tabrizian@mcgill.ca

<sup>#</sup>First author - equal contribution.

current outlook and trends in the development and commercialization of microfluidic NA analysis platforms are discussed. Much of the focus of current work is on the integration issues, which includes the introduction of novel NA analysis techniques. Finally, the major inventors and companies are identified, as to provide a glance of the microfluidic NA analysis market.

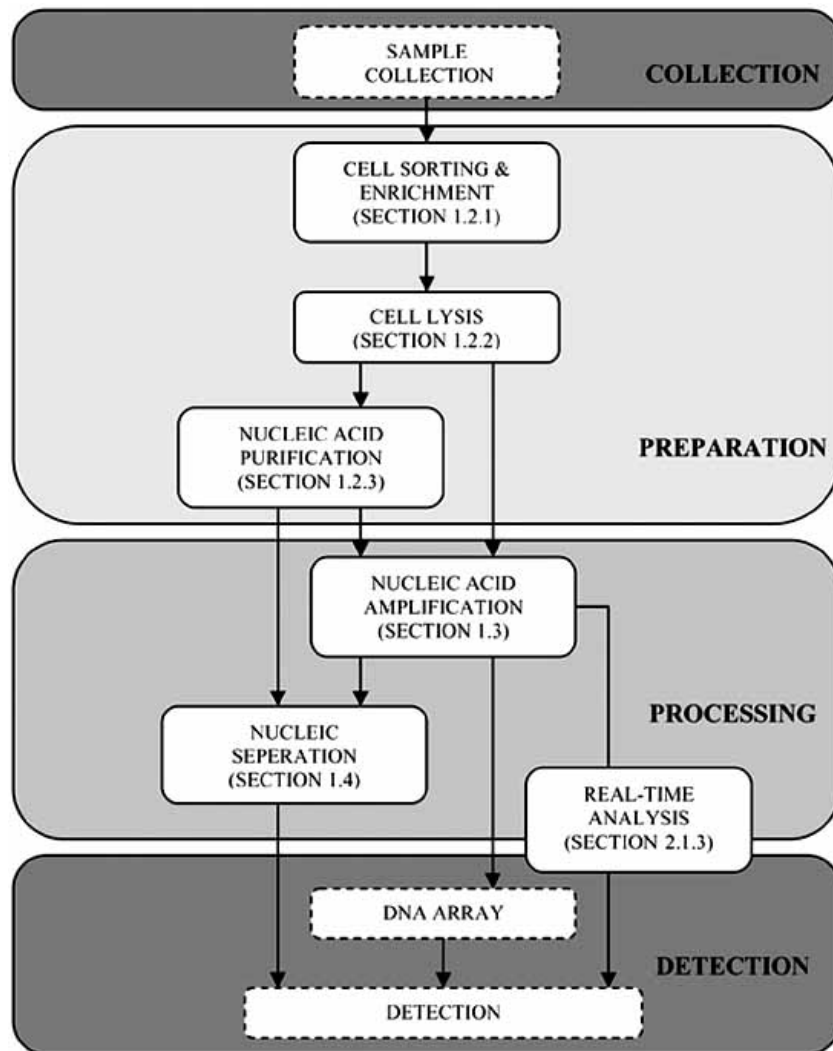
## 1. INTELLECTUAL PROPERTY FOR NUCLEIC ACID ANALYSIS

### 1.1. Principles of Nucleic Acid Analysis

The analysis of the NA content of a given sample requires a succession of manipulation steps, as illustrated by Fig. (1). The sequence of individual steps varies according to the chosen method of analysis and the required information. The procedure starts with the collection of a sample, which contains the genetic material to be analyzed. This sample can be a body fluid, such as blood, urine, saliva; cultured cells in their medium; solubilized grinded tissue or an environmental water sample. For more complex samples that contain higher

quantities of potential contaminants, it is critical to prepare the sample prior to analysis.

The first step for sample preparation is the sorting and enriching of the cells and organisms of interest. In this review, cell sorting will be discussed. Generally, these techniques can be applied to eukaryotic cells, bacteria, viruses as well as other NA containing organisms. Although not always necessary, cell sorting and enrichment is often desired. For instance, to isolate white blood cells from the more abundant red blood cells and platelets, or to concentrate bacterial cells from a diluted water sample, to collect rare stem cells, or to study the genetic expression profiles of healthy cells versus cancer cells, this initial step must be performed. After the cells of interest have been isolated in sufficient number, the genetic material is extracted by disrupting the cellular membranes or other protective layers. This particular step is called *cell lysis*. Although it is an essential step in sample preparation, during cell lysis, cellular debris is generated and harmful substances are released along with the NA material, which can hinder



**Fig. (1).** Manipulation steps for nucleic acid analysis. The steps depicted in boxes with solid lines are covered in this review. The numbers provided in each box refer to the section in which the particular topic is presented. The boxes with dotted lines are beyond the focus of the review.

subsequent manipulations. Consequently, the released NAs are often purified before they can be further processed.

After the genetic material has been extracted and purified, it can be processed by a variety of techniques. The processing greatly depends on a given application. For instance, the NAs can be readily separated by electrophoresis for sequencing purposes or single nucleotide polymorphism identification. Due to the limited amount of sample available, sensitive instruments such as laser induced fluorescence or mass spectrometry are often required for detection. Alternatively, a specific NA sequence can be amplified by polymerase chain reaction (PCR) prior to the separation to facilitate the detection. NA amplification of a particular nucleic acid sequence can also be monitored in real-time (RT-PCR) to study for example the level of expression of a disease-associated gene or to identify the presence of a particular microorganism. Additionally, for more exhaustive studies such as the comparison of the expression profiles of healthy versus cancer cells, or cells following a particular treatment, the whole NA extract can be amplified and subsequently hybridized on a microarray chip.

The following sections examine the different microfluidic approaches to NA analysis, as described in patents or patent applications, focusing on sample preparation and processing. Although the detection methods are necessarily associated to the analysis [11], they are issued from different technologies and thus are discussed only as a part of a system where they are integrated with one or several of the previous steps of the NA analysis.

## 1.2. Microfluidics for Sample Preparation

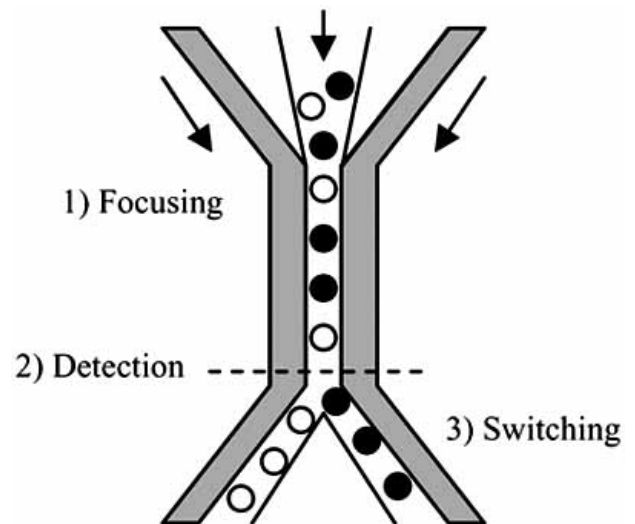
### 1.2.1. Cell Sorting and Enrichment

For cell sorting and enrichment, the conversion of laboratory instruments into a microfluidic format, as well as specifically adapted microfluidic solutions have been described. These inventions are presented in the following section.

#### 1.2.1.1. Fluorescence Activated Cell Sorting

Fluorescence Activated Cell Sorting (FACS) is frequently used in biological and medical laboratories for its high efficiency and specificity. However, the associated costs, the requirement for a highly qualified operator, and the risk of cross-contamination between samples are important limitations, which impair the routine use of this technique. Therefore, researchers have naturally concentrated their efforts on developing an inexpensive, easy-to-use and disposable device based on the same principles as FACS.

FACS involves a sequence of three independent events to sort cells as depicted in Fig. (2). Individual events or their integration into a functional device have all been the subject of recent patents and patent applications. First, the cells are separated and aligned into a ribbon of cells, a process called *cell focusing*. In microfluidic systems, this initial step is achieved by generating three parallel laminar streams with a central stream large enough to allow the passage of only one cell at a time. In 2000, Wada *et al.* from Caliper



**Fig. (2).** Microfluidic Fluorescent Activated Cell Sorting (FACS). Sequence of the three independent events for cell sorting with microfluidic FACS. The grey streams represent the focusing buffer, while the middle white stream represents the cell containing sample. Two types of cells are depicted by white and black circles

Technologies proposed several methods for cell focusing in microfluidic systems [12]. In 2003, Micronics filed a patent application for an injection device, a so-called sheath injector, for hydrodynamic focusing and sorting of cells [13]. A year later, they patented a device for the separation of white blood cells from a whole blood sample [14].

Following the focusing step, positive cells, namely the cells that are separated from the other components of the flowing solution, are detected. Several methods for positive *cell detection* have been elaborated. The large majority of systems however use fluorescent detection, whether the cells are tagged with specific fluorescently-labeled antibodies or they express a modified fluorescent protein. The detection event actuates a switch, whose activation directs the positive cells into a separate collection channel. *Flow switching* can be implemented in several manners such as electromechanical valves [15,16], pressure-driven [17] or electroosmotic switches [18-20]. In particular, electroosmotic force (EOF) has been frequently employed as it is a robust, rapid and easily miniaturized means of directing flows in microfluidic channels. In 1999, Quake *et al.* patented a microfabricated FACS device based on fluorescent detection and electroosmotic flow switching to separate, as a proof-of-principle, GFP expressing *E. coli* [18,19]. More recently, Liu *et al.* elaborated a very similar device with integrated optics for cell detection, seeking further miniaturization and portability [20]. In 2005, Evotec also released two patent applications describing the operation of similar FACS devices [21,22].

Nevertheless, the biggest challenge yet to be overcome in microfluidic FACS design lies in the optimization of the

separation rate while minimizing the occurrence of false positive events. Essentially, the faster the flow of cells, the lesser time is available for accurate detection and sorting. In this regard, the use of microfluidics permits to increase the separation rate by parallelizing multiple microsystems rather than by accelerating the flow. Moreover, multiple embranchments can be laid in a sequence to perform multi-step sorting with re-sorting decisions, thereby decreasing the number of false-positive events [16].

Although the majority of reported devices use planar channels, mostly due to fabrication constraints, other schemes have been proposed. Foster J.S., for instance, patented a device in which the cells are isolated and detected in parallel vertical channels and then oriented toward the appropriate outlet by electromechanical valves [15]. The smaller planar area occupied by vertical channels, as compared to an array of horizontal channels, allows for massive multiplexing, which in turn greatly increases the overall separation rate. The patented micromechanical actuator is capable of sorting hematopoietic stem cells individually at an operation rate of 3.3 kHz. With the massively parallel 1024-fold device, a throughput of 3.3 million events per second was demonstrated.

#### **1.2.1.2. Magnetic Activated Cell Sorting**

Besides fluorescence, other types of labeling are also available. In particular, magnetic labeling is often used as it represents a more affordable alternative method for cell separation. By analogy, cell sorting using magnetic labeling is referred to as MACS. In one embodiment of the aforementioned patent application from Micronics [13], the flowing cells are attached to magnetically-labeled antibodies. After focusing, the positive cells are attracted into a secondary channel by a magnet positioned on one side of the *T-junction*. This approach could be described as *dynamic*, as opposed to a *static* approach, where the positive cells are not only rerouted into a secondary channel but are rather magnetically trapped inside a unique linear channel. For instance, Cosman *et al.* described a device, in which magnetic beads with capture moieties are immobilized inside the channel prior to the injection of the cell containing solution [23]. The cells are selectively trapped and can later be collected by demagnetizing the trapping device and releasing the beads. Such trapping device can consist of external permanent magnets or more conveniently of microfabricated electromagnets, such as described by Whitesides G.M. [24] or Ahn *et al.* [25].

#### **1.2.1.3. Filter-based Cell Sorting**

Various strategies, which do not require prior labeling of cells, have also been adapted to microfluidic systems. Despite a lower specificity, they offer the possibility of readily sorting cells from a complex sample without further manipulations or introduction of foreign labeling species. Among these techniques, microfabricated filters (or sieves), which separate cells based on their size, shape and deformability, are very popular [26-30]. The company AVI-VA Biosciences, for instance, disclosed a patent application on several methods, compositions, and automated systems for separating rare cells from fluid samples [28]. Although microfabricated filters are easily implemented in micro-

fluidic systems, their inherent small area increases the susceptibility to clogging. In this regard, Sethu and Toner developed a device for the removal of red blood cells and platelets from blood, featuring a junction with a lateral sieve rather than a frontal filter [27]. In this device, a force generator perpendicular to the main stream leads the red blood cells and platelets through the lateral sieve into a secondary channel, while the larger white blood cells continue their course into the main stream, thus avoiding clogging the filter. Alternatively, Wilding and Kricka included a secondary flow channel connected with the separation zone, which allows the discharge of collected cells [29]. Less conventional methods, such as differential cell disruption in a serpentine channel [26], separation based on gravitational acceleration [31], or the separation of white blood cells based on lateral migration due to frequent collisions with red blood cells, have also been proposed [32].

#### **1.2.1.4. Dielectrophoresis**

Another common approach for cell separation is based on dielectrophoresis (DEP). DEP is the translational motion of charge-neutral matter caused by polarization effects in non-uniform electric fields. Positive DEP occurs when a cell is more polarizable than the surrounding medium, and results in the cell being drawn toward a region of higher field gradient. Conversely, negative DEP occurs when a cell is less polarizable than the medium, and results in the cell being drawn toward a region of lesser field gradient. Due to the ease of integration of microelectrodes, DEP provides an especially attractive method for on-chip cell manipulation. Moreover, the limited height of microfluidic channels constrains the flow of cells to few tens of microns above the electrodes, where the electric field is the strongest. The electrodes can be arranged in a perpendicular array to the main stream, thereby differentially changing the linear velocity of various types of cells, either separating or trapping a specific cell population [33]. They can also be disposed in a parallel array, displacing the cells laterally toward distinct outlet channels [34,35]. Yet another advantage of this technique relies on the possible utilization of the same electrodes for subsequent steps such as the extraction of the genetic material and further amplification as described by Nanogen [36] and Iliescu *et al.* [37].

Interestingly, the dielectric properties of cells have also been employed to separate various populations by optical trapping or guiding. In two patent applications, Arryx described the use of holographic laser steering for this purpose [38,39]. Essentially, this technique uses the gradient forces of a beam of light to trap a particle based on its dielectric constant. Particles with a slight dielectric constant differential with their surroundings are sensitive to this gradient and are either attracted to or repelled from the point of highest light intensity. When the beam is shined perpendicular to a microfluidic channel, the flowing cells are separated and collected at various lateral positions.

#### **1.2.2. Cell Lysis**

Standard laboratory methods for cell lysis employ chemical, mechanical, thermal and electrical means to disrupt cellular membranes. Although similar approaches have been used to lyse the cells in microfluidic systems, the

miniaturization of these techniques is not always straightforward and fluidic designs often have to be adapted.

### 1.2.2.1. Chemical Cell Lysis

Chemical lysis methods are often used in microfluidic systems, as many well defined protocols, which are adapted to break down specific types of eukaryotic cells, bacteria, viruses, etc., have been developed over the years. Furthermore, no large external device that requires independent controls, space and power supply is needed. For instance, Kayyem J.F. proposed to use lysing agents such as guanidium chloride, chaotropic salts and lysozymes [40]. Parthasarathy *et al.* from 3M Innovative Properties Company have likewise suggested freeze/thawing, hypotonic shock an alkaline treatment with sodium hydroxide to lyse cells from a blood sample [41]. However, stringent buffers containing the lytic agent might interfere with the subsequent NA analysis by inhibiting the enzymes necessary to perform, for example, a PCR amplification. Thus the lytic agent needs to be neutralized or replaced without losing the genetic material that was previously released. This manipulation often leads to the undesirable dilution of the sample, and frequently demands a more complex fluidic design with integrated valves [42]. Hong *et al.* from the California Institute of Technology, for instance, described a microfluidic system that uses the two-layer PDMS valves developed by Quake's group [43]. In particular, they realized a parallelized isolation and lysis of cells, DNA affinity purification on beads and recovery of the purified DNA on a single chip.

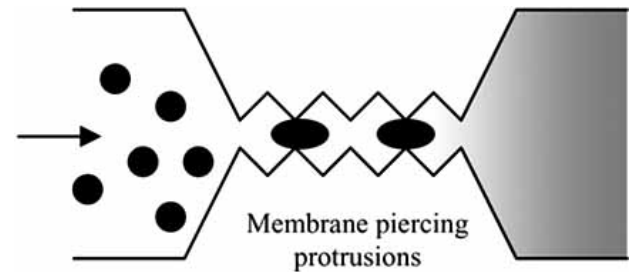
More recently, Irimia D. and Toner M. developed an ingenious device capable of trapping and chemically lysing a single cell within an extremely limited volume of 50-pL, thus greatly reducing the dilution factor of the released molecules [44]. In this device, a cell is first trapped between two air-bubbles delimiting a 25-pL chamber. This chamber is connected via a thin channel to a second 25-pL compartment containing the lysis buffer. The two solutions are then mixed together and the cellular membrane is disrupted. The released molecules are free to diffuse inside the second compartment while cell debris remains in the cell-trapping chamber.

### 1.2.2.2. Mechanical Cell Lysis

Mechanical cell lysis methods comprise a large variety of means to break down cellular membranes and organisms. One of the most popular methods in conventional laboratories uses ultrasonication. The generation of ultrasonic waves creates microscopic bubbles in the medium, which burst out violently thus creating holes in membranes and lysing the cells [45]. Although certain limitations are associated with the utilization of sonication in microfluidic systems, such as the resulting presence of foam and bubbles, as well as high power consumption and lack of portability, Cepheid currently commercializes a microfluidic cartridge, which coupled to an ultrasonic horn, delivers ultrasonic energy to the sample in the lysing region [46,47]. In addition to ultrasonic agitation, solid particles, such as glass beads, can be injected in the system thereby adding mechanical stress and increasing the lysing efficiency [47]. Very

recently, Yuan *et al.* from Microfluidic Systems described a similar device [48].

Another mechanical approach, more specific to microfluidic systems, consists of the intrinsic design of cell membrane piercing protrusions, that extend from the surface of the channel inside which the cells are forced through and ruptured by shearing forces, as patented by Wilding *et al.* [49] and schematized in Fig. (3).



**Fig. (3).** Mechanical cell lysis in a specially designed microfluidic channel. The cells (black circles) are forced through a thin channel designed with membrane piercing protrusions. The protrusions disrupt the cellular membranes and the intracellular medium containing the nucleic acid material is released in the downstream compartment.

### 1.2.2.3. Thermal and Electrical Cell Lysis

Other methods, which are particularly attractive for NA analysis on microfluidic chips, are based on the thermal or electrical disruption of cellular membranes. Heating the cells at a high temperature, for instance, can be compatible with the subsequent on-chip NA amplification, using the same integrated heating element [50]. Elevated temperature in the solution can be achieved, for example, by radiating microwaves from a monolithic microwave integrated circuit (MMIC) [51], or can be induced at a precise location inside a microchannel by an external laser beam [52]. Likewise, electrolysis, also called irreversible electroporation, necessitates only microfabricated electrodes that can be also used for fluid handling and subsequent NA separation by capillary electrophoresis [53-55]. In particular, Motorola patented in 2004 a microfluidic device featuring embedded metal conductors to perform both cell lysis and NA amplification on a single chip [56]. The same company also released a patent on a multilayered microfluidic DNA analysis system in which cell lysis is performed by subjecting the cells to pulses of high electric field strength [57]. Similar to ultrasonication, the major drawback of both the thermal and electrical approaches is associated with the formation of bubbles inside the microfluidic system due to the elevation of the temperature. The generation of joule heating can however be minimized by applying electrical pulses instead of a continuous current. Techniques such as the local electro-generation of hydroxide ions can also help reduce the high

field strength and voltage required for breaking the cells [58].

In recent years, many approaches have been described for microfluidic cell lysis. The chosen technique mostly depends on the type of cells or organisms from which the NAs have to be extracted and its compatibility with the subsequent steps of the analysis. Moreover, different methods can be combined to achieve the differential lysis of a specific cell type as proposed in the microfluidic differential extraction cartridge developed by Microfluidic Systems [59].

#### **1.2.2.4. Nucleic Acid Extraction and Purification**

Following the cell lysis, the next step in NA analysis is the extraction and purification of the NA material from the cell lysate. On a macro-scale, NA extraction is commonly completed by using various chemicals, precipitation and centrifugation. However, these conventional methods are time consuming, difficult to automate and to scale down to small sample volumes. In particular, the centrifugation and precipitation processes often involve the use of large-scale apparatus, such as that disclosed by Osanai *et al.* [60]. From this point of view, the solid phase extraction (SPE) method is expected to become mainstream in the future where downsizing is required. Various techniques of SPE involving the use of magnetic particles, filters, silica gels and beads, and micro- and nanoengineered surfaces have been developed. Novel devices utilizing these methods are discussed next.

#### **1.2.2.5. Filters**

Several microfluidic-based extraction devices that employ filters in lateral-flow devices [61-66] and in vertical-columns [67-70] have been described. While vertical column-based devices have the advantage of high throughput due to the high-level integration of columns, their fabrication does not benefit from microfabrication-batch process, hence only lateral-flow devices are considered in this section. In lateral-flow devices, a suitable sample extraction matrix comprises filters to block the impurities resulting from the cell lysis process while permeating the NA material [61,62]. Alternatively, a filter paper can be employed to temporarily entrap the NAs present in the sample, while washing the undesired contaminants [63,64]. Here, the NAs entrapped within the matrix may be eluted and then transported into the next channel for further processing. Affymetrix patented a system where the extraction device comprises a deformable porous material for NA binding and pneumatic ports for fluid transport [64]. As such, it overcomes the fluidic problems encountered with high surface area packed systems. However, as the filter is added after the microfluidic channel formation, it does not have the advantages of batch fabrication that arise from a monolithic design.

Although most of the reported inventions use external filters inserted into the microfluidic chamber at the assembly level, efforts have been made to monolithically integrate filtering functions at the fabrication level and thus simplify the system assembly. For instance, Microtechnology Ct Man presented a monolithic integration of a polymeric filter produced by ablation or stamping in microchannels, which also allows for precise control of pore size during fabrication [65]. Alternatively, Nanostream incorporated filter-holding

structures to easily achieve fitting and tight seal of the filter element [66]. While on-chip filter-based extraction method has been demonstrated, unresolved issues concerning lateral-flow saturation at the filter and the resultant slow assay time have led to the development of other SPE methods.

#### **1.2.2.6. Magnetic Beads**

The use of magnetic beads is an alternative extraction method, where the target NA molecules can be captured by complementary binding with molecular probes immobilized on the surface of the beads. Subsequently, the beads are collected within a channel using an applied magnetic field and washed with an elution buffer. The NA strands, dissociated from the surface of the magnetic beads, are retrieved along with the buffer.

Several inventions have been reported utilizing this method; they vary on the arrangement and the number of external magnets and microchannels. For instance, a single magnetic field for collection of magnetic particles was shown by Yang *et al.* [71] and Kusumoto *et al.* [72]. In the latter invention, a multi-channel network is implemented to increase throughput. To enable more efficient mixing of the sample with magnetic beads, Kreuwel *et al.* from Biomerieux presented a microfluidic device utilizing two separate magnetic fields applied simultaneously in different directions [73]. In further attempts to improve the efficiency of target capture, Canon disclosed a patent application where an aggregate of magnetic particles is formed thus facilitating the capture without the need to apply a large magnetic field [74]. Nelson *et al.* from Aclara Biosciences patented an extraction-separation device consisting of a network of channels and electrodes that control the fluid flow electrokinetically, while the magnetic bead collection inside the SPE channel is carried out with a single magnet [75].

#### **1.2.2.7. Silica Beads and Gels**

Several microfluidic inventions tailored to the extraction by silica-beads have been disclosed in patents and patent applications. Norchip demonstrated the use of packed silica beads on a PDMS microfluidic platform for NA extraction [42]. In the presence of chelating agents, the NAs bind to the packed silica particles. After washing, the NAs can be released with an elution buffer. To enhance the extraction, the device uses several electrodes adjacent to the packed silica beads-channel to reversibly bind and pre-concentrate the eluted NAs on-chip. A different invention disclosed in a patent application by Quake's group uses a densely packed channel network with a set of control valves instead of electric fields to trap and release the affinity-beads [43]. However, a disadvantage of the packed beads NA-extraction method is that the free beads are not strongly linked to the channel, thus they can be accidentally exhausted with the flow of solution.

As an alternative, sol-gel methods for improving NA extraction have been developed. Landers *et al.* used immobilized beads in a gel matrix to alleviate the problem of free beads in the solution flow [76]. A different invention, utilizing gel-extraction method in the form of microfluidic disk, was developed by Gyros [77]. This device relies on centrifugal forces for fluid transport and integrates other necessary components for NA analysis.

### 1.2.2.8. Micro- and Nano-engineered Surfaces

Engineered surfaces employ different micro- [78-82] and nano- [83] fabricated structures to improve the NA extraction yield. They may be used to capture affinity beads [81-83] or be coupled with a specific surface chemistry [78-80] to increase NA binding capacity and retention. For instance, Microfluidic Systems has a patent pending on a handheld portable extraction device that incorporates pillars configured in a gradient of silica-on-silicon structures, as represented in Fig. (4) [79]. In addition to increasing the extraction efficiency, the gradient acts as a filter to block physical debris present within the sample. By positioning the pillars less densely near the input port, they block more effectively the debris without becoming clogged. With the debris removed, the fluidic sample passing the more densely configured pillars is better prepared for NA extraction and collection. A similar invention utilizing pillars etched in silicon substrate to capture and retain NA affinity beads was also disclosed [82]. In this device, pillars of different heights and widths are etched to form a filter that traps the beads onto which NAs are subsequently immobilized. An advantage of the micropillar chip for NA purification and concentration, besides providing increased binding surface area, lies in the ability to produce multiplexed channels containing compact arrays of micropillar on a small footprint for high throughput purification.

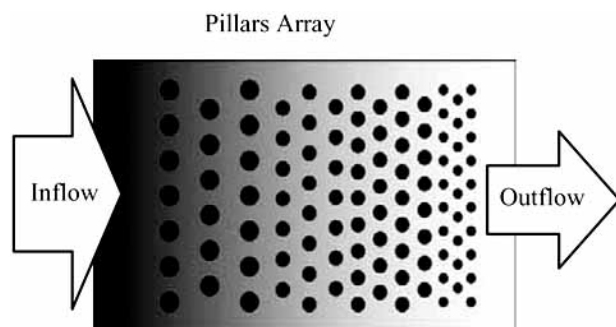


Fig. (4). Gradient of increasing density of pillars on substrate for nucleic acid extraction and purification.

However, to fabricate pillars in a silicon substrate, expensive DRIE (Dry Reactive Ion Etching) equipment and complex fabrication methodology are required. To simplify the fabrication process, smooth-walled silica channel with electrically controlled fluid-flow [84], or thermally grown silicon-dioxide on porous silicon substrate with an appropriate surface chemistry for enhanced NA absorption [85] have also been demonstrated. Further developments in this arena have led to novel devices with improved surface chemistries that employ dendrimers on glass or polymer channels [86] and minor groove binders for simultaneous detection on glass channels [87].

### 1.3. Microfluidics for Nucleic Acid Amplification

Polymerase Chain Reaction (PCR) is the most commonly used method for NA amplification. It is a three-step

amplification process requiring three temperature transitions. The process starts with a *denaturation* step, during which the hydrogen bonds of a double stranded NA (*template*) are broken to form single stranded NAs. The process occurs at temperatures between 94°C and 96°C. In the *annealing* step, the temperature is lowered to 50-65°C, allowing primers which limit the NA region to be replicated to bind to the single stranded template. The temperature is then raised to 70-75°C to allow the enzymatic replication of the template by a DNA polymerase in the *extension* step. Several repeats of this three-step thermocycle result in millions of copies of the NA template.

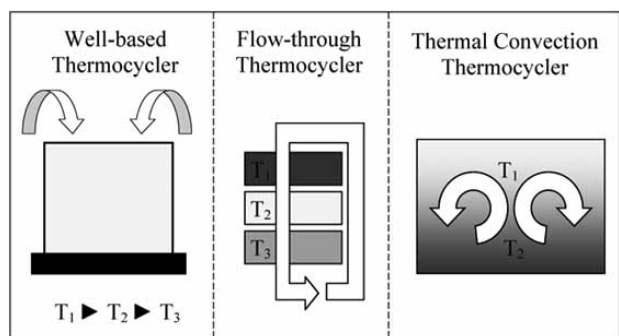
The miniaturization of the PCR system brings particular advantages to the amplification process. Specifically, it offers a significant thermodynamic advantage as small device dimensions and reduced sample volume exhibit less inertia to temperature change. Conventional PCR thermocyclers achieve heating and cooling rate in the range of 2-10°C per second, while miniaturized thermocyclers can reach rate of 15-40°C per second. This thermal advantage remains the key driving force in the development of microfluidic PCR allowing for rapid amplification. Yet, the thermal requirement for PCR constrains the design of microfluidic chambers and channels for optimal heat flow and isolation, as it limits the fabrication to thermally stable materials.

As the first PCR patent issued in 1983 to Cetus, is set to expire in 2006, an extensive catalogue of patents exists describing integrated PCR systems on microfluidic platforms. Since Northrup *et al.* in 1993 first introduced on-chip PCR devices [88], they and many other inventors have successfully patented systems that can be categorized into *well-based PCR*, *flow-through PCR* and *thermal convection PCR*, shown in Fig. (5). Various heating principles, relying on both contact and non-contact techniques have also been described. The general operation of the PCR starts from extracted NA samples from upstream processing to which primers and reagents are added. After mixing, the homogeneous solution can be thermally cycled for amplification.

#### 1.3.1. Well-based PCR

Early microfluidic PCR systems consisted of reaction chambers or wells, where the PCR reagents (NA template, primers, and polymerase) are kept stationary and the temperature is cycled between the three PCR temperature ranges. The amplified NAs are recovered from the chambers for post processing upon completion of the reaction.

Northrup A. and co-workers at the University of California patented various well-based PCR designs starting from 1996 [89-91]. These patents describe microfabricated reactors that incorporate reagent agitators and mixers, heaters, pumps, and later, optical or electromechanical sensors to the PCR chamber. The inventions focus on the design of silicon chambers that combine a critical ratio of silicon and silicon nitride to ensure uniform heating. Wilding and Kricka also patented and disclosed similar approaches to silicon PCR reaction chambers in 1996 [92,93] and 2005 [94]. They describe chambers designed with high surface to volume ratio to facilitate thermal regulation. The thermal cycling occurs in successive chambers set at different



**Fig. (5).** Schematic of 3 different strategies for nucleic acid amplification on-chip.  $T_1$ ,  $T_2$  and  $T_3$  represent 3 specific temperatures required for nucleic acid amplification by Polymerase Chain Reaction (PCR).

temperatures. The latter patent application describes a chamber design for use in conjunction with the collection and analysis of cell samples. A multi-chamber thermocycling device patent was also granted to Biometra Biomedizinische Analytik and the Institut für Physikalische Hochtechnologie, which incorporates a heat sink on the substrate and heating elements constructed in the chamber wall [95].

In another direct adaptation of the semi-conductor fabrication technology, a patent by Tamiya *et al.* presents a rapid implementation of an array of hydrophilic microwells etched into a hydrophobic substrate via anisotropic bulk etching [96]. Recently, other materials have been considered for chamber fabrication. Briscoe *et al.* implemented a PCR device, using ceramic multilayer technology, embedding conductive and isolation layers to provide thermal control [97]. Similarly, Agilent Technologies patented a micro-reactor device in polymer materials [98]. Furthermore, the work at Fluidigm on elastomeric materials has led to the disclosure of a device for PCR with  $N \times M$  reaction cells connected to samples and reagent inlets [99]. The approach is currently used for genetic analysis in their commercial system Biomark™.

### 1.3.2. Flow-Through PCR

Generally, a well or stationary approach to PCR limits its efficiency due to the time required for uniform temperature transition in the reaction chamber. An alternative approach consists of a dynamic, continuous sample flow system, where solutions are moved through successive zones of constant temperatures. The small sample volume allows the solution to reach the temperature equilibrium quickly, while the transition from temperature zones is only limited by the flow rate. In an early description of this technique in 1999 [100] and patented in 2005 [101], Koehler *et al.* show a microfluidic device constituting of three substrate platelets maintained at different temperatures. A fluidic path meanders through each zone successively, each passage equivalent to a PCR cycle. A similar concept was patented by the French Commissariat Energy Atomique in 2000 [102]. In 2001, Micronics described a PCR device where the samples and PCR reagent are mixed through a diffusion process in the PCR channel [103]. Later, Franzan J. from Bruker Daltonik patented an improved method of the

temperature zone technique for very fast NA replication by simply dividing the PCR reaction solution into fine capillary arrays in each temperature zone [104]. The close proximity to the heating source and small volume further reduce the thermal inertia, allowing for a 3-step PCR thermocycle in only a few seconds.

The meander design limits the PCR reaction to a given number of cycles, defined by the number of loops. An alternative method consists of the circular path approach. The patent for a microfluidic continuous flow PCR device was granted to the California Institute of Technology [105]. The device includes a rotary channel featuring multiple temperature regions along the circular path. Agilent Technology also published a similar approach, but instead of channels, the PCR chamber is compartmentalized into 3 portions arranged in a cyclic manner [106]. The PCR solution flows from portion to portion during the duplication process in a continuous fashion. A linear version of the continuous flow rotary concept also exists. Auroux *et al.* with the Imperial College of London described a sample-shutting PCR device in which the sample is passed back and forth over the three thermal zones in a straight channel [107].

In a different implementation of the continuous flow PCR technique, the samples and reagents for NA amplification are manipulated as microdroplets. The discrete microdroplets are moved on a silicon based chip via electrowetting through different temperature zones for the thermal cycling and propelled into channels for post-processing [108].

### 1.3.3. Thermal Convection PCR

In 2003, Benett *et al.* from the University of California were granted a patent for a PCR microfluidic device based on thermal convection [109]. By creating a chamber reaction with different temperature zones and utilizing the differential temperature to create a convection current, rapid amplification can be realized with a simple design employing minimal pumping mechanisms and electronic control components.

### 1.3.4. Thermal Control and Novel PCR Approaches

In the description of PCR microfluidic systems, the design of an integrated heater, temperature sensor and control components are central. Variations of popular techniques using resistive elements made of platinum or chromium are often implemented [110]. Peltier-assisted thermocycling was also disclosed by the University of California [111]. A few patents are worth noting as they describe approaches to increase the heating/cooling efficiency in order to rapidly provide stable and uniform temperature zones. To improve thermal control, Aclara Technology described the incorporation of heat transfer films in the structure of the microfluidic channel [112]. Li D. and Erickson D. also disclosed a patent for a low power micro-channel reactor that incorporates in-channel heating elements instead of the conventional in-wall approach [113]. In conjunction with a capillary PCR method, Lee *et al.* disclosed a device in which a conductive polymer that emits heat under an applied current is molded around and in-between capillaries for fast heating [114]. In other patents, the focus is on novel non-contact techniques for temperature

control. IR/UV heating and air cooling for multiplexed microfluidic PCR devices have also been disclosed [115-117]. The integration of parallel microfluidic channels and reservoirs into a PCR microfluidic system to transport and carryout exothermal and endothermal chemical reactions was also described. Such a system was disclosed by Micronics [118]. Stichting Voor De Technische Waterschappen published a similar concept in which one of its embodiments uses the evaporation of acetone for cooling and the dissolution of sulphuric acid in water to generate heat [119].

Alternative PCR methods are considered in other patents. Techniques such as isothermal NA amplification (e.g. NASBA, SDA, etc [120,121]) was described using microfluidic devices. In many instances, devices designed for a 3-step PCR are well suited for these methods. Burns *et al.*, at the University of Michigan, for example, disclosed a microfabricated device dedicated to low temperature isothermal NA amplification [122].

Improved handling of the PCR products were also proposed with the use of microcarriers or microspheres on which the NA template is immobilized. In 1995, Hitachi patented a capillary PCR device to amplify trace amount of NA on microspheres [123]. In 2005, Mathies *et al.* with the University of California, described an on-chip genomic analysis system using microspheres to move the NAs through a matrix thermocycling reaction chambers to a detection area, incorporating an optical scanner for the detection of the microcarriers [124].

Interestingly, Goel recently disclosed a novel approach to PCR without thermocycling, referred to as Nano-PCR [125]. Denaturation, annealing and extension of the NAs can be induced by a mechanical, hydrodynamic or electromagnetic stress of different intensities in a NA strand, analogous to changing the temperature. The disclosed microfluidic device incorporates microchannels in a linear or rotary configuration, treated for the anchoring of the NAs, in which hydrodynamic flow provides tension to denature, anneal and extend the NA strands.

#### 1.4. Microfluidics for Electrophoresis and Nucleic Acid Separation

Traditionally, efficient electrophoretic separations are performed in either slab-gel or capillary configurations. While slab-gel electrophoresis exhibits higher resolution, capillary electrophoresis (CE) is more suited for high throughput sequencing and genotyping, since it does not require casting of a new gel prior to each separation experiment. Although significant advances have been made in electrophoretic analysis in silica capillaries over the past decade, the difficulty in assembling very large numbers of capillary arrays that would enable large-scale screening redirected the research focus on the development of microfabricated electrophoresis systems [126].

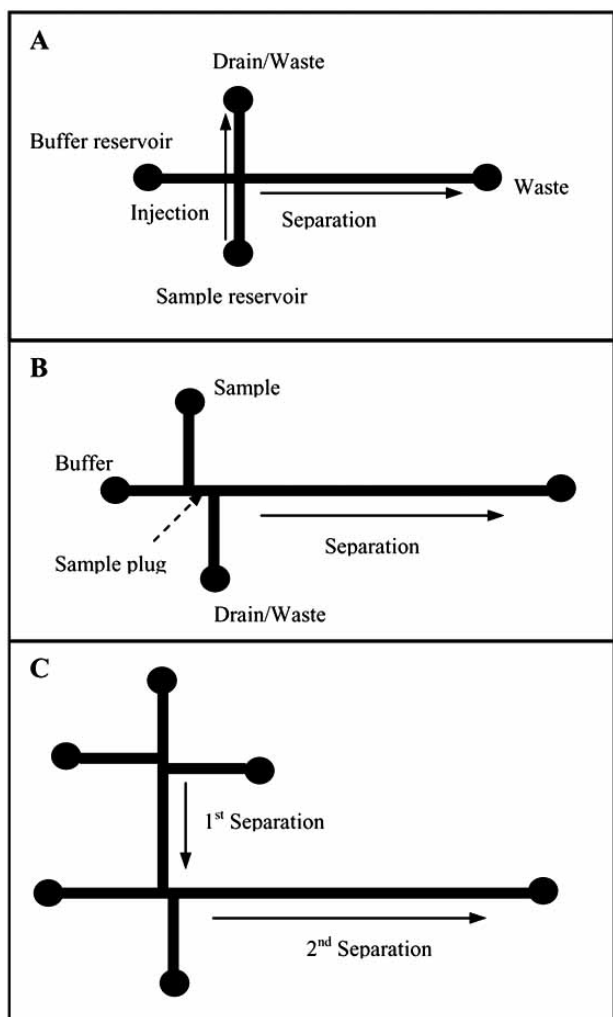
Generally, in CE microchips, the substrate defines at least one elongate capillary channel which extends between opposed cathode and anode ports and contains the electrophoretic medium. A typical chip-based electrophoretic system with cross-injection and separation channel is shown in Fig. (6A). When a biological fluidic sample is deposited in

the sample port, electrical potential is applied to direct a portion of the fluid sample first into the elongate microchannel and then towards the opposed anode port. The different chemical species within the sample migrate at distinct rates towards the oppositely charged end of the capillary; the rate of migration is dependent on the electrophoretic mobility of the chemical substance in the separation medium (polymeric solution or gel such as polyacrylamide and agarose gels). As a result of their distinct rates of migration, the various chemical components become separated as they progress along the electrophoresis channel and, thus, can be separately detected. The design and construction of electrophoretic microfabricated systems however pose challenges such as achieving high-resolution separation within a compact footprint, which requires optimization of electrokinetic manipulations, channel geometry and sieving media.

##### 1.4.1.1. Capillary Gel Matrix Electrophoresis

Electrophoresis in planar chips was first patented by Salvatore from Du Pont in 1990 using a simple straight channel geometry etched in silicon substrate [127]. Following this invention, Manz from Ciba Geigy fabricated both silicon and glass microfluidic chips to demonstrate the use of the electrophoretic functions [128,129]. In the latter patent, channel length was increased using a closed-loop arrangement with multiple cross-injections to make the device more suitable for NA separation. However, the regular cross-injection method could alter the original sample composition in the separation channel due to sample leakage at the intersection by diffusion. Hence, another electrokinetic injection method using a disjointed sample and drain channel in a shape of a double-T to inject a well-defined sample plug was patented (Fig. 6B) [130,131]. Another effort by Manz and Effenhauser was directed toward increasing the separation efficiency by integrating two separation paths in microfabricated devices having the shape of a double-T piece (Fig. 6C) [131]. To increase throughput of microfabricated CE chips, Manz and Zhang have used a cross-injection scheme for sample loading in a multichannel electrophoretic separation device fabricated in glass and PDMS substrates comprising of eight separation paths [132]. Burns *et al.* have also addressed sample injection issue by patenting a novel electrode-driven injection scheme and precise interface gel casting techniques to achieve sample focusing [133]. By fabricating an array of on-chip electrodes to apply the electric field in the sample-loading region, the NAs are forced to migrate and are collected at an anode located just outside the gel interface. Next, the collected NAs are released and enter the gel as a narrow well-defined band, thus enhancing sample injection and enabling low-voltage operation. Another electrically assisted injector-concentrator CE-device has been described by Swierkowski S.P [134]. In this patent, sample injection and pre-concentration is achieved with a single input port, a feature desirable in highly packed array systems.

Simultaneously, other efforts have been directed towards developing polymer-based chips suitable to capillary electrophoresis. Aclara Biosciences has used a double-T injection scheme for a chip fabricated by hot-embossing



**Fig. (6).** Designs for microfluidic capillary electrophoresis-based nucleic acid separation. (A) Typical cross-section design (B) Sample injection through double-T design (C) CE chip integrating two separation paths.

PMMA [135] and Zeonor [136]. In particular, the use of a Zeonor substrate alleviates the problems related to complex polymer surface chemistries that tend to aggravate sample adsorption to the capillary walls and generate non-uniform electroosmotic flow resulting in reduced separation resolution.

In order to increase the length of the separation channels while maintaining throughput, Liu S. from Molecular Dynamics developed a microfabricated capillary electrophoretic DNA sequencing device that consists of an array of sixteen 7cm long channels that are fanned out on a 10cm diameter wafer [137]. Each channel has its own cathode, sample and waste wells and they all converge on a common high-voltage anode. An average of 457 bases per channel can be acquired in 15min with 99% accuracy [138]. Another high-throughput microfabricated CE system was presented by Davidson J. and Balch J. [139], which uses a high density

array of microchannels with extended read lengths enabled by the channel geometry. To increase the effective length of the separation channels while minimally impacting the packing density, they use sinusoidally-shaped microchannels, and microchannels etched on both sides of the substrate, connected by a via. Similarly, Mathies *et al.* provided a capillary array electrophoresis microplate with an array of 96 radially configured separation channels [140]. The necessary separation length is provided by four hyperturns in the center of the device, which minimizes the geometric band dispersion introduced by turns in the separation path. This device is able to acquire 41,000 bases in 24 min with 99% accuracy [141].

In terms of integrated CE-detection systems, Motorola patented a capillary electrophoresis device in which an optical waveguide system transmits excitation radiation from a source port into each one of the electrophoresis channels, while a detector optical system images the fluorescence radiation onto a CCD or photodiode array, enabling individual monitoring of each channel [142]. An alternative CE-detection platform that fully integrates electrochemical detection and high voltage electrodes with a portable power supply was disclosed by Keyton *et al.* [143].

Finally, one of the recent inventions disclosed by Lee and Devoe demonstrates an automated, high-throughput, 2-dimensional DNA gel electrophoresis system. They patented a 2-dimensional plastic microfluidic network capable of rapidly and accurately resolving DNA fragments based on their differences in size and in sequence [144]. Increased throughput is achieved by rapid size-based separations in the first dimension, followed by simultaneous transfer of the size-separated DNA fragments together with the parallel sequence-dependent separations in the second dimension.

#### **1.4.1.2. Nano-Electrophoresis**

Many devices rely on sieving gel matrices for electrophoretic NA separation. Alternative methods utilizing nanostructured materials are also being explored for rapid microchannel electrophoretic separation. For instance, Yager *et al.* from Visible Genetics have demonstrated a dense separation chip comprising of 50 channels on a 1cm wide substrate [145]. The device uses an extended series of microelectrodes (i.e. multiple anodes and cathodes in each channel to permit greater control of the electrophoretic process) to move a NA sample through a homogeneous separation medium to a desired location for detection or further reaction. When a voltage is applied across two or more of the microelectrodes, the charged molecules are induced to move and separate according to the electric field density, the type of solvent film, the charge, the shape and the size of the molecule. Additionally, they patented a novel separation matrix that overcomes problems associated with gel, such as the need for hydration and the randomness of the structure [146]. The separation matrix comprises a solid support with a plurality of nanofabricated posts and pores that form an obstacle course for the NA strands.

Another interesting invention that relies on *entropic recoil* for length-based separation of NA molecules was developed by Craighead's group. [147]. The 3-dimensional microfluidic device is formed by placing a polymer

membrane, consisting of thick (1.5-3 $\mu$ m) and thin (75-100nm) regions, between two micropatterned plastic chips. Size-dependent trapping of NA occurs at the onset of each constriction. The separation is achieved by moving the NA sample with short electric pulses through the porous membrane. Short NA molecules pass completely through the membrane, while longer NA molecules are only partially moved into the holes. By switching off the voltage, longer NA molecules recoil out of the holes, resulting in the separation of the NA molecules by length. The membrane may be a filter or artificial gel produced using *electron beam lithography* to define an array of entropic traps (holes, pillars, etc) [148,149].

Further developments in microfabricated capillary electrophoresis systems have been directed towards device optimization, such as new low-viscosity polymer sieving matrices [150] and novel designs of electrical circuitry for delivery of uniform electric fields in two dimensions [151]. Other optimizations include sample pre-concentration methods for enhanced electrophoretic separation, using semi-permeable porous silicate membranes [152] and nanofabricated obstacles [153].

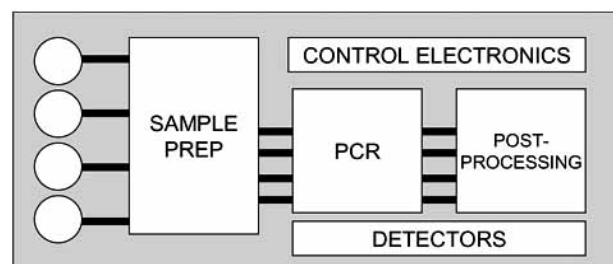
## 2. TOWARD INTEGRATED MICROFLUIDIC NUCLEIC ACID ANALYSIS

In the previous sections, primary functional components necessary to perform microfluidic NA analysis have been reported. Many inventors described adaptations of standard laboratory approaches onto a miniaturized format, while others presented innovative microfluidic-oriented techniques. With the recent development in microfabricated valves and pumps for liquid handling, the next step lies in the integration of individual components into a self-sufficient device capable of delivering application-specific information.

In this regard, the following section discusses inventions incorporating some level of sample preparation, processing and detection. In some applications, the integration of sample preparation with NA separation by electrophoresis, followed by laser induced fluorescent detection or mass spectrometry, is sufficient. However, such systems are limited to applications where the detection can be carried out with only a small amount of genetic material. By reviewing inventions that incorporate PCR amplification in the processing of the samples, the following discussion is focused on the challenges in implementing more complex integrated systems (Fig. (7)).

The integration is necessarily application specific, as it determines which functional components are to be included. For example, a microfluidic platform for NA-based diagnosis requires sample preparation to extract the NAs from a blood sample, an amplification step, and a real-time detection via a sequence-specific molecular probe. This is opposed to an application in drug discovery, where the post-treatment variation in expression profiles of numerous genes is recorded following the non-specific amplification of the total NAs and the hybridization on a microarray chip.

Although theoretically straightforward, the integration of individual functional components has encountered many practical issues as the technological requirements for each



**Fig. (7).** Schematic of an integrated lab-on-a-chip device containing individual functional microfluidic components for sample preparation, nucleic acid amplification and post-processing. The detection system and the control electronics should ideally be integrated on-chip.

component may be conflicting. Additional limitations to microfluidics, ranging from micro-to-macro interface compatibility to the resistance of end-users (both psychological and economical), must also be considered in order to understand the challenges in bringing such devices to market.

### 2.1. Integration of Functional Components

#### 2.1.1. Sample Preparation and Detection

The inclusion of sample preparation is an important first step toward integration. Many devices described in the earlier sections for cell sorting, cell lysis, and NA extraction can be readily integrated onto a single substrate. Subsequently, the products of these processes can be carried to an amplification step, as described by Norchip [42]. This invention features a lab-on-a-chip platform for NA amplification displaying chambers for performing cell lysis and NA extraction. Similarly, Micronics described an integrated sample preparation and amplification system with a lateral flow strip for the visual detection of the PCR product [154]. Also, as reported in section 1.2.2.3, thermal and electrical cell lysis have been integrated with a subsequent NA amplification step in several inventions [50,56,57].

#### 2.1.2. Electrophoresis

An extension to the above integration is the incorporation of controls and components for the purification and processing of the PCR products. In recent years, Mathies *et al.* have disclosed many examples of the integration of PCR with capillary electrophoresis and detection. For instance, they developed a PCR-CE glass chip that achieves the enzymatic digestion and the affinity capture of DNA for detection, patented by Affymetrix in 2001 [155]. In a more recent invention, another device incorporating sample preparation, PCR amplification, CE separation and detection was disclosed [156]. Here, the detection is done using a 4-color rotary confocal scanner described in a different patent [157]. Mathies *et al.* also released a patent application for an integrated capillary electrophoresis-electrochemical detection system fabricated in borosilicate glass substrate to detect DNA restriction fragments and size PCR products [158]. Furthermore, they described in several patent applications a microfabricated electrophoretic platform for sequencing that includes sample desalting, PCR template removal, precon-

centration and CE analysis [159-161]. The device comprises a four layer stack of PDMS and glass to form a membrane used to modulate the fluid flow and allow for less interfacing components to control very large number of valves.

Other integrated devices have been developed by Caliper Technologies and are currently being commercialized. In particular, the company presented a microfluidic DNA sequencing device that integrates sample preparation, mixing, amplification, size separation and detection [162-164]. In the latter system, the CE device allows size-separation of DNA in 75 sec separation time. In a different Caliper Technologies invention, simultaneous NA separation of a sample in 32 channels with minimal leakage was demonstrated [165].

Gyros also patented a device for sample purification, amplification and separation by CE using their centrifugal force disk approach [77]. Cardy and Allen expand on the electrophoresis or chromatographic approach by developing a lateral flow device for the detection of a specific NA sequence [63]. The device consists of linearly positioned zones containing reagents required to carry out the reactions for NA extraction, amplification, and detection.

### 2.1.3. Real-time PCR Detection

The use of intercalators the likes of SYBR<sup>®</sup> Green or ethidium bromide, and more recently sequence-specific fluorogenic probes, such as TaqMan<sup>®</sup>, enables real-time detection of the NA products during the replication process. The amplification kinetics are measured, from which the initial concentrations of target NA fragments can be calculated. Sequence specific probes also eliminate the need for electrophoresis separation and further detection. Real-time techniques are well-suited for microfluidic PCR as many implementations feature materials that are transparent at optically relevant wavelengths. As an example of a successful platform, Cepheid now commercializes a real-time PCR apparatus (GeneXpert<sup>®</sup> System) with fully integrated sample preparation. Its patented approach consists of a fabricated reaction chamber with optically transmissive walls through which the excitation and detection are carried out [166]. Another example of integrated real-time PCR is given by Oh *et al.* [167]. Alternatively, access ports can also be incorporated in the microfabricated substrate for the insertion of an optical system. Such a system, for instance, was patented by Bennett *et al.* at the University of California [168].

### 2.1.4. DNA Array and Hybridization Detection

Nucleic acid assays using hybridization are also widely used in gene-expression analysis. The integration of large DNA arrays to microfluidic PCR has been developed; as exemplified by Lipshutz *et al.* in an Affymetrix's 1999 patent [169]. The device features several chambers for performing sample preparation and amplification connected to a hybridization chamber that includes a DNA array. Olympus Optical also realized the integration of a DNA array to a PCR chip that features electrophoresis for extraction and an elliptically polarized light system for detection of DNA hybridization [170]. Quake S.R. and Chou H.P. presented a modified approach to the DNA hybridization detection in which the DNA probes are immobilized

on a closed loop channel (*active flux microfluidic*) [171]. The samples, with or without previous amplification, can be moved into the loop and exposed repeatedly to the probes. In a microfluidic device by Blackburn G., microchannels are sectioned into regions in which DNA probes are bound to a porous polymer or beads [172]. The hybridization is detected optically by fluorescence. Similarly, Mathies *et al.* described a miniature device featuring multiple functional components with NA probes immobilized on the substrate of the chambers. After hybridization, the substrate is removed for measurements under a microscope [156].

In other systems, electrical detection methods have been implemented. Miles *et al.* used impedance measurements for the detection of the end-products of PCR amplification [173]. For sequencing, Ouchi *et al.* from the Tokyo Shibaura Electric Company, patented a detection system in which a DNA array features electrical probes for the electrochemical measurement of hybridization [174].

## 2.2. Challenges of Component Integration

One major limitation impeding the integration of individual components lies in the intrinsic properties of the material chosen for the fabrication. The choice of material must meet all design requirements in terms of cost effectiveness, ease of fabrication, chemical functionality, thermal stability and optical transparency. More often than not, a compromise must be made to satisfy all the requirements of an integrated functional device. Simplicity in the design of an integration system is crucial. The development of novel methods of analysis or processing protocols may alleviate integration challenges by removing certain steps in the sequence of NA analysis. Specifically, new techniques, such as isothermal or non-thermal amplifications (discussed in section 1.3.4) avoid the need to design heating/cooling elements and thermal sensors. In this case, the formation of air bubbles in microchannels due to heating is also bypassed, along with the limitations related to the thermal stability of the material.

The majority of inventions described previously are *microscale* devices that need coupling to *macroscale* equipment. In order to achieve a higher degree of system portability and functionality required for on-field applications, it is desirable to decrease the reliance on external instruments. For instance, fluorescence lasers, lamps and microscopes used for detection can be replaced with arrays of optical or electrochemical detectors that are more suitable to miniaturization. Rapid and automatic dispensing and handling of small sample volumes is also needed to reduce the level of human intervention, which in particular requires improved *micro-to-macro* interface with innovative hardware interconnection technologies. In this regard, integrated microfluidic devices on CD, or *lab-on-a-CD*, are advantageous due to their versatility in handling fluids, their simple rotational motor requirements and inexpensive fabrication methods [175]. Additionally, the CD format is already adapted to miniaturized optical detection platforms used to image CDs at the micron resolution and soon at the submicron resolution with the advent of DVD and HD-DVD technologies. Several companies such as Gyros, Burstein Technologies and Abaxis are currently commercializing CD-based systems. However, an appropriate technological

solution is not necessarily commensurate with a commercial success. Tecan, for example, has discontinued the development of its LabCD technology as it foresaw difficulties in achieving “significant economical competitiveness and substantial market share in the required time-frame” (Thomas Bachmann, CEO of Tecan Group Ltd).

Several additional non-technological barriers can impede the introduction of microfluidic products to the market. One of them is the reluctance of some instrument-supplier companies to incur additional costs and loss of profit due to the commercialization of a new technology that is intended to replace the equipment they are currently marketing and for which great costs were already incurred. Additional impediments include the reluctance of end-users to adopt new technologies (due to the costs associated with equipment replacement and laboratory personnel training) and the general uneasiness of the public toward micro/nano-technologies applications in life-science.

### 3. CURRENT & FUTURE DEVELOPMENTS

#### 3.1. Trends for Integration

In developing the systems described in the earlier sections, inventors have managed to circumvent the issues related to the integration of individual functional components with some success. Many of these devices employ electromagnetic fields for liquid handling by electroosmosis, cell sorting by DEP, electrolysis, NA electrophoretic separation and for heating in PCR. This approach allows the implementation of the device using fewer materials, thus minimizing the cost and number of fabrication steps. However, as for most current technologies, such integration necessitates a certain level of trade-off in performance of each functional component.

Additionally, micro-sized particles and beads, either made of silica, polymers or magnetic materials, have been successfully incorporated in microchips at different steps of the analysis. Microbeads dramatically increase the surface area available for the capture of cells or NA; they are compatible with a subsequent amplification step, and can also be used for detection purposes [176]. Hence, microbeads represent a valuable approach in the integration of individual components.

#### 3.2. Nanoengineering

Recent advancements in nanotechnology have opened new avenues for the development of nanodevices that promise to overcome some of the limitations in miniaturized NA analysis systems. For instance, nanostructures and nanomaterials have been explored for NA separation in microchip electrophoretic devices as an alternative to polymer sieving matrices (see section 1.4.2). Densely spaced nanofabricated structures (e.g. nanopillars) or nanopacking medium (e.g. nanosphere solution inside a microchannel) can replace the viscous polymers that are often unsuitable for separation of long DNA and are difficult to inject into narrow channels. These nanostructures can be produced in highly parallel fashion in each separation channel as to maintain throughput. Additionally, surface functionalization using self-assembled monolayers of capturing biomolecules can provide pre-concentration and highly specific separation of target NA molecule [177].

Although these nanoengineered systems offer advantages of high speed, potentially increased sensitivity and specificity due to their unique material properties and processing capabilities, they still require costly and complex fabrication processes. Nevertheless, the proliferation of nanotechnology in genomic analysis is certainly expected [178].

#### 3.3. Main Contributors and Intellectual Property Market

Among the major contributors to the development of microfluidic NA analysis technologies, many academic researchers have played a critical role in the pioneering work, including Manz, Northrup, Quake, Toner, Wilding, and Kricka. Other contributors to the wider field of microfluidics, such as Whitesides G.M., Madou M. and Delamarche E., have developed alternative fabrication methods, microfluidic components (valves, pumps, etc.), and surface chemistries, enabling the more recent progress in microfluidics for NA analysis [179]. Many of them have licensed their inventions to start-up and affiliated companies, presented in Table (1).

The development of platforms for microfluidic NA analysis has also been carried out by small and large companies, seeking to find a niche in an increasingly crowded microfluidic market. Table (2) lists the companies holding patents and patent applications related to

**Table 1. Major Academic Researchers Identified Contributing to the Development of Intellectual Property in the Field of Microfluidic Nucleic Acid Analysis**

| Name           | Academic affiliation   | Company affiliation                                    |
|----------------|--|--|
| Manz Andreas   | Imperial College (UK), Institute for Analytical Sciences (Germany) | Ciba Geigy (now Novartis)                              |
| Northrup Allen | University of California (USA)                                     | Cepheid, Microfluidic Systems                          |
| Quake Stephen  | Stanford University, California Institute of Technology (USA)      | Fluidigm   |
| Wilding Peter  | University of Pennsylvania (USA)                                   | Aviva Biosciences, Chemcore (now Caliper Technologies) |
| Kricka Larry   | University of Pennsylvania (USA)                                   | Chemcore (now Caliper Technologies)                    |

**Table 2. Major Companies Identified Contributing to the Development of Intellectual Property in the Field of Microfluidic Nucleic Acid Analysis and Sorted by Categories\***

| <b>Microfluidic</b>                                   |
|---|
| Aclara Biosciences                                    |
| AVIVA Biosciences                                     |
| Caliper Life Sciences (formerly Caliper Technologies) |
| Cepheid   |
| Fluidigm (formely Mycometrix)                         |
| Gyros   |
| Handylab  |
| Microfluidic Systems                                  |
| Micronics   |
| Nanogen   |
| Nanostream  |
| Norchip   |
| <b>Life Sciences</b>                                  |
| Becton Dickinson                                      |
| Biomerieux  |
| Bruker Daltonik                                       |
| Ciba Geigy (now Novartis)                             |
| Evotec  |
| <b>Diversified with Life Sciences</b>                 |
| 3M Innovative Properties                              |
| Agilent technologies                                  |
| Du Pont   |
| Hitachi   |
| <b>Electronics</b>                                    |
| Canon   |
| Motorola  |
| Olympus Optical                                       |
| ST Microelectronics                                   |
| Tokyo Shibaura Electronic                             |

\***Microfluidic**: companies specialized in the development and commercialization of microfluidic products; **Life Sciences**: companies specialized in life sciences such as biotechnology, pharmacology, etc.; **Diversified with Life Sciences**: companies not specialized in Life Sciences but with a significant Life Sciences department; **Electronics**: companies specialized in electronics and microelectronics.

microfluidic NA analysis technologies. The listed companies are classified into four distinct groups according to their field of expertise.

Several companies, well established in their field of competency, such as Canon and ST Microelectronics, have

applied their technological strengths to the field of molecular biology. Their interest is driven by the capacity of their technology to provide solutions to unmet application areas. In many cases, their competencies in microfabrication, electronic design, optical system components and packaging are essential in the development of microfluidic integrated platforms. For companies already well established in the life sciences providing instrumentations and laboratory equipments, microfluidic technology offers a particularly enticing opportunity. Mindful of the significant cost in replacing existing equipments and the consumer reluctance to accept new technology, they are able to leverage benefits of miniaturization by integrating microfluidic with their pre-existing technology.

A large contingent of companies currently working toward providing integrated NA analysis systems are smaller companies highly specialized in microfluidic technology, as listed in Table (3). Many originate from academia while other from larger companies (e.g. Gyros from Amersham Biosciences). Their competitiveness and success lies in providing the advantage of microfluidics to current laboratory equipment in terms of cost and performance, as well as in bringing portability and user-friendliness for new devices in point-of-care diagnosis or on-field instrumentations, where traditional technologies cannot provide viable solutions. As a group, these microfluidic companies, however, represent a small segment of the overall intellectual property market, with less than 1% of the share (Fig. (8A)). It should be noted that the numbers in this analysis must be taken qualitatively, as the comparison is based on a keyword search against a worldwide patent and patent application database rather than a company size, sales or market value. Nonetheless, these numbers highlight the major role that the smaller companies play as developers and innovators in the field of microfluidics, holding over 80% of the granted and pending patents worldwide, as shown in Fig. (8B).

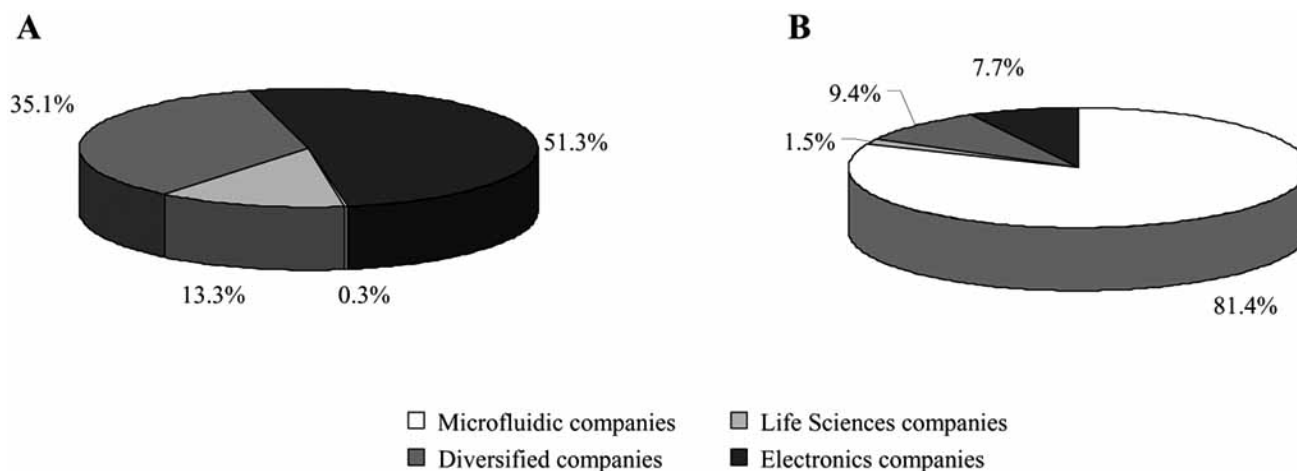
The microfluidic market remains young and in constant evolution and the introduction of commercial products featuring microfluidic systems are on the rise. Many companies have been created in the late 90s or early 2000s, and have since merged or have established partnerships with larger companies. Increasingly, collaborations between companies with complementary competencies are becoming more common, as exemplified by ST Microelectronics joint development with Mobidiag for microfluidic lab-on-chip for NA-based diagnosis.

Geographically, Japan and Europe are important players in microfluidics; however the great majority of the companies identified in this review, involved in microfluidic NA analysis, are American. Thus, considering the US patent market as indicative of worldwide trends, one can observe a year to year growth of microfluidic granted patents and patent applications disclosed by NA analysis-orientated microfluidic companies (Fig. (9)). The growth in the late 1990 to 2003 stems from the rapid introduction of novel technologies and materials, and increased public awareness. In recent years, a stabilization of the number of granted patents is observed, pointing to a maturing microfluidic technology. In view of the number of recent patent applications and the lengthy process of patents prosecution,

**Table 3. Description of Major Identified Companies Specialized in Microfluidics and Active in the Field of Nucleic Acid Analysis\***

| Name                 | Creation date | HQ Location         | Number of patent |     | Commercialized product    |
|----------------------|---------------|---------------------|------------------|-----|---------------------------|
|                      |               |                     | USG              | USA |                           |
| Caliper              | 1995          | Hopkinton, MA       | 222              | 128 | LabChip                   |
| Nanogen              | 1991          | San Diego, CA       | 76               | 41  | NanoChip                  |
| Nanostream           | 1999          | Pasadena, CA        | 36               | 44  | Brio Cartridge            |
| Cepheid              | 1996          | Sunnyvale, CA       | 27               | 42  | GeneXpert, SmartCycler    |
| Gyros                | 2000          | Uppsala, Sweden     | 21               | 21  | Gyrolab Bioaffy           |
| Aclara Biosciences   | 1995          | San Francisco, CA   | 16               | 58  | LabCard                   |
| Fluidigm             | 1999          | San Francisco, CA   | 12               | 34  | Topaz, BioMark            |
| AVIVA Biosciences    | 1999          | San Diego, CA       | 12               | 0   | SealChip                  |
| Micronics            | 1996          | Redmond, WA         | 9                | 9   | Microflow, Microcytometer |
| Handylab             | 1999          | Ann Arbor, MI       | 4                | 3   | Integrated cartridge      |
| Norchip              | 1998          | Klokkarstua, Norway | 0                | 1   | NucliSens                 |
| Microfluidic Systems | 2001          | Pleasanton, CA      | 0                | 3   | Biolyser                  |

\*USG: number of US granted patent; USA: number of US patent application since 2002.



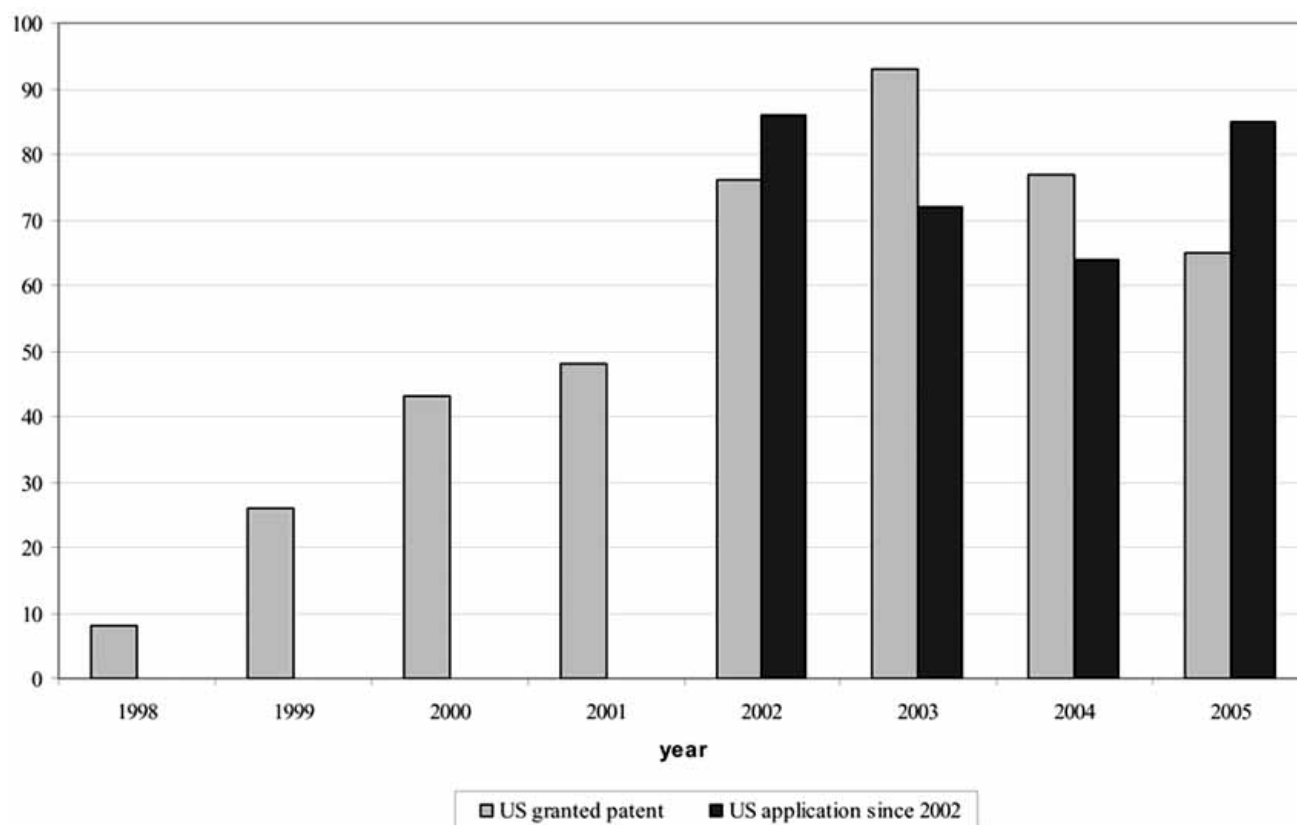
**Fig. (8).** Pie charts representing the distribution of intellectual property by company category. The search includes worldwide patents and patent applications of the companies listed in table 2 without any time limitation. **(A)** General intellectual property. **(B)** Intellectual property in the field of microfluidics as defined by the presence of the words “microfluidic”, “microchannel” or “microfabricated channel” in the title or abstract of the document.

one can expect the current trend to continue in the coming years.

## CONCLUSION

In this paper, recently disclosed patents and patent applications of microfluidic devices in key subject areas of NA analysis have been presented. As evident from this review, the major focus has been on the development of individual components for sample preparation, NA amplification, separation, and detection schemes on-chip, and great success has been reached in the miniaturization of each of these areas. However, to take full advantage of

miniaturization technology for on-field applications, the ability to achieve full integration among different system components is essential in order to construct self-contained lab-on-a-chip NA analysis system. In this regard, the technical challenges (fabrication materials and micro-to-macro interconnects) along with non-scientific issues related to the introduction of new technology to the market, still need to be overcome. The more recent trends have therefore focused on the use of electromagnetic fields, bead and particles proven to be compatible with individual steps of NA analysis. Additionally, exploration of nanoengineered surfaces promises to solve some of the material-related



**Fig. (9).** Annual total number of US patents and patent applications (since 2002) of the companies specialized in microfluidics listed in tables 2 and 3.

challenges. Despite this work, the progress toward integrating many or all functions of an assay on-chip is difficult, since the conditions needed for successful completion of all steps of NA analysis, from cell sorting to detection, are not necessarily compatible. Nonetheless, several companies identified in this review are marketing commercial products which exhibit a certain level of integration. A continued collaboration between researchers with different expertises is needed in this highly multidisciplinary field, to enable rapid technological innovations that will overcome the integration challenges and allow the commercialization of self-contained lab-on-a-chip nucleic acid systems.

## REFERENCES

### \* Patents of Special Interest

- [1] Schulte TH, Bardell RL, Weigl BH. Microfluidic technologies in clinical diagnostics. *Clin Chim Acta* 2002; 321(1-2): 1-10.
- [2] Ahn CH, Choi JW, Beaucage G, *et al.* Disposable smart lab on a chip for point-of-care clinical diagnostics. *Proceedings of the IEEE* 2004; 92(1): 154-73.
- [3] Beebe DJ, Mensing GA, Walker GM. Physics and applications of microfluidics in biology. *Annu Rev Biomed Eng* 2002; 4: 261-286.
- [4] Fiorini GS, Chiu DT. Disposable microfluidic devices: fabrication, function, and application. *Biotechniques* 2005; 38(3): 429-46.
- [5] Duffy DC, McDonald JC, Schueller OJA, Whitesides GM. Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). *Anal Chem* 1998; 70: 4974-84.
- [6] Pallandre A, de Lambert B, Attia R, Jonas AM, Viovy JL. Surface treatment and characterization: Perspectives to electrophoresis and lab-on-chips *Electrophoresis*. 2006; 27(3): 584-610.
- [7] Makamba H, Kim JH, Lim K, Park N, Hahn JH. Surface modification of poly(dimethylsiloxane) microchannels. *Electrophoresis* 2003; 24(21): 3607-19.
- [8] Delamarche E, Donzel C, Kamounah FS, Wolf H, Geissler M, Stutz R. Microcontact Printing Using Poly(dimethylsiloxane) Stamps Hydrophilized by Poly(ethylene oxide) silanes. *Langmuir* 2003; 19: 8749-58.
- [9] Auroux PA, Koc Y, deMello A, Manz A, Day PJ. Miniaturised nucleic acid analysis. *Lab Chip* 2004; 4(6): 534-46.
- [10] Sun Y, Kwok YC. Polymeric microfluidic system for DNA analysis. *Analytica Chimica Acta* 2006; 556: 80-96.
- [11] Mogensen KB, Klank H, Kutter JP. Recent developments in detection for microfluidic systems. *Electrophoresis* 2004; 25(21-22): 3498-12.
- [12] Wada, H., Kopf-Sill, A., Alajoki, M., Parce, J., Wang, B., Chow, A., Brow, R.: WO020070080 (2002).
- [13] Weigl, B., Bardell, R., Hayenga, J.: WO03078972 (2003).
- [14] Bardell, R., Weigl, B.H., Battrell, F.C.: US20046674525 (2004).
- [15] Foster, J.: US20056838056 (2005).
- [16] Leary, J., Frederickson, C.: WO03060486 (2003).
- [17] Brody, J.: US5726404 (1998).
- [18] Quake, S., Fu, A., Arnold, F., Spence, C.F.: WO9961888 (1999).
- \*[19] Spence, C.F., Fu, A.Y., Quake, S.R., Arnold, F.H.: US20036540895 (2003).
- [20] Liu, A.Q., Liang, X.J., Zhang, X.M., Sun, Y., Droge, P.: WO05108963 (2005).
- [21] Schnelle, T., Mueller, T.: WO05075958 (2005).
- [22] Schnelle, T., Pfennig, A., Mueller, T.: WO05075957 (2005).
- [23] Cosman, M., Kapur, R., Carvalho, B., Barber, T., Balis, U., Toner, M., Huang, L.: WO05084374 (2005).

- [24] Whitesides, G.: WO02093125 (2002).
- [25] Ahn, C., Cho, H., Choi, J.W.: WO0187458 (2001).
- [26] Toner, M., Truskey, G., Kapur, R.: WO04029221 (2004).
- [27] Sethu, P., Toner, M.: WO04113877 (2004).
- [28] Yamanishi, D., Hujisak, P., Yang, Z., Xu, J., Wang, X., Wu, L., Huang, M., Tao, G., Cheng, J.: WO03031938 (2003).
- [29] Wilding, P., Kricka, L.J.: US5726026 (1998).
- [30] Austin, R.H., Volkmath, W.D., Rathburn, L.C.: US5427663 (1995).
- [31] Takayama, S., Huh, D., Grothberg, J.: WO02084276 (2000).
- [32] Shevkoplyas, S., Munn, L., Bitensky, M., Yoshida, T., Sun, C.: WO06031385 (2006).
- [33] Betts, W., Hawkes, J.: US5569367 (1996).
- [34] Ager, C., Dames, A., Purvis, D., Safford, N.: US20016310309 (2001).
- [35] Soh, H.T., Wang, J., Kim, U., Hu, X., Qian, J., Meinhart, C.: WO06058245 (2006).
- [36] Cheng, J., Sheldon, E., Wu, L., O'Connell, J.: WO9938612 (1999).
- [37] Iliescu, C., Xu, G., Tay, F.: WO06004558 (2006).
- [38] Gruber, L., Bradley, K., Lopes, W., Lancelot, R., Plewa, J., Grier, D.: WO04012133 (2004).
- [39] Anderson, A., Gruber, L., Mueth, D., Plewa, J., Shireman, J., Rosenbaum, N.: WO05023391 (2005).
- [40] Kayyem, J.F.: US20056942771 (2005).
- [41] Parthasarathy, R.V., Ericson, K., Bedingham, W.: WO05068626 (2005).
- [42] Schonfeld, F., Von Germar, F., Karlsen, F., Lichtenberg, J., Verpoorte, S.: WO05073691 (2005).
- \*[43] Hong, J., Studer, V., Anderson, W., Quake, S., Leadbetter, J.: WO04040001 (2004).
- [44] Irimia, D., Toner, M.: WO05010147 (2005).
- [45] Marmottant P, Hilgenfeldt S. Controlled vesicle deformation and lysis by single oscillating bubbles. Nature 2003; 423(6936): 153-56.
- [46] Pourahmadi, F., McMillan, W., Ching, J., Chang, R., Christel, L., Kovacs, G., Northrup, M., Petersen, K.: WO9933559 (1999).
- [47] Taylor, M., Belgrader, P., Pourahmadi, F., McMillan, W., Chang, R., Sakai, S., Ching, J., Petersen, K., Northrup, M.: EP1180135 (2002).
- [48] Yuan, B., Northrup, A., Pourahmadi, F.: WO06060566 (2006).
- \*[49] Wilding, P., Kricka, L.J., Zemel, J.N.: US5635358 (1997).
- [50] Wu, B., Handique, K., Parunak, G., Kehrer, A., Ganesan, K.: WO05011867 (2005).
- [51] Barenburg, B.F., Burdon, J., Chan, Y.T., Dai, X., Gallagher, S., Grodzinski, P., Marrero, R., Nair, V., Rhine, D., Smekal, T.: US20036605454 (2003).
- [52] Baeummer, A.J., Dhawan, M.D.: US20046815209 (2004).
- [53] Culbertson, C.T., Jacobson, S.C., McClain, M.A., Ramsey, J.M.: US20046783647 (2004).
- [54] Lee, H.J., Kim, J.H., Yoo, C.E., Lim, H.K., Hwang, K.Y., Ma, S.M., Min, J.H.: JP2006166916 (2006).
- [55] Albritton, N., Li, G.-P., Bachman, M., Sims, C., Han, F.: WO03093791 (2003).
- \*[56] Rhine, D.B., Smekal, T.: US20046787339 (2004).
- [57] Briscoe, C.G., Yu, H., Grodzinski, P., Marrero, R., Burdon, J.W., Huang, R.F.: US20036544734 (2003).
- [58] Di Carlo D, Ionescu-Zanetti C, Zhang Y, Hung P, Lee LP. On-chip cell lysis by local hydroxide generation. Lab Chip 2005; 5(2): 171-78.
- [59] Belgrader, P., Yuan, B., Aflatooni, N.: WO05028635 (2005).
- [60] Osanai, T., Yamamoto, E., Tomaru, K., Fujimoto, J., Watanabe, K., Matsuki, T., Miyamoto, Y.: US2004248130 (2004).
- [61] Oikawa, Y.: JP2005261389 (2005).
- [62] Nakayama, H., Inuzuka, H., Tawara, S.: JP2003202347 (2003).
- [63] Cardy, D., Allen, G.: US2006160078 (2006).
- [64] Anderson, R.C., Lipshutz, R.J., Rava, R.P., Fodor, S.P.A.: US20016168948 (2001).
- [65] Atkin, M.: WO04074169 (2004).
- [66] Karp, C.D.: US20046811695 (2004).
- [67] Gjerde, D., Hanna, C.: WO0400708 (2004).
- [68] Kozwicz, D., Gerdes, J.: WO0012675 (2000).
- [69] Fujimoto, K., Torisawa, N.: US2005161377 (2005).
- [70] Fujishiro, M., Togashi, A., Tani, Y., Ishii, T.: US5824224 (1998).
- [71] Yang, M., Chen, X., Zhao, J.: CN1634965 (2005).
- [72] Kusumoto, M., Nishiya, Y., Kishimoto, M., Umebayashi, N.: WO05008209 (2005).
- [73] Kreuwel, H., Verwimp, E., Beerling, B., Spee, F.: BV: WO06010584 (2006).
- [74] Okamoto, H., Canon K.K.: JP200604262 (2006).
- [75] Nelson, R.J., Hooper, H.H., Hauser, A.K., Singh, S., Williams, S.J., Sassi, A.P.: US20026344326 (2002).
- [76] Landers, J., Norris, P., Power, M., Ferrance, J., Shrinivasan, S., Wolfe, K., Breadmore, M.: WO03104774 (2003).
- [77] Tooke, N., Anderson, P., Gyros AB.: US20056884395 (2005).
- [78] Pourahmadi, F., McMillan, W.A., Ching, J., Chang, R., Christel, L.A., Kovacs, G.T.A.: US20026440725 (2002).
- [79] Yuan, B., Aflatooni, N.: WO06029387 (2006).
- [80] Samper, V., Hongmiao, J., Yu, C., Kiat, H., Lim, T.: WO05066343 (2005).
- [81] Harrison, D., Oleschuk, R., Shultz-Lockyear, L., Skinner, C., Li, P.: WO0138865 (2001).
- [82] Anderson, H., Stemme, G., Van Der Wijngaart, W.: AB: WO0185341 (2001).
- [83] Yamaguchi, Y.: JP2005130726 (2005).
- [84] Ikeda, N., Kato, S., Hatsuzawa, T.: JP2006078475 (2006).
- [85] Long, A., Gill, P., Cox, T.: WO04071662 (2004).
- [86] Kazuhisa, F., Saya, S., Tadashi, M., Haruko, T.: US2005130191 (2005).
- [87] Reed, M.W., Weigl, B.H., Bardell, R.L.: US2006166223 (2006).
- [88] Northrup MA, Ching MT, White RM, Watson RT. DNA amplification with a microfabricated reaction chamber. Transducers 1993; 93: 924.
- [89] Northrup, M.A., Mariella, J., Carrano, A.V., Balch, J.W.: US5589136 (1996).
- [90] Northrup, M.A., White, R.M.: US5639423 (1997).
- [91] Northrup, M.A.: US20036602473 (2003).
- [92] Wilding, P., Kricka, L.J.: US5587128 (1996).
- [93] Wilding, P., Kricka, L.J.: US5498392 (1996).
- [94] Wilding, P., Kricka, L.J.: US20056953676 (2005).
- [95] Baier, V., Bodner, U., Dillner, U., Koehler, J., Hler, J., Poser, S., Schimkat, D.: US5939312 (1999).
- [96] Tamiya, E., Yokoyama, K., Murakami, Y., Sakaguchi, T., Morita, S., Nagai, S., Idegami, K.: JP2000236876 (2000).
- [97] Briscoe, C., Burdon, J., Chan, T., Barenburg, B., Grodzinski, P., Hawkins, G., Huang, R.F., Kahn, P., Marcero, R., McGarry, M., Tuggle, T., Yu, H.: US2004043479 (2004).
- [98] Tso, J., Swedberg, S.A., Wolber, P.K.: US20036613560 (2003).
- [99] Goodsaid, F., Unger, M., Huang, J., Quan, E., Grossman, R., Lam, P., Chou, H.P., Kimball, J., Pieprzyk, M., Facer, G.: WO05107938 (2005).
- [100] Kohler, J., Mokansky, A., Poser, S., Schulz, T.: WO9941015 (1999).
- [101] Kohler, J., Mokansky, A., Poser, S., Schulz, T.: US20056896855 (2005).
- [102] Caillat, P., Fouillet, Y., Vauchier, C., Clerc, J.F.: FR2795426 (2002).
- [103] Weigl, B.H.: WO0189692 (2001).
- [104] Franzen, J.: US20016180372 (2001).
- \*[105] Enzelberger, M.M., Liu, J., Quake, S.R.: US20056960437 (2005).
- [106] Gottwald, E., Preckel, T.: WO05094981 (2005).
- [107] Aurox, P.-A., Manz, A., Day, P.: WO04073863 (2004).
- [108] Burns, M.A., Mastrangelo, C.H., Sammarco, T.S., Man, F.P., Webster, J.R., Johnson, B.N., Foerster, B., Jones, D., Fields, Y., Kaiser, A.: US6057149 (2000).
- [109] Benett, W.J., Richards, J.B., Milanovich, F.P.: US20036586233 (2003).
- [110] Zhang C, Xu J, Ma W, Zheng W. PCR microfluidic devices for DNA amplification. Biotechnol Adv 2006; 24(3): 243-84.
- [111] Northrup, M.A., Beeman, B.V., Benett, W.J., Hadley, D.R., Landre, P., Lehew, S., Krulervitch, P.: WO9850147 (1998).
- [112] Smith, T., Bjornson, T., Ricco, A.: WO0131053 (2001).
- [113] Li, D., Erickson, D.: WO03006161 (2003).
- [114] Lee, M.A., Bird, H.: US20016312886 (2001).
- [115] Northrup, M.A., Mariella, R., Koo, J., Davidson, J.: WO9641864 (1996).
- [116] Landers, J.P., Huehmer, A., Oda, R.P., Craighead, J.R.: US20016210882 (2001).
- [117] Landers, J.P.: WO04033099 (2004).

- [118] Breidford, W., Lancaster, C.A., Hayenga, J., Bardell, R., Tonn, J., Weigl, B.H.: WO04108287 (2004).
- [119] Guijt, R., Dodge, A.: WO04040645 (2004).
- [120] Jing C, Shoffner MA, Mitchelson KR, Kricka LJ, Wilding P. Analysis of ligase chain reaction products amplified in a silicon-glass chip using capillary electrophoresis. *Journal of Chromatography A* 1996; 732(1): 151-58.
- [121] Anja G, Lars S, Frank K, Henrik R, Eivind H, Trine Nm, Reidun S. Real-time nucleic acid sequence-based amplification in nanoliter volumes. *Anal Chem* 2004; 76(1): 9-14.
- [122] Burns, M.A., Denuzzio, J., Burke, D., Johnson, B.N.: WO9822625 (1998).
- [123] Kuno, N., Uchida, N., Harada, Y.: JP07107962 (1995).
- [124] Mathies, R.A., Blazej, R., Liu, C., Kumaresan, P., Yeung, S.: WO05118867 (2005).
- \*[125] Goel, A.: WO06076022 (2006).
- [126] Ugaz VM, Elms RD, Lo RC, Shaikh FA, Burns MA. Microfabricated electrophoresis systems for DNA sequencing and genotyping applications: current technology and future directions. *Philos Transact A Math Phys Eng Sci* 2004; 362(1818): 1105-29.
- [127] Pace, S.J.: US4908112 (1990).
- [128] Manz, A.: US5180480 (1992).
- [129] Manz, A.: US5296114 (1994).
- [130] Manz, A., Harrison, D.J., Effenhauser, C.S., Zeptosens AG: US20016280589 (2001).
- [131] Manz, A., Effenhauser, C.S.: US5599432 (1997).
- [132] Manz, A., Zhang, C.X.: US2003150766 (2003).
- [133] Burns, M., Brahmaandra, S., Ugaz, V.: US20037005050 (2003).
- [134] Swierkowski, S.P.: US20036558523 (2003).
- [135] Soane, D.S., Soane, Z.M., Hooper, H.H., Amigo, M.G.A.: US20006054034 (2000).
- [136] Lackritz, H.S., Cruzado, I., Tan, H.R.: WO0208744 (2002).
- \*[137] Liu, S.R.: WO0168898 (2001).
- [138] Liu S, Ren H, Gao Q, Roach DJ, Loder RT, Armstrong TM, *et al* Automated parallel DNA sequencing on multiple channel microchips. *Proc Natl Acad Sci USA* 2000; 97: 5369-5374.
- [139] Davidson, J., Balch, J.: US6153076 (1999).
- \*[140] Simpson, P.C., Mathies, R.A., Woolley, A.T.: US20046749734 (2004).
- [141] Paegel BM High throughput DNA sequencing with micro-fabricated 96-lane capillary array electrophoresis bioprocessor. *Proc Natl Acad Sci USA* 2002; 99: 574-79.
- [142] Foley, B., Sawyer, J., Briscoe, C.: US20016592733 (2001).
- [143] Keyton, R., Baldwin, R., Naber, J., Walsh, K., Edelen, J., Jackson, D., Roussel, T., Crain, M.: WO03066514 (2003).
- [144] Lee, C.S., Devoe, D.: US2006054504 (2006).
- [145] Yager, T.D., Waterhouse, P., Izmailov, A.M., Maruzzo, B.C., Stevens, J.K., Larson, M.T.: US20016261430 (2001).
- [146] Yager, T.D., Waterhouse, P., Izmailov, A.M., Maruzzo, B.C., Stevens, J.K., Larson, M.T.: US20006110339 (2000).
- \*[147] Turner, S.W., Kameoka, J., Park, H.Y., Craighead, H.G.: US2003180711 (2003).
- [148] Craighead, H.G., Turner, S.W.: US2004209392 (2004).
- [149] Han, J., Craighead, H.G.: US2004035701 (2004).
- [150] Baba Y., Kataoka, K., Tabuchi, M., Nagasaki, Y., Kuwahara C.: US2006040396 (2006).
- [151] Huang, L.R., Sturm, J.C., Austin, R.: WO03035228 (2003).
- [152] Jacobson, S.C., Ramsey, J.M., Culbertson, C.T., Whitten, W.B., Foote, R.S.: US20046685809 (2004).
- [153] Huang, L., Sturm, J.C.: WO04037374 (2004).
- [154] Battrell, C.F., Shen, M., Weigl, B.H., Houkal, J.M., Lancaster, C.A., Breidford, W.: US20040013732 (2004).
- [155] Mathies, R.A., Simpson, P.C., Williams, S.J.: US6261431 (2001).
- [156] Mathies, R.A., Lagally, E.T.: US2005019902 (2005).
- [157] Mathies, R.A., Scherer, J.R., Wexler, D.: US6100535 (1999).
- [158] Mathies, R.A., Emrich, C., Singhal, P., Ertl, P.: WO2005103672 (2005).
- [159] Mathies, R.A., Grover, W.H., Paegel, B., Skelley, A., Liu, C.N., Lagally, E., Blazej, R.: US2006073484 (2006).
- [160] Mathies, R.A., Grover, W.H., Paegel, B.M., Skelley, A., Lagally, E.T., Liu, C.N., Blazej, R.: US2006287572 (2006).
- [161] Mathies, R.A., Grover, W.H., Paegel, B., Skelley, A., Lagally, E., Liu, C.N., Blazej, R.: US2004209354 (2004).
- [162] Knapp, M., Parce, J.W., Bousse, L.J., Kopf-Sill, A.R.: EP0972082 (2000).
- [163] Knapp, M., Parce, J.W., Bousse, L.J., Kopf-Sill, A.R.: US2003104466 (2003).
- [164] Ausserer, W., Bousse, L.J., Dubrow, R., Sundberg, S., Chow, A., Wang, B.: WO0210732 (2002).
- [165] Parce, J.W., Kopf-Sill, A.R., Bousse, L.J.: US2003003026 (2003).
- \*[166] Chang, R., Christel, L., Kovacs, G., McMillan, W., Northrup, M.A., Petersen, K., Pourahmadi, F., Young, S., Yuan, R., Dority, D.B.: US20036565815 (2003).
- [167] Oh, K.W., Kim, J.-T., Namkoong, K., Park, C.S., Cho, Y.K.: US2005164281 (2005).
- [168] Benett, W.J., Richards, J.B., Stratton, P.L., Hadley, D.R., Milanovich, F.P., Belgrader, P., Meyer, P.L.: US20046699713 (2004).
- [169] Lipshutz, R.J., Rava, R.P., Anderson, R.C., Fodor, S.P.A.: US5856174 (1999).
- [170] Makino, T. Co Ltd: JP2001149097 (2001).
- [171] Quake, S.R., Chou, H.P.: US20046767706 (2004).
- [172] Blackburn, G.: US20056875619 (2005).
- [173] Miles, R.R., Belgrader, P., Fuller, C.K.: US2006136466 (2006).
- [174] Ouchi, S., Okada, J., Hongo, S.: JP2004061427 (2004).
- [175] Madou M, Zoval J, Jia G, Kido H, Kim J, Kim N. Lab on a CD. *Annu Rev Biomed Eng* 2006; 8: 601-28.
- [176] Pamme N. Magnetism and microfluidics. *Lab Chip* 2006; 6(1): 24-38.
- [177] Lin YW, Huang MF, Chang HT. Nanomaterials and chip-based nanostructures for capillary electrophoretic separations of DNA. *Electrophoresis* 2005; 26(2): 320-30.
- [178] Mohamadi MR, Mahmoudian L, Kaji N, Tokeshi M, Chuman H, Baba Y. Nanotechnology for genomics & proteomics. *Nanotoday* 2006, 1(1): 38-45.
- [179] Kamholz AE. Proliferation of microfluidics in literature and intellectual property. *Lab Chip* 2004; 4(2): 16N-20N.