The DNA-chip gold rush

As mentioned above, and if we take as generic the definition of DNA-chips, the first working DNA-chip prototypes were only erratic leaps of µ-TAS researchers into the genetic analysis application field. Following the work of *Manz* and others in capillary electrophoresis and their own work in confocal fluorescence microscopy detection [Quesada1991], the first capillary gel-electrophoresis chips were introduced by *Mathies* and his team in 1992 [Mathies1992]. Almost at the same time, *Northrup et al.* were working on a different idea of DNA analysis systems, conducting the pioneering work that led to the conception of the first PCR-chips [Northrup1993]. All the while, in the backstage, *Fodor et al.* were working on the basic steps of light-directed DNA synthesis [Fodor1991]. Their work, which ultimately led to the first hybridization chips [Pease1994], introduced for the first time a novel analytical procedure based on the exploit of micro-fabrication techniques. The thus uncovered potential of microsystems in the DNA analysis field stirred the imagination of both researchers and market analysts, propping up a gold rush for (the thereafter called) DNA-chips that still abides today.

1.3.2. TYPES OF DNA-CHIPS

The massive social and economical impact of hybridization chips, and their mainly distinctive origins and technology, has led to the division of DNA-chips into two different subgroups: DNA-arrays and lab-on-a-chip schemes, even though, as we will see, the current gold rush is centered on the merging of these converging technologies (see p. 58) to produce a working DNA µ-TAS.

Hybridization chips

The main coiners and inheritors of the term DNA-chip have had (and still keep) a wild variety of nicknames. DNA-arrays, oligonucleotide arrays (ONA), hybridization arrays or hybridization chips are all terms that refer to the basic scheme developed by *Fodor's* team at *Affymetrix* [Pease1994], even though its inherent technology has developed into multiple different approaches. Nonetheless, even if most of their monikers suit them well, the newer application of these systems to other analytical niches (such as protein [Haab2001] or antigen [De Wildt2000] analysis) and the myriad technological methods surrounding them, makes the generic term *hybridization chips* the best suited to encompass all their possible variations

while still preserving a definition that unites both function, method and substrate.

Principles

The underlying principles of hybridization chips as an application product are easy to appreciate. Independently of the production technology they rely on, the primary hybridization chips are passive slabs of glass, plastic or silicon onto which short strands of DNA (oligonucleotides) have been fixed at known positions on the slab surface (see Figure 31). After the array of oligonucleotides has been put into place, hybridization is carried on by depositing a drop of labeled probe-DNA solution onto the array. Subsequent to a rinsing step, the chip is placed under a fluorescence scanner to locate hybridization sites.
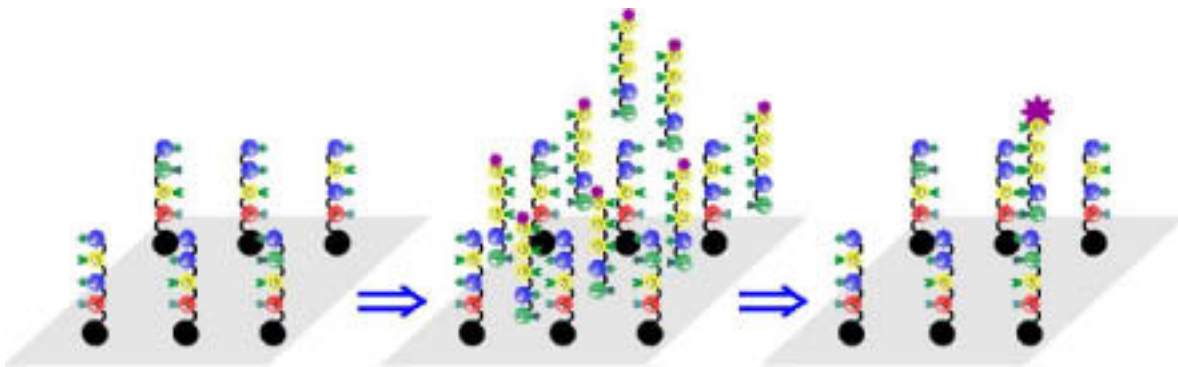

Figure 31 - Hybridization chips: probe introduction, rinsing and detection.

The success of hybridization chips (up to date, the only solid commercial success of DNA-chips) does not only rely on their inherent simplicity and their relative technological feasibility when compared to other DNA-chip approaches. With up to 65.000 hybridization sites per chip [Noble1995], hybridization chips provide the means to carry out massively parallel hybridization assays, in a matter of minutes and in a highly reproducible way. This has opened up the way for the high-throughput studies required in genome analysis ([Cutler2001], [Mei2000]), gene expression profiling [Courcelle2001], genetic diagnosis [Wikman2000] or surveillance [Kuritzkes2000], and even for quick response, sensitive, biowarfare agent detection [Edelstein2000].

Technologies

It is a curious historical fact that hybridization chips began their venture with, possibly, the most complex production method devised up to date [Fodor1991]. Following the light-addressed chemical synthesis approach of *Fodor's* team, electronically-addressed binding and arrayer depositing have come off age at a surprising pace, flooding the market with a myriad of devices and applications.

Arrayer depositing

Given its inherent simplicity, it is remarkable that arrayer depositing was the last technique for hybridization chip production to be commercially approached. Instead of dealing with complicate systems for light or electrically directed binding of the oligonucleotides to the chip, arrayer technologies make use of mechanical robot arms with micro-metric control and ink-jet systems of pico-liter precision (see Figure 32) to deposit previously synthesized oligonucleotides onto the chip surface. The deposited DNA strands are then typically bound to the surface by means of an UV-activated chemical link [Eisen1999].
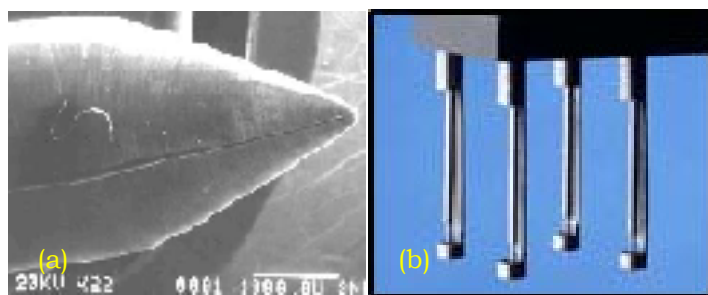

Figure 32 - Arrayer tips: (a) fountain-pen like [Eisen1999] (b) pin&ring by Affymetrix™.

Even though the chips produced by arrayer technologies cannot compete in resolution with electronically addressed binding or light-addressed synthesis chips (at their best, they provide ~200 µm dots with 136 µm inter-spacing [Dudoit2002]), their ease of use, their low fungible cost and their higher versatility (cells [Fejzo2001], proteins [Haab2001] or antibodies [De Wildt2000] can be also deposited with this methodology) have made arrayers such a killer-app that even companies holding patents for more complex hybridization chip systems, like *Affymetrix*, have made their move into the arrayer market [Dalton2000].

In the bioinformatics sector, the massive success of arrayer hybridization chips has prompted the necessity for powerful image analysis and normalization techniques [Steinfath2001] to correctly interpret the complex images (see Figure 33) generated by these assays, and has also made plain the urge for authoritative database management of the vast amounts of data produced [Brazma2001].
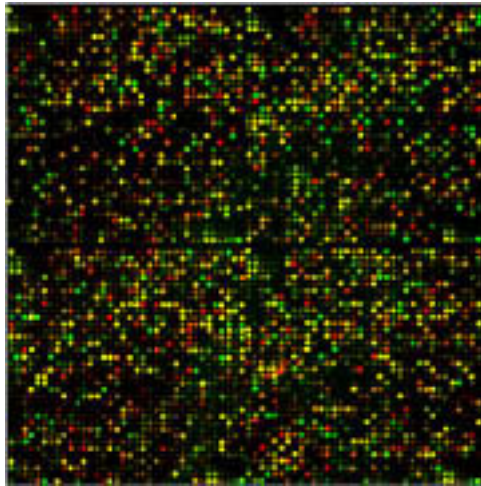

Figure 33 - Typical cDNA arrayer hybridization chip image. Two differently marked probes (A and B) have been exposed to the chip. Green dots reveal gene *A* expression while red dots show evidence of gene *B* expression. Yellow dots represent mixed expression of *A* and *B*. Robust image analysis and normalization are of crucial importance in the correct interpretation of DNA array data [Beißbarth2000].

Electronically-addressed binding

Far more complex than the competing arrayer technologies, electronically-addressed binding is a system for oligonucleotide chip production that makes extensive use of microelectronics technology. Using active electrodes deposited below the chip surface, electronically-addressed binding generates the oligonucleotide array by selectively attracting or repelling to each site the oligonucleotide molecules.

Figure 34 - Line-by-line electronic addressing and stringency control. Source: *Nanogen™*.

Although more expensive than arrayer technology, electronic addressing produces high-density arrays [Edman1998] and introduces the means for active hybridization stringency control (i.e. forcing non-optimal hybridization matches to break up), thus improving hybridization specificity (see Figure 34b).

Light-addressed synthesis

Even thought it was the first technique to be commercially developed, light-addressed synthesis is the ultimate step in the hybridization chip merging of micro-fabrication technologies and biochemical techniques, since it calls for selective synthesis of oligonucleotides on the chip surface [Fodor1993]. Light-addressed synthesis thus solves one of the problems implied by the previously seen schemes, for it eliminates the need to buy previously synthesized oligonucleotides by creating them directly onto the chip. Conversely, light-addressed synthesis makes the production of DNA-arrays more complex and expensive, as it requires the integration of DNA-synthesizer machinery with semiconductor-type photolithographic steppers, and the creation of a mask set for each array to be completed.
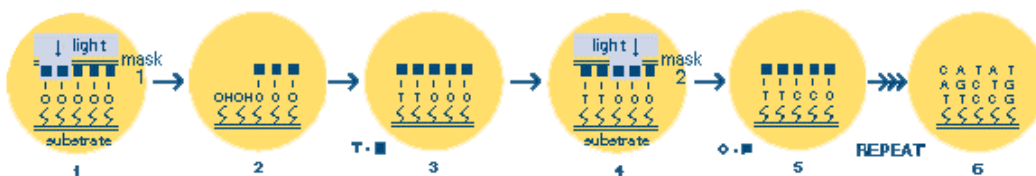

Figure 35 - Light-addressed synthesis steps. Source: *Affymetrix™*.

The basic procedure for light-addressed synthesis (see Figure 35) is also quite straightforward if the required complex technological setup is assumed [Fodor1991]. A slab with initial light-directed bound DNA nucleotides serves as a starter. A solution containing a specific nucleotide (i.e. A, G, T or C) the analyst wants to enlarge certain oligonucleotides on the chip with is then dropped on the slab. Under patterned UV light, the incoming nucleotide attaches itself only to illuminated spots, enlarging only the selected oligonucleotides. After washing, another specific nucleotide is introduced, and the process is repeated using the appropriate masks, thus selectively growing the oligonucleotide chains on the chip.

Lab-on-a-chip

The term lab-on-a-chip (LOAC), a synonym to μ-TAS in the DNA-chip field, was used until recently as a generic label to designate all the microsystem approaches to DNA analysis that were not based on hybridization techniques. Although there have been attempts at generic DNA manipulation by di-electrophoresis (see [Lin2000a], [Lin2000b]) and specific designs targeting purification and detection techniques (like [Belgrader2000]), the main flag-ships of the lab-on-a-chip approach have been PCR-chips (which due to their relevance to the work here discussed will be extensively dealt with in a separate section (see p.96)) and capillary gel-electrophoresis chips.

Capillary gel-electrophoresis chips

As mentioned before (see p.34), DNA electrophoresis demands the presence of a sieving matrix to achieve linear charge/length separation. Following the work of *Manz* in capillary electrophoresis chips ([Manz1990], [Manz1992]), the challenge of creating capillary gel-electrophoresis chips for DNA separation was diversely approached in the early 1990's.

Advantages

The advantages of chips for conducting capillary gel-electrophoresis assays are based mainly on their heat-dissipation properties. Firstly, the use of established and exquisitely controlled micro-fabrication techniques allows and guarantees the highly reproducible creation of electrophoresis channels with micrometric or sub-micrometric dimensions. The use of micrometric channels (or capillaries) is of special relevance in gel-electrophoresis, since the gel matrix is a poor heat conductor and its surface/volume ratio reflects on poorer resolutions by inducing Joule effects and thus limiting the strength of the electric field that can be applied (see p. 37). In addition, the same substrate of the chip (be it glass or, preferably, silicon) acts as a powerful heat dissipater, distributing heat in a homogeneous ways across the substrate and thus facilitating heat-exchange with the environment while minimizing the occurrence of local Joule effects. Finally, micro-fabrication techniques permit the creation of complex capillary manifolds, which can be exploited to provide precise plug injections (another limiting factor on resolution).

Gel-electrophoresis chips

The first working prototypes of capillary gel-electrophoresis chips were demonstrated by *Mathies* and his group in 1992 [Mathies1992]. Working on the cross-injection scheme developed by *Manz* [Manz1990] (see Figure 36), arrays of 30-120 μm capillary channels were filled with cross-linked poly-acrylamide and a denaturing buffer to conduct high-speed separation of DNA restriction fragments [Woolley1994].
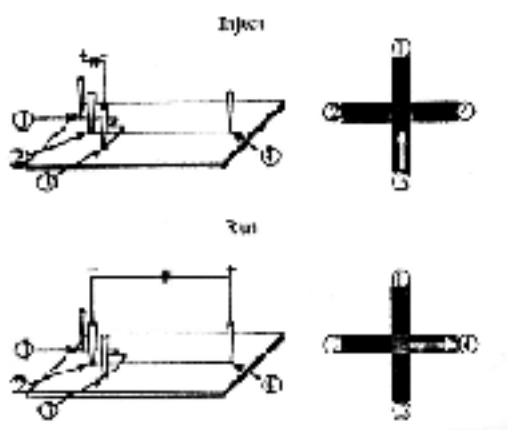

Figure 36 - Cross-injection method for low dead-volume plugs. Source: [Woolley1995].

A similar scheme, combined with the use of linear poly-acrylamide gels for chip reuse and a four-color confocal fluorescence scanner [Kheterpal1996], provided the means for carrying out high-resolution, sequencing-resolving separations on electrophoresis chips [Woolley1995] in an astonishing short time (540 s for a 150 bp fragment) using kV range voltages. Similar results have also been reported by various research groups in the last few years ([Liu2000], [Koutny2000], [Paegel2002]), and research on more versatile sieving matrices (such as thermo-viscous matrices [Buchholz2001]) is under way to optimize these systems [Albarghouthi2000].

Silicon-matrix electrophoresis chips

Nonetheless, the use of cross-linked or even replaceable linear poly-acrylamide for capillary gel-electrophoresis chips poses some direct and indirect problems in chip production. For instance, the acrylamide gel must be inserted and polymerized in the chip, with often non-reproducible results due to the presence of air bubbles and in-homogeneities in the medium or to injection problems that are aggravated by capillary and surface tension

effects. Moreover, polymerized acrylamide is a perishable product, thus limiting the scope for efficient mass-production and distribution of gel-electrophoresis chips.

An alternative to the approach of filling micro-fabricated channels with acrylamide gels was early devised by *Austin* and *Volkmuth* [Volkmuth1992]. Using conventional micro-lithography techniques, they created capped quasi-two-dimensional obstacle courses in silicon oxide (SiO$_2$). Unfortunately, their spike-arrays yielded a low sieving effect, similar to that of low-concentration (~0.05%) agarose, and could only be used to separate very large DNA fragments. Nevertheless, their work produced interesting results in the theoretical study of DNA electrophoresis ([Austin1993], [Duke1998]) and has later been pursued to produce mixing elements for μ-TAS [Knight1998]. More recent approaches to the subject of producing sieving matrices with micro-photolithographic methods have relied on the creation of entropic trap arrays (capped arrays of saw-teeth like arrangements) [Han2000] and macro-porous silicon [Ohji2000].

Integrated systems

As in its parent field, μ-TAS, system integration is a key issue in DNA-chips. Although different chip analytical approaches offer remarkable improvements over traditional systems (see p.57, p.97), and even though special kinds of chips, as hybridization chips, provide brand-new analytical procedures, the advantages of separate chip approaches are difficult to justify if previous and ulterior analytical procedures need to be carried off-chip. For instance, one of the most acclaimed universal feature of chips, their low reagent and sample requirements, which is of fundamental importance in certain clinic [Krishnan2001] and forensic [Verpoorte2002] applications, is not of too much avail if preparation and detection procedures are conducted off-chip and require comparatively large amounts of reagents and sample. In the case of DNA-chips, integration has been approached mainly with two different objectives: to create generic DNA analysis systems (a closer match to μ-TAS) and to produce standardized closed-kits for diagnostic and drug-targeting applications.

Generic analysis systems

As mentioned before (see p.31), the combination of selective amplification and separation techniques is the main key to generic DNA analysis

systems. With such two techniques (as, for instance, PCR and capillary gel-electrophoresis) the analyst can conduct a wide variety of experiments that include fragment sizing, gene expression profiling, SNP detection and even biotechnology's *primma donna*: sequencing. Therefore, it is not surprising to find out that most research in the field of DNA chip integration for generic analysis systems has dealt with the combination of an amplification technique (typically PCR, although SDA and LCR have also been approached) and a high-resolution separation technique (mainly a capillary gel-electrophoresis system).

Early attempts at this kind of integration were conducted by joint teams of researchers working in gel-electrophoresis and PCR-chips. *Northrup* and *Mathies* teams demonstrated the feasibility of an integrated PCR plus high-resolution capillary gel-electrophoresis system by gluing together both kinds of chips in 1996 [Woolley1996]. Although the complete system was closer to a multi-chip module than to an integrated chip, the effort proved the viability of integrating these different techniques (addressing reagent disposal and cross-interaction problems) in a monolithic, if hybrid, system. More recently, separate work by other key researchers of the electrophoresis-chip field showed that real integrated systems for PCR-plus-electrophoresis can be produced, even though the PCR system setup (see p.68, p.114) was external, using a commercial thermal cycler [Waters1998a] or a Peltier cell [Khandurina2000] to induce the PCR cycles on the whole chip. *Ramsey's* team has also demonstrated [Waters1998b] the possibility of on-chip cell lysis for *in-situ* PCR and electrophoresis, a feat also achieved in PCR-chips [Wilding1998]. Other work has shown similar results in non-chip [Soper1998] and hybrid (capillaries and chips) capillary systems [Soper1999].

In the last few years, two teams ([Burns1998], [Lagally2001]) have succeeded in creating fully integrated PCR-plus-electrophoresis systems using similar approaches (see Figure 37). Making combined use of micro-fluidic valves, hydrophobic vents, electrokinetic injection and integrated or off-chip thermal elements, both groups have produced functional monolithic chips for PCR-plus-electrophoresis analysis. Hence, even if many hindrances still need to be addressed in order to produce functional generic analysis systems for DNA, the goal of creating a sort of DNA μ-TAS is getting closer and, combined with the advances in closed-kit analysis systems, its implications in research and clinical biology could certainly be as

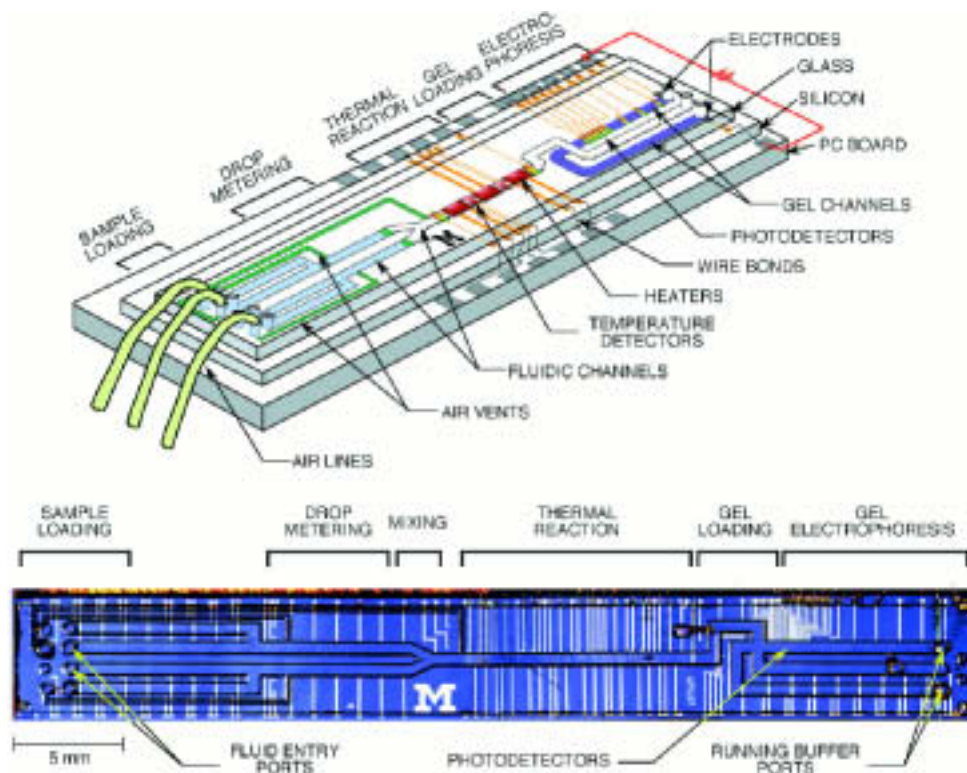revolutionary as the introduction of integrated circuits was to the computer sciences arena [Stipp1997].

Figure 37 - An integrated system for DNA analysis providing reagent mixing, amplification and separation by gel electrophoresis. Source: [Burns1998].

Closed-kit systems

Hybridization chips are, to date, the sole successful commercial exploit of DNA-chip technology. Hence, research on technology integration within this sub-field of DNA-chips has not focused much on the creation of generic multipurpose analysis systems, but, rather, has concentrated its efforts on the addition of suitable capabilities to hybridization chips, with the aim of delivering closed, benchmarked systems to the clinical diagnosis and drug-targeting industry. In particular, different attempts have closed on the following areas: (1) providing amplification power both to sample input and output, (2) integrating reagent delivery and sample pretreatment, optimizing hybridization procedures and, in a lesser degree, (4) probing for alternative substrate materials and (5) integrating detection technologies.

For instance, *Nanogen, Inc.* has developed systems that integrate sample amplification in high specificity hybridization assays ([Westin2000],

[Radtkey2000]). Amplification is carried out by *in-situ* strand displacement amplification (see p.41), while hybridization specificity is accomplished by selective electronic stringency control (see Figure 34, p.54). Using similar technology, ultra-sensitive electronic detection in hybridization chips ([Yu2001], [Umek2001]) has been attained by *Motorola LifeSciences* (formerly *Clinical Microsensors*) by using hybridized DNA as a conducting gate between two strands attached to an electrode and reading the capacitance differences between hybridization and non-hybridization sites.
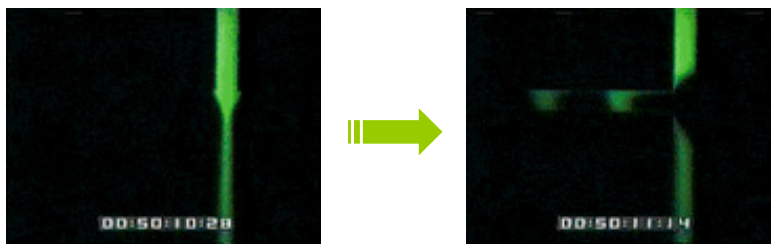

Figure 38 - Electrokinetic injection in micro-channels. Source: *Caliper Technologies.*

Precise electrokinetic and pressure-driven systems like those developed at *Caliper Technologies* ([Chien2001], see Figure 38), have recently been used at *Affymetrix* to create a polycarbonate device capable of cell lysis, RNA purification, reverse-transcript PCR, fragmentation and subsequent hybridization in a proprietary GeneChip Array (see Figure 39, [Anderson2000]). Sample preparation and purification have also been largely addressed by *Cepheid, Inc.* ([Christel1998], [Belgrader2000]), taking advantage of the surface interaction properties of DNA molecules in silicon microstructures.
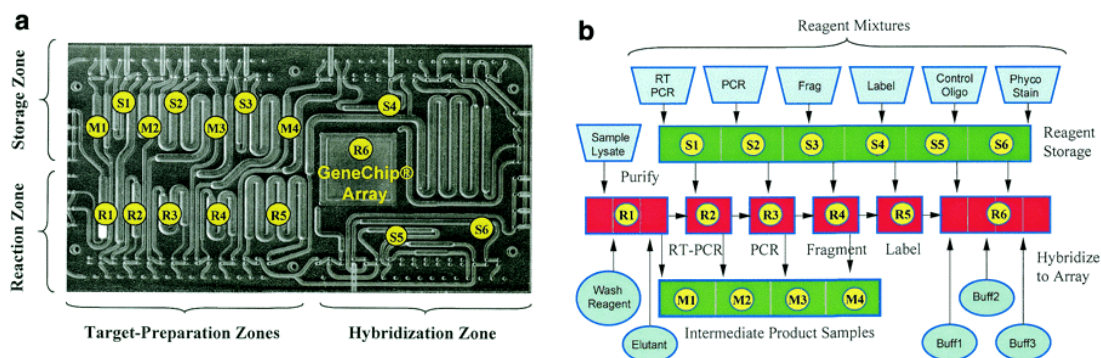

Figure 39 - Integrated polycarbonate system for cell lysis, RNA purification, RT-PCR amplification and hybridization. Source: [Anderson2000].