

Sequencing

Sequencing is the *prima dona* of the analytical techniques the molecular geneticist has at hand. Not only does it provide the ultimate information on the DNA molecule (sc. its nucleotide sequence), but it is also a troublesome technique to handle.

Conventional sequencing

Traditional or conventional sequencing consists mainly in the combination of a modified PCR procedure (the sequencing reaction) and a high-resolution electrophoresis run.

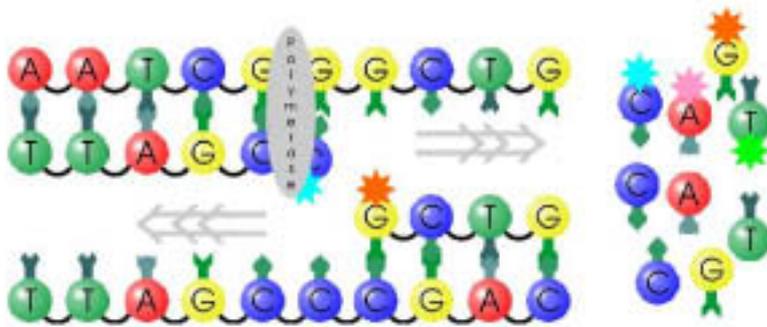


Figure 30 - Extension step in a modified PCR / sequencing reaction.

Sequencing reaction

In the current variety of the sequencing reaction, a new element is introduced into the standard PCR technique. Conveniently mixed with normal nucleotides (deoxyribonucleotides, dNTP), terminator nucleotides (di-deoxyribonucleotides, ddNTP) are fluorescently labeled nucleotides that interrupt the polymerase extension process when the DNA polymerase takes them up as building blocks (see p.38). Hence, at each PCR cycle, some strands of DNA will be replicated only until a terminator is used, producing DNA fragments of different lengths (Figure 30). Moreover, these randomly sized DNA fragments will be labeled with the fluorescent dye of their terminator nucleotide, revealing (since there is a different wavelength label for each of the four possible nucleotides) the base that terminated the sequence at that precise position. Ultimately, and due to the sheer numbers of molecules involved in a PCR reaction, an equal statistic amount of

terminated and labeled DNA will be produced for all possible strand lengths, with every strand being labeled with its terminator wavelength. Alternatively, the sequencing reaction can be divided into four sequencing reactions, using four labeled primers and non-labeled terminators.

High-resolution electrophoresis

After the sequencing reaction has taken place, its products are inserted in a single-base resolution acrylamide gel, that is, a sieving matrix that can discriminate between fragment lengths differing in only one base-pair (see p.34). A four-color laser-induced fluorescence scanner is used to detect the different fragments as they migrate through the gel and thus the underlying DNA sequence is read backwards (the first read fragment, the smallest, being the one with the starting base of the sequence). The need for efficient high-resolution electrophoresis assays and fast operation in DNA sequencing is one of the main drives behind the outbreak of capillary electrophoresis (see p.56) and PCR chips (see p.93).

Re-sequencing

Due to the specific requirements of high-resolution gel electrophoresis and the possibility of errors during the sequencing reaction, conventional sequencing remains a delicate technique of elaborate setup and limited reproducibility. But, although conventional sequencing is the only available method to decipher completely unknown sequences, there are many applications in which researches just require sequencing to check out specific mutations and variants. For instance, re-sequencing is of crucial importance for tracking HIV mutations in AIDS patients ([Coffin1996], [Hirsch2000]). Since, unfortunately, HIV has an astounding mutation rate, physicians need to regularly check HIV strains in patients to assess whether drug-resistance mutants have appeared and to adjust the drug-cocktail treatment accordingly. With conventional sequencing, the process is excessively laborious and unreliable to attain the throughput required in a day-care attention center. In other fields, such as the screening for carcinogenic mutations, this problem can be overcome by the use of hybridization targeted on single-base polymorphisms (SNP), but in the case of AIDS, the harsh mutation rate of HIV renders this approach unfeasible. Fortunately, the surge of massive hybridization chips (see p.51) has provided physicians and other researchers with a powerful tool for quick and reliable re-sequencing [Anderson1998]. Re-sequencing on a hybridization

chip consists on the parallel hybridization of a probe DNA sequence to all possible short (8 to 20 nucleotides) combinations of target sequences, which, of necessity, present overlaps between them [Noble1995]. By looking at sequence overlaps of the targets where the probe has successfully hybridized, researchers can reconstruct the DNA sequence if a previous consensus sequence is already known (i.e. re-sequencing). This fragmentary approach is also currently being used with some success to reduce graph complexity in the assembling of multiple sequence-shot reads for massive sequencing projects, such as the *Human Genome Project* [Pevzner2001].

1.3. DNA-CHIPS

1.3.1. INTRODUCTION

From their humble origins as sporadic plunges of μ -TAS researchers into the genetic analysis field, DNA-chips have become one of the most important market niches of microsystems, with prospects of even rivaling the investment levels attracted by the automotive application sector [Frost1997]. Although this doctoral work is centered mainly on the development of PCR-chips, a basic understanding on the diverse nature, evolution and intermingling of the different types of DNA-chips is a necessary prerequisite to ascertain the options, difficulties and global framework that circumscribes the design and development of functional PCR-chip prototypes.

Origins

Even though they are usually seen as a single and unified field, DNA-chips comprise a myriad of molecular and technological solutions for the implementation of DNA analysis at the microscopic scale by use of micro-fabrication techniques. Thus, in order to clarify matters and trace their different origins, DNA-chips must be envisaged in the womb of their parent field: μ -TAS.

μ -TAS, an acronym for micro-total-analysis-systems, is a long sought application goal of microsystems. The term, introduced by *Andreas Manz* [Manz1990], implies the integration of sensor and actuator elements in a complex microsystem to develop functional devices that should be able to automatically take care of all the conventional steps (sample introduction,

handling, purification, filtering, mixing, analysis and detection) involved in a typical analytical procedure.

The advantages of integrating and downscaling analytical procedures on a microsystem are many and closely related to the ones posed by microprocessors in the computing field [van den Berg1995]. For instance, when physical laws hold true at the micro-scale (which is not always the case [Hsu2001]), analytical processes will tend to occur faster and require lower quantities and concentrations of reagents. Furthermore, even in the case in which thermodynamic or kinetic laws become altered by the downsizing, the μ -TAS designer can still take advantage of these new features (such as increased surface/volume ratios [He1998], superficial tension factors [Attiya1998] or sonic forces [Belgrader2000]) to provide the analyst with new tools and analysis methods. Nonetheless, on the whole, the main advantage of μ -TAS resides on its automation capabilities and the exclusion of the always prone to error human intervention in the analytical process [van den Berg1995]. Thus, μ -TAS should not only provide faster and less expensive assays, but also new, safer and more reproducible analyses, which are also the main targets of DNA-chips.

History

The μ -TAS quest

The first approach to μ -TAS was carried out long before *Manz's* definition of the term. In 1979, *Terry et al.* produced the first demonstrator of a viable μ -TAS: a working micro-machined device for gas chromatography [Terry1979]. As it is bound to happen with revolutionary ideas, the contest for μ -TAS remained unchallenged for more than ten years, until *Manz* published his results on a capillary electrophoresis chip and firmly established the term μ -TAS [Manz1992]. Following his lead, the inflow of articles on the subject has been steady and rising during the last decade, and μ -TAS research has made a notch in many different analytical applications. Although there is still a long trek to cover on the way to a really *total* μ -TAS, researchers have demonstrated the separated viability of almost all the different aspects involved in a fulfilled μ -TAS.

Concerning separation techniques, working demonstrators have been produced for gas [Terry1979] and liquid chromatography ([Ocvirk1995], [He1998]), capillary zone [Manz1992] and gel electrophoresis (see. p56), free

flow electrophoresis [Raymond1994] and di-electrophoresis [Yang1999]. This last methodology, based on the use of inter-digitized [Wang1997a] or spiral [Wang1997b] electrodes, has also produced a number of applications for electric cell and particle directed manipulation ([Washizu1990], [Kricka1993]) and even centrifugation [Duffy1999]. Liquid handling for reagent mixing and arbitrary manipulation has also been demonstrated using a number of different approaches that range from thermodynamic pumps and valves [Burns1996] to conventional pressure driven [Shoji1994], air-bubble [Jun1998] or electro-osmotic [Seller1994] systems. Filtering and purification have also been approached in many different ways. μ -TAS researchers have developed systems that can filter molecules and cells using aperiodic arrays of micro-fabricated structures and that exploit superficial tension and adsorption properties [Christel1998]. These same principles, together with heat [Yu2000] or electric [Cheng1998] shocks can be applied to provide sample preparation, purification or extraction [Pagán2002]. Regarding detection, a myriad of lines of attack has been deployed. For instance, optical detection methods have produced systems that integrate avalanche photodiode detectors [Pauchard2000] and optical filters [Wolffenbuttel1987]. On the other hand, electrical detection has also been demonstrated for many applications using a wide variety of techniques that comprise amperometric [Hilmi2000], capacitive [Sohn2000] and conductivity sensors ([Klampfl1998], [Zemann1998], [Meller2000]). Lastly, the up-to-date main hitch of μ -TAS, their interface capabilities with the macroscopic world of analysts, has also been extensively dealt with, providing some inviting results. Ink-jet technologies [Luginbuhl2000] and electrical ([Fan1994], [van der Moolen1997]) or mechanical [Shoji1994] miniaturized injectors have been widely explored to convey the required interface between the macroscopic world and the problems posed by superficial tension and weird liquid behavior in the microscopic world of μ -TAS.

All in all, even though much pioneering work has been done in all these areas, technological integration problems between these different approaches and methods still abide in the quest for producing a complete μ -TAS. Moreover, methods and processes for easy and automated dispensing of reagents must be developed to allow mass-production of non-perishable systems and, all the while, the problems posed by the necessary integration of detection systems (*in-situ* calibration, durability and reliability) still call for a major commitment.