Pyrosequencing - a new approach to DNA analysis

A novel genetic code-screening technology is breaking new ground in the field of DNA sequence-based analysis.

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The final step in the preparation of a new company and product in the emerging field of applied DNA analysis was marked at the Genome Sequencing and Analysis Conference (GSAC) held in Miami in September 1999. The company is the Swedish-based Pyrosequencing AB, the product is the Luc 96 System - considered to be the world’s first dedicated system for SNP (single nucleotide polymorphism) analysis. Both have emerged with a speed that is unusually rapid, even in today’s business environment, and both have received early, award-wining recognition on the way.

The proprietary technology, Pyrosequencing™, for automated DNA sequence-based analysis is based on ‘sequencing-by-synthesis’, a method which originates from breakthrough research undertaken at the Royal Institute of Technology (Stockholm, Sweden) under the guidance of Mathias Uhlén, Professor of Microbiology, and Pål Nyrén, Associate Professor of Biochemistry. Despite the widely held belief that sequencing-by-synthesis was not possible - and difficulties in attracting funding during the 1990s - the researchers persisted with their work. They were rewarded in the late summer of 1996 when a new way of sequencing DNA was conceived, literally in a flash - a flash of light made possible by copying the mild luminescence emanating from the firefly. The method, real-time pyrophosphate detection for DNA sequencing (now commonly known as Pyrosequencing), is strongly patented and forms the basis of the products under development by the company. The technology is recognised as offering unparalleled flexibility in designing dedicated products to fulfil the growing needs of applied DNA sequencing.

DNA analysis at the speed of light

The generation of light plays a key role in how the technology works. The fact that base pair bonds holding together two DNA strands only form between adenine (A) and thymine (T), and between cytosine (C) and guanine (G), means that the base sequence of each single-strand template can be deduced from that of its complementary sequence. Pyrosequencing’s technology hinges on the principle of ‘sequencing-by-synthesis’, whereby four enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) are formulated so that light is produced whenever an added nucleotide (A, T, G or C) forms a base pair and is incorporated into the growing DNA strand. This signal is detected, the base is registered and the next nucleotide is then added. (For a review, see Reference 1). The light-producing reaction that occurs following the chain of biochemical reactions resulting from the incorporation of a nucleotide is illustrated in Figure 1.

The initial starting sample consists of single-stranded DNA of unknown sequence with a short annealed primer. The enzymes are automatically dispensed into the sample well and the four nucleotide bases are then added in a defined order, for example, A, G, T, C. If A does not form a base pair with the first available base on the DNA template, it is rapidly removed by the apyrase and G is added. When a base pair is formed (G with C), the polymerase incorporates G into the primer strand and in the process releases pyrophosphate (PPi), which is immediately converted to the energy source ATP by ATP sulfurylase. Luciferase uses this energy to produce light that is detected by a camera under the sample well. The light pulse...
Two different phases

Pyrosequencing can be performed in two different phases - liquid and solid. In the liquid phase, the template to be sequenced and a suitable annealed primer are dispensed (without immobilisation into a small well such as a micro-titer plate well) together with the four different enzymes; the nucleotides (A, T, G or C) are then dispensed into the wells one at a time. Incorporation of a nucleotide is accompanied by the release of PPI which can be detected using ATP-sulfurylase and luciferase, generating photons. Apyrase then degrades the ATP and other di- and trinucleotides, and the generated light is ‘switched off’. The cycle is repeated - no washing is required between additions. Pyrosequencing in liquid phase is currently the company’s principle focus.

Pyrosequencing on solid phase has the potential to become the leading technology for ultra high-throughput DNA sequencing on micro-fabricated structures. The template to be sequenced with the annealed primer is immobilised on a solid support and exposed to DNA-polymerase and one of the four different nucleotides. As in the liquid phase, incorporation of a nucleotide results in the release of PPI, which, as previously, is detected using ATP-sulfurylase and luciferase. After a wash that removes the excess nucleotide, the next dNTP can be added and the cycle is repeated until the sequence of the template has been determined. The advantage of reactions performed with solid phase chemistry is the potentially much lower consumption of reagents.

Figure 1. Pyrosequencing step-by-step.

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signalling incorporation of a base is shown as a series of peaks in real time on a PC.

Apyrase rapidly breaks down the excess G not used to form the base pair and the ATP is also quickly consumed, thus ‘switching off’ the light. T is then added; this either forms a pair with the next available base on the template, initiating a new chain reaction resulting in another light signal being detected, or it is quickly broken down by apyrase. By repeatedly adding A, G, T and C and recording which produce light, the correct sequence of bases in the DNA template is built up.

On average, one base is read every 90 seconds. As the technique is commonly performed in a 96-well microtiter plate, 96 samples can be read in parallel. For SNP analysis, this means that the technology is capable of generating data for up to 96 SNPs in less than 10 minutes. This capacity translates into thousands of SNPs per working day.
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• Chemical Synthesis
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A main theme should be:

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If implementation of new and innovative technologies is necessary for you a comprehensive knowledge in the field of Laboratory Automation is needed.

Breaking new ground

Following the scientific breakthrough, a new company - Pyrosequencing AB - was formed in Uppsala, Sweden, in 1997. Major financial commitments were received from HealthCap, a Swedish healthcare investment fund, together with other well-known financial institutions. Key employees were recruited and the first generation system prototype based on Pyrosequencing technology was designed. Today, Pyrosequencing AB employs 31 people and has recently introduced its first generation of commercial products. The company’s overall objective is to make DNA sequencing a widely available core technology that is routinely used in both academic and commercial life science R&D, as well as a standard technique in clinical diagnostics.

Pyrosequencing was determined that not only would its technology be revolutionary, but its way of working would also need to break new ground. With Erik Walldén as President and CEO, the company pursued a policy of outsourcing all its non-core business; it built up a network of experienced partners for optical detection, software development, mechanical design and manufacturing. This way of working showed itself to be very productive. Second and third generation instrument prototypes were soon completed and the software was significantly enhanced.

In 1998, Pyrosequencing technology received a major boost when the magazine, Science, published a review article describing the sequencing method and highlighting its potential benefits (1). The article concluded that it “should be possible to analyse thousands of samples daily with little or no manual intervention”. In June 1999, the company enjoyed further recognition for its efforts - this time for its approach to starting business - when it was voted “Biotech start-up of the month” by European BTi (Bio Technology International) magazine. 1999 also saw the first instruments being tested in the field; tests of the alpha prototypes were concluded successfully in terms of both installation and operation.
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Company interest

The beta evaluation programme that followed involved leading companies engaged in DNA-related research. DuPont Agricultural Genomics (Newark, Delaware, USA) is using the system for internal SNP analysis projects and for comparison with established techniques already operational in-house. The system was successfully installed and tested by Pyrosequencing’s installation team in just one day in June 1999. The work included analyses of test samples at the specified rate of 96 samples in ten minutes. The DuPont team, which has substantial experience of testing newly marketed instrumentation in the genomics area, was pleasantly surprised by the trouble-free installation. After testing the SNP analysis functions, Ada Ching, one of their scientists, was happy to report that “they looked beautiful”.

A similar story can be told at Incyte Pharmaceuticals (Palo Alto, California, USA). Once again, installation, testing and basic training plus sign-off were completed in just one working day. Like their counterparts at DuPont, the team of scientists at Incyte will be using the system for SNP applications; the work they have completed so far has shown great promise.

The most recent beta evaluation installation was carried out at SmithKline Beecham’s research facility at Harlow, UK, in September 1999. Here again, SNP analyses are the main focus of attention and the first results of the research team are eagerly awaited.

The Luc 96 instrument - which has won the Swedish Design Award for Industrial Design - was shown in public for the first time at the Genome Sequencing and Analysis Conference in September 1999. The product was the only major new technology on display and generated a great deal of interest - both in the system for Pyrosequencing and the dedicated software for SNP analysis (Figures 2 and 3). Results presented at the conference by scientists from DuPont Agricultural Genomics and Incyte Pharmaceuticals further raised interest among the many representatives of genomics and pharmaceutical companies attending the conference.

Areas of application

Typical areas of application for Pyrosequencing’s technology were also demonstrated at GSAC, including the results of analysing polymorphisms in the renin, angiotensin, aldosterone system (RAAS) pathway. The results of analysing ACE (angiotensin converting enzyme) exon 15 are shown in Figure 4. The homozygote for the A/G polymorphism (a) has peak heights corresponding to single base peak heights. The heterozygote (b) shows only half peak heights for both of the first two bases (A and G), reflecting the 50:50 mixture of the two alleles. The additional bases scored around the SNP serve as an internal control that verifies the identity of the locus. The bar charts represent a theoretical outcome as calculated by the software’s SNP entry tool, which automatically generates a nucleotide dispensation order from an entered sequence.

In applied genomics - for example, in drug response analysis and population screening - there is an urgent need for high throughput methods. In pharmaceutical research, there are several applications which would benefit from the speed of Pyrosequencing’s technology, for example SNP analysis and tag sequencing, as well as checking clones and insertions and deletions. SNPs are particularly powerful as genetic markers for research into disease susceptibility, drug target identification and drug response. Enormous efforts are being made to discover and characterise SNPs, and Pyrosequencing, with its high throughput capacity and ease-of-use, is well suited for this fast-growing application area. Beyond these immediate needs, there is also the prospect of the emerging clinical diagnostics market which is expected to form the largest part of the DNA sequencing market; a need for rapid and robust ways of analysing DNA is expected to make Pyrosequencing the method of choice for clinical diagnostics. With its strong proprietary technology, sound financial backing and clear business strategy, Pyrosequencing is well poised to enter all these sectors of the market.

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