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The History of Pyrosequencing®

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Summary

One late afternoon in the beginning of January 1986, bicycling from the lab over the hill to the small village of Fullbourn, the idea for an alternative DNA sequencing technique came to my mind. The basic concept was to follow the activity of DNA polymerase during nucleotide incorporation into a DNA strand by analyzing the pyrophosphate released during the process. Today, the technique is used in multidisciplinary fields in academic, clinical, and industrial settings all over the word. The technique can be used for both single-base sequencing and whole-genome sequencing, depending on the format used.

In this chapter I will give my personal account of the development of Pyrosequencing®—beginning on a winter day in 1986, when I first envisioned the method—until today, nearly 20 yr later. I will conclude with future prospects for the method.

Key Words: Pyrosequencing; sequencing; bioluminescence; pyrophosphate.

1. Introduction

Pyrosequencing® is a DNA sequencing technique that utilizes enzyme-coupled reactions and bioluminescence to monitor the pyrophosphate release accompanying nucleotide incorporation, in real-time. The Pyrosequencing method *(1,2)* is the first and only currently available commercial alternative to the well-known Sanger method for *de novo* DNA sequencing. Several hundred scientific papers comprise the literature describing the development and applications of Pyrosequencing (www.ncbi.nlm.nih.gov/gquery/gquery.fcgi; www.biotage.com). The method can be used for single-nucleotide polymorphism (SNP) analysis and tag sequencing (up to 100 bases), as well as for whole-genome sequencing *(3)*.

It was during my post-doctoral period in Cambridge when it occurred to me that detection of pyrophosphate could be used to sequence DNA. While working with Sir John Walker (later awarded the 1997 Nobel Prize for Chemistry)

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at the Medical Research Council Laboratory of Molecular Biology (LMB), one of my projects was to isolate and sequence the gene for the bovine mitochondrial phosphate-carrier protein *(4)*. Sanger sequencing was performed manually, using radioactive-labeled nucleotides and handmade thin gels for detection and separation. Sequencing was an arduous business at the time, requiring weeks of practice to learn the procedure. Several steps were involved in the method, leaving time for the mind to wander in between. As a newcomer, I was not always successful with the handling of very thin acrylamide gels. I remember thinking that it would be great if the method could be simplified or if sequencing could be performed in some other way. During my time as a PhD student at Stockholm University, I worked extensively with modification and simplification of a variety of methods, as well as with the development of new procedures, so it was natural for me to look for ways to improve methods that I found cumbersome and labor intensive.

One late afternoon in the beginning of January 1986, bicycling from the lab over the hill to the small village of Fullbourn, the idea for an alternative DNA sequencing technique came to my mind. It was late, dark, and rainy as I hurried home to tell my wife Maija about the new idea. She later told me that when I explained the new idea to her, she thought that I looked like Gyro Gearloose's little helper—the bright-headed assistant with a light bulb as a head. I had difficulty sleeping that night and was eager to go home to Sweden to test my new idea. What I could not expect that day was that 10 yr would pass before the method was fully developed.

The basic concept was to follow the activity of DNA polymerase during nucleotide incorporation into a DNA strand by analyzing the pyrophosphate released during the process. Why pyrophosphate detection? As a PhD student I worked in the fields of bioenergetics and photosynthesis. My PhD project was to isolate and study an enzyme endogenous to a photosynthetic bacterium. The enzyme, proton-translocating inorganic pyrophosphatase, is involved in photophosphorylation and catalyzes light-driven pyrophosphate synthesis. In order to follow the light-driven process, I developed a very sensitive luminometric method *(5)* capable of following pyrophosphate synthesis in real-time, both after continuous illumination *(6)* and after short light flashes *(7)*. This was a break-through in bioenergetics research. The new idea was to follow the DNA polymerase activity using the pyrophosphate method, thereby detecting whether or not a base is incorporated during DNA synthesis. Because Watson–Crick base pairing rules that nucleotide G will always pair C and T with A, DNA polymerase activity will decipher the base composition of the template when known nucleotides are utilized.

As stated previously, it required nearly 10 yr to get the sequencing method working, involving much struggle to get financing and support for my idea,

and to solve several problems that turned up as the project evolved. In the following sections, I will describe the most important parts of the developing process.

2. Envisioning Pyrosequencing

As I previously mentioned, I wanted to go back to Sweden as quickly as possible to test my new idea in the lab. However, we could not leave Cambridge before my post-doctoral period was completed. Of course I also wanted to accomplish something significant with my work at LMB before leaving. To expedite the process, I wrote a letter to my friend Åke Strid, asking him to advise a new student to purchase the necessary chemicals and set up a pilot study of the DNA polymerase assay. When I called after a month to check how things were going, he informed me that he could not get the suggested assay to work. I had to wait until the end of November 1986 before I could investigate what went wrong with my idea.

Back in Stockholm, I applied for research funding for two projects: my old bioenergetics project and the new sequencing concept. Unfortunately, I obtained financing only for the old project. The committee did not share my enthusiasm for the new idea, reasoning that ATP is a substrate for DNA synthesis and therefore would interfere with the luciferase assay. However, this point is wrong: ATP is not a substrate for DNA polymerase. The head of the department at that time had already advised me (without reading my project plans) not to apply for two separate projects because of the tough competition. Later, when I had already moved to the Royal Institute of Technology (KTH), and the project was shown to be successful, he alleged that it was a pity that I did not have the idea during my time at the Stockholm University.

Although I did not get research money for the DNA sequencing project, I decided to use a small portion of money from the other project to buy a few necessary chemicals and enzymes. I then spent evenings and weekends developing the DNA polymerase assay ***(8)***. I eventually found that the method worked. I do not know why it had not worked for the student. I have learned that it is always better to do things yourself, if possible, rather then try to persuade another person, who might already be distracted by other projects. Even after the paper was published, I continued to apply for research funding without success. The project was shelved and I had to wait several years before I could continue to develop the method for use in DNA sequencing.

Much later, I learned that Bob Melamede ***(9)***, whom I met in Stockholm in 1997, had described the general principles of DNA sequencing-by-synthesis in a previously obtained patent. The method was based on detection of the decrease in nucleotide absorbance upon nucleotide incorporation. The method's sensitivity was not sufficient for normal DNA concentrations. The

pyrophosphate approach had an important advantage in terms of sensitivity; DNA quantities obtained by standard PCR procedures are adequate for analysis with this firefly-luciferase-based method. The sensitivity with absorbance–difference measurements is probably 100–1000 times lower. When I later met Bob, he was very happy to hear that his sequencing-by-synthesis concept worked and that I had circumvented the problem of DNA polymerase-activity monitoring.

3. Solid-Phase Pyrosequencing and the α-S-Nucleotide

In 1990, I moved to the KTH in Stockholm to join Professor Jan Rydstöm's group. Together with Åke Strid, studies of the bovine membrane-bound transhydrogenase were initiated, and we were also able to continue our old projects in Rydstöm's lab. As a newcomer at KTH, I spent some time reading old theses and articles published by the Biochemistry department. One especially attracted my interest: it described a method for solid-phase DNA sequencing ***(10)***. Paramagnetic beads were utilized for DNA template preparation before sequencing. I theorized that if I could combine the magnetic bead technique with my DNA analysis system, a DNA sequencing procedure would be possible. I consulted Professor Mathias Uhlén, who was the principle investigator for the magnetic bead project, and shortly thereafter I collaborated with Bertil Pettersson, a student from Mathias Uhlén's group. Bertil taught me everything that I needed to know about working with magnetic beads. He synthesized oligonucleotides and prepared DNA by PCR. I worked with the magnetic beads and the pyrophosphate detection system. During the first 4 yr I worked 1–2 d a week with the new project.

A problem I faced during the initial stages of the project was high background during the luminescence measurement. The enzymes and the nucleotides were the source of most of the background signals. One approach I used to lower the background was preincubation with apyrase immobilized on magnetic beads; the method was published several years later ***(11)***. I also used to pretreat the nucleotides with pyrophosphatase to decrease the pyrophosphate content. Today, this is a standard procedure. The most severe problem I observed was that dATP functioned as a weak substrate for luciferase giving light corresponding to 2–3% of an equal amount of ATP. I mediated this difficulty by starting all reactions by adding DNA polymerase and not by adding nucleotides. This procedure made it unnecessary to subtract a high background from a low signal. Because most polymerases were delivered in phosphate buffers, these enzymes also had to be pretreated with pyrophosphatase.

Although solutions to several of the mentioned problems had now been found, I still had difficulty getting strong, clear signals. Bertil then came up with the brilliant proposal of setting up the analysis system quite differently.

We used run-off signals to increase signal intensity, and dideoxynucleotides were used to facilitate reading the first base position. This new method was the first proof-of-principle for the DNA sequencing concept, and the success was encouraging for future developmental work ***(12)***. Later, we published an alternative method for single-base change detection ***(13)***. The concept relies on measuring the differences in primer extension efficiency by a DNA polymerase of a matched over a mismatched 3'-terminal utilizing α-thiotriphosphate analogs. One important feature of the DNA sequencing concept is that DNA polymerases lacking 3'–5' activity are required, which limited us to the use of Sequenase and a modified Klenow enzyme. When we used dideoxynucleotides, we could only use Sequenase because of their hindrance to Klenow's activity.

To be able to sequence multiple bases, we had to work on decreasing the dATP background. I made a literature search for alternative nucleotides and decided to test an α-*S*-modified nucleotide. Nucleoside thiophosphates comprised a new class of modified nucleotides in which one nonbridging oxygen atom in the α-phosphate of the nucleoside 5'-triphosphate is replaced by a sulfur group. I found that the modified nucleotide was a good substrate for the polymerase and in addition, a poor substrate for luciferase. This finding was made during 1995 and was published 1996 ***(14)***. We were able to sequence 15 bases with our new approach—a new world record for the sequencing-by-synthesis principle.

From 1991 to 1994 I was engaged in several other projects ***(15–17)***, and the development speed for the DNA sequencing method was low. It was essentially a one-man project with little or no funding, and I spent about 25% of my time with research and the rest with teaching obligations. From 1994 to 1998 my one-man group increased to several people, at most eight, working with different aspects of the DNA method. No one was particularly enthusiastic about the new idea during the earlier period—when I presented my idea at a conference in Stockholm very few people showed interest in my poster. However, one well-known scientist (an adviser for the government) approached my poster, looked it over, and then turned to me, saying, "I don't think this will ever work." That comment made me a bit anxious, as she was an expert in nucleotide metabolism. Regardless, I continued to believe in my idea.

Up to this point, we had used magnetic beads and manual sequencing, but our aim was to automate the procedure. Together with Professor Johan Roeraade, PhD, Sean Waters and my students Mostafa Ronaghi, Tommy Nordström, and Atefeh Shakri, I started to explore the possibility of immobilizing enzymes and DNA on silica with the goal of constructing an automated sequencing procedure utilizing a capillary flow system. At that time, we also started a collaboration with Björn Ekström at Pharmacia Biotech. We were able to immobilize both luciferase and ATP sulfurylase and successfully ana-

lyze ATP and pyrophosphate continuously in the capillary system (unpublished results). We also made preliminary studies of nucleotide incorporations on immobilized DNA.

4. Apyrase

While attempting to solve problems such as low-signal intensity and nucleotide contamination associated with the capillary flow system, another idea occurred to me. Instead of including a washing step between the nucleotide additions, it might be possible to utilize a nucleotide-degrading enzyme. My first thought was to use my earlier published ***(11)*** concept of apyrase immobilized on magnetic beads. The immobilized apyrase could be separated from the assay after each degradation step by simply using a magnet. Alternatively, if the kinetics were properly adjusted, it might be possible to omit the separation step altogether. However, it remained to be seen whether apyrase could degrade nucleotides other than ATP and if all four deoxynucleotides were equally well degraded. At that time, no one was especially impressed or excited with this proposal. I asked one of my students to test the concept, but he later told me that it did not work. I could not let the idea go, so during the summer 1996 vacancies I went back to the lab bench and started to set up some really exciting experiments.

After about 6 wk in the lab I had preliminary data for a new DNA sequencing procedure ready. The main obstacle that I had to handle was false signals, which I theorized were a result of nucleoside diphosphate kinase activity. I identified the ATP sulfurylase preparation as the main contamination source for this activity. As predicted, the false signals decreased with lower concentrations of ATP sulfurylase. Two months later I had evidence supporting my theory ***(18)***. I started using a new source of ATP sulfurylase purified from dry yeast (bought from a nearby food store) by my student Nader Nourizad. Another student, Samer Karamohamed, was later able to produce a recombinant form of the enzyme in *Escherichia coli* ***(19)***. Both the purified enzyme and the recombinant ATP sulfurylase improved the sequencing results dramatically. Together with Ronaghi, I started to optimize all parameters of the method, and in early 1998 the protocol was ready ***(1,2)***. Only minor changes have since been made to the protocol.

One problem we encountered during the optimization process was that some templates produced significantly better results than others. We hypothesized that some DNA templates formed secondary structures because of the relatively low temperature (22°C) that was used. We tried to solve this issue by adding different substances such as glycerol, proline, and DMSO, but none of these cheaper substances helped with this problem. Ronaghi then suggested that we should test the effects of adding a single-stranded DNA-binding pro-

tein (SSB). Most organisms utilize this protein to decrease secondary-structure formation, thereby improving DNA synthesis. I had earlier found that SSB could be used to improve DNA priming with two or three unligated hexamers, so the substance was still available in our lab. SSB was a hit; it dramatically improved the sequence quality for difficult templates and for less difficult templates to some degree as well *(20,21)*.

5. Automation

With the aim of commercializing the new DNA sequencing technique, the company Pyrosequencing AB (today Biotage AB) was founded in 1997 by Pål Nyrén, Mathias Uhlén, Mostafa Ronaghi, Bertil Pettersson, and Björn Ekström. The first commercially available automated system was sold 1999. The first noncommercially available automated system was developed by myself, Tommy Nordström, and Mostafa Ronaghi *(22)*. It included an LKB-1251 luminometer, two dispenser controllers, a power unit, a computer, a recorder, and four separate dispensers. At 1–5 μL, the dispensed volume was rather large, as was the sample volume at 0.4 mL. The system was also restricted to sequencing one sample at a time. In contrast, the commercial systems developed later sequence 96 samples in parallel, and both dispensation volumes (0.05–0.2 μL) and sample volumes (10–50 μL) are much smaller, substantially decreasing the cost and time for a DNA sequencing project.

6. Improvements

In addition to the development process, several aspects of the Pyrosequencing method have been improved during the last few years. I will briefly mention a few of the most important alterations. We had observed major decreases in apyrase activity following the substitution of an S-modified variant for the standard dATP during longer sequencing projects. The new nucleotide used was a mixture of two isomers—one of which, the R-isomer, is not a substrate for the DNA polymerase. We therefore decided to test a nucleotide solution consisting of only the active S-isomer. By utilizing a pure isomer, we could decrease the nucleotide concentration by one-half and thereby dramatically decrease the apyrase inhibition. We obtained much longer reads using the new approach *(23)*; up to 153 bases of sequence information could be analyzed on one of the studied templates.

It was obvious from the previously mentioned experiment that the modified A nucleotide had a negative effect on apyrase activity. Although the problem was decreased by the use of the pure isomer we could still observe apyrase inhibition, especially during long sequencing projects. After a deep literature search, I came to the conclusion that only a few nucleotide analogs were potentially appropriate for the Pyrosequencing method. We tested 7-deaza-2'-

deoxyadenosine-5'-triphosphate and observed positive effects on the sequencing data. The nucleotide was incorporated by the polymerase with good efficiency, and in addition it had no negative effect on apyrase activity even after many nucleotide additions *(24)*. We surmise that this nucleotide might be a good choice for use in future Pyrosequencing protocols.

Another improvement of the sequencing method, especially for templates with homopolymeric T-regions, has been the introduction of Sequenase. For example, a template containing an 8-mer poly(T) track could be easily read when Sequenase was used, but not when Klenow polymerase was used *(25)*. Sequenase could also be used in combination with 7-deaza-2'-deoxyadenosine-5'-triphosphate *(24)*.

We had showed earlier that SSB improved the sequence quality for some templates but the effect of temperature had not been investigated because of the thermo-instability of firefly luciferase. By introducing glycine betaine we were able to increase the temperature for the sequencing procedure by approx 10°C, up to 37°C *(26,27)*. At this higher temperature, the activity of the different enzymes was doubled and the sequence quality for some templates improved.

Template preparation for Pyrosequencing has been substantially improved by the introduction of a Sepharose bead/vacuum system that allows preparation of 96 samples within a few minutes. Another strategy, introduced by Tommy Nordström, allows double-stranded DNA to be used as a template for DNA sequencing after a simple preparation procedure *(28–30)*. Using a combination of apyrase, pyrophosphatase, and blocking oligonucleotides, the template can be prepared by a one-step procedure within a few minutes *(30)*.

Preprogrammed dispensation order was introduced to improve read length *(31)*. This strategy has applications in resequencing projects, such as mutation detection in cancer research and clone checking *(31,32)*.

A major improvement of the Pyrosequencing method was the introduction of the multiprimer DNA sequencing concept by my student Baback Gharizadeh *(33–35)*. In this method, two or more sequencing primers, combined in a pool, are added to a DNA sample of interest. The oligonucleotides that hybridize to the DNA sample will function as primers during the subsequent DNA sequencing procedure. This new strategy is suitable for sequencing and typing of samples harboring different genotypes (coinfections with multiple genotypes) and samples yielding nonspecific amplifications. This therefore eliminates the need for nested PCR, stringent PCR conditions, and cloning. The new approach has also proved to be useful for amplicons containing low yields or subdominant types.

7. Applications

DNA sequencing has become an invaluable tool in such disparate fields as medicine, agriculture, and forensic studies. Pyrosequencing can be used for both single-base sequencing and whole-genome sequencing, depending on the format used. The recently developed picotiterplate format allows whole-genome sequencing with very high throughput: a 100-fold increase over current Sanger sequencing technology *(3,36)*. The conventionally used microtiterplate format, developed by Biotage AB (www.biotage.com), is used to sequence 96 samples in parallel for up to or more than 100 bases *(23,34)*. Earlier used primarily for SNP analyses *(28,37)*, the method now has numerous different applications: analysis of allele frequency in pooled samples *(38)*, methylation analyses *(39)*, molecular haplotyping *(40)*, sequencing of heteroplasmic DNA *(41)*, forensic analyses *(42–44)*, bacterial typing *(34)*, fungal typing *(45)*, and viral typing *(46)*. Furthermore, Pyrosequencing facilitates clinical research in areas such as Alzheimer's disease, autoimmune disorders, bowel disease, cardiovascular disease, coronary heart disease, dermatology, diabetes, gynecology, hematology, hearing loss, hematopoietic chimerism, immunology, mitochondrial disorders, nephrology, neurology, obesity, oncology, orthopedics, psychiatric genetics, and trauma (for references *see* www.ncbi.nlm.nih.gov/gquery/gquery.fcgi; www.biotage.com). The method is also used for animal *(47)* and plant studies *(48)*.

8. Future Prospects

With the completion of the human genome-sequencing project, there is now a focus on developing rapid new methodologies that will enable routine genomics studies and molecular testing in clinical settings. The future prospects for Pyrosequencing are encouraging, but present numerous challenging possibilities. The method can be used in many different formats, from single-sample analysis to analysis of millions of samples in parallel, utilizing volumes ranging from the milliliter-to-picoliter scale. Pyrosequencing is now established as a very reliable method for diagnostic sequencing of short DNA stretches. The information it provides about sequence context for SNP analysis makes it the most robust method available at this time. The procedure has also been shown to be appropriate for whole-genome sequencing with very high throughput. Increasing read length is among the greatest challenges related to Pyrosquencing. In forensics, being able to analyze short tandem repeat markers by sequencing might help to overcome problems encountered with traditional size determination methods.

The Pyrosequencing method relies on the cooperation of four different enzymatic reactions. One way to aid understanding of this complicated process is to use mathematical models. Once a reliable model has been developed, it can be used to find ways of improving the Pyrosequencing process itself. This could, for instance, involve modifications of the substrate concentrations, or use of other enzymes with different kinetic properties. Performing full tests like these in the laboratory is often both expensive and time-consuming, which is why an *in silico* model may help to identify especially promising configurations. The model would be designed to find the parameters producing an optimal sequencing result given certain input criteria.

We have recently constructed a mathematical model of the Pyrosequencing reaction system utilizing the assumptions of irreversible Michaelis–Menten rate equations ***(49)***. In this first published model the dynamic of a single light pulse was captured with great accuracy, as well as the overall characteristics of a whole Pyrogram®. The simulation results of the mathematical model show significant potential, motivating further development of the model for detail and accuracy. The approaching task is to find a way to replace the artificial factors of incorporation efficiency and plus-shifts with a nonfixed variable. Incorporation efficiency should, for instance, be dependent on polymerase velocity, nucleotide concentration, time, and quantity of complementary DNA. In order to include these parameters in the first model, a more sophisticated description of the polymerase's kinetics is likely needed. Another factor is a polymerase's processivity—the number of nucleotides the enzyme can incorporate before it releases them from the DNA strand. For the Klenow fragment, the processivity is approx 20 when the enzyme is saturated by nucleotides. The first model utilizes a processivity of one. With these features incorporated into the model, information about the benefits and drawbacks of using a different polymerase, with other kinetic properties, can be investigated. We believe that there is great potential for increasing Pyrosequencing's read length, thereby extending the method's applicability in new and different fields.

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