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Review

Pyrosequencing: History, biochemistry and future

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Abstract

Background: Pyrosequencing is a DNA sequencing technology based on the sequencing-by-synthesis principle.
Methods: The technique is built on a 4-enzyme real-time monitoring of DNA synthesis by bioluminescence using a cascade that upon nucleotide incorporation ends in a detectable light signal (bioluminescence). The detection system is based on the pyrophosphate released when a nucleotide is introduced in the DNA-strand. Thereby, the signal can be quantitatively connected to the number of bases added. Currently, the technique is limited to analysis of short DNA sequences exemplified by single-nucleotide polymorphism analysis and genotyping. Mutation detection and single-nucleotide polymorphism genotyping require screening of large samples of materials and therefore the importance of high-throughput DNA analysis techniques is significant. In order to expand the field for pyrosequencing, the read length needs to be improved.
Conclusions: Th pyrosequencing system is based on an enzymatic system. There are different current and future applications of this technique.

Keywords: Pyrosequencing; Real-time monitoring; SNP; Bioluminscence

Contents

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1. Introduction

1.1. Overview of DNA sequencing technologies

During the latter part of the 20th century, the innovation of a range of DNA sequencing techniques enabled a revolution in the field of molecular biology. In the 1970s, technologies for sequence determination of DNA were invented, both by Maxam-Gilbert [1] and Sanger [2], and these techniques enormously increased the possibilities of genetic research. The complete DNA sequences of whole genomes are currently known for an increasing number of organisms including the human [3,4]. Detection of genetic variations in a large number of samples representing a broad range of biological material give insight into genetic mechanisms of different diseases. Even with an increasing number of genomes already sequenced, the importance of technical developments in the field of DNA analysis is evident. The number of DNA sequencing technologies is currently high. Different techniques are advantageous over others depending on the application and therefore, a general ranking of the technologies may be incorporated or misleading. The invention of the Sanger DNA sequencing technique in 1977 [2] revolutionized DNA sequencing technology. This sequencing technology is undoubtedly, by far the most frequently used, exemplified by sequencing of various genomes such as the human. The Sanger DNA sequencing technique is based on DNA synthesis with incorporation of normal dNTPs as well as ddNTPs causing a termination of the newly synthesized DNA molecule. Thus, the prematurely ended fragments can be analyzed with regard to size. The size separation of the Sanger fragments are usually performed by electrophoretic separation although mass spectrometry analysis has also been described [5,6]. Since the different dideoxy nucleotides are used in different tubes or alternatively marked with different fluorophores the DNA sequence can be deduced from these results. Another DNA sequencing technique presented by Maxam and Gilbert in 1977 is based on sequencing by chemical cleavage [1]. In this technique, the DNA fragments are generated either by digestion of the sequencing template by restriction enzymes or PCR amplification with the ends of the fragments labeled, traditionally by radioactivity. Single stranded DNA fragments radioactively labeled at one end are isolated and subjected to chemical cleavage of base positions. Four parallel cleavage reactions are performed, each one resulting in cleavage after one specific base. The sequence is deduced from the gel separation pattern like in the Sanger DNA sequencing method. In 1975, Ed Southern [7] presented a technique for detection of specific DNA sequences using hybridization of complementary probes. This principle lays the foundation for the sequencing by hybridization (SBH) technology presented in 1988 [8,9]. Sequencing by hybridization utilizes a large number of short nested oligonucleotides immobilized on a solid support to which the labeled sequencing template is hybridized. The target sequence is deduced by computer analysis of the hybridization pattern of the sample DNA. DNA sequences can also be analyzed by sequencing by synthesis. Pyrosequencing is a sequencing method based on real-time monitoring of the DNA synthesis. It is a four-enzyme DNA sequencing technology monitoring the DNA synthesis detected by bioluminescence [10]. The system is thoroughly described in this paper.

1.2. Different techniques–different applications

Sequencing technologies like Sanger, Maxam and Gilbert and pyrosequencing have the ability to determine unknown DNA sequence, de novo sequence determination. In contrast, sequencing by hybridization (SBH) is mainly suitable for detection of genetic variations within known DNA sequences, re-sequencing. SBH may also be employed for certain applications such as genotyping samples with well-characterized genetic variations such as single nucleotide polymorphisms (SNPs). However, the extremely small differences in duplex stability between a perfect match and a one-base mismatch duplex may limit the reliability and applicability of this technology [11]. This difficulties can be relieved by use of probes made of Peptide Nucleic Acid, PNA, or Locked Nucleic Acids, LNA, which forms duplexes with DNA with higher melting point than the corresponding DNA–DNA duplex [12–14]. However, currently the price of these molecules is significantly higher compared to DNA.

The read length and accuracy of the obtained sequences is of crucial importance for the choice of sequencing technology. In the case of the Maxam and Gilbert technique read length up to 500 bp has been achieved [15]. Nevertheless, the occurrence of incomplete reactions usually decreases the read length. Using Sanger sequencing followed by separation by capillary gel electrophoresis, the average read-length obtained is typically between five hundred and thousand bases. Several commercial systems

are available for this technology and development in capillary electrophoretic equipment has enabled rapid and accurate determination of up to significantly above thousand bases [16]. However, when using sequence technology for identification of genetic variants such as SNP genotyping, bacterial- or virus typing, detection of specific mutations, gene identification in transcript analysis, etc., the read-length required is much shorter. In these cases, running a several hour experiment for obtaining sequences of several hundred bases is not meaningful. In such cases, faster sequence analysis methods like pyrosequencing are very attractive and have been successfully used [17–32]. Moreover, the use of directed base dispension in pyrosequencing analysis of SNPs in close proximity to each other enables haplotype profiling that is not possible using Sanger DNA sequencing.

One important argument for the choice of sequencing technique is the amount of work and time required as well the possibility for automation of different steps. In the sequencing methods described above a step of template amplification performed by Polymerase Chain Reaction, PCR [33] is generally required. A PCR clean up prior to sequence analysis is necessary and a vast number of commercial solutions are available for this purpose. If using the pyrosequencing technology, the purified PCR samples are directly analyzed by real-time monitoring of the DNA synthesis. In turn, when using Sanger DNA sequencing, the sequencing reaction is followed by purification and thereafter separation of the Sanger DNA fragments. The fragment purification has mainly been performed by ethanol precipitation which includes several manual operations and therefore does not readily lend itself to automation. However, alternative techniques such as separation using magnetic beads are available [34]. Although the Sanger DNA sequencing methods can be highly automated, the higher analysis time compared to pyrosequencing decreases its suitability when only shorter sequences are required. The chemical reactions in the Maxam and Gilbert technique are slow and involve hazardous chemicals that require special handling in the DNA cleavage reactions. Therefore, this technology has not been suitable for large-scale investigations. Sequencing by hybridization would, if the accuracy and reliability of the technique were sufficient, provide a very fast analysis of a specific sequence.

2. Sequencing by synthesis

2.1. History

The real time monitoring of DNA synthesis, the sequencing-by-synthesis principle, was first described in 1985 [35]. The technique is based on sequential addition of nucleotides to a primed template and the sequence of the template is deduced from the order different nucleotides are incorporated into the growing DNA chain which is complementary to the target template. In 1987, P. Nyrén described how DNA polymerase activity can be monitored by bioluminescence [36,37]. Recently fluorescently labeled nucleotides have been used for sequencing by synthesis. In order to minimize unwanted termination and to be able to measure the incorporated nucleotides the labeling group is removable. This has been accomplished both by photocleavage and cleavage by reduction [38,39]. When detecting the incorporation of nucleotides with luminescence, a number of different enzymes are needed. In the early days this sequence technology utilized six different sequential columns with immobilized enzymes to pass the nucleotides through upon each base addition [37]. Ten years later, the pyrosequencing DNA sequencing method was presented [10] enabling faster bioluminometric real-time sequence determination in solution.

2.2. The pyrosequencing principle

The 4 enzymes included in the pyrosequencing system are the Klenow fragment of DNA Polymerase I [40], ATP sulfurylase [41], Luciferase [42] and Apyrase [43] (Fig. 1). The reaction mixture also contains the enzyme substrates adenosine phosphosulfate (APS), D-luciferin and the sequencing template with an annealed primer to be used as starting material for the DNA polymerase. The four nucleotides are added one at a time, iteratively, in a cyclic manner and a CCD camera detects the light produced.

The enzymatic reactions exploited in the pyrosequencing technology, with catalyzing enzyme given in the reaction in parentheses, are the following. The first reaction, the DNA polymerization, occurs if the added nucleotide forms a base pair with the sequencing template and thereby is incorporated into the growing DNA strand.

$$(\mathrm{DNA})_n + \mathrm{dNTP} \rightarrow (\mathrm{DNA})_{n+1} + \mathrm{PP_i}(\mathrm{Polymerase}). \quad (1)$$

The inorganic pyrophosphate, PP_i, released by the Klenow DNA polymerase serves as substrate for ATP Sulfurylase, which produces ATP:

$$\mathrm{PP_i} + \mathrm{APS} \rightarrow \mathrm{ATP} + \mathrm{SO_4^{2-}}(\mathrm{ATP\ Sulfurylase}). \quad (2)$$

Through the third and fourth reactions, the ATP is converted to light by Luciferase and the light signal is detected. Hence, only if the correct nucleotide is added to the reaction mixture, light is produced.

$$\mathrm{Luciferase} + \mathrm{D-luciferin} + \mathrm{ATP} \rightarrow \mathrm{Luciferase-luciferin-AMP} + \mathrm{PP_i} \quad (3)$$

$$\mathrm{Luciferase-luciferin-AMP} + \mathrm{O_2} \rightarrow \mathrm{Luciferase} + \mathrm{oxyluceferin} + \mathrm{AMP} + \mathrm{CO_2} + \mathrm{light} \quad (4)$$

Apyrase removes unincorporated nucleotides and ATP between the additions of different bases.

$$\mathrm{ATP} \rightarrow \mathrm{AMP} + 2\mathrm{P_i}(\mathrm{Apyrase}) \quad (5)$$

$$\mathrm{dNTP} \rightarrow \mathrm{dNMP} + 2\mathrm{P_i}(\mathrm{Apyrase}). \quad (6)$$

This degradation between base additions is crucial for synchronized DNA synthesis asserting that the light signal

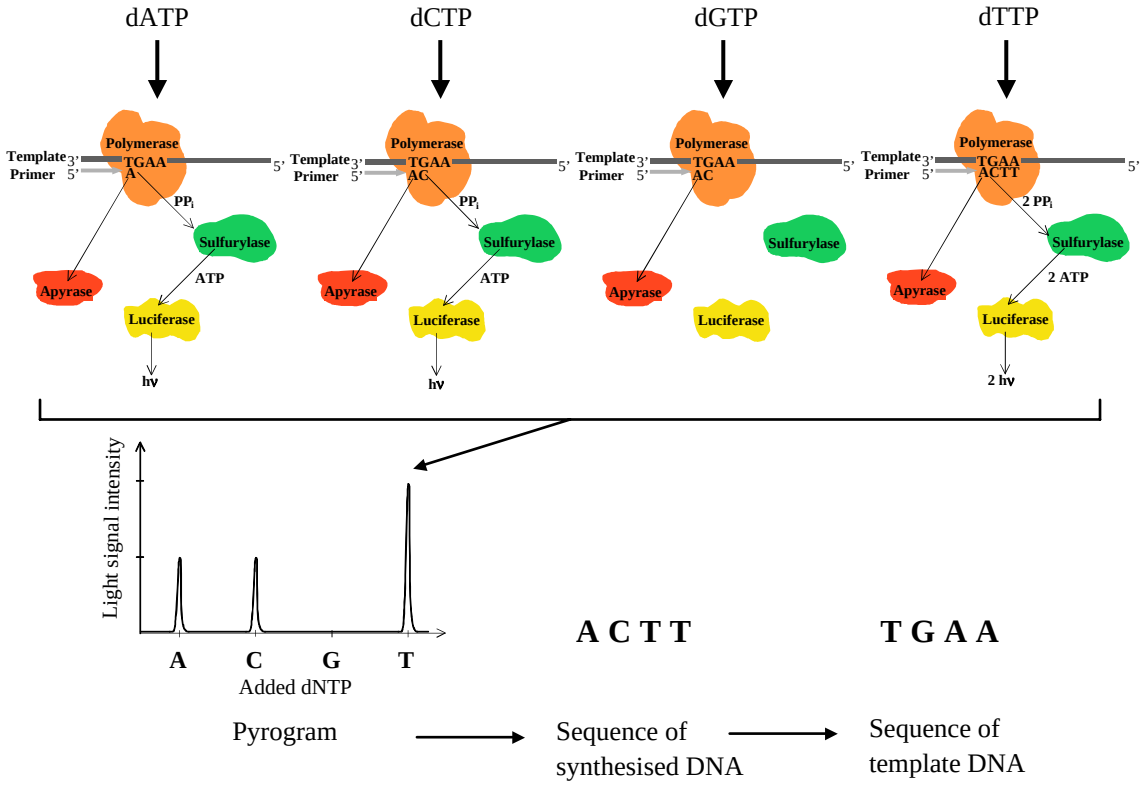

Fig. 1. Schematic representation of the pyrosequencing enzyme system. If the added dNTP forms a base pair with the template, Klenow Polymerase incorporates it into the growing DNA strand and pyrophospate (PPi) is released. ATP Sulfurylase converts the PPi into ATP which serves as substrate for the light producing enzyme Luciferase. The produced light is detected as evidence of that nucleotide incorporation has taken place.

detected when adding a certain nucleotide only arises from incorporation of that specific nucleotide.

2.3. Pyrosequencing enzymes

The performance of the four enzymes is crucial for the accuracy of this DNA sequencing technology. Their basic characteristics and influence on the quality of the pyrosequencing result is therefore discussed below.

2.3.1. Klenow DNA polymerase

DNA polymerases (E.C. 2.7.7.7) catalyze DNA polymerization in replication and repair and are thus crucial for survival of all living cells [44]. *Escherichia coli* DNA polymerase I is the most extensively studied polymerase and possess, in addition to polymerase activity, both 3′→5′ and 5′→3′exonuclease activity. Proteolytic cleavage of the native 109 kDa polymerase by subtilisin results in one smaller proteolytic fragment harboring 5′→3′ exonuclease activity and one larger fragment, called Klenow polymerase, that possess both polymerase and 3′→5′ exonuclease activity [40]. However, by mutating only two amino acids, an exonuclease deficient (exo$^-$) Klenow with intact structure and polymerase activity variant has been created [45]. In pyrosequencing, the (exo$^-$) Klenow polymerase is used for extension of the primer and simultaneous release of PP_i. The deficiency of the 3′→5′ exonuclease activity is important in order to avoid unsynchronized DNA polymerization. Although, the (exo$^-$) Klenow used in pyrosequencing is devoid of the proofreading 3′→5′ exonuclease activity from DNA polymerase I, several mechanisms in the DNA extension ensure high fidelity of base insertion. Firstly, the binding of the correct nucleotide is stronger than binding of an incorrect one [46]. Secondly, the necessary conformational change from open to closed conformation takes place only upon binding of the correct nucleotide. This conformational change positions the 3′-OH and the dNTP for the nucleophilic attack and thereby determines the rate of phosphodiester bond formation [47–50]. After formation of the phosphodiester, a conformational change slows dissociation of the incorrect DNA products from Klenow and in use of (exo$^+$) Klenow, 3′→5′ exonuclease activity removes the incorrect base [51]. However, in pyrosequencing with (exo$^-$) Klenow, the slower kinetic mechanism for mismatch incorporation is exploited by the use of Apyrase so that mismatch incorporation is efficiently eliminated [52].

2.3.2. ATP sulfurylase

The second reaction in pyrosequencing technology, namely the production of ATP from PP_i released upon DNA polymerization, is catalyzed by ATP sulfurylase (E.C. 2.7.7.4). ATP sulfurylase is involved in vivo in sulfur

activation by producing APS from ATP and SO_4^{2-} [41]. The produced adenosine phosphosulfate, APS, is further phosphorylated by APS kinase into adenosine 3′-phospate 5′-phosphosulphate, PAPS, which is used for synthesis of various sulfur containing compounds. The equilibrium of the reaction catalyzed by ATP sulfurylase is naturally very unfavorable for APS production but the removal of APS and PP_i by APS kinase and inorganic pyrophosphatase pulls the reaction to the right [41]. Hence, being uncoupled, the ATP Sulfurylase catalyzed reaction is favorable for ATP synthesis from PP_i and this is exploited in the second reaction in the pyrosequencing technology (2).

ATP sulfurylase has been found in a broad range of organisms such as yeast and filamentous fungi [41], spinach leaf [53] and rat [54]. However, the first ATP sulphurylase was cloned from the *MET3* gene on chromosome X of *Saccharomyces cerevisiae* yeast and this is currently the only commercially available enzyme. This enzyme is a 315 kDa homo hexamer [41] that has been successfully produced intracellularly in *E. coli* for use in pyrosequencing technology [55].

2.3.3. Luciferase

Luciferase (E.C. 1.13.12.7) catalyses the light production from ATP detected in pyrosequencing. Variants of the enzyme are used for light production in all bioluminescent organisms. The light emission from each species is characterized by the color and the flashing pattern. The color of the emitted light, which is determined by the active site of the luciferase, varies between species from green ($\lambda_{max} \approx 543$ nm) to red ($\lambda_{max} \approx 620$ nm). Moreover, each beetle emits a distinct flashing pattern that is recognized by the opposite sex of the species [56–60].

The most extensively used luciferase that first was cloned and is the only commercial variant originates from the North American firefly *Photinus pyralis* [57]. This luciferase is a 61 kDa enzyme which produces light in the green-yellow region (550–590 nm) with an emission maximum at 562 nm at physiological pH [61]. The light production performed by the *P. pyralis* luciferase is rather efficient with 0.88 photons produced per luciferin molecule consumed [62]. In the first luciferase-catalyzed reaction, the enzyme undergoes a conformational change upon forming a complex with D-luciferin in presence of magnesium ions according to Eq. (3). Successively, light production takes place through oxidative carboxylation of the luciferyl-adenylate (Eq. (4)).

Since luciferase can produce light from dATP but no other nucleotides, a modified A nucleotide, dATP-S, is used instead of dATP in the pyrosequencing polymerization [10]. In pyrosequencing technology, the low thermostability of luciferase limits the reaction temperature to 25 °C. Since the temperature optimum for several other enzymes is higher, an increased reaction temperature might shorten the analysis time and decrease background signals. However, various strategies have been used to increase the thermostability of luciferase such as addition of stabilizing compounds [63,64] and site-specific mutagenesis [65,66].

P. pyralis luciferase was originally purified from the firefly tails but extensive isolation was required since contaminant in the tails interfered with the light production [67–72], After cloning of the gene, recombinant luciferase production in *E. coli* has been performed [57,73]. By use of gene fusion technology, purification tags such as protein A [74,75] has enabled rapid purification as well as immobilization of the enzyme on solid supports.

2.3.4. Apyrase

Apyrase (E.C. 3.6.1.5) is included in the pyrosequencing technology for degradation of unincorporated nucleotides and excess ATP between base additions. Apyrases and ecto-ATPases are E-type ATPases, a group of enzymes different from other ATPases by several aspects. Most important, their activity is dependent on divalent cations, mainly Ca^{2+} or Mg^{2+} [76,77]. E-type ATPases play diverse important roles in biological processes as modulation of neural cell activity [78], prevention of intravascular thrombosis [79,80], regulation of immune response [81], protein glycolyzation and sugar level control [82] as well as regulation of membrane integrity [83]. However, apyrases differ from ecto-ATPases since they can hydrolyze nucleoside tri-, di- and mono-phosphates and thus have lower substrate specificity [77]. Apyrases have been described in various animal tissues and organisms [84,85]. However, the only commercially available apyrases, which are the most extensively studied, originate from potato tubers *Solanum tuberosum*. Several isoenzymes from different clonal varieties of *S. tuberosum* have been isolated and characterized although the best known are those of the Pimpernel and Desirée types [86]. The 2 apyrases have the same size (49 kDa) but different isoelectric points, pI [76]. The most important characteristic for use in pyrosequencing technology is the ratio between ATP and ADP hydrolysis rates since a high ratio increases the efficiency of nucleotide degradation [87]. Thus, since the ratio is ten for apyrase from the Pimpernel and one for that from Desirée, Pimpernel apyrase from *S. tuberosum* is used in pyrosequencing.

3. Analytical performance

In order to increase the use of the pyrosequencing technology platform, further improvements were made. One problem addressed was the limited read length obtained. The use of pyrosequencing technology is currently restricted to analyses of short DNA sequences exemplified by mutation detection [24–27] and single nucleotide polymorphism, SNP, analysis [17–20,22,23]. The factors limiting read length in pyrosequencing can be divided into three major groups: background sequence, plus and minus frameshift [32]. However, to extend the use of the

pyrosequencing technology to applications such as sequencing DNA libraries for gene identification, prolonged read lengths are required for acquisition of sufficient amounts of data.

3.1. SSB as a means for prolonging read length

E. coli single-stranded DNA binding protein, SSB, stabilizes single stranded DNA, ssDNA, by binding to it and thereby protecting it from degradation and formation of secondary structures [88,89]. This property has been exploited in several DNA applications [90–94] including pyrosequencing [26,29,31,32,95,96] where the sequence quality has been improved by addition of the protein. In one study, a systematic effort was made to analyze the positive effect of SSB on template with different characteristics [32]. The template material used consisted of PCR products from a cDNA library used in a transcript profiling study where the pyrosequencing technology had been compared to Sanger sequencing [32]. The read length in pyrosequencing both with and without SSB was correlated to the PCR product length (Fig. 2). Results from these experiments show that the pyrosequencing read length is dramatically increased by addition of SSB. Furthermore, the sequence quality decreases with increasing PCR product length (Fig. 2). In the following analysis, the causes of low quality sequences from different templates were characterized by thorough investigation of the pyrograms. The data show that the major limiting factor for longer DNA templates (>600 bp) is background sequence that occurs very early in the program, thereby limiting the read length significantly. These background disturbances are probably caused by primer mis-annealing and their occurrence can efficiently be suppressed by SSB addition. Plus frameshift is caused by insufficient apyrase activity causing incomplete nucleotide degradation between subsequent base dispensions. Small amounts of nucleotide from preceding base dispensions cause non-synchronized extension with a population of templates being ahead of the majority. Addition of SSB decreases the intensity of the plus frameshift peak as well as suppresses the unspecific background so efficiently that the risk of interpreting the plus shift peak as a normal signal is minimized. Minus shift is caused by insufficient Klenow activity. In homopolymeric stretches of more than three or four identical nucleotides, poor Klenow activity might result in incomplete template extension during the base dispension. Further polymerization of the template will not take place before the second dispension with the identical nucleotide where a minus frameshift peak is detected. The SSB protein also reduces the minus shift phenomenon. This positive effect of SSB might be due disruption of secondary structures in the ssDNA by SSB. Since shift phenomena are sequence dependent, these problems occur on templates of all length although the probability of finding homopolymeric stretches in a PCR product increases with the length of the DNA fragment. For short and intermediate DNA templates, SSB decreases the limiting effects of all factors. For longer templates, background sequence is completely suppressed by SSB and longer reads are obtained. Since the read length increases for these clones, other limiting factors occur although for 50% of the clones, more than 25 bases can be read from the pyrograms.

Attempts were made to increase the primer annealing efficiency and thereby signal intensity through use of elevated amounts of primer in the annealing reaction [32]. Although the signal intensity was increased, aggravated background disturbance and non-proportional signals rad-

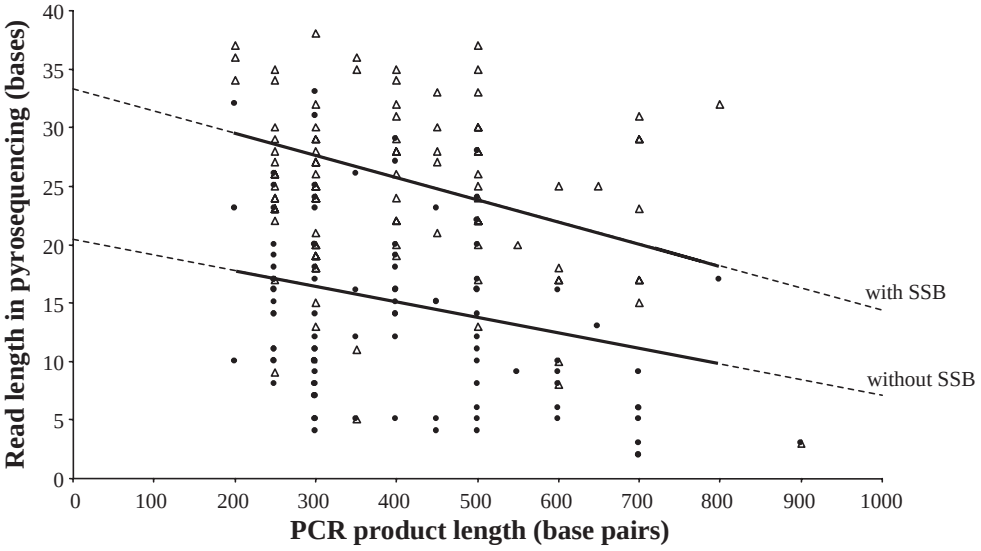

Fig. 2. Correlation between read length in pyrosequencing and length of PCR products with and without SSB is shown. Samples with SSB in the pyrosequencing reaction are marked as triangles and samples without SSB are shown as black circles. The plotted lines with (higher line) and without SSB (lower line) are linear fits of average read length and PCR product length.

ically reduced the read length. Inclusion of an extra washing step after primer annealing partially relieved those problems but the read length was still much lower than when using less primer. These results show that SSB addition is a simple and rapid measure to be taken for obtaining a general improvement of sequence data quality. Moreover, the attempts to increase primer annealing efficiency indicate that non-hybridized ssDNA in solution disturbs the pyrosequencing enzymes and impair their performance.

4. Applications

An important feature of the pyrosequencing technique is its ability to sequence at least 20 bases. This characteristic allows numerous applications such as sequencing and determining known as well as unknown polymorphic positions, microbial typing and tag sequencing.

4.1. Scanning for undefined mutations

Detection of unknown mutations in known sequences is normally denoted re-sequencing. However, pyrosequencing has in most cases been used for re-sequencing of a few number of selected hotspot codons [97–99] but in one study the ability of using pyrosequencing for sequencing exons 5 to 8 of the p53 gene has been investigated [26]. In the study published by Garcia et al. [26], a set of sequencing primers was designed to cover the 4 exons of the p53 gene. Pyrosequencing was performed on an amplified DNA template and overlapping sequences were assembled to determine the sequence of the p53 gene. Two forms of nucleotide dispensation strategies were used, cyclic and programmed. In the cyclic dispensation strategy, nucleotides were repeatedly added in the order A, G, T and C while in the programmed strategy the order of nucleotide dispensation was pre-defined according to the wild type consensus sequence. The use of programmed dispensation approach allows longer read length with fewer primers, faster readout and less frame-shifts in the determined sequence. Although using pyrosequencing in re-sequencing permits detection of the mutation, non-synchronized extension may appear in the sequence after the polymorphic position, which generally yields non-interpretable data. Nevertheless, the detected mutation can readily be quantified and in the cases of heterogeneous tumor material mutation signals as low as 10–20% may be detected [24].

4.2. SNP genotyping

The problems with read-length and appearance of non-synchronized sequences after a polymorphic position has so far limited the use of pyrosequencing in re-sequencing and detection of unknown mutations. However, if the polymorphic position is known (e.g. single nucleotide polymorphisms (SNPs)), the number of sequenced bases usually does not need to exceed 4–10 bases and thus the read-length of pyrosequencing does not become a limiting factor. In addition, it is possible to use a programmed nucleotide delivery to maintain synchronized extension of alleles and thereby obtain high quality sequence peaks even after the polymorphic position. This factor has strongly contributed to associate the pyrosequencing method with analysis of single nucleotide polymorphisms (SNPs) [17,19,20,22,25,31,100,101]. A feature of pyrosequencing in typing SNPs is that each allele combination (homozygous, heterozygous) confers a specific pattern compared to the two other variants (Fig. 3). Thus, it is rather easy to score the allelic status by pattern recognition software. Even here, two different orders of nucleotide additions, cyclic and sequential (programmed), may be tried. In both cases, SNP determination starts with analysis of nucleotide(s) preceding the investigated position. This step serves as a positive control of the amplification process as well as calibration of peaks and reaction conditions. The advantage of using cyclic addition of nucleotides is that it results in three distinctive patterns at the polymorphic sites due to non-synchronized extensions. This is also a unique feature of this technology, which allows haplotype determination when two or more SNPs are in vicinity of each other [24,102]. In contrast, the sequential nucleotide addition generates differences in three peak positions and is designed so that the individual allele extensions are in phase. Thereafter, further nucleotide additions will give the consensus sequence of the target and can improve raw data interpretation.

4.3. Bacterial genotyping

The identification of bacterial strains usually involves sequencing of the 16S rRNA gene. The 16S rRNA gene (16S rDNA) contains highly conserved regions that are flanking variable and species specific sequences [103]. Several studies have reported the usefulness of pyrosequencing technology for bacterial typing by sequencing the variable region of the 16S rRNA gene. The typing involves a nucleic acid amplification using universal primers targeting the conserved sequences and sequencing of the flanking variable region by one of the amplifying primers. Monstein et al. [28] employed the technique to identify and subtype variable V1 and V2 regions of *Helicobacter pylori*. The same group pyrosequenced 16S rRNA to distinguish pathogenic bacteria from commensals or saprophytic bacteria found in the same habitat [104] and to identify contaminated bacteria in industrial water systems. However, in order to distinguish closely related bacterial strains by pyrosequencing, longer read-lengths may be necessary. To overcome the read-length limitation in 16S rRNA studies, Gharizadeh et al. [105] suggest the use of multiple group-specific sequencing primers. In this approach, a pool of

5’-GTTTTCTGTTGTAAATGCC[T/G]TTTACAAA

3’-……CAAAAGACAACATTTACGG[A/C]AAATGTTTGTAACT……-5’

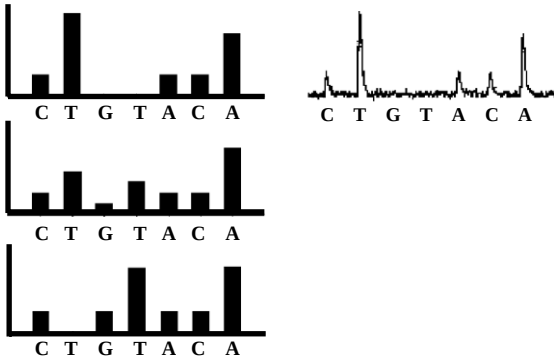

Fig. 3. Analysis of single nucleotide polymorphisms (SNPs) by pyrosequencing. The amplified target DNA (indicated by black letters) is primed by a sequencing primer (green letters). Pyrosequencing (red letters) is initiated by incorporation of a C nucleotide (serving as calibration of signals) and the order of nucleotide addition after this nucleotide is T-G-T-A-C-A. The polymorphic position in this case is situated after the first incorporated nucleotide and is a T to G substitution. This order of nucleotide addition renders three distinct pyrosequencing patterns (one for each genotype). The raw data (right) is then compared to theoretical patterns (left) and the genotype can easily be assessed.

pyrosequencing primers is added but only one of the primers hybridizes to the template and functions as sequencing primer. The hybridized primer from the pool is specific for a semi-conservative region and enables primer hybridization closer to a variable region leading to “win of read-length”.

4.4. Viral genotyping

The pyrosequencing technology has been employed in genotyping of viruses with heterogeneous sequences. In an early report, O’Meara et al. [29] investigated the feasibility of using pyrosequencing as a genotyping tool to characterize the presence of drug resistance mutations in protease inhibitors (PIs) of human immunodeficiency virus type 1 (HIV-1). The study included use of 12 pyrosequencing primers to cover 33 codons that are implicated in 52 drug resistance mutations. Gharizadeh et al. [106] used pyrosequencing for genotyping of human papillomaviruses (HPV) that are believed to be the major cause of cervix cancer. Since more than 100 HPV types with extreme heterogeneous sequences are reported to exist, the amplification needs to be carried out on semi-conservative regions by the use of degenerative general primers and the pyrosequencing is performed by one of these PCR primers. However, the use of a PCR primer as pyrosequencing primer causes difficulties when unspecific amplifications occur. In addition, occurrence of clinically important multiple HPV types are quite common and in these cases, mixed sequence peaks make the analysis very difficult. In a later publication [107], the authors suggest the use of a pool of 4 type-specific primers (4 clinically interesting HPV types) to avoid problems associated with unspecific PCR fragments. Nevertheless, the specific typing will require special “pattern recognition” software or separate sequencing reactions for each of the 4 type-specific primers, if the sample comprises more than one HPV type of interest. Other applications of pyrosequencing involve genotyping of hepatitis C virus (HCV) [108,109] and hepatitis B virus (HBV) [110].

4.5. Tag sequencing

Expressed sequence tag (EST) sequencing has had an enormous influence on expression profiling and identification of differentially expressed genes that have not been present in databases. It has been proposed that short gene specific nucleotide sequence tags (approximately 8–13 bases) should give sufficient information to identify a transcript [111]. However, to uniquely identify a transcript, longer read-length than 8–13 bases may be needed and thus pyrosequencing can be an alternative method. In two studies, the feasibility of pyrosequencing for tag sequencing has been investigated [32,95]. In both reports an average read length of 25–30 bases was achieved, demonstrating the reliability of the technique to uniquely identify transcripts in complex organisms.

5. Future

The pyrosequencing technology is currently used in 96 or 384 plate format but to be a high throughput technology, an improved sample capacity would be beneficial. One way of doing this would be to use micromachined filter-chamber arrays where parallel analyses of nano-liter samples can be monitored in real-time. In this experimental setup, the DNA sample is immobilized on beads trapped in a filter chamber that allows for injection of solutions into the chamber and transport through the cell to the outlet. Microfluidic systems containing several parallel chambers have been produced. Moreover, SNP analysis by the use of pyrosequencing

chemistry has been performed in filter-chamber systems [112,113]. However, since the filter chamber allows passage of all pyrosequencing enzymes through the chamber, the light produced in the SNP analysis was detected in the sample outlet. This lack of light localization within the chamber is a great disadvantage since only one analysis can be run at the time and the light signal becomes diluted. This problem has been addressed by designing a strategy in which the luciferase was genetically fused to a DNA binding domain (Klenow or SSB) and purification handle (Z_{basic}) that could specifically be removed by enzymatic cleavage [114] (Fig. 4). After purification the fusion proteins were analyzed by complete extension using pyrosequencing chemistry. In these experiments, paramagnetic beads with attached ssDNA to which a primer had been annealed were incubated with the fusion proteins, Z_{basic}-SSB-Luciferase, SSB-Luciferase, Z_{basic}-Klenow-Luciferase and Klenow-Luciferase, respectively. The proteins were allowed to bind the immobilized DNA and free protein was removed. A pyrosequencing mixture devoid of Luciferase and in the case of Klenow fusions also lacking Klenow, was added to the beads. The mixture contained all four nucleotides so that, DNA synthesis would take place on the primed template in presence of polymerase and resulting in light emission in case of luciferase activity. All tested proteins bound selectively to the immobilized DNA and their enzymatic domains were active. These results promisingly suggest that with more sophisticated detection systems, such as a highly sensitive CCD camera, these proteins could by used on miniaturized formats such as nano-liter filter chamber or ultimately on DNA microarrays. Alternatively, these fusion proteins may be employed in another microfluidic format introduced by 454 Life Sciences (http://www.454.com). Based on the pyrosequencing chemistry and the use of thousands of pico-liter wells on a PicoTiter Plate, 454 Life Sciences has created a massively parallel sequencing system that claims to be able to sequence 10 Mbp genomes.

6. Concluding remarks

Pyrosequencing is a DNA sequencing technology based on real-time detection of DNA synthesis monitored by bioluminescence. Four enzymes are exploited by the technology and a fifth protein, SSB, can be included to enhance the quality of the obtained sequences and thereby prolong the read length. The pyrosequencing technology has been used in a broad range of applications such as SNP genotyping, *de novo* mutation detection, gene identification and microbial genotyping. We believe that the pyrosequencing technology possesses several unique features which

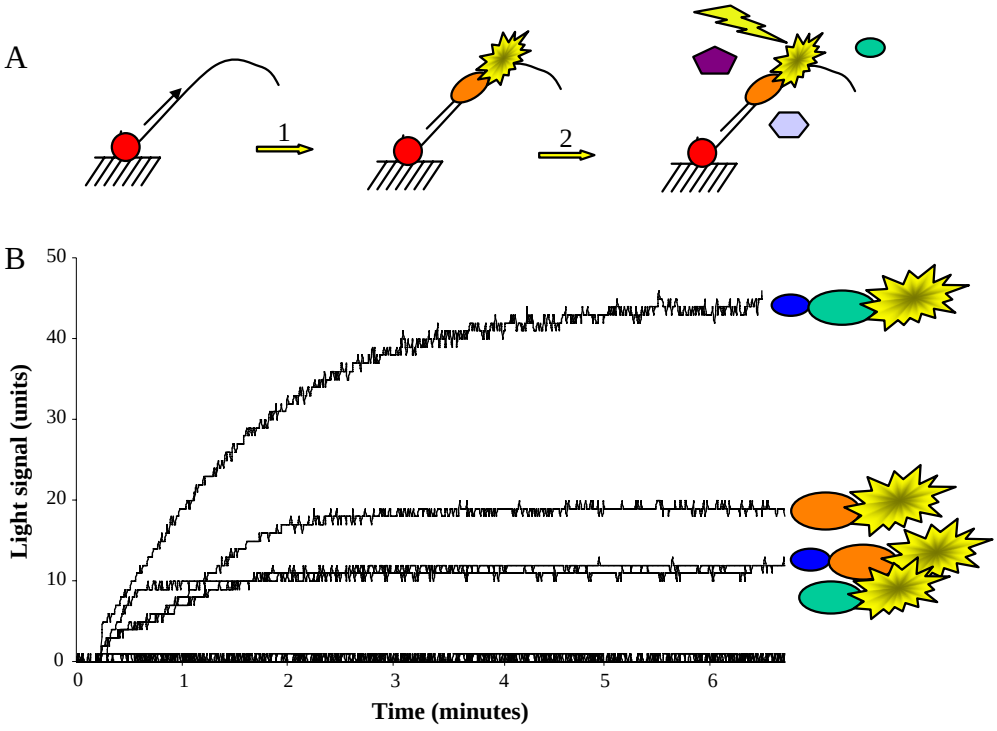

Fig. 4. (A) A schematic overview of the principle of using DNA-anchoring proteins for pyrosequencing. First the template is immobilized on a solid matrix (1). Thereafter the fusion protein is added, comprising of a DNA-anchoring part (Klenow (orange) or SSB (light blue)) and the luciferase (yellow). In the third step all other needed enzymes and nucleotides are added (3) and following primer extension measurable light will be produced. (B) A complete primer extension assay performed on DNA immobilized on magnetic beads is shown. The *x*-axis time in seconds and the *y*-axis denotes measured light signal in arbitrary units. Reactions where all four fusion proteins have been incubated with DNA-free beads is shown as curves parallel to the *x*-axis. The purification tag Z_{basic} is colored blue.

make this technique advantageous compared to other sequencing methods in a number of applications. In order to further expand the field of utility, increased throughput and sequence read length as well as the use of smaller reagent volumes are necessary and are currently developed. This amelioration of the technology very promisingly shows that the competitiveness of pyrosequencing is likely to increase in the future.

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