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## TECH.SIGHT

### DNA SEQUENCING:

## A Sequencing Method Based on Real-Time Pyrophosphate

Mostafa Ronaghi, Mathias Uhlén, and Pål Nyrén\*

DNA sequencing is one of the most important technologies in bioscience today. Whole-genome approaches (1) and human expressed sequence tag (EST) sequencing (2) have started to exert profound influence on biology and medicine. The need for robust, high-throughput methods to replace the elegant Sanger method, described more than 20 years ago (3), has led to the development of several new principles, such as array methods based on sequencing by hybridization (4). New applications, such as population-based biodiversity projects and brute-force genotyping using single-nucleotide polymorphism, make such efforts even more urgent, in particular, for simple and robust methods for sequencing short "tags" (1 to 20 bases) such as ESTs or biallelic markers and methods suitable for routine diagnostic applications.

Sequencing-by-synthesis is based on the detection of nucleotide incorporation, using a primer-directed polymerase extension. The sequence can be deduced iteratively (5). During the last decade, many researchers, including the groups of Rosenthal (6), Gibbs (7), and Jones (8), described various protocols based on fluorescently labeled nucleotides. The level of incorporation of these fluorescent nucleotides is low, however, as shown by Metzker *et al.* (7), and therefore, the protocols only permit detection of a few bases.

Recently, Ronaghi *et al.* (9) showed that natural nucleotides can be used to obtain efficient incorporation during a sequencing-by-synthesis protocol. The detection was based on the pyrophosphate (PPi) released during the DNA polymerization, the quantitative conversion of pyrophosphate to ATP by sulfurylase, subsequent production of visible light by firefly luciferase. However, sequencing method is not without drawbacks: The template must

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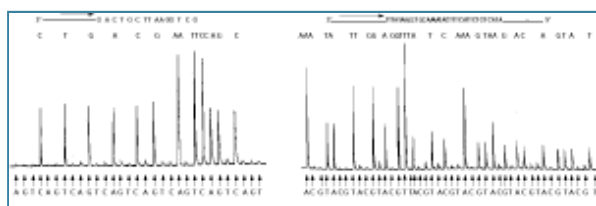
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Recently, Ronaghi *et al.* (9) showed that natural nucleotides can be used to obtain efficient incorporation during a sequencing-by-synthesis protocol. The detection was based on the pyrophosphate (PPi) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to ATP by sulfurylase, and the subsequent production of visible light by firefly luciferase. However, this PPi-based sequencing method is not without drawbacks: The template must be washed



faster than the rate of ATP hydrolysis to obtain ATP concentrations and light production in proportion to the amount of PPi released.

To optimize the assay, several parameters were tested by using synthetic oligonucleotides as template ([11](#)). The assay solution consisted of the template-primer plus varying amounts of the four enzymes ([12](#)). The protocol consisted of simply adding a new nucleotide every other minute in an iterative manner and detecting the visible light produced. In Fig. 2 (Left), an example of the results is shown. Clear specific signals can be observed with low background. Note the higher signals obtained at cycles 21, 23, and 24 when two nucleotides were incorporated because of the presence of two identical adjacent bases in the template. Similar high-quality sequencing results were obtained for other oligonucleotide templates ([13](#)).



**Fig. 2.** Pyrosequencing was performed on a 35-base-long oligonucleotide template (Left) and a 130-base-long PCR product (Right). About 2 pmol of the template-primer was used in the assay. The reaction was started by the addition of the indicated deoxynucleotide, and the PPi released was detected by the described method. The DNA sequence after the primer is indicated.

For direct sequencing of polymerase chain reaction (PCR) products, single-stranded template was obtained by biotin capture on magnetic beads ([14](#)). In Fig. 2 (Right), the amount of light produced during approximately 40 subsequent cycles is shown. Sequence data covering 34 bases is obtained ([15](#)), and the signal-to-noise ratio remains relatively high even at 40 cycles. The low signals in the early cycles (2, 3, 6, and 7) show that the misincorporation of bases, which would produce PPi and consequently light, is not a major problem, despite the presence of "wrong" noncomplementary nucleotides. The background does increase during the later cycles, which is not surprising because relatively crude enzyme preparations were used in this work. Low amounts of contaminating enzymes such as exonucleases or kinases will give rise to nonsynchronized extensions on some templates, causing increased background and lower specific signal. Obviously, more work is needed to purify these enzymes from present contaminants. In addition, apyrase activity is decreased in later cycles, which is because of accumulation of intermediate products (such as deoxynucleoside diphosphate, or dNDP) and eventually undegraded dNTP. Removal of these nucleotides by enzymes such as nonspecific nucleoside diphosphatases ([16](#)) will increase the efficiency of apyrase in degradation of nucleoside triphosphates and thereby allow longer reads. However, it is reassuring that the nonoptimal enzyme mixture used here allows accurate determination of more than 20 bases for many different PCR products tested ([13](#)).

An inherent problem with the described method is the difficulty in determining the number of incorporated nucleotides in homopolymeric regions due to the nonlinear light response following incorporation of more than three or four identical nucleotides. This can be demonstrated by the relatively low signal at cycle 16 (Fig. 2, Right) when four T bases are incorporated. However, this nonlinear response can most likely be compensated for by software algorithms. In addition, for most

tag-sequencing applications, such as brute-force EST-sequencing, biallelic marker analysis, and confirmatory sequencing, this problem is not a major concern, because the number of bases, if present, will be known.

With this method, parallel processing of large numbers of samples can easily be envisioned with the use of high-density microtiter plates and microinjector technology. An automated instrument has recently been developed based on the precise delivery of submicroliter volumes of the four nucleotides by "ink-jet" technology into a microtiter plate coupled with simultaneous detection of all samples by a single CCD unit (17). Together with a robot (17) performing single-strand template preparation (from double-stranded PCR products), ready for pyrosequencing, it would be possible to analyze thousands of samples daily with little manual intervention.

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11. The oligonucleotides E3PN (5'-GCTGGAATTCGTCAGACTGGCCGTCGTTTTACAAC-3'), NUSPT (5'-GTAAAACGACGGCCAGT-3'), and JA80 (5'-GATGGAAACCAAAATGATAGG-3') were synthesized by phosphoramidite chemistry (Interactiva).
12. The oligonucleotide E3PN and the PCR product generated from cloned HIV-V3 were used as templates for DNA sequencing. The oligonucleotides and single-stranded PCR product were hybridized to the primers NUSPT and JA80, respectively. The hybridized DNA fragments were incubated with  $\text{exo}^-$  Klenow or  $\text{exo}^-$  T7 DNA polymerase (Sequenase 2.0), respectively (Amersham). The sequencing procedure was carried out by stepwise elongation of the primer-strand upon sequential addition of the different deoxynucleoside triphosphates and simultaneous degradation of nucleotides by apyrase (nucleoside 5'-triphosphatase and nucleoside 5'-diphosphatase; EC 3.6.1.5) (Sigma). The sequencing reaction was performed at room temperature and was started by adding a specific amount of one of the deoxynucleotides. The PPi released due to nucleotide incorporation was detected as described (9).
13. M. Ronaghi and P. Nyrén, data not shown.
14. The biotinylated PCR products were immobilized onto streptavidin-coated super paramagnetic beads [Dynabeads M280-Streptavidin (Dyna)]. Elution of single-stranded DNA and hybridization of sequencing primers was carried out as

described (9).

15. The sequencing data obtained from the pyrosequencing method was confirmed by semiautomated solid-phase Sanger sequencing (18).
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17. The pyrosequencing instrument was based on a cassette containing the four separate nucleotides on an x-ray robotic arm (B. Ekström, M. Ronaghi, T. Nordström, P. Nyrén, M. Uhlén, unpublished data). The sample preparation robot was based on streptavidin-coated magnetic particles for PCR-capture and handling (A. Holmberg and M. Uhlén, unpublished data).
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