

Pyrosequencing – a service provided by varionostic®

Strengths and Applications

The Strengths of Pyrosequencing

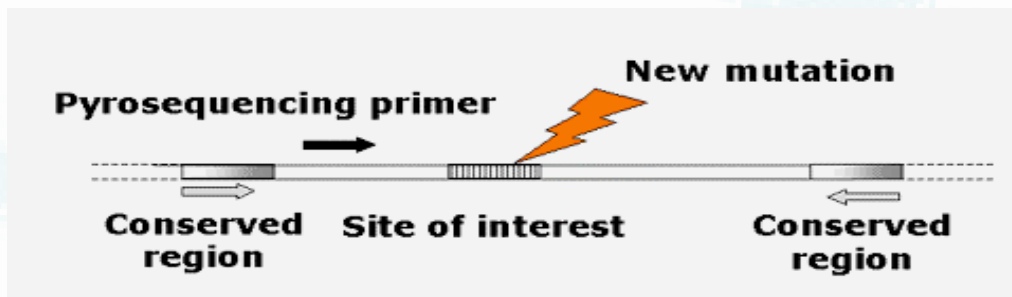
- Pyrosequencing delivers the "gold standard" of genetic analysis: the sequence itself. Other methods only provide a "Yes/No" signal.
- With Pyrosequencing, there is great flexibility in primer placement. Therefore it is easy to design a Pyrosequencing assay to analyze virtually any genetic marker. So far, Pyrosequencing has an unbeaten track record in analysing any SNP.
- Pyrosequencing assays are mutation-tolerant. Unlike hybridisation-based assays, Pyrosequencing generates a correct sequence regardless of the appearance of a new, unexpected mutation. This is very important to microbiological applications: hybridisation-based assays can give false negatives in the presence of a new mutation.
- With Pyrosequencing, you not only obtain sequence information, but the data is also fully quantitative, ideal for measuring the relative amounts of alleles. This property allows the quantification of DNA methylation, heterozygosity, ploidy levels, multi-copy genes, pooled DNA samples, hematopoietic chimerism, and mixed genotypes in heterogeneous samples (e.g. tumor and normal cells).

Pyrosequencing addresses a wide range of genetic analyses

<p>Sequence analysis</p> <ul style="list-style-type: none"> • Sequence identification • Microbial typing • Cloned DNA re-sequencing • mtDNA • Transgenetics • Expression profiling • Oligo ID • Microsatellite 	<p>Mutation detection</p> <ul style="list-style-type: none"> • Known positions • Unknown positions within hot spots • Point mutations • Insertions/deletions • Multiple mutations • Multiplexing
<p>Genetic variation</p> <ul style="list-style-type: none"> • SNPs and DIPs <ul style="list-style-type: none"> - Di-, tri- and tetra allelic - Multiple - Multiplexing • Haplotype <ul style="list-style-type: none"> - Out-of-phase - Allele-specific PCR 	<p>Quantification</p> <ul style="list-style-type: none"> • Allele frequency assessment <ul style="list-style-type: none"> - SNP frequency - Tri/tetra allelic SNP frequency - Indel frequency • CpG-methylation • Gene copy number • Loss of heterozygosity • Polyploid genomes • Expression analysis

Pyrosequencing Strengths and Applications

Pyrosequencing assays are mutation-tolerant



Consequence of new mutation:

- With hybridization-based probe, no hybridization occurs in the presence of a mutation
- With Pyrosequencing, new mutations are tolerated and detected

Unlike the majority of genotyping technologies, which depend on a probe hybridizing to a specific site of interest, Pyrosequencing assays are tolerant of unexpected mutations. When PCR primers are placed in conserved regions of DNA, Pyrosequencing assays produce meaningful results, regardless of the mutation.

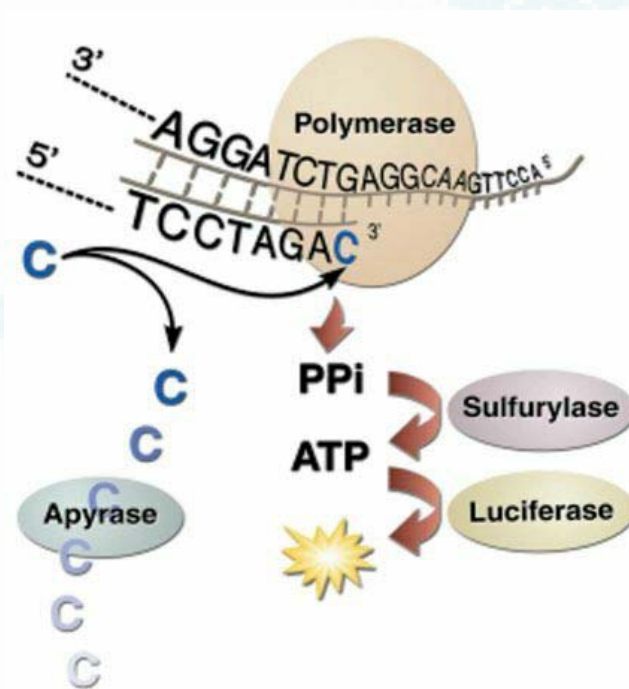
Pyrosequencing is the method of choice for simple and robust assays in studies of **antibiotic resistance** and the identification of new microbial mutations.

Pyrosequencing data is quantitative

Pyrosequencing not only gives real sequence information, but the data is also a quantitative measure of each detected nucleotide. The data is ideal for measuring the relative amounts of alleles. This property allows the quantification of DNA methylation, heterozygosity, ploidy levels, multi-copy genes, pooled DNA samples, hematopoietic chimerism, and mixed genotypes in heterogeneous samples (e.g. tumor and normal cells).

⇒ **Pyrosequencing uniquely presents qualitative sequence data with quantitative allele representation**

Pyrosequencing Step by Step

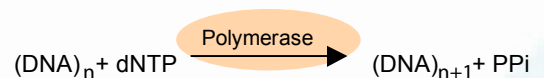


Step 1

A sequencing primer is hybridized to a single stranded, PCR amplified, DNA template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5' phosphosulfate (APS) and luciferin.

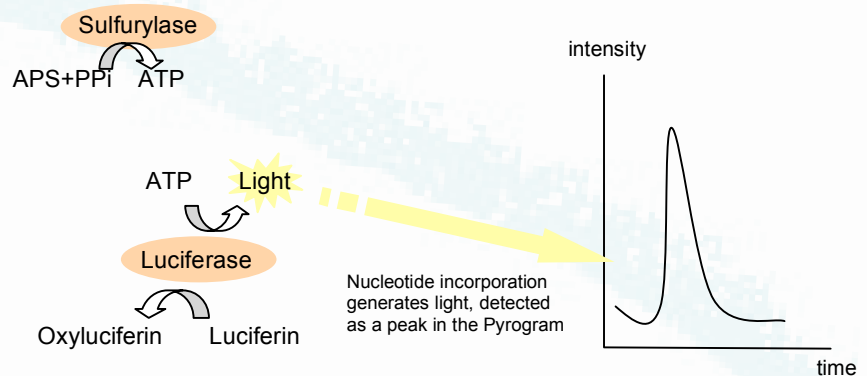
Step 2

The first of four deoxyribonucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribo-nucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.



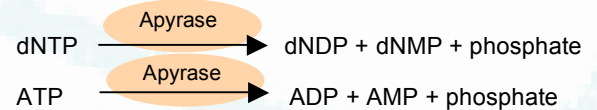
Step 3

ATP sulfurylase quantitatively converts PPI to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a Pyrogram™. The height of each peak (light signal) is proportional to the number of nucleotides incorporated.



Step 4

Apyrase, a nucleotide degrading enzyme, continuously degrades ATP and unincorporated dNTPs. This switches off the light and regenerates the reaction solution. The next dNTP is then added.

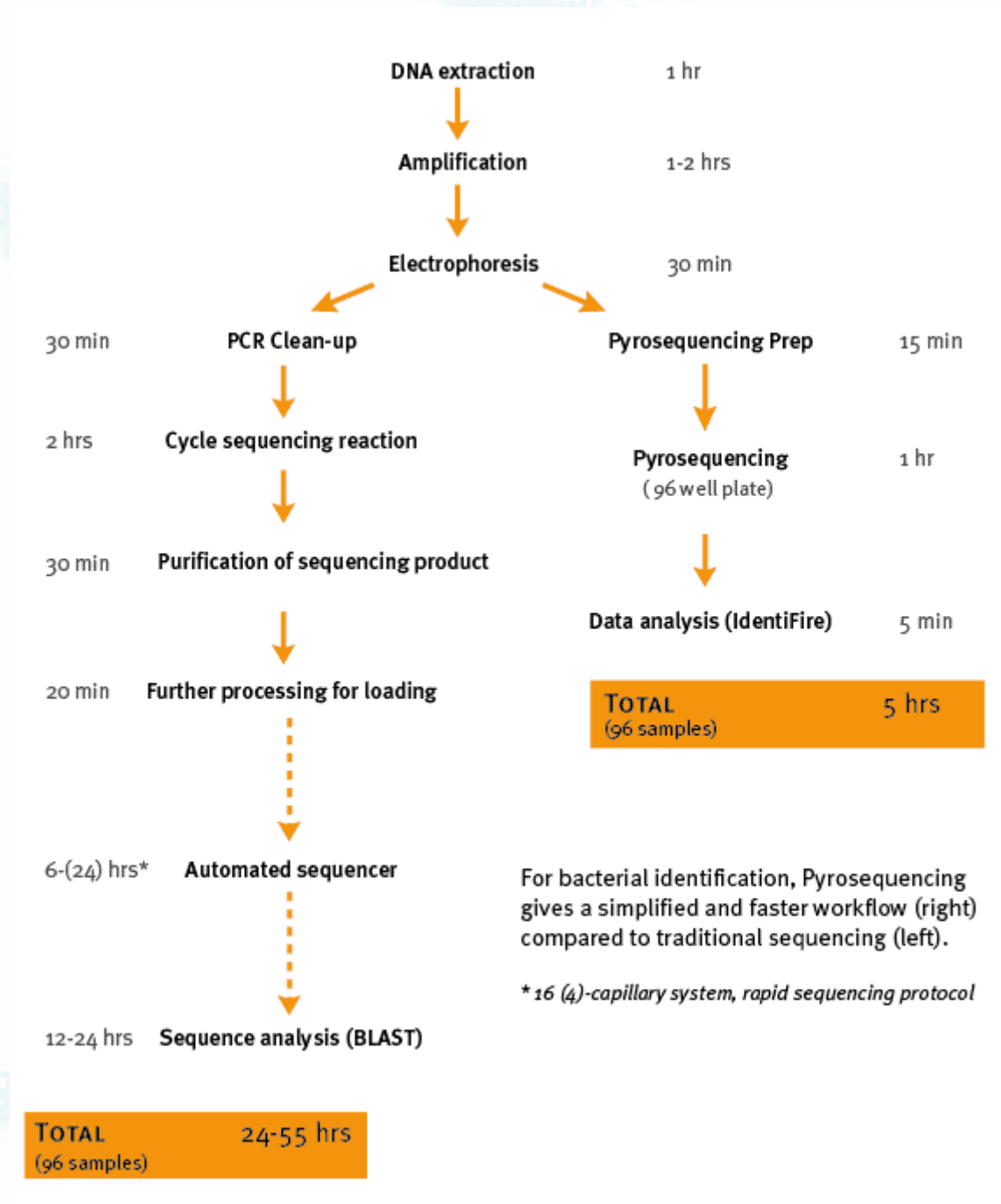


Step 5

Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate (dATPaS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase.

As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram.

Pyrosequencing The Principle Workflow



Our Service - the analytical workflow

Step by step to reliable results

- 1) Sampling**
- 2) DNA isolation**
 - 2a) Bisulfite conversion in case of methylation analyses**
- 3) Assay design**
- 4) Primer design and -syntheses**
- 5) PCR optimization with inhouse controls**
- 6) Pyrosequencing optimization on controls**
- 7) PCR + Pyrosequencing with customers samples**
- 8) Data evaluation**
- 9) Analyzing report**
- 10) Data interpretation within scientific context**