Pyrosequencing® and its applications

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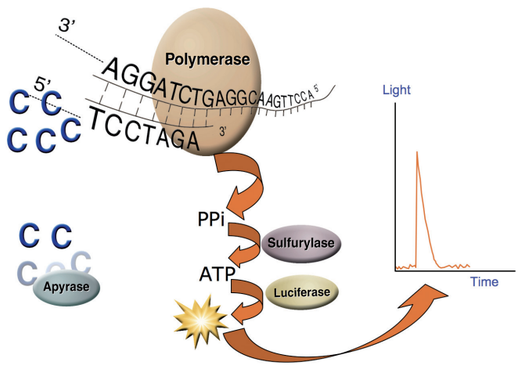
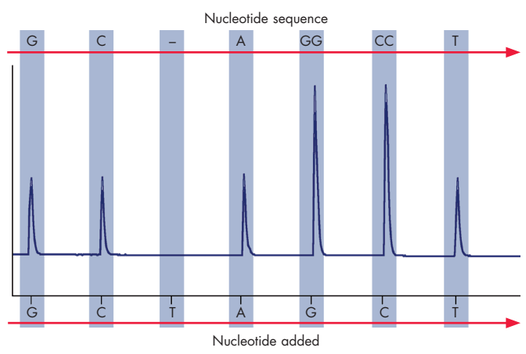
Pyrosequencing principle

Pyrosequencing technology is based on the sequencing by synthesis principle. After successful incorporation of a nucleotide by a polymerase using a single-stranded PCR (or RT-PCR) fragment as template, the released PPi is converted to light by an enzyme cascade: ATP sulfurylase converts PPi to ATP in the presence of APS. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light, which is detected by CCD sensors and seen as a peak in the raw data output (Pyrogram®). Apyrase continuously degrades unincorporated nucleotides and ATP. The height of each peak (light signal) is proportional to the number of nucleotides incorporated. Sequential addition of nucleotides allows quantitative decoding of the sequence to analyze.



The enzyme cascade of the Pyrosequencing technology.



Sequential addition of nucleotides.

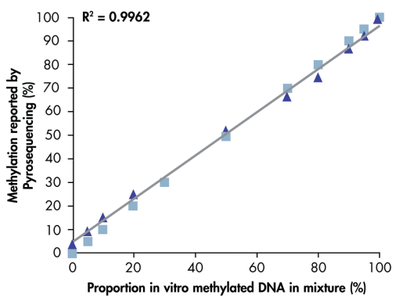
Pyrosequencing workflow

Assay design ~5 min	Amplification ~2h	Sample prep ~15 min	Pyrosequencing ~10–60 min
Rapidly create your own assay using the Assay Design Software 2.0, or use predesigned tests or QIAGEN's GeneGlobe® assays	Amplify your region of interest with a biotinylated primer using the PyroMark® PCR Kit or PyroMark One-Step RT-PCR Kit	Prepare your samples using the easy-to-handle PyroMark Q24 vacuum workstation or PyroMark Q96 vacuum workstation	Run your samples on the PyroMark Q24, PyroMark Q96 ID, or PyroMark Q96 MD instrument

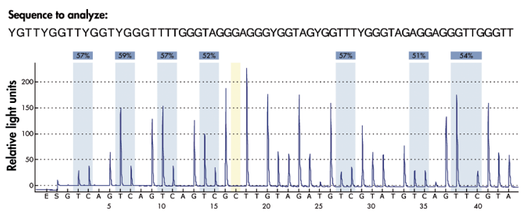
Methylation analysis

Pyrosequencing generates highly reproducible quantification of methylation frequencies at individual consecutive CpG sites. Pyrosequencing can detect and quantify even small changes in methylation levels. Other features include the inherent quality control afforded by the sequence context of results. Built-in bisulfite treatment controls eliminate manual estimation of non-converted DNA levels and prevent false-positive methylation detection, ensuring reliable results.

Pyrosequencing has been used to correlate DNA methylation with tumor type and gene expression, to measure cellular response to treatment with demethylating agents, and to assess changes in methylation state in relation to tumorigenesis, genetic imprinting, and exposure to environmental toxins.



Linearity of methylation quantification by Pyrosequencing. PCR products from varying mixtures of unmethylated genomic DNA and methylated DNA (EpiTect® Control DNAs) were analyzed by Pyrosequencing. A tight correlation between the known percentage of methylated DNA in the mixtures (light blue) and the methylation percentage reported by Pyrosequencing (dark blue) was observed (r^2 = 0.9962). The graph represents the quantification of methylation at a single CpG site in the p16 gene.



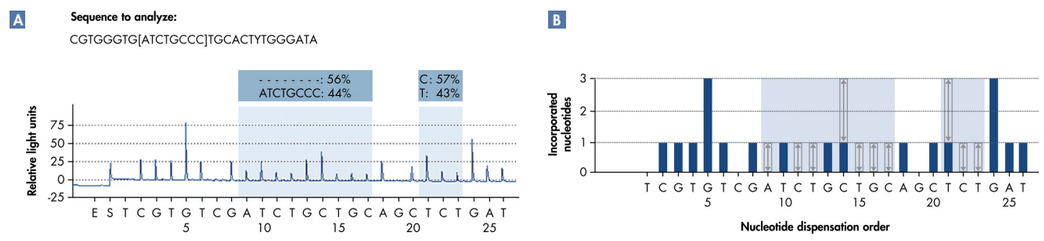
Analysis of multiple contiguous CpG sites. Methylation at 7 independent CpG sites (highlighted in blue) is quantified in a single Pyrosequencing run. Position-specific information in the context of an analyzed sequence presents broad-sequence methylation patterns. Note the built-in quality control sites (highlighted in yellow) consisting of cytosines converted to thymines, demonstrating full bisulfite conversion of the treated DNA.

Mutation analysis

Pyrosequencing ensures precision and accuracy when performing highly sensitive mutational analysis or when quantifying alleles in mixed cell populations. Data are presented in a sequence context which serves as a built-in quality control.

- Reliable detection and quantification of sequence variation, down to at least the 5% mutation level
- Characterization of contiguous and multivariable mutations
- Discovery of unknown mutations
- Allele quantification in loss of heterozygosity analyses
- Unambiguous, fully quantitative genotyping that distinguishes multi-site variations from single nucleotide polymorphisms (SNPs)

The PyroMark product line is growing and includes analysis of KRAS, EGFR, BRAF, NRAS, APOE, HFE, and MTHFR. In addition, easy assay design with PyroMark software enables detection and quantification of virtually any sequence variation.

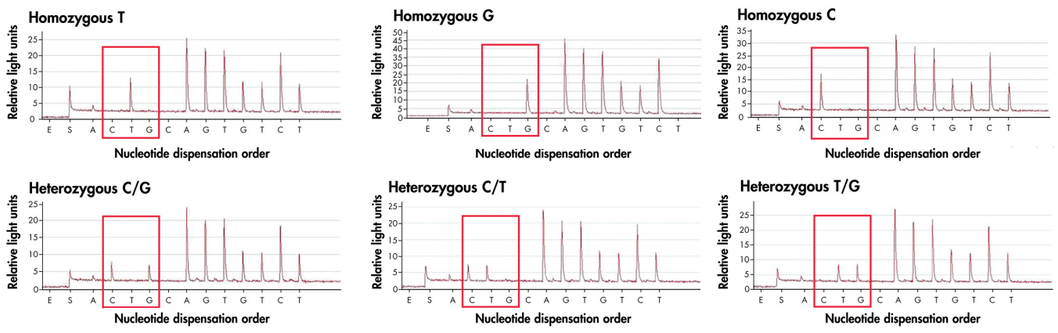


2 mutation types quantified in a single Pyrosequencing reaction. A Pyrogram of a DNA sequence featuring an insertion-deletion mutation (ATCTGCCC) and a somatic mutation involving a single base pair substitution (C vs. T). The variable regions are highlighted in blue and the allele frequencies are given above the indicated sites. B The histogram shows the number of nucleotides incorporated at each nucleotide dispensation. The dark blue bars represent the nucleotide positions conserved between alleles and arrowed empty bars portray the quantified variation.

Genetic testing

Genetic testing is an important component of many applications. For example, developing effective therapeutic agents requires information about how gene polymorphisms impact metabolism; understanding genetic contributions to a disease involves characterizing linked SNPs; finally, analysis of forensic DNA evidence relies on accurate detection of sequence variation.

Because of the flexibility of primer placement in Pyrosequencing reactions, virtually all genetic markers can be assayed. Alleles of variable loci are accurately quantified, and heterozygosity is easily resolved. In addition, because Pyrosequencing delivers sequence information, various types of genetic variation can be evaluated — insertion-deletions, SNPs, short tandem repeats, and variable gene copy number — and it is possible to assay several contiguous sequence variants in a single run.



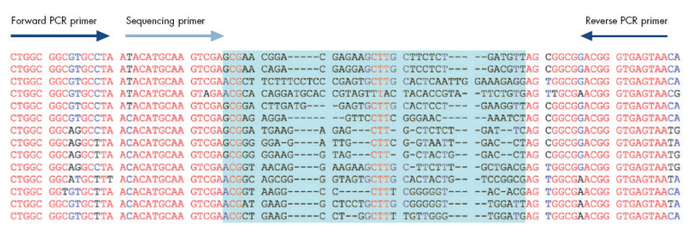
Analysis of a tri-allelic SNP. Detection of tri- and tetra-allelic SNPs can be difficult with commonly used methods. This series of Pyrograms illustrates the ease of Pyrosequencing-based detection of a tri-allelic SNP (red outline). C, T, and G are serially dispensed in the Pyrosequencing reaction and only the incorporated nucleotides will elicit a signal peak. The result is a different peak pattern for homozygous samples of each allele (Pyrograms on the top) or compound peak patterns for heterozygous samples (Pyrograms on the bottom).

Microbial identification

Sequence information provides reliable data for microbial genotyping applications. Since Pyrosequencing sequences by synthesizing new copies of the DNA template, results provide unambiguous information. Users can check the sequence surrounding the variable site ensuring that the correct DNA region was analyzed.

Unlike hybridization techniques, Pyrosequencing allows the identification of a large number of species using a single conserved primer. Consequently, DNA extracted from multiple microbe species can be sequenced in the same Pyrosequencing run. PyroMark IdentiFire Software compiles local sequence database against which imported Pyrosequencing outputs are rapidly aligned. The raw data, matched hits, and percentage concordance of each hit are presented in detailed reports.

Depending on assay design, the sequence can be used to discriminate microbial species, types and strains, or detect genetic mutations.



Pyrosequencing-based bacterial identification. PCR primers were designed for conserved regions of 16S rRNA and the sequencing primer was placed immediately upstream of the hypervariable region V1 (blue). One set of PCR and sequencing primers is sufficient to discriminate the listed species (as well as others not included in the alignment).

The Pyrosequencing applications presented here are for research purposes. Not for use in diagnostic procedures.

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Sample & Assay Technologies