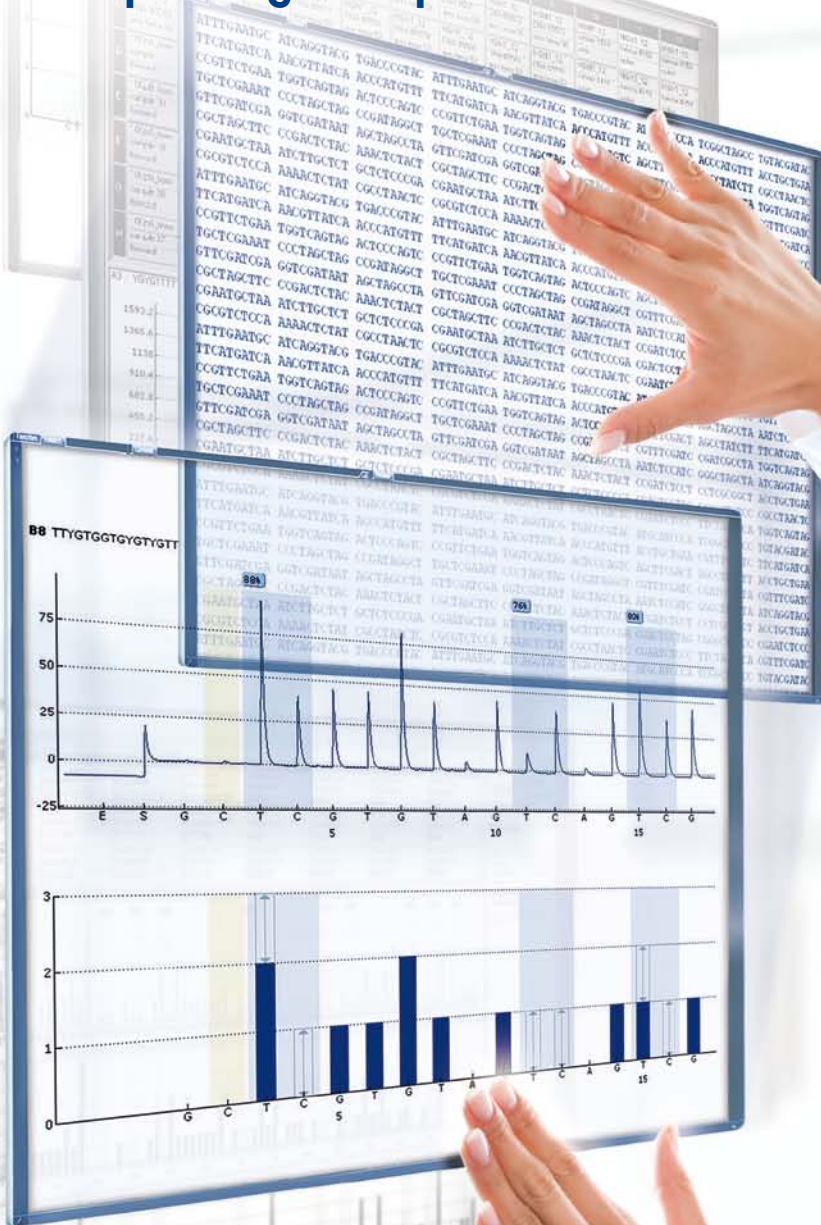


A New Standard in Epigenetic and Genetic Analysis!

Pyrosequencing® — the synergy
of sequencing and quantification



Sample & Assay Technologies

Quantitative data for new horizons in genetic research

The development of sequencing technology has greatly expanded our understanding of how biological information is coded in the sequence of nucleotides of DNA. This knowledge has also led to new questions: What is the significance of subtle sequence changes, especially in non-coding regions? When do interactions of regulatory elements impact gene expression? How does nonsequence-based information — epigenetic information — contribute to developmental programming and cell differentiation? Finally, how can answers to these questions benefit applied sciences? These questions are the new horizons in genetic research. Researchers are uncovering new layers of biological information stored and inherited in genetic and epigenetic modifications of DNA. Characterization of this information requires next-generation research tools that enable application flexibility and acquisition of quantitative data.

Pyrosequencing with QIAGEN® PyroMark® products meets these requirements

Integrating detection and quantification of genetic variation into one powerful system, Pyrosequencing with the PyroMark platform outperforms other sequence-based solutions in the analysis of targeted short DNA sequences. For a range of applications, this means reduced cost and time investment (Figure 1).

Advantageous features of Pyrosequencing include:

- [Quantification of alleles](#), even when found at low frequencies
- Detection of [unknown sequence](#) variants
- [Assay versatility](#) on the same instrument and even in the same run
- [Built-in assay controls](#) guaranteeing data accuracy and reliability
- Fast delivery of direct and unambiguous sequence data
- [Intuitive software](#) that enables powerful analyses and user-designed assays



Figure 1. The advantages of Pyrosequencing in different areas of research.

Elegant chemistry and sensitive technology enable quantification of sequence variation

Step 1

A DNA segment is amplified and the strand to serve as the Pyrosequencing template is biotinylated. After denaturation, the biotinylated single-stranded PCR amplicon is isolated and allowed to hybridize with a sequencing primer.

Step 2

The hybridized primer and single-stranded template are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the substrates adenosine 5' phosphosulfate (APS) and luciferin.

Step 3

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

Step 4

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 in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by CCD sensors and seen as a peak in the raw data output (Pyrogram®). The height of each peak (light signal) is proportional to the number of nucleotides incorporated.

Step 5

Apyrase continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.

Step 6

Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alpha-thio triphosphate (dATP α S) is used as a substitute for natural deoxyadenosine triphosphate (dATP) since it is efficiently used by DNA polymerase, but not recognized by luciferase. As the process continues, the complementary DNA strand is elongated and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.

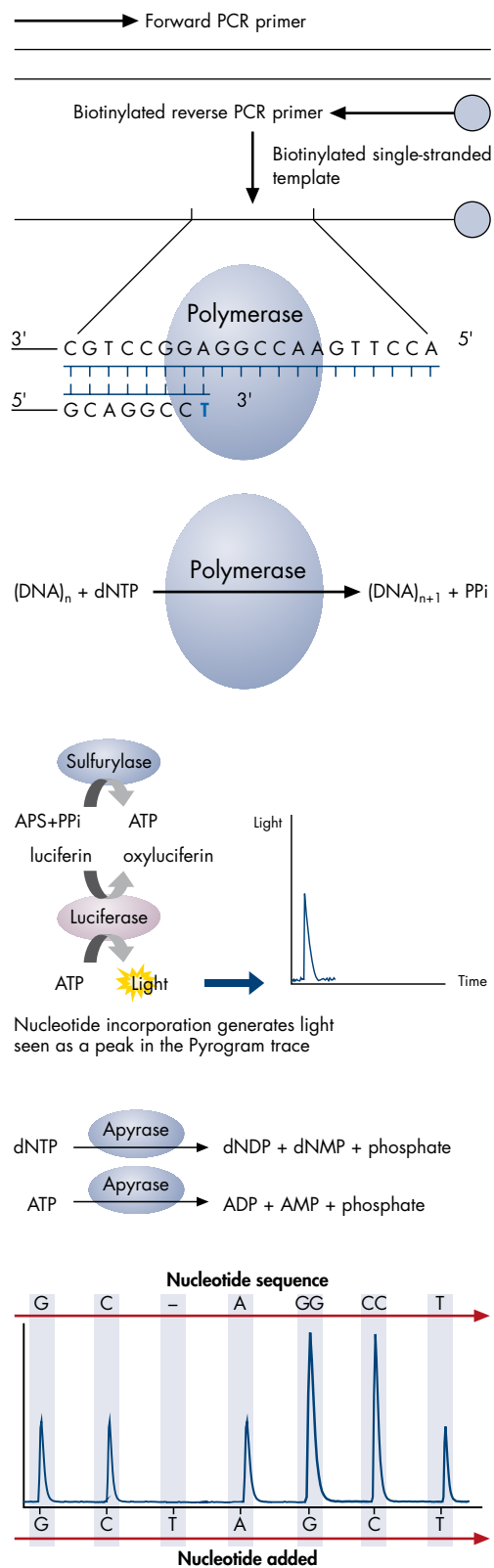


Figure 2. The Pyrosequencing reaction cascade.



DNA methylation analysis

Standard methylation analysis methods provide only qualitative or semi-quantitative data, which can lead to inaccurate conclusions regarding the effects of [epigenetic DNA methylation](#) on cell cycle and metabolism. Without quantification of methylation levels, it is not possible to distinguish physiologically relevant methylation from background methylation.

Pyrosequencing solves this limitation by generating highly reproducible quantification of methylation frequencies at individual consecutive CpG sites (Figures 3 and 4). As such, Pyrosequencing can detect and quantify even small changes in methylation levels. Other valuable features include the inherent quality control afforded by the sequence context of results and the ability to compare results to expected methylation levels. Built-in controls for the bisulfite treatment eliminate manual estimation of non-converted DNA levels and prevent false-positive methylation detection, thereby ensuring the reliability of results.

These features have established Pyrosequencing as the gold standard for DNA methylation analysis. This technology has been used to correlate DNA methylation to 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 (1).

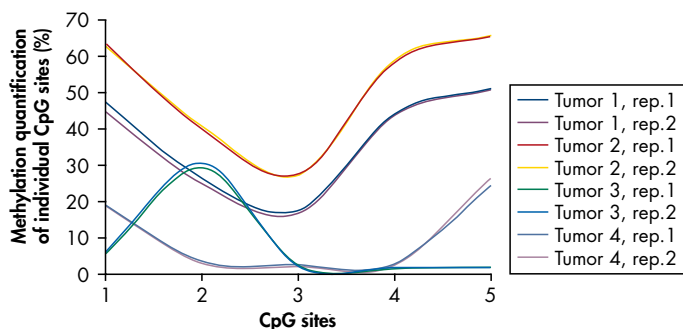


Figure 3. Pyrosequencing analysis of CpG methylation pattern in the RASSF1A gene. Variation in methylation levels at five different CpG sites in four tumor samples. Sample replicates were run on different days and still show high repeatability.

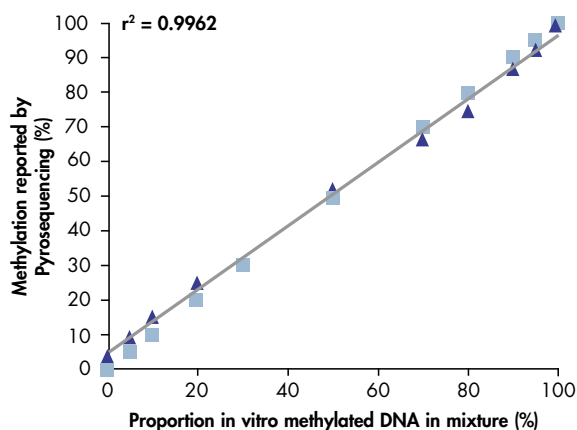


Figure 4. Linearity of methylation quantification by Pyrosequencing. PCR products from varying mixtures of unmethylated genomic DNA and methylated DNA (EpiTect Control DNAs) were analyzed by Pyrosequencing. A tight correlation between the known percentage of methylated DNA in the mixtures (■) and the methylation percentage reported by Pyrosequencing (▲) was observed ($r^2 = 0.9962$). The graph represents the quantification of methylation at a single CpG site in the p16 gene.



Valuable quality control of bisulfite conversion

To characterize the methylation status of a DNA sequence via Pyrosequencing, the DNA is first incubated with sodium bisulfite. As a result, unmethylated cytosine residues are converted into uracil while methylated cytosines remain unchanged, giving rise to two different sequences that can be distinguished. An internal control for bisulfite treatment is incorporated into analysis. Cytosines that are not followed by guanine in template sequences are not methylated, and should therefore be converted to thymine by bisulfite treatment and PCR (Table 1). Full bisulfite conversion is confirmed if all templates show thymine and no cytosine in these positions. With unique DNA Protect technology, QIAGEN [EpiTect® Bisulfite Kits](#) facilitate complete conversion and minimal degradation of the treated DNA.

Table 1. Sequences resulting from bisulfite conversion and PCR

	Original sequence	After bisulfite treatment	After PCR amplification
Unmethylated DNA	A-C-G-T-C-G-T-C-A	A-U-G-T-U-G-T-U-A	A-T-G-T-T-G-T-T-A
Methylated DNA	A-C-G-T-C-G-T-C-A	A-C-G-T-C-G-T-U-A	A-C-G-T-C-G-T-T-A

Flexible analysis of methylation patterns

The analysis of methylation status exploits the quantitative nature of Pyrosequencing data. Unlike Sanger sequencing, the peak heights in the resulting Pyrogram report the ratio of cytosine to thymine at each analyzed CpG site, which reflects the proportion of methylated DNA. Assay design is flexible — it can be performed in forward or reverse orientations, on either the top or the bottom strands. In addition, contiguous CpG sites are analyzed independently and within the same run, which enables assessment of sequence-wide methylation patterns while retaining details of position-specific methylation (Figure 5).

Sequence to analyze:

C/TGTTTGC/TGTTTC/TGAC/TGTTT/TGAGGTTTTC/TGC/TGGTGC/TGATC/TGTT

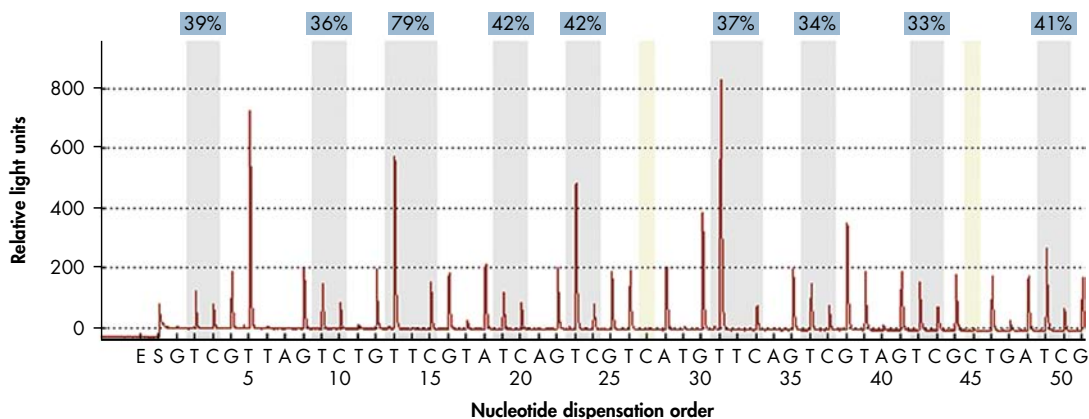


Figure 5. Analysis of multiple contiguous CpG sites. Methylation at nine independent CpG sites (highlighted in gray) is quantified in a single Pyrosequencing run. Position-specific information in the context of an analyzed sequence presents broad sequence methylation patterns. Note the built-in quality control sites (highlighted in yellow) consisting of cytosines converted to thymines, demonstrating full bisulfite conversion of the treated DNA.



Insertion-deletion and SNP analysis

[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 mutations; finally, analysis of forensic DNA evidence relies on accurate detection of sequence variation.

Many mutation types are utilized for these applications and new markers are continuously being validated in areas such as epidemiology, pharmacogenetics, and animal husbandry. Correspondingly, a variety of analysis methods are used to detect this genetic variability. This can complicate comparison of results obtained by different methods and different researchers. Ideally, a single technology could be adapted for all applications to standardize results. Pyrosequencing offers precisely that versatility. Because of the flexibility of primer placement in Pyrosequencing reactions, virtually all genetic markers, those currently used and those to be identified, can be assayed. Alleles of variable loci are accurately quantified, and heterozygosity is easily resolved (Figure 6 and 7). In addition, because Pyrosequencing delivers sequence information, various types of genetic variation can be evaluated — insertion-deletions, single nucleotide polymorphisms, single tandem repeats, and variable gene copy number — and it is possible to assay several contiguous sequence variants in a single run.

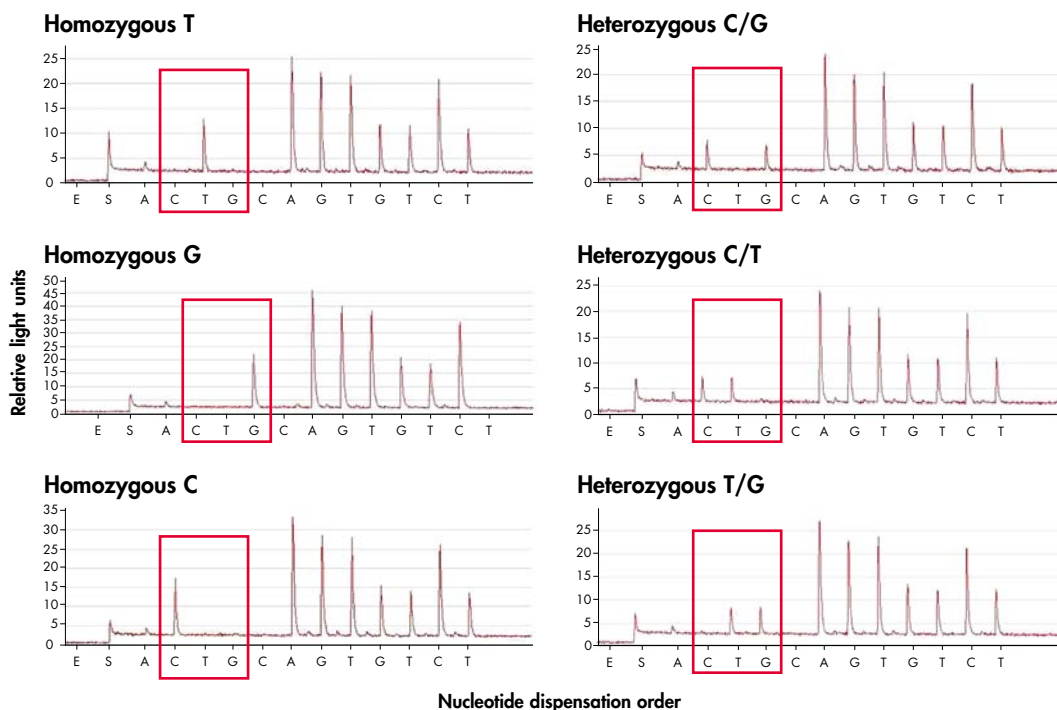


Figure 6. Analysis of a tri-allelic SNP. Detection of tri- and tetra-allelic SNPs can be difficult with commonly used methods. This series of Pyrograms illustrates the ease of Pyrosequencing based detection of a tri-allelic SNP (red outline). **C**, **T** and **G** are serially dispensed in the Pyrosequencing reaction and only the incorporated nucleotides will elicit a signal peak. The result is a different peak pattern for homozygous samples of each allele (three Pyrograms on the left) or compound peak patterns for heterozygous samples (three Pyrograms on the right).



Versatility at your fingertips

Regardless of the marker or mutation being analyzed, preparation of templates for Pyrosequencing and the subsequent analysis of the resulting sequence information are quick and easy, saving time and valuable resources. However, the strength of Pyrosequencing for genetic testing lies in the elegance of its output. Because results are simply the true sequence of the DNA in a sample, the user can examine multiple mutation sites within a specified region, and even multiple variation types, all in the same run (Figure 8). Furthermore, the straightforward results are easily interpreted. Pyrosequencing enables de novo sequencing which, coupled with the built-in control afforded by the sequence surrounding the variable site, is a guaranteed way of validating newly identified markers. The high throughput facilitates rapid compilation of the population data needed to establish reference databases for these markers. This feature makes Pyrosequencing a powerful and versatile tool for the development of pharmacogenetic and forensic markers (2).

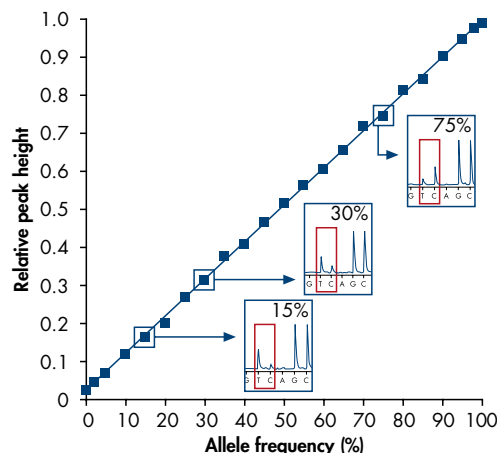


Figure 7. Quantitative mutation analysis. Pyrogram peak heights are proportional to the frequency of an allele in the sample. Therefore, it provides accurate measures of the proportion of, for example, a mutation in a blood sample.

Sequence to analyze:

CGTGGGTG[ATCTGCC]TGC ACTY TGGGATA

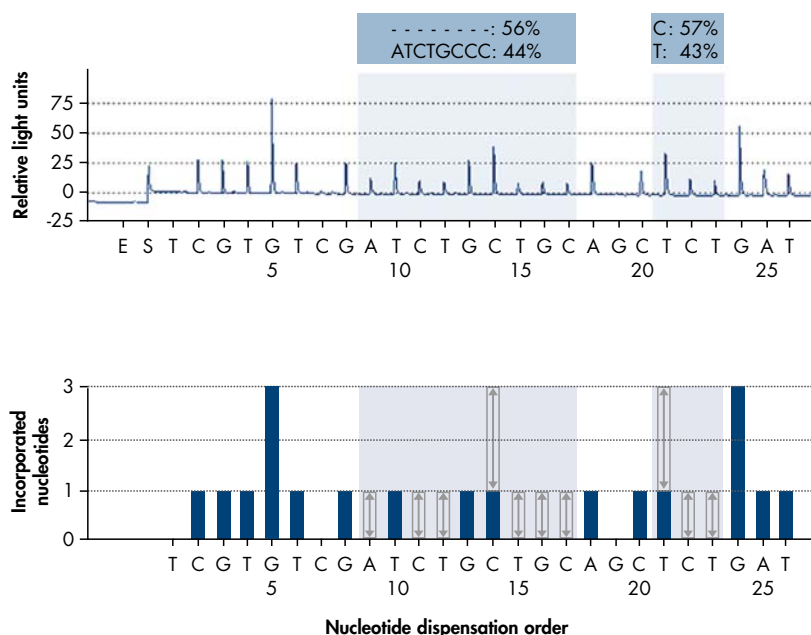


Figure 8. Two mutation types quantified in a single Pyrosequencing reaction. Pyrogram of a DNA sequence featuring an insertion-deletion mutation (ATCTGCC) and a somatic mutation involving a single base pair substitution (C vs. T). The variable regions are highlighted in blue and the allele frequencies are given above the indicated sites. The histogram (lower graph) indicates 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.



Microbial identification and drug resistance typing

Sequence information provides reliable data for microbial genotyping applications. However, standard methods used to assess discriminatory regions of microbial genes can be time-consuming, may require species-specific probes or gel electrophoresis, or are susceptible to the presence of unknown mutations (e.g., hybridization). Pyrosequencing is a rapid and accurate alternative method. Because this technology [sequences by synthesizing](#) new copies of the DNA template, the results provide unambiguous information since users can check the sequence surrounding the variable site to ensure that the correct DNA region has been analyzed.

Identify multiple species in one run and with one primer set

Unlike hybridization techniques, Pyrosequencing allows the identification of a large number of species using a single conserved sequencing primer (Figure 9). Consequently, DNA extracted from multiple microbe species can be sequenced in the same Pyrosequencing run. PyroMark Identifire SW 1.0 facilitates the compilation of a local sequence database against which imported Pyrosequencing output are rapidly aligned. The raw data, matched hits, and percent concordance of each hit are presented in detailed identification reports.

These reliable data are provided within short timeframes. In less than one hour, Pyrosequencing reads a discriminatory stretch of DNA of up to 96 samples in parallel (Figure 10). Depending on assay design, the sequence can be used to discriminate microbial species, types and strains, or detect genetic mutations that confer resistance to antibiotics and antiviral drugs.



Figure 9. 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).



Tracking drug resistance development

Multiple samples can be concurrently assayed for common drug resistance mutations (Figure 11). Since Pyrosequencing reports the actual sequence of the locus, a single assay can detect a range of possible mutations including new and unexpected mutations. Instrumentation and optimization of assays make Pyrosequencing technology routinely capable of detecting sequence variants present at frequencies as low as 5%, and potentially lower for some targets. Furthermore, heterogenic variation among several gene copies, which accounts for different resistance patterns, is reliably quantified. Visualizing the mutation in the context of the DNA sequence permits the user to verify the analysis. Coupled with the short time to obtain results and the ease of use, this analysis flexibility and detection sensitivity make Pyrosequencing an informative complement or even alternative to culture-based and hybridization-based resistance screening.

A growing library of species-specific assays

While broad-range identification strategies using signature sequences in well-characterized hypervariable regions of microbial genes have been described, there are a growing number of assays that specifically target commonly studied pathogenic species and strains of bacteria, viruses, and fungi.

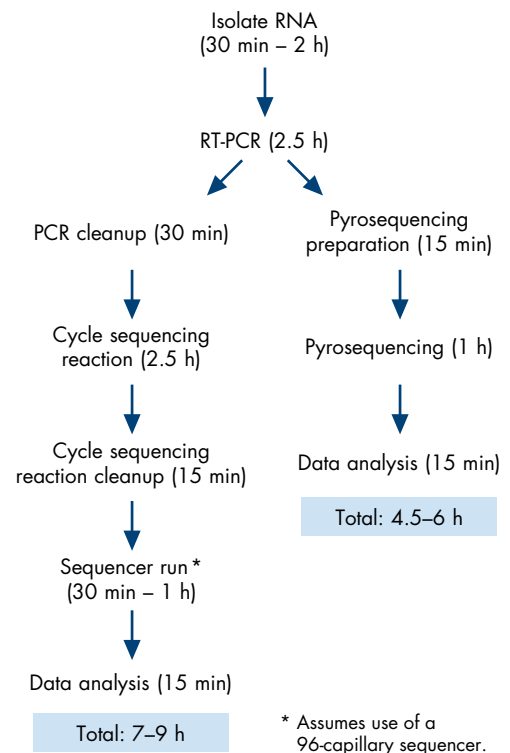


Figure 10. Time saving afforded by Pyrosequencing.

From RNA isolation to data analysis, with Pyrosequencing detection of resistance-conferring mutations in a RNA virus can easily be completed within a single workday. Times given reflect sequencing of a 44 base-long region in 96 viral samples.

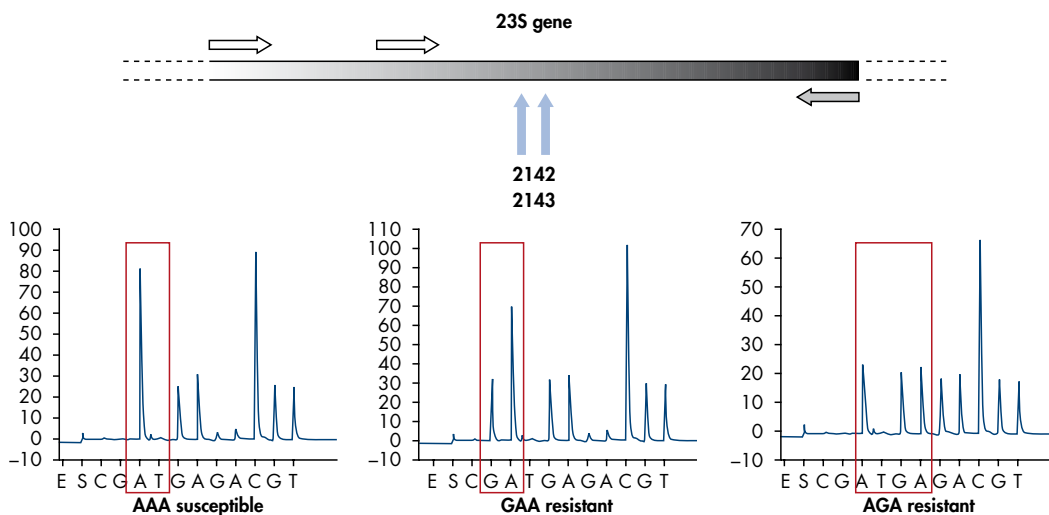


Figure 11. Analysis of mutations in the 235 genes that confer antibacterial resistance in *Helicobacter pylori*.

PyroMark Q24 — small footprint, great versatility

Pyrosequencing of up to 24 samples for a multitude of applications is the strength of the [PyroMark Q24](#). PyroMark Q24 Software offers three assay types to accommodate your research needs — allele quantification (AQ), methylation analysis (CpG), and sequence analysis (SQA). Thus, in minutes you can characterize DNA methylation patterns, quantify allele frequencies, detect rare mutations, perform de novo sequencing, test for markers linked to specific phenotypes, and assay for disease-related mutations. This compact sequencing platform occupies little workbench space and yet streamlines all workflow steps, from PCR products to sequence variant detection and quantification, so that several runs can be completed in a workday. Furthermore, assays designed on the PyroMark Q24 are compatible with the large-format PyroMark instruments, giving you the option to validate new assays on this smaller platform and then upgrade to high throughput.

A screen on the instrument displays real-time sequencing information for a selected well as samples are processed, so you can track the progress of a run. All collected data are stored simultaneously on the instrument hard drive and on a USB stick. This allows you to transfer run files to any computer with an installed copy of PyroMark Q24 Software for analysis.

In addition to the sequencing instrument and software, the PyroMark Q24 System features a [vacuum workstation](#) to rapidly prepare the single-stranded DNA templates for the Pyrosequencing reactions. The system also includes [PyroMark Gold Q24](#) Reagents that optimize Pyrogram peak clarity, and a variety of test kits of QIAGEN Quality® (Figure 15).

If the trade-off between workbench space, time limitation, and application diversity is an issue in your lab, the PyroMark Q24 offers an optimal solution to meet the needs of varied research and testing programs.



Figure 15. PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation — a complete Pyrosequencing system.