Pyrosequencing in genotyping and epigenetic studies

Gerald Schock, PhD.
Associate Director Pyrosequencing
QIAGEN GmbH
1. Introduction into Pyrosequencing Technology and Workflow
2. Pyrosequencing for Quantitative Mutation Analysis and SNP genotyping
3. Pyrosequencing for Methylation Analysis and Verification of Genomewide Data
4. Summary
5. PyroMark Instruments and Kits
Pyrosequencing at a glance

Variety of applications addressed by Pyrosequencing

- **Methylation Studies**
  - Quantify methylation level of multiple CpG sites in one assay

- **SNP Confirmation**
  - Di-, tri & tetra SNPs in up to 10 x 96 sample throughput format

- **Cancer Mutations**
  - Detect and quantify complex mutations

- **Microbial ID**
  - Identification and sub-typing of varies microbial organism

- **Forensics**
  - Y-STR markers and SNPs, tissue specific methylation detection

- **Resistance Typing**
  - Detect and quantify complex mutations leading to drug resistance

- **Biomarker verification**
  - Validation & verification of GWAS & NGS data
## Traditional Sequencing vs Pyrosequencing

<table>
<thead>
<tr>
<th></th>
<th>Traditional Sequencing</th>
<th>PyroMark Pyrosequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Application</strong></td>
<td>Whole genome sequencing&lt;br&gt;Research for unknown genes&lt;br&gt;Sequencing of long stretches of DNA</td>
<td>Detection of short sequences of DNA for&lt;br&gt;SNP (Mutation) Analysis&lt;br&gt;Methylation Analysis&lt;br&gt;Microbial Identification</td>
</tr>
<tr>
<td><strong>Starting material</strong></td>
<td>Unknown sequences</td>
<td>Known sequences (but with unknown mutations or methylation)</td>
</tr>
<tr>
<td><strong>Regulatory</strong></td>
<td>No standardized system</td>
<td>Commercial available kits</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>6 h up to several days</td>
<td>~ 30 – 60min</td>
</tr>
</tbody>
</table>
Pyrosequencing at a glance

Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle*

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- 4 enzymes present in the system at all time
  - DNA-Polymerase
  - ATP-Sulfurylase
  - Luciferase
  - Apyrase
- Only one nucleotide (dNTP) is added at a time


Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Nucleotide incorporation by DNA-Polymerase generates Pyrophosphate (PPI)


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**The Pyrosequencing reaction**

- Nucleotide incorporation by DNA-Polymerase generates Pyrophosphate (PPI)
- Pyrophosphate(PPI) is converted into ATP by ATP-Sulfurylase


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Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Nucleotide incorporation by DNA-Polymerase generates Pyrophosphate (PPi)
- Pyrophosphate(PPi) is converted into ATP by ATP-Sulfurylase
- ATP is converted into light by Luciferase


Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
Pyrosequencing at a glance

Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- The light is seen as peak in the Pyrogram trace


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Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Excess nucleotides are degraded by Apyrase before addition of the next base


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Pyrosequencing at a glance

Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Light signals are proportional to the amount of nucleotides incorporated
  - signals are automatically converted into a sequence information

G A A A C C ...

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Pyrosequencing at a glance

Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Light signals are proportional to the amount of nucleotides incorporated
  - Intensity of the signals are used for quantification of sequencing results

Di-, tri- and tetra allelic mutations / SNP

Pyrosequencing at a glance

Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Light signals are proportional to the amount of nucleotides incorporated
  - Intensity of the signals are used for quantification of sequencing results


Insertions / Deletions
Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

The Pyrosequencing reaction

- Light signals are proportional to the amount of nucleotides incorporated
- Intensity of the signals are used for quantification of sequencing results

DNA methylation of multiple CpG sites

Pyrosequencing at a glance

Principle and key features

Based on SEQUENCING-by-SYNTHESIS Principle

- Stepwise synthesis of DNA by addition of nucleotides
- Special enzyme cascade generates the light signal upon incorporation of nucleotides

QIAGEN Pyrosequencing:
Real-time, quantitative DNA sequencing without gels, labels, or probes

- Precise
- Fast (results in 10 – 60 min post PCR)
- Flexible in
  - Throughput (1 – 96 samples)
  - Assay design (ADSW, validated, pre-designed or custom assays)

Pyrosequencing is the method of choice whenever short or medium DNA sequences need to be analyzed with high precision and in a quantitative manner

Pyrosequencing workflow

Assay Design
- ~ 5 min

PCR
- ~ 2h

Sample prep
- ~ 15 min

Pyrosequencing
- ~ 10-60 min

PyroMark Assays Design SW 2.0
PyroMark Custom Assays

PyroMark PCR Kit
PyroMark OneStep RT-PCR Kit

PyroMark Q24/Q96 Vacuum Prep Workstation

PyroMark Q24
PyroMark Q24 MDx
PyroMark Q96 ID
PyroMark Q96 MD

Pyrosequencing provides quantitative results in a sequence context in as little as 1 hour after PCR
**Assay design**

- Two PCR primers (one is biotinylated)
  - Amplification of a region of 70 bp – 500 bp (70 - 300 bp for bisulfite converted DNA)
    - PyroMark PCR Kit / PyroMark OneStep RT-PCR Kit
  - Biotin-labeled strand is isolated using Vacuum Prep Workstation

- Sequencing primer
  - Placed in front of region of interest
  - Annealed to single stranded DNA before Pyrosequencing reaction
Pyrosequencing Workflow

Assay design

Custom Assay Design
- PyroMark Assay Design Software 2.0
- PyroMark Custom Assays\textsuperscript{NEW}

Pre-designed Assays
- PyroMark CPG Assays\textsuperscript{NEW} (genomewide coverage of CPG islands)
- PyroMark RUO Tests (selected CPG or mutation targets)
- Therascreen Pyro Assays (KRAS, BRAF, EGFR, etc.)
Custom Assay Design
- PyroMark Assay Design Software 2.0
- PyroMark Custom Assays
  - Dedicated assay format - No tedious optimization of primer concentrations
  - Suitable for any type of Pyrosequencing analysis (epigenetics, mutation, etc.)
Pre-designed Assays

- PyroMark CpG Assays (genomewide coverage of CpG islands)
  - Dedicated assay format - No tedious optimization of primer concentrations
  - Primers are checked against the entire bisulfitome - Highest PCR specificity
  - Include built-in bisulfite conversion controls if the sequence allows

1. Search

2. Select

3. View gene map
PyroMark CpG Assays

Release Information

- Human Ver. 1.3 (Nov 2010)
  - Number of assays: over 30,000
  - Number of CpG Islands with assay: ~12,000 (~80%)

- Mouse Ver. 1.0 (April 2011)
  - Number of assays: over 30,000
  - Number of CpG Islands with assay: ~11,000 (>80%)

- Rat Ver. 1.0 (Dec 2011) NEW
  - Number of assays: over 24,000
  - Number of CpG Islands with assay: ~7,500

- Human Ver. 2.0 in preparation
- Common NGS targets planned
Pyrosequencing Workflow

PCR

**PCR / RT-PCR**
- Can use any PCR machine
- PyroMark PCR Kit / PyroMark OneStep RT-PCR Kit
- Amplify relevant region by PCR (70 - 500 bp)
- Can use very short PCR products if desired (i.e. degraded DNA)
- One primer has to be biotinylated

"If you can run a PCR, you can sequence with Pyrosequencing"
Jon Jonasson, University Hospital, Linköping, Sweden
10 ng template DNA was amplified using the PyroMark PCR Kit, in parallel with PCR master mixes from Suppliers R, I, and AII. Primers were specific for Cyclin A1, BARHL1, or DNMT3b, and an annealing temperature gradient ranging from 48.4°C to 60.1°C was used. M: 50 bp ladder.

Superior PCR results with QIAGEN PyroMark PCR Kit
After PCR amplification the PCR product is transferred to a new plate containing a suspension of streptavidin coated sepharose beads in a buffer that facilitates the binding from biotin to streptavidin.

Under mixing the biotinylated amplicons bind to the sapharose beads. This way the sample DNA can easily be manipulated in the next steps by immobilizing the sepharose beads.

Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
The next steps take place on a PyroMark Vacuum Workstation which consists of a series of troughs containing solutions that will denature and cleanse the sample DNA. Vacuum applied to the vacuum tool captures the sepharose beads on the surface of the filter probes.

The plate with biotinylated amplicons bound to sepharose beads is placed on the Vacuum Workstation. After turning on the vacuum the vacuum tool is gently lowered into the plate wells.

Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
Pyrosequencing Workflow

Sample preparation

- As the sample fluid flows through the filter probes, sepharose beads are caught on the filter probes.

- The vacuum tool as transferred to the first trough containing ethanol.

Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
In the ethanol any PCR by-products that were not removed while capturing the sepharose beads are rinsed away.

The vacuum tool is then lowered into the trough with denaturation solution. As the strands of DNA separate, the strand lacking biotin, now no longer bound to the sepharose beads, is washed away.

Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
Sample preparation

- A final rinse in the trough containing wash buffer flushes away denaturation solution and any remaining non-biotinylated strands of DNA.

- To completely clear the filter probes of fluid, the vacuum tool is briefly raised to over 90 degrees vertical before releasing the now single-stranded DNA templates into the PyroMark plate.
Sample preparation

- The filter probes are lined up with the wells of the PyroMark plate and the vacuum is switched off before lowering the vacuum tool into the plate containing the sequencing primers.

- No longer held by vacuum pressure the sepharose beads and attached single stranded DNA enter the solution with the sequencing primers.

- Having released the sepharose beads into the PyroMark plate the Vacuum Tool is rinsed thoroughly and returned to its storage position.

Watch the complete Pyrosequencing animation at: www.qiagen.com/overview/pyrosequencingvirtual.aspx.
The Pyromark plate is transferred to a heating block. Template DNA and sequencing primers are heated to 80 degrees Celsius for two minutes and then allowed to cool to room temperature. During cooldown, the primers anneal to the single-stranded templates.

The final product of this fast, straightforward process is single-stranded DNA specifically primed for sequencing. This is the starting material for the Pyrosequencing reaction.
The Pyrosequencing Workflow

The Pyrosequencing reaction

- One nucleotide (dNTP) is added at a time
- Nucleotide incorporation generates Pyrophosphate (PP$_i$)
- Pyrophosphate (PP$_i$) is converted into light seen as peak in the Pyrogram trace
- Excess nucleotide is degraded before addition of the next base.

The amount of generated light is proportional to the amount of incorporated nucleotides
# Agenda

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| 3 | Pyrosequencing for Methylation Analysis and Verification of Genomewide Data |
| 4 | Summary |
| 5 | PyroMark Instruments and Kits |
PyroMark for Genetic Testing

One platform for many genetic analyses

- Mutation analysis
  - Known and unknown positions
    - Point mutations
    - Multiple mutations
    - Insertions/Deletions

- SNP analysis
  - Di-, tri- and tetra allelic SNPs
  - Multiple SNPs

- Allele Quantification
  - SNP frequency
  - Di-, tri- and tetra allelic mutations
PyroMark for Genetic Testing

Ins/Del & Allele Quantification analysis in the same run

Dispensation order: CGTGGGTG[ATCTGCCC]TCACCTYGGGATA

relative light units

-25 0 25 50 75

E S T C G T G T C G A T C G C T G A C T G C A T G 5 10 15 20 25

incorporated nucleotides

0 1 2 3

T C G T G T C G A T C G C A C T C G A C T C G A T 5 10 15 20 25

Nucleotide dispensation order

ATCTGCCC: 44%
C: 57%
T: 43%
PyroMark for Genetic Testing

One platform for many genetic analyses

- Mutation analysis
  - Known and unknown positions
    - Point mutations
    - Multiple mutations
    - Insertions/Deletions

- SNP analysis
  - Di-, tri- and tetra allelic SNPs
  - Multiple SNPs

- Allele Quantification
  - SNP frequency
  - Di-, tri- and tetra allelic mutations
Detection of tri-allelic SNPs
PyroMark for Genetic Testing

Quantitative peak heights to measure allele frequencies

Allele frequency (%)

0% 20% 45% 70%
2% 25% 50% 75%
5% 30% 55% 80%
10% 35% 60% 85%
15% 40% 65% 90%
50%
85%
Quantitative peak heights to measure allele frequencies

Even as little as 2% of one allele in 98% of the other could be detected
One platform for many genetic analyses

- **Mutation analysis**
  - Known and unknown positions
    - Point mutations
    - Multiple mutations
    - Insertions/Deletions

- **SNP analysis**
  - Di-, tri- and tetra allelic SNPs
  - Multiple SNPs

- **Allele Quantification**
  - SNP frequency
  - Di-, tri- and tetra allelic mutations
Detecting all mutations in the KRAS gene

- Erbitux and Vectibix are known to be effective only when the human KRAS gene is not mutated.
- Mutation in the KRAS gene occurs in around 35% of all patient samples
- PyroMark KRAS Kit detects all known mutations

Example for single base mutations

<table>
<thead>
<tr>
<th>Codons</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>GGT</td>
<td>GGC</td>
</tr>
<tr>
<td></td>
<td>TGT</td>
<td>AGC</td>
</tr>
<tr>
<td></td>
<td>GCT</td>
<td>CGC</td>
</tr>
<tr>
<td></td>
<td>AGT</td>
<td>TGC</td>
</tr>
<tr>
<td></td>
<td>GAT</td>
<td>GAC</td>
</tr>
<tr>
<td></td>
<td>GCT</td>
<td>GTC</td>
</tr>
<tr>
<td></td>
<td>GAT</td>
<td>CAT</td>
</tr>
</tbody>
</table>

Normal: GGT

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

mt: GCC

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>mt</td>
<td>44%</td>
<td>0%</td>
<td>56%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Pyrosequencing technology provides quantitative results and sequence information for sensitive detection of all key KRAS mutations.
## Agenda

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3. Pyrosequencing for Methylation Analysis and Verification of Genomewide Data
4. Summary PyroMark Instruments and Kits
Why is bisulfite treatment required?

Bisulfite converts all unmethylated cytosines into uracil

- Methylated cytosines in CpG sequences remain unaffected
- Uracil is replaced by thymine during PCR reaction

Bisulfite converted DNA can then be analyzed by Pyrosequencing

Recommended products:

- EpiTect Plus DNA Bisulfite Kit (59124) – for gDNA
- EpiTect Plus FFPE Bisulfite Kit (59144) – for FFPE tissue slices
- EpiTect Plus LyseAll Bisulfite Kit (59126) – for cells, fres/frozen tissue and whole blood
Pyrosequencing for DNA Methylation Analysis

A range of analysis possibilities

**Any single CpG site**

A4: TAYGGTTTGTAT

- One or several genes at a time (up to 96 different assays in a single run)
- The sequence context confirms that the assay worked properly

**Multiple consecutive CpG sites**

A1: TYGGGATYGGGTGTTAGTTYGTTGTTGGATTTTTTYGGGAYTTTGAAGGAGAAAGGGAAGGTGTTGG

- Global methylation level
  - Estimate the global methylation levels using repetitive elements (LINE1)
Built-in quality control of bisulfite treatment

RASSF1A gene

Before bisulfite treatment
CCGACATGGCCCGTGTTGGGCCGCTGCTTTGGGCGCTAGCAAGCGCGGGCCGCGGGG

Analyzed sequence
TYGATATGGTTYGTTGTTGGTGTTYGTTTGGGTYGTTACGTAAGYGGGTGGYGGYGGGT

Any C not followed by a G gives bisulfite QC
**Pyrosequencing for DNA Methylation Analysis**

**Built-in quality control of bisulfite treatment**

**Sequence to be analyzed:**
AGTTACGACA

**After Bisulfite conversion:**
AGTTACGA and AGTTACmGACA

**Analyzed sequence:**
AGTTAT/C/CGATA

**Biotinylated PCR strand:**
TCATAGCTAT...

**Nucleotides added:**

**Built-in Quality control: Successful Bisulfite conversion**

27%
DNA Methylation Analysis

Technologies used at various stages within a project

Screening / Identification / Discovery

Genome-wide analysis

- Illumina Genome Analyzer
- Life Technology ABI - Solexa
- Roche 454 Roche 454 Junior
- PacBio
- Oxford Nanopore Technologies
- Life Technology IonTorrent

Regional analysis

- EpiTect Methyl qPCR**
- High Resolution Melting (HRM)

Verification / Functional analysis

Single/Multiple site analysis

- MSP*
- MethyLight
- Pyrosequencing
- (Bisulfite) Sanger Sequencing
- MassSpec Sequenom EpiTyper

Combining 454 sequencing and Pyrosequencing for verification of genome-wide methylation results.

*MSP: Methylation-Specific PCR; ** EpiTect Methyl qPCR formerly SABiosciences Methyl Profiler qPCR
Verification of Genome Wide Methylation Results

Cross validation of 454 sequencing and Pyrosequencing

Systematic cross-validation of 454 sequencing and pyrosequencing for the exact quantification of DNA methylation patterns with single CpG resolution

*BMC Biotechnology* 2011, 11:6  

Anna Potapova¹, Cord Albat¹, Britta Hasemeier¹, Katrin Häußler², Stella Lamprecht³, Sebastian Suerbaum³, Hans Kreipe¹, Ulrich Lehmann¹

¹Institute of Pathology, Medizinische Hochschule Hannover, D-30625 Hannover, Germany  
²Institute of Biometrics, Medizinische Hochschule Hannover, D-30625 Hannover, Germany  
³Institute of Medical Microbiology and Hospital Epidemiology, Medizinische Hochschule Hannover, D-30625 Hannover, Germany

“Overall, the mean methylation levels obtained by pyrosequencing and 454 sequencing showed an excellent correlation for every gene in all samples”

“Therefore, in our opinion conventional pyrosequencing and 454 sequencing are not competing but complementary methodologies fulfilling different functions in the field of DNA methylation analysis.”
Verification of Genome Wide Methylation Results

Cross validation of 454 sequencing and Pyrosequencing
"Excellent concordance between both methods"

Potapova et al.
BMC Biotechnology 2011, 11:6
Cross validation of 454 sequencing and Pyrosequencing

Regression Analysis

Regression analysis of the methylation levels of all individual CpG sites (n=869) obtained independently by the two methods revealed a very good concordance.
Combining methylation arrays and Pyrosequencing for verification of genome-wide methylation results

*MSP: Methylation-Specific PCR; ** EpiTect Methyl qPCR formerly SABiosciences Methyl Profiler qPCR
DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients

Michael Volkmar¹, Sarah Dedeurwaerder¹, Daniel A Cunha², Matladi N Ndlovu¹, Matthieu Defrance¹, Rachel Deplus¹, Emilie Calonne¹, Ute Volkmar³, Mariana Igoillo-Esteve², Najib Naamane², Silvia Del Guerra⁴, Matilde Masini⁴, Marco Bugliani⁴, Piero Marchetti⁴, Miriam Cnop²,⁵, Decio L Eizirik² and François Fuks¹,*

¹Laboratory of Cancer Epigenetics, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium, ²Laboratory of Experimental Medicine, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium, ³Department of Molecular Evolution, Institute for Cellular and Molecular Biology (IZMB), University of Bonn, Bonn, Germany, ⁴Metabolic Unit, Department of Endocrinology and Metabolism, University of Pisa, Pisa, Italy and ⁵Division of Endocrinology, Erasmus Hospital, Brussels, Belgium

"Using PyroMark Q24 we've successfully established Pyrosequencing for the verification of Infinium DNA Methylation data without the need of tedious cloned Bisulfite Sanger Sequencing" M. Volkmar
Verification of Genome Wide Methylation Results

Bisulfite Sanger Sequencing vs. Pyrosequencing


Infinium:: HumanMethylation27
BS: Bisulfite Sequencing
Verification of Genome Wide Methylation Results

Bisulfite Sanger Sequencing vs. Pyrosequencing

Infinium:

- Using PyroMark Q24 we've successfully established Pyrosequencing for the verification of Infinium DNA Methylation data without the need of tedious cloned Bisulfite Sanger Sequencing" M. Volkmar

Volkmar et al. (2012)
DNA methylation profiling of type 2 diabetic islets, EMBO Journal 2012; 1-22

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Bisulfite Sanger Sequencing vs. Pyrosequencing


Infinium:: HumanMethylation27
BS: Bisulfite Sequencing
BPS: Bisulfite Pyrosequencing

Methylation values obtained by Infinium assay and BPS show high correlation (Pearson’s correlation coefficient R=0.927)
PyroMark CpG Assays

Release Information

- **Human Ver. 1.3 (Nov 2010)**
  - Number of assays: over 30,000
  - Number of CpG Islands with assay: ~12,000 (~80%)

- **Mouse Ver. 1.0 (April 2011)**
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- **Rat Ver. 1.0 (Dec 2011) **
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- **Human Ver. 2.0 in preparation**
- **Common NGS targets planned**
Introduction into Pyrosequencing Technology and Workflow

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Summary
PyroMark Instruments and Kits
Sample & Assay Technologies

PyroMark Instrument Platforms

Three Instruments for Pyrosequencing

PyroMark Q24

PyroMark Q96 ID

PyroMark Q96 MD
## PyroMark Instrument Overview

### Software functionality and application areas

<table>
<thead>
<tr>
<th></th>
<th>PyroMark Q24 &amp; PyroMark Q24 MDx</th>
<th>PyroMark Q96 ID</th>
<th>PyroMark Q96 MD</th>
<th>PyroMark Q96 MD Automated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Throughput</strong></td>
<td>1–24 samples</td>
<td>1–96 samples</td>
<td>1–96 samples</td>
<td>1–10x96 with automated option</td>
</tr>
<tr>
<td><strong>Running volume</strong></td>
<td>25 µl</td>
<td>40 µl</td>
<td>12 µl</td>
<td>12 µl</td>
</tr>
<tr>
<td><strong>PCR requirements</strong></td>
<td>5–10 µl (~0.5–3 pmol of product)</td>
<td>20–40 µl (2–4 pmol of product)</td>
<td>5–10 µl (0.5–1.5 pmol of product)</td>
<td>5–10 µl (0.5–1.5 pmol of product)</td>
</tr>
<tr>
<td><strong>Read lengths (estimates)</strong></td>
<td>SQA ~50 – 100 bp</td>
<td>SQA ~40 – 70 bp</td>
<td>SNP ~10 – 140 bp</td>
<td>SNP ~10 – 100 bp</td>
</tr>
<tr>
<td></td>
<td>SNP ~10 – 100 bp</td>
<td>SNP ~10 – 100 bp</td>
<td>SNP/AQ ~10 – 100 bp</td>
<td>SNP/AQ ~10 – 100 bp</td>
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<tr>
<td></td>
<td>AQ ~10 – 100 bp</td>
<td>AQ ~10 – 100 bp</td>
<td>AQ ~10 – 100 bp</td>
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<tr>
<td></td>
<td>CpG ~10 – 120 bp</td>
<td>CpG ~10 – 120 bp</td>
<td>CpG ~10 – 120 bp</td>
<td>CpG ~10 – 140 bp</td>
</tr>
<tr>
<td><strong>Main applications</strong></td>
<td>Genetic testing Epigenetics Microbiology</td>
<td>Genetic testing Epigenetics (*) Microbiology</td>
<td>Genetic testing Epigenetics*</td>
<td>Genetic testing Epigenetics* (SNP/AQ only in batch mode)</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>2% mutation 98% wt</td>
<td>2% mutation 98% wt</td>
<td>2% mutation 98% wt</td>
<td>2% mutation 98% wt</td>
</tr>
</tbody>
</table>

* Additional Software (SW) (PyroMark CpG SW) needed on MD instruments and on ID instruments with Q96 ID Application SW older than Version 2.5
Summary

**PyroMark instruments:**

- Unique detection and quantification platform technology
- Rapid, easy-to-use, and cost effective
- Suitable for analysis of fresh, frozen, fixed and paraffin-embedded specimens
- Quality assessment of individual sites and sequence context
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**Pyro Kits and assays for mutation and methylation analysis:**
- Pre-designed Assays for KRAS, BRAF, NRAS, EGFR & UGT1A1 mutation detection
- Pre-designed PyroMark CpG Assays for human, mouse, and rat
- Flexible Assay Design Software for virtually and target
Pyrosequencing Web Resource

http://www.qiagen.com/pyrosequencing
Pyrosequencing Web Resource

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Application Pages

Genetic Testing

Features
- Testimonials
- Scientific articles

Resources
- Pyrosequencing for quantitative genetic testing
- Strength of Pyrosequencing
- etc.

More Links
- Genotyping Resource site
- FAQs
- etc.

DNA Methylation Analysis

Features
- Testimonials
- Scientific articles

Resources
- Pyrosequencing for quantitative methylation analysis
- Strength of Pyrosequencing
- etc.

More Links
- Epigenetics Resource site
- FAQs
- etc.

Microbial Identification & Drug resistance typing

Features
- Testimonials
- Scientific articles

Resources
- Pyrosequencing for Microbial Identification & Drug resistance typing
- Strength of Pyrosequencing
- etc.

More Links
- FAQs
- etc.