Pyrosequencing™

New Standard in Epigenetic and Genetic Study

April 2010

Robert Rice, PhD.
Director Market Development, Asia-Pacific
Pyrosequencing

- Introduction
  - Pyrosequencing at a glance
  - Pre-screening with HRM
  - The principle of Pyrosequencing

- Three Applications for Pyrosequencing
  - Genetic Testing
  - Epigenetics
  - Microbial Analysis

- Three Instruments for Pyrosequencing
  - PyroMark Q96 ID
  - PyroMark Q96 MD /MD Automated
  - PyroMark Q24 / Q24 MDx

- Summary
Pyrosequencing at a glance
Applications that require quantification

- Pooling Strategies for Association/Linkage studies
- SNP Confirmation: >1% presence in a population indicates a true SNP
- Cancer Mutations: Presence of normal cells in tumor samples give mixed genotypes
- Methylation Studies: In combination with bisulphite treatment
- Polyploidy: Detection and assessment of SNPs in Polyploid organisms
- Heteroplasmy: Quantitation of mutated and wild type mt-DNA
High Resolution Melt - Prescreening

Prescreening with High Resolution Melt
### High Resolution Melting

<table>
<thead>
<tr>
<th>Features &amp; Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial, viral, fungal DNA/RNA</td>
</tr>
<tr>
<td>Specific &amp; sensitive</td>
</tr>
<tr>
<td>Closed-tube analysis after qPCR</td>
</tr>
<tr>
<td>Cost effective</td>
</tr>
<tr>
<td>High throughput</td>
</tr>
<tr>
<td>Uses same equipment as qPCR</td>
</tr>
<tr>
<td>Handling similar to qPCR</td>
</tr>
<tr>
<td>Can detect genetic variations</td>
</tr>
<tr>
<td>Allows further downstream sequencing</td>
</tr>
</tbody>
</table>

### Pyrosequencing

<table>
<thead>
<tr>
<th>Features &amp; Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial, viral, fungal DNA/RNA</td>
</tr>
<tr>
<td>Specific &amp; sensitive</td>
</tr>
<tr>
<td>Maximum sequence resolution</td>
</tr>
<tr>
<td>Highest confidence</td>
</tr>
<tr>
<td>Flexible throughput (1-96 samples)</td>
</tr>
<tr>
<td>Multiple answers with single run</td>
</tr>
<tr>
<td>De novo sequencing for new variants</td>
</tr>
<tr>
<td>Characterization includes detection</td>
</tr>
</tbody>
</table>

Choice and mix of methods depends on laboratory focus
**The critical instrumental parameters:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>Well-to-well temperature variation must be minimal for optimal reproducibility</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Set temperature must be equal to actual temperature</td>
</tr>
<tr>
<td>Equilibration time</td>
<td>Determines time lag to collect data</td>
</tr>
<tr>
<td>Ramping rate</td>
<td>Influences speed of analysis</td>
</tr>
<tr>
<td>Illumination</td>
<td>Must be identical for consistent well-to-well signals</td>
</tr>
<tr>
<td>No. of channels</td>
<td>Determines multiplexing capabilities</td>
</tr>
</tbody>
</table>

**The Rotor-Gene Q specifications:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>0.01 °C; 20x better than any other cycle r</td>
</tr>
<tr>
<td>Accuracy</td>
<td>0.25 °C, at the top of its class</td>
</tr>
<tr>
<td>Equilibration time</td>
<td>Zero seconds, as rotor spins continuously</td>
</tr>
<tr>
<td>Ramping rate</td>
<td>15-20 °C/sec, at the top of its class</td>
</tr>
<tr>
<td>Illumination</td>
<td>100% identical for each tube</td>
</tr>
<tr>
<td>No. of Channels</td>
<td>Up to 6 channels, at the top of its class</td>
</tr>
</tbody>
</table>
Prescreening with High Resolution Melt
Basic HRM principle: Five steps from PCR to result

Step 1: Amplification
Was the amplification successful?

Step 2: Melt curve analysis
Optional step to test amplification specificity

Step 3: Normalisation
Select suitable samples and analysis range

Step 4: Difference plot
Select the reference genotype

Step 5: Autocalling genotypes
unknowns will be either related to known genotypes or will be marked as variation

<table>
<thead>
<tr>
<th>SNP Class 1</th>
<th>SNP 1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Genotype</td>
</tr>
<tr>
<td>TT SNP 1.1</td>
<td>WT</td>
</tr>
<tr>
<td>Unknown WT</td>
<td>99.62</td>
</tr>
<tr>
<td>Unknown VT</td>
<td>99.95</td>
</tr>
<tr>
<td>Unknown WT</td>
<td>99.62</td>
</tr>
<tr>
<td>Unknown VT</td>
<td>99.95</td>
</tr>
<tr>
<td>TT SNP 1.1</td>
<td>Hetero</td>
</tr>
<tr>
<td>Unknown Hetero</td>
<td>99.97</td>
</tr>
<tr>
<td>Unknown Hetero</td>
<td>99.92</td>
</tr>
<tr>
<td>Unknown Hetero</td>
<td>99.02</td>
</tr>
<tr>
<td>Unknown Hetero</td>
<td>99.75</td>
</tr>
<tr>
<td>TT SNP 1.1</td>
<td>Mut</td>
</tr>
<tr>
<td>Unknown Mut</td>
<td>92.7</td>
</tr>
<tr>
<td>Unknown Mut</td>
<td>99.74</td>
</tr>
<tr>
<td>Unknown Mut</td>
<td>99.97</td>
</tr>
<tr>
<td>Unknown Mut</td>
<td>99.71</td>
</tr>
</tbody>
</table>
Advanced Statistical Data Analysis Workflow
Rotor-Gene ScreenClust HRM Software ONLY

Raw data → Normalisation → Residual plot

- Line-of best fit
- Differentiation
  - Averaging
  - Subtraction

Autocalling genotypes

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Cluster</th>
<th>Typicality</th>
<th>Prob - Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample 1</td>
<td>Wild Type</td>
<td>0.51018410</td>
<td>1.00000000</td>
</tr>
<tr>
<td>2</td>
<td>Sample 2</td>
<td>Wild Type</td>
<td>0.13595388</td>
<td>1.00000000</td>
</tr>
<tr>
<td>3</td>
<td>Sample 3</td>
<td>Wild Type</td>
<td>0.74975411</td>
<td>1.00000000</td>
</tr>
<tr>
<td>4</td>
<td>Sample 4</td>
<td>Wild Type</td>
<td>0.50040615</td>
<td>1.00000000</td>
</tr>
<tr>
<td>5</td>
<td>Sample 5</td>
<td>Wild Type</td>
<td>0.45418789</td>
<td>1.00000000</td>
</tr>
<tr>
<td>6</td>
<td>Sample 6</td>
<td>Wild Type</td>
<td>0.01705666</td>
<td>1.00000000</td>
</tr>
<tr>
<td>7</td>
<td>Sample 7</td>
<td>Wild Type</td>
<td>0.00030817</td>
<td>1.00000000</td>
</tr>
<tr>
<td>8</td>
<td>Sample 8</td>
<td>Wild Type</td>
<td>0.00100477</td>
<td>1.00000000</td>
</tr>
</tbody>
</table>

Clustering

Principal component analysis

Significantly more accurate and faster determination of mutations
Prescreening with High Resolution Melt
Macrolide resistance in *Mycoplasma pneumoniae*


**Target:** *Mycoplasma pneumoniae*

**Clinical Isolates:** 30 oropharyngeal/nasopharyngeal swabs

**Resistant Strains:** 5/100 with erythromycin resistance (2,048 fold)

**Template:** DNA

**Gene Region:** Domain V of 23S rRNA

**Amplicon Size:** 112 base pairs

**Mutation:** A2063G or A2064G

**Cycler:** Rotor-Gene Q
Various ratios of methylated and unmethylated DNA-APC

- Standard normalized melt curve
- Rotor-Gene HRM + EpiTect HRM PCR Kit
- Difference plot normalized to a 50% methylated sample

Excellent performance for quantitative methylation screening (fast & cost effective)
HRM is an emerging detection technology for fast and cost-effective genotyping

Outperforming any competition
- New Type-it HRM PCR Kit for fast and accurate HRM analysis on every cycler
- RotorGene Q and Screen Clust HRM Software: THE instrumentation solution in the market!

QIAGEN offers the most comprehensive portfolio for HRM analysis with unique features
- Most accurate
- Fast & Easy
- For many applications
  - SNPs, mutation detection, mutation scanning, pathogen detection

For ALL genotyping customers reliable results are fast and easy to achieve
- HRM beginners
- HRM customers with competitive equipment
- HRM Customers with Rotor-Gene equipment
Pyrosequencing at a Glance
Pyrosequencing at a glance

What is Pyrosequencing?

- Sequence based technology in real time
- Simple and robust
- No separation, gel, label or probes
- Flexible
  - in throughput
  - in assay design
  - in type of applications

Sequencing by Synthesis
Real-Time Pyrophosphate Detection for DNA Sequencing.
*Science* **281**:363-365

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

### Positioning of HRM and Pyrosequencing

<table>
<thead>
<tr>
<th>HRM</th>
<th>Pyrosequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost &amp; Throughput</td>
<td>Resolution</td>
</tr>
<tr>
<td>Handling &amp; Equipment</td>
<td>Accuracy</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Highest confidence</td>
</tr>
<tr>
<td>Quantitation</td>
<td>Quantitation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Simple Genotype</th>
<th>First choice HRM</th>
<th>First choice Pyrosequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>(low/medium sequence variability)</td>
<td>SNPs, mutations</td>
<td>Reliable results &amp; specificity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrosequencing added when independent verification needed (e.g. diagnostic setting)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Moderate Genotype</th>
<th>HRM and/or Pyrosequencing</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(higher sequence variability)</td>
<td>Multiple SNPs, complex mutations</td>
<td>Depending on sequence, throughput and/or biological/clinical context both are applicable.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complex Genotype</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(rapid sequence identification)</td>
<td>Multiple SNPs, complex mutations</td>
</tr>
<tr>
<td></td>
<td>Multiple mutations, complex structure</td>
</tr>
<tr>
<td></td>
<td>First choice Pyrosequencing</td>
</tr>
<tr>
<td></td>
<td>Multiple answers with single run</td>
</tr>
<tr>
<td></td>
<td>HRM added when pre-screening needed (throughput, costs)</td>
</tr>
</tbody>
</table>
The Principle of Pyrosequencing
Dye-terminator sequencing

- Utilizes labelled chain terminator dideoxynucleotides (ddNTPs)
- Each ddNTP is labelled with a fluorescent dye with different wavelengths of fluorescence and emission
- Single tube reaction of sequencing master mix + template + sequencing primer
- The electronic DNA sequence trace (chromatogram) is determined by capillary electrophoresis

Dye-terminator sequencing -- Limitations

- Dye effects due to differences in the incorporation of the dye-labelled ddNTPs into the DNA fragment which results in unequal peak heights and shapes in the chromatogram
- Dye blobs
- Does not provide quantitative data in respect to SNPs revealed in sequencing
Sequencing of Human K-Ras Precursor Sub-region - Codon 12 Position 2

Wild Type Sequence

181 AATGACTGAATATAAACCTTGTTGAGCTGGGCCTGAGCAAGAGTGCCTTGAC

Sequence Chromatograph

GGT>GTT
Gly12Val

Sequence Pyrograph
The Principle of Pyrosequencing Technology

Workflow

Step 1: Assay Design
- PyroMark Assay Design Software 2.0

Step 2: PCR
- Region of interest amplified with a biotinylated primer (~100-300 bp)

Step 3: Sample prep
- Separation to single stranded DNA using streptavidin-coated beads.
- Annealing of sequencing primer.

Step 4: Pyrosequencing
- Sequencing-by-synthesis. Sequence data generated from the first base next to the sequencing primer.
- Sequence context as built in control

---

~ 2h
~ 15 min
~ 10-60 min
Assay design

- PyroMark Assay Design Software 2.0
- PyroMark Assay Database
  - Free Online Access for customers
- PyroMark RUO Test
- PyroMark CE marked Kit (Europe only)

“If you can run a PCR, you can sequence with Pyrosequencing”
Jon Jonasson, University Hospital, Linköping, Sweden
Assay design

- PyroMark Assay Design Software 2.0
  - easy to use software for simultaneous design of PCR and sequencing primers
  - Assay designs optimized for all PyroMark instruments
  - Dedicated functionality for SNP Genotyping, Allele Quantification, Sequence analysis, and DNA Methylation analysis
    - Three simple steps from the original DNA sequence to an assay
    - Select Pyrosequencing assay design based on quality scoring
Assay design

PCR

- Amplify relevant region by PCR (100 - 300 bp)
- One primer is biotinylated
The Principle of Pyrosequencing Technology
PCR using PyroMark PCR Kit

✔ Assay design

PCR

- PyroMark PCR Kit
  - Larger signal heights
  - More reliable quantification results (e.g. blue vs. red)

Temperature

PyroMark PCR Kit

10 µl

Temperature

Standard Hotstart PCR

10 µl

Temperature

Sample & Assay Technologies
Assay design

PCR

Sample preparation

- Immobilize biotinylated PCR products onto streptavidin coated beads
- Separate strands by denaturation in NaOH
- Wash /neutralize the immobilized strand
- Anneal sequencing primer
The Principle of Pyrosequencing Technology
Sample Preparation

- PCR product immobilized on Sepharose beads
- Denaturation Solution (NaOH)
- EtOH
- Washing buffer
- Water
- PSQ plate with sequencing primer
Pyrosequencing Workflow

Enzyme Cascade

-Assay design
-PCR
-Sample preparation

Pyrosequencing

- One species of nucleotide (dNTP) is added at a time
- Nucleotide incorporation generates Pyrophosphate (PPi)
- Pyrophosphate (PPi) is converted into light seen as peak in the Pyrogram trace
- Excess nucleotide is degraded before addition of the next base.
The Principle of Pyrosequencing Technology
Generating quantitative data in a sequence context

- Assay design
- PCR
- Sample preparation
- Pyrosequencing

Data analysis

- Sequence and quantitative information
- Allows complex analyses including DNA Methylation and heterozygote analysis

PyroMark Software – dedicated software for easy and comprehensive data analysis

Pyrogram – provides sequence information, mutation information and quantitative CpG methylation in a sequence context
The Principle of Pyrosequencing Technology
Example: SNP Genotyping

- Sequence to analyze: a/g C T G C C T
- Dispersion order (done by SW): C A G T C T G C T
- Genotype analyzed: A/G Heterozygote

![Diagram showing pyrosequencing peaks and controls](image-url)
The Principle of Pyrosequencing Technology
Example: SNP Genotyping

Homozygous A

Homozygous G

Heterozygous A/G

The Principle of Pyrosequencing Technology
Example: SNP Genotyping
Analyzing variable position

- Genotyping (SNP, mutations)
- Allele quantification
- CpG Methylation Quantification

Analyzing unknown sequences

- sequencing with automatic base-calling

SNP

Genetic Testing

Epigenetics

Microbiology
Pyrosequencing
Applications, Instruments, and Markets

Analyzing variable position

- Genotyping
  (SNP, mutations)
- Allele quantification

CpG Methylation Quantification

Analyzing unknown sequences

- sequencing with automatic base-calling

SNP
AQ
Genetic Testing

AQ
Genetic Testing

AQ
Epigenetics

SQA
Microbiology

PyroMark Q24
PyroMark Q96 ID
PyroMark Q96 MD
PyroMark Q24
PyroMark Q96 ID
PyroMark Q96 ID
PyroMark Q24
Three Applications for Pyrosequencing

- Genetic Testing
- Epigenetics
- Microbial Analysis
Genetic Testing
PyroMark – 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

*Currently only available on PyroMark Q96 ID and MD
Genetic Testing
PyroMark – one platform for many genetic analyses

- Mutation analysis
  - Known and unknown positions
    - Point mutations
    - Multiple mutations
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- SNP analysis
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  - Multiple SNPs

- Allele Quantification
  - SNP frequency
  - Di-, tri- and tetra allelic mutations

*Currently only available on PyroMark Q96 ID and MD
The EGFR signal transduction pathway is known to have an important role in cell proliferation and survival.

The EGFR signal transduction pathway is activated by growth factors binding to the EGF receptor.

Activation of the EGFR pathway results in cell proliferation and survival.

KRAS serves as a downstream signaling effector in the EGFR signal transduction pathway.

The KRAS protein is involved in many aspects of the development and progressions of cancer, including abnormal cell growth, proliferation and differentiation.
The EGF receptor has become an important target for cancer therapies:
- Anti-EGFR antibodies for treatment of colorectal cancer (CRC): Erbitux® (Imclone), Vectibix® (Amgen)
- Successful anti-EGFR therapy blocks cell proliferation and survival

Mutations in KRAS leads to resistance to anti-EGFR antibodies due to constantly active KRAS
- KRAS mutations are complex, multiposition mutations
- The most common KRAS activating mutations are found in codons 12, 13 and 61
- The KRAS status of a tumor may be indicative of prognosis and predictive of response to certain drugs
Mutations of importance within codons 12, 13 and 61 of the KRAS gene

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Mutations of importance within codons 12, 13 and 61 of the KRAS gene

Example of analysis of position 2 in codon 12

Sequence to analyze:   GGT   GGC   GTA   GG

Codon 12 & 13

Codon 61
Mutations of importance within codons 12, 13 and 61 of the KRAS gene

Example of analysis of position 2 in codon 12

Sequence to analyze: GGT GGC GTA GG

Normal GGT GGC

Wild type KRAS

Mut Gly12Asp GGT>GAT GGC

Mutant KRAS codon 12

Codon 12 mutation: GGT > GAT

Pyrosequencing technology provides quantitative results and sequence information for sensitive detection of all key KRAS mutations.
Genetic Testing
PyroMark – 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

*Currently only available on PyroMark Q96 ID and MD
Genetic Testing
Detection of tri- and tetra allelic SNPs


seq1518ser10728 2000-07-28 15:04:39 : C10


Pyrosequencing offers:
- SNP analysis
  - Single variable position AG/TC
  - Multiple variable positions AG/TCAG/TCAG/T/AC
  - Di- Tri- or Tetra-allelic mutations GA/C/G/TA
- Quality assessment of individual sites and sequence context
- Analysis of SNPs in the presence of CpG sites
- Frequency calculations of variable positions in sequence context
- Suitable for analysis of fresh-frozen, fixed and paraffin-embedded specimens
- 24/96 samples analyzed in parallel in 1 hour after PCR

Suitable instruments:
- PyroMark Q24 / PyroMark Q96 ID and PyroMark Q24 MD
Pyrosequencing for Genetic Testing

Workflow

Nucleic Acid Isolation
- QIAamp Kits
- DNAeasy Kits
- QIAcube/EZ1
- QIAsymphony

Assay Design
- PyroMark Assay Design SW 2.0
- PyroMark Pre-designed Assays

PCR
- PyroMark PCR Kit

Sample prep
- PyroMark Q24 Vacuum Workstation
- PyroMark Q96 Vacuum Workstation

Pyrosequencing
- PyroMark Q24
- PyroMark Q96 ID
- PyroMark Q98 MD
- PyroMark Q24 SW
- PyroMark Gold Reagents

Time Estimates
- ~ 2h
- ~ 15 min
- ~ 10-60 min
Three Markets for Pyrosequencing

Genetic Testing

Epigenetics

Microbial Analysis
DNA methylation

- Adding of a methyl group on cytosine residues of CpG dinucleotides
- Reversible process

CpG islands

- High frequency of CpG dinucleotides: One CpG per 10 nucleotides
- CpG islands are often found in promoter regions
  - CpG islands co-localize with 60% of all promoters

Function

- Genes in methylated DNA are silenced
  - In humans 70-80% of CpGs are methylated
  - Most non methylated CpGs are found in regulatory elements
- Relevance on Cancer
  - Hypermethylation and silencing of important tumour suppressor genes
  - Hypomethylation and activating of oncogenes (e.g. K-ras, C-myc)
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:
- C to T conversion means unmethylated C
- C remains as C means methylated C
Methylation patterns in genomic DNA change due to factors such as environment, age, disease (cancer and others), etc. These changes can affect

- All CpG sites (e.g. change from unmethylated CpG to methylated CpGs)
Methylation patterns in genomic DNA change due to factors such as environment, age, disease (cancer and others), etc. These changes can affect:

- All CpG sites (e.g. change from unmethylated CpGc to methylated CpGs)
- Only some CpG sites (resulting in a mosaic of methylated and unmethylated CpGs)

DNA Methylation Analysis

Why analyzing CpG methylation in detail?

![Diagram showing gene expression and CpG methylation in normal and cancer states]
Methylation patterns in genomic DNA change due to factors such as environment, age, disease (cancer and others), etc. These changes can affect

- All CpG sites (e.g. change from unmethylated CpGc to methylated CpGs)
- Only some CpG sites (resulting in a mosaic of methylated and unmethylated CpGs)

**RASSF1A CpG Methylation**

- Tumor suppressor gene on chromosome 3p21.3
- RASSF1A is inactivated in a variety of tumors by hypermethylation
- Methylation levels vary among consecutive sites
Methylation patterns in genomic DNA change due to factors such as environment, age, disease (cancer and others), etc. These changes can affect

- All CpG sites (e.g. change from unmethylated CpGc to methylated CpGs)
- Only some CpG sites (resulting in a mosaic of methylated and unmethylated CpGs)

Methylation pattern in the tumorsupressor RASSF1A in neighboring CpG sites in 4 tumor samples (duplicate runs)
The Principle of Pyrosequencing Technology
Analyzing a pyrogram for DNA-methylation

Sequence to be analyzed: \[ \text{AGTTACGAC} \]
\[ \text{AGTTACGAC} \quad \text{and} \quad \text{AGTTACGAC} \]

After Bisulfite conversion: \[ \text{AGTTATGAT} \quad \text{and} \quad \text{AGTTACGAT} \]

Biotinylated PCR strand: \[ \text{TCAATA/GCTA....} \]

Analyzed sequence: \[ \text{AGTTACmGAT} \]

CpG methylation level: 27%

Nucleotides added: \[ \text{AGTAATCGACT} \]

Built-in Quality control: Successful Bisulfite conversion
**Sequence to analyze (bisulfite converted DNA):**

\[ C/TGTTTTGC/TGTTTC/TGAC/TGTTC/TGTAGGTTTTTC/TGC/TGGTGC/TGTATC/TGTTTGC/TGA \]

**Grey areas:**
Variable positions (CpG sites), are individually quantified by software

**Yellow area:**
Bisulfite treatment control, will provide automatic control for complete conversion
(Any C not followed by a G serves as quality control for the bisulfite reaction)
Pyrosequencing for DNA Methylation Analysis

Pyrosequencing assay results

Sequence to analyze (bisulfite converted DNA):

C/TGTTTTG C/TGTTTC/TGAC/TGTTT C/TG TTAGGTTTTTC/TGC/TGGTGC/TGTATC/TGTTTG C/TGA

Grey areas:
Variable positions (CpG sites), are individually quantified by software

Yellow area:
Bisulfite treatment control, will provide automatic control for complete conversion
(Any C not followed by a G serves as quality control for the bisulfite reaction)
CpG Methylation analysis of adjacent CpG sites of the MGMT gene

Pyrosequencing confidently measure the individual degree of methylation in adjacent CpG sites, even at long distances from the sequencing primer

<table>
<thead>
<tr>
<th>CpG position</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Methylation*</td>
<td>76.3%</td>
<td>72.5%</td>
<td>75.1%</td>
<td>77.4%</td>
<td>75.7%</td>
<td>77.3%</td>
<td>60.4%</td>
<td>60.7%</td>
<td>76.6%</td>
<td>66.6%</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.2</td>
<td>1.2</td>
<td>1.9</td>
<td>0.8</td>
<td>1.8</td>
<td>1.6</td>
<td>1.4</td>
<td>1.5</td>
<td>1.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Each methylation value is the mean of 13 replicates
CpG Methylation analysis of 73 bp of the MLH1 gene

Methylation levels are consistent even when using different sequencing primers

<table>
<thead>
<tr>
<th>CpG position</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq. Primer 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seq. Primer 2</td>
<td>27,3%</td>
<td>28,2%</td>
<td>28,7%</td>
<td>25,8%</td>
<td>25,9%</td>
<td>28,2%</td>
<td>27,0%</td>
<td>28,4%</td>
<td>26,5%</td>
<td>27,2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seq. Primer 1</td>
<td>27,5%</td>
<td>27,8%</td>
<td>29,2%</td>
<td>28,2%</td>
<td>28,2%</td>
<td>25,7%</td>
<td>24,3%</td>
<td>27,4%</td>
<td>25,7%</td>
<td>28,6%</td>
<td>26,0%</td>
<td>26,1%</td>
</tr>
</tbody>
</table>

Each methylation value is the mean of 3 replicates
Pyrosequencing
Rapid results compared to classical sequencing methods

- Reproducible quantification of all analyzed sites
- Only semiquantitative
- Highly labour intensive

*PSQ: Pyrosequencing

Time saving – Fast and easy results
Pyrosequencing for CpG Methylation analysis

Benefits

Pyrosequencing offers:

- Reliable quantification of consecutive CpG sites
- Analysing methylation in the presence of SNP’s
- Suitable for analysis of fresh-frozen, fixed and paraffin-embedded specimens
- Quality assessment of individual sites and sequence context
- Mean methylation for the whole assay shown for quick overview of results
- Present deviation from predefined methylation ranges
- 24/96 samples analyzed in parallel in 1 hour after PCR

Suitable instruments:

- PyroMark Q24 / PyroMark Q96 ID and PyroMark Q24 MD
Pyrosequencing for CpG Methylation Analysis

Workflow

- gDNA Isolation
  - QIAamp Kits
  - DNAeasy Kits
  - QIAcube/EZ1
  - QIAasympiohy

- Bisulfite Conversion
  - EpiTect Bisulfite Kits
  - EpiTect 96 Bisulfite Kits

- Assay Design
  - PyroMark Assay Design SW 2.0
  - PyroMark Pre-designed Assays

- PCR
  - PyroMark PCR Kit

- Sample Prep
  - PyroMark Q24 Vacuum Workstation
  - PyroMark Q96 Vacuum Workstation

- Pyrosequencing
  - PyroMark Q24
  - PyroMark Q24 SW
  - PyroMark Gold Reagents
  - PyroMark Q96 ID
  - PyroMark Q96 MD
  - PyroMark Gold Reagents
  - PyroMark CpG SW
Three Markets for Pyrosequencing

Genetic Testing

Epigenetics

Microbial Analysis
Microbial Identification
- Bacterial / Viral / Fungal ID
- Microbial typing

Examples
- General/universal: 16S bacteria, general fungi assays
- Panel-based: meningitis, Candida, Gram-positives, Mycobacteria
- Species-specific: e.g. H. pylori, Bordetella parapertussis/pertussis; Listeria monocytogenes
- Subtype-specific: e.g. B. anthracis, M. tuberculosis, N. gonorrhoeae

Resistance typing
Examples
- Resistance mutation in H3N2
- Monitoring SWINE FLU resistance
- Rifampicin resistance
- Linezolid resistance in Enterococci
Microbial Identification

- Bacterial / Viral / Fungal ID
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Resistance typing

Examples

- Resistance mutation in H3N2
- Monitoring SWINE FLU resistance
- Rifampicin resistance
- Linezolid resistance in *Enterococci*
Example of a general target: 16S gene

- All primers are placed in conserved regions
- Sequencing primers are placed in conserved regions up front the variable region

**Species-specific region in 16S gene**

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR-forward</th>
<th>Sequencing primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAA CGA-----C GAGAAGCTTG CTTCCTC--- --GATGTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAA CAGA-----C GAGGAGCTTG CTTCCTC--- --GACGGTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG AGGA----- --GTTCCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Streptococcus constellatus</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Clostridium sp.</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Shigella boydii</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Eikenella Corrodens</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Propionibacterium p.</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Arcanobacterium h.</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
<tr>
<td>Actinomyces odontolyticus etc.</td>
<td>CTGGCC GCGGGGCTTA ATACATGCAA GTCGAGCGAG GAGA----- --CTAGCTTG GGGAGA--- --AAATCTAGG CGGCCGACGG GTGAGTAAACA</td>
<td></td>
</tr>
</tbody>
</table>
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Resistance typing
Examples
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- Monitoring SWINE FLU resistance
- Rifampicin resistance
- Linezolid resistance in Enterococci
Update: Drug Susceptibility of Swine-Origin Influenza A (H1N1) Viruses, April 2009

Since April 21, 2009, CDC has reported cases of respiratory infection with a swine-origin influenza A (H1N1) virus (S-OIV) that is being spread via human-to-human transmission (1). As of April 28, the total number of confirmed S-OIV cases in the United States was 64; these cases occurred in California (10 cases), Kansas (two), New York (15), Ohio (one), and Texas (six). The viruses contain a unique combination of gene segments that had not been reported previously among swine or human influenza viruses in the United States or elsewhere (1). Viruses from 13 (20%) of 64 patients have been tested for resistance to antiviral medications. To date, all tested viruses are resistant to oseltamivir and zanamivir but are susceptible to amantadine and rimantadine. The purpose of this report is to provide detailed information on the drug susceptibility of the newly detected S-OIVs, which will aid in making recommendations for treatment and prophylaxis for swine influenza A (H1N1) infection. These data also will contribute to antiviral-resistance monitoring and diagnostic test development.

Adenoviral susceptibility was assessed by conventional sequencing or pyrosequencing assay (2) with modifications (3), using viral RNA extracted from original clinical specimens and/or virus isolates. Susceptibility of virus isolates to the neuraminidase inhibitors (NAIs), including oseltamivir and zanamivir and two investigational NAIs (peramivir and A-315675), was assessed by chemoluminescent neuraminidase inhibition assay using the NASstar Kit (Applied Biosystems, Foster City, California) (4). The generated IC50 values (i.e., drug concentration needed to inhibit 50% of neuraminidase enzyme activity) of test viruses were compared with those of sensitive seasonal control viruses. In addition, because H274Y is the most commonly detected mutation in oseltamivir-resistant viruses (4,5), a set of new primers for pyrosequencing of the N1 gene was designed to monitor a residue of the neuraminidase protein at 274 (275 in N1 numbering) in viruses of swine origin (6,7) (Table 1).

All 13 specimens tested contained the S31N mutation in the M2 protein, which confers cross-resistance to the adamantane class of anti-influenza drugs (Table 2). In addition, a partial sequence deduced from the M2 protein revealed changes characteristic for the M gene of S-OIVs. Existing primers used for the detection of adamantane resistance in seasonal viruses do not work with all tested S-OIVs. Optimized primers have been designed and are currently being validated. All 13 tested virus isolates exhibited IC50 values characteristic of oseltamivir- and zanamivir-sensitive influenza viruses. A/Georgia 17/2006 (H1N1), which is a seasonal virus, was used as a control (Table 2). The IC50 for oseltamivir ranged from 0.28 nM to 1.41 nM, whereas those for zanamivir ranged from 0.30 nM to 1.34 nM. All tested viruses also were susceptible to peramivir and A-315675. A subset of viruses (n = 2) tested in the fluorescent neuraminidase inhibition assay showed IC50 for oseltamivir and zanamivir ranging from 1.50 nM to 2.40 nM, similar to the sensitive control. Among the 36 specimens tested to date with pyrosequencing for the H274Y mutation in N1, none had mutations at residue 274.

Reported by: L. Guire, PhD, M. Okomo-Adhiambo, PhD, V. Deyde, PhD, K. Frye, MD, T. Sheu, R. Garten, PhD, J. Smith, J. Barnes, A. Myrick, M. Hillman, J. Shaw, PhD, C. Bridges, MD, A
Resistance to Adamantanes

**Molecular Epidemiology Team**
Virus Surveillance and Diagnosis Branch

*Centers for Disease Control and Prevention, NCIRD/CCID*
Influenza Division

SOP Title: Swine-H1N1 M2 detailed pyrosequencing protocol
Effective Date: May 11, 2009

**Pyrosequencing of M2 gene to detect resistance to adamantanes (amantadine and rimantadine)**

*RT-PCR primers (20 uM):*
sw-M2-F670, AGC TCC AGT GCT GGT CTG AAA G
sw-M2-R900-biot, GAC TCA GGC ACT CCT TCC GTA GAA

*Sequencing primer (100 uM):*
sw-M2-F747-seq, GCG ATT CAA GTG ATC C
Pyrosequencing for Resistance Detection
Monitoring SWINE FLU resistance

Resistance to Tamiflu

Molecular Epidemiology Team
Virus Surveillance and Diagnosis Branch

Centers for Disease Control and Prevention, NCIRD/CCID
Influenza Division

SOP Title: Swine-H1N1 NA-H274 detailed pyrosequencing protocol
Effective Date: May 12, 2009

Pyrosequencing of NA gene to detect oseltamivir resistance-conferring mutation
H274Y (H275Y in N1 numbering)

RT-PCR primers (20 μM):
Uni-sw-N1-B-F780, GGG GAA GAT TGT YAA ATC AGT YGA
Uni-sw-N1-B-R1273-biot, CWA CCCA GAA RCA AGG YCT TAT G

Sequencing primer (100 μM):
Uni-sw-N1-B-F804seq, GYT GAA TGC MCC TAA TT
Pyrosequencing for Microbial Analysis

Workflow

- gDNA Isolation
  - QIAamp Kits
  - DNAeasy Kits
  - QIAcube/EZ1
  - QIAsymphony

- Assay Design
  - PyroMark Assay Design SW 2.0
  - PyroMark Pre-designed Assays

- PCR
  - PyroMark PCR Kit

- Sample prep
  - PyroMark Q24 Vacuum Workstation
  - PyroMark Q96 Vacuum Workstation

- Pyrosequencing
  - PyroMark Q24
  - PyroMark Q24 SW
  - PyroMark Gold Reagents

- Sequence Identification
  - PyroMark Q96 ID SW
  - PyroMark Q96 ID SW
  - PyroMark Gold Reagents
  - PyroMark Identifire SW 1.0
Pyrosequencing Summary
PyroMark product line
Product Offering Overview

- Software
- PCR Kits, Reagents, Assay Kits
- Instruments
- Sample preparation

PyroMark Q24 Workstation
PyroMark Q96 MD
PyroMark Q24
PyroMark Q96 ID
<table>
<thead>
<tr>
<th></th>
<th>PyroMark Q24</th>
<th>PyroMark Q24 MDx</th>
<th>PyroMark Q96 ID</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>10x96 with automation 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 Needed</strong></td>
<td>10-20ul</td>
<td>20-40ul</td>
<td>5-10ul</td>
<td>5-10ul</td>
</tr>
<tr>
<td><strong>SW Functionality</strong></td>
<td>SQA, SNP, AQ, CpG methylation</td>
<td>SQA, SNP, AQ, CpG methylation**</td>
<td>SNP, AQ, CpG methylation**</td>
<td>SNP, AQ, CpG methylation**</td>
</tr>
<tr>
<td><strong>Main Applications</strong></td>
<td>Genetic testing, Epigenetics</td>
<td>Microbiology, Genetic testing, Epigenetics</td>
<td>Epigenetics, Genetic testing</td>
<td>Genetic testing (SNP)</td>
</tr>
<tr>
<td><strong>Additional SW</strong></td>
<td>Assay Design sw 2.0</td>
<td>Assay Design sw 2.0, PyroMark IdentFire (SQA), PyroMark CpG sw</td>
<td>Assay Design sw 2.0, PyroMark CpG sw</td>
<td>Assay Design sw 2.0, PyroMark CpG sw</td>
</tr>
</tbody>
</table>

** Additional sw (PyroMark CpG sw) needed on ID and MD instruments
Pyrosequencing — the unique detection and quantification technology

Pyrosequencing technology, which is based on the principle of sequencing by synthesis, provides quantitative data in sequence context within minutes. It enables real-time, sequence-based detection and quantification in genetic testing and epigenetic DNA methylation analysis, as well as microbiology applications. Find all the information you need in our dedicated Web resource.

- Find out what researchers are saying about Pyrosequencing!
Workflow Comparison/Integration
HRM and Pyrosequencing

Common Instrument Process

Nucleic Acid Isolation
- QIAamp Kits
- DNAeasy Kits
- QIAcube/EZ1
- QIAsymphony

Assay Design
- PyroMark Assay Design SW 2.0
- Public Domain e.g. Primer3
- QIAcube/EZ1
- QIA asymphony

PCR/QPCR
- QuantiTect Reverse Transcription Kit
- Type-it HRM PCR Kit

High Resolution Melt Analysis
- RotorGene Q + HRM Analysis

ScreenClust Analysis
- ScreenClust HRM Software Ver 1.0

Unique to RGQ

Workflow Link: HRM to Pyrosequencing

Sample prep

Pyrosequencing
- PyroMark Q24
- PyroMark Q96 ID
- PyroMark Q98 MD
- PyroMark Q24 SW
- PyroMark Gold Reagents
Thank You!