High Resolution Melting: History, Technology, and Utility

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Field Applications Consultant
High Resolution Melting

What is it?

- Melting Curve Analysis is well established as a method to characterize amplicons with SYBR Green I, HybProbe (FRET) or SimpleProbe probes.

- High resolution melting analysis is an extension of melting curve analysis...
  - enables not only detection of SNPS but also their discovery
  - requires special fluorophores, a high-performance instrument (block homogeneity, suitable filters, optical sensitivity and resolution) and special analysis algorithms.
History - Background

- Evolved from need to monitor sequence variation of entire amplicon
  - Single-strand conformation polymorphism
  - Heteroduplex migration
  - Denaturing gel electrophoresis
  - Temperature gradient gel electrophoresis
  - Enzymatic or chemical cleavage
  - Cycle sequencing and gel electrophoresis
  - Denaturing HPLC
  - Mass Spectrophotometry
  - Array analysis
History – Melting Curve Analysis

• Melting Curve Analysis
  – Introduced in 1997 in conjunction with real time PCR
  – With SYBR Green, provides a rough characterization of what product is amplified, and purity of product, indicating specificity of PCR reaction
    • Heterozygote detection possible only with addition of subsequent steps such as amplicon purification and addition of high concentrations or urea
  – With hybridization probes or ‘Simple probes’, can interrogate and detect specific regions of amplicon for sequence alterations
    • Difficult and expensive to screen for unknown mutations due to multiple probes required to span region
  – With a high resolution dye, can detect amplicon and oligonucleotide denaturation, allowing for product identification and SNP detection or discovery in same run.
    • Quantification not possible with HRM Dyes
Melting Curve Analysis

Established Applications

SYBR Green I for product identification
Melting Curve Analysis

Established Applications

SYBR Green I for product identification

Fluorescence labeled Probes for Genotyping

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Melting Curve Analysis
Established and New Applications

SYBR Green I for product identification
High Resolution Melting Dye for Gene Scanning
Fluorescence labeled Probes for Genotyping

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History – High-Resolution Melting

• Traditional genotyping methods versus high resolution melting
  – Ideal for screening 1000s of samples for sequence variations

Previous gene scanning techniques

- dHPLC
- Sequencing
- Real-Time PCR

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SNP Discovery and Genotyping Methods

Melting Curve Analysis

Specimen → Amplify DNA → Load on matrix

Clean up
Enzymatic reactions
Clean up

Electrophoresis
dHPLC
Mass Spec
Arrays
History – High-Resolution Melting

Why High Resolution Melting?

Robust, non-destructive closed-tube method with many applications; highly informative and flexible. More convenient and cost-effective than current technologies, such as sequencing or dHPLC.
High Resolution Melting - Technology

• Principles
• Prerequisites
• Dyes
• Instrumentation
• Data Analysis
High Resolution Melting
Amplicon Melting

DNA with heterozygote SNP

PCR

Denaturation reannealing

Intercalating fluorescent dye

Increasing temperature

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High Resolution Melting

Raw Data
# Amplicon Melting

## Variation in Melting Temperature (Tm)

- The Tm of an amplicon depends mainly on GC content. Alterations in the amplicon may influence the Tm.

<table>
<thead>
<tr>
<th>Highest Stability</th>
<th>Lowest Stability</th>
</tr>
</thead>
</table>

- Amplicon Melting of homozygote samples (containing homoduplexes of wildtype or mutant DNA) give very similar curve shapes.
- Amplicon Melting of heterozygote samples (containing homo and heteroduplexes) give curve shapes which are highly distinct.
Technology - Prerequisites and Innovations

What Is Needed to Perform HRM?

- **Novel intercalating dye to identify heteroduplex DNA**
  - saturating, non-inhibitory, ds DNA binding without redistribution during melting

- **Precise Instrument** to allow genotyping and/or mutation scanning of whole PCR products.
  - homogenous temperature profile and temperature control
  - high sensitivity optical system (light source, filters and detection system)

- **Flexible Data Analysis Software**
  - Sensitive and specific algorithms to distinguish detected differences
  - Easy to use, easy to adjust
  - Melt-standard compatible
High Resolution Melting

*Non-Saturating vs Saturating Dyes*

Fluorescent ds-DNA specific dyes (e.g., SYBR Green I)
- individual curves not sharp
- overlap is the same for homo- and heteroduplexes

Saturating dye
- uniform, sharp signals
- only sequence but not dye makes a difference
High Resolution Melting

High Resolution Melting Dye in Action

Non-Saturating Dye - SYBR Green I

Heat → No decrease in fluorescence

Saturating Dye - LightCycler HRM Master

Heat → Decrease in fluorescence
High Resolution Melting

Dyes

• Gundry et al tried a number of common and uncommon dyes for HRM
  – SYBR Green 1
  – SYBR Gold
  – Ethidium bromide
  – Pico Green
  – TOTO-1
  – YOYO-1

• Requirements:
  – Saturating
  – non-inhibitory to PCR reaction
  – Sufficient fluorescent levels for detection
  – Allows heteroduplex detection
High Resolution Melting

Dyes

- Very few dyes meet the requirements
  - LC Green – Idaho Technologies – somewhat inhibitory
  - R27 – Biolight – limited heteroduplex detection
  - EvaGreen – Biotium – somewhat inhibitory, though less than SYBR
  - ResoLight – Roche
    - Signal 7x higher than LC Green
    - No PCR inhibition within 8x concentration range
    - Improved stability over LC Green or R27
    - Well suited to heteroduplex differentiation
Prerequisites and Innovations

What Is Needed to Perform Hi Res Melt?

• **Precise Instrument** to allow genotyping and/or mutation scanning of whole PCR products.
  – homogenous temperature profile and temperature control
  – high sensitivity optical system (light source, filters and detection system)
**LightCycler® 480 System**

**Thermocycler**

- Six Peltier elements: semi-conductors where direction of current either cools or heats the thermoblock.

- Includes Therma-Base™ for optimized heat exchange which results in excellent overall temperature homogeneity.

- Allows to finish a PCR run:
  - 96 wells in < 1 hour
  - 384 wells in < 40 min.

- New technology for thermocyclers

  | Unique to LC480 |
Thermal Uniformity

Intra-Run Reproducibility of 96 Replicates

<table>
<thead>
<tr>
<th></th>
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### LightCycler® 480 Performance
#### Absolute Quantification, SYBR Green I

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<tr>
<th>Total RNA per reaction</th>
<th>100ng</th>
<th>10 ng</th>
<th>1 ng</th>
<th>100 pg</th>
<th>10 pg</th>
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<tr>
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<td>0,63</td>
<td>1,06</td>
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2-step RT-PCR
Target: h-HPRT
Thermal Uniformity

*Instrument Comparison - 96 wells*

LightCycler® 480 Instrument  Standard Instrument
Thermal Uniformity

*Instrument Comparison – 384 wells*

LightCycler® 480 Instrument  
Standard Instrument
LightCycler® 480 Instrument

Optical System - Lightpath

Folded optical path to reduce height
LightCycler® 480 Optical System

*Sensitivity and Homogeneity*

- Xenon lamp
- CCD camera
- Five excitation filters
- Six detection filters
- Optimized arrangement of optical components
- Homogeneous excitation and fluorescence detection
LightCycler® 480 Instrument

Optical Properties

- Light source: high intensity xenon lamp
- Highest intensity of light over a broad electromagnetic spectrum
- Degrades (ages) in linearly, without spectral shift.

Excitation filters

450, 483, 523, 558, 615 nm

Emission filters

500, 533, 568, 610, 640, 670 nm
LightCycler® 480 System
Assay Formats and Dyes

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<th>Excitation filters</th>
<th>450</th>
<th>483</th>
<th>483</th>
<th>523</th>
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<td>533</td>
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<td>610</td>
<td>640</td>
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<table>
<thead>
<tr>
<th>Dyes (Examples)</th>
<th>LightCycler® Cyan 500</th>
<th>SYBR Green I</th>
<th>Fluorescein (Fluos / FAM)</th>
<th>HEX (VIC)</th>
<th>LightCycler® Red 610</th>
<th>LightCycler® Red 640</th>
<th>Cy5</th>
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<tbody>
<tr>
<td>Detection formats</td>
<td>Hydrolysis probes (R), HybProbe probes (D)</td>
<td>SYBR Green I</td>
<td>Hydrolysis probes (R), HybProbe probes (D), SimpleProbe probes (R)</td>
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<td>Hydrolysis probes (R), HybProbe probes (A)</td>
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</tr>
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</table>

Legend: Reporter (R), Donor (D), Acceptor (A).
External evaluation
ARUP (Salt Lake City) study of hardware features


- Heterozygote scanning: LightCycler® 480 equals LightScanner
- LightCycler® 480 advantages: data density, signal-to-noise ratio, melting rate, speed
High Resolution Melting

Software and Data Analysis

• Wittwer et al (2003) demonstrated a useful and robust analysis methodology that has the capability to reveal both homo- and hetero-duplex DS DNA configurations
• Utilizes fluorescence normalization, temperature shift adjustment, and derivative melting curve plots
• Can reveal extremely minor differences in DS DNA melting curve shape
• Allows for comparison and adjustment to use melting standards for genotyping
High Resolution Melting

*Data Analysis*

- **Raw Data**
- **Normalization**
- **Temperature Shift**
- **Difference Plot**

*Normalized, Tm-shifted Difference Plot*
Wt/Homo/Heterozygote Differentiation

Example:
Sequence variations (SNP G→T) in the LPLH3 gene
72 samples, 164 bp amplicon
Melting Curves

Heterozygote Amplification

Two Heteroduplexes

Observed Combination of 4 Duplexes

Two Homoduplexes

Normalized Fluorescence

Temperature

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Unlabeled Probe Genotyping and Amplicon Melting *Simultaneous genotyping and scanning*
Unlabeled Probe Melting

*Principle of Genotyping by Hi Res Melt*

High-Resolution Melting with intercalating dye and unmodified oligo specific for known mutation site
Combined Unlabeled Probe and Amplicon Melting

Example 1: TNFα

Probe for SNP
C→T

Amplicon 136 bp
96 samples

1st Derivative

Normalization, Difference Plot

Wildtype
Mutation
Heterozygotes

Homozygotes (not separated)

Heterozygotes for another SNP (A→G) in this amplicon
High Resolution Melting

Utility

• Optimization requirements
• Data and Results
• Possibilities
• References and Papers
High Resolution Melting

Utility

• Optimization requirements
• Data and Results
• Possibilities
• References and Papers
Optimizing a Gene Scanning Experiment

**MgCl₂ Concentration**

Amplification Curves

<table>
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<th>Color</th>
<th>Name</th>
<th>CP</th>
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<tbody>
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<td>&gt;90.00</td>
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<tr>
<td>Gray</td>
<td>1.5 mM MgCl₂</td>
<td>91.45</td>
</tr>
<tr>
<td>Blue</td>
<td>2.0 mM MgCl₂</td>
<td>29.89</td>
</tr>
<tr>
<td>Red</td>
<td>2.5 mM MgCl₂</td>
<td>28.85</td>
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<tr>
<td>Green</td>
<td>3.0 mM MgCl₂</td>
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<tr>
<td>Pink</td>
<td>3.5 mM MgCl₂</td>
<td>27.79</td>
</tr>
<tr>
<td>Black</td>
<td>4.0 mM MgCl₂</td>
<td>26.13</td>
</tr>
</tbody>
</table>

167 bp PCR Fragment
MgCl₂ Titration 1.0 – 4.0 mM
PCR Primers: 200 nM each
Touchdown PCR Protocol (64 – 54° C)

Agarose Gel 2%

<table>
<thead>
<tr>
<th>MWM</th>
<th>PCR Products (+ NTC 4.0 mM)</th>
<th>MWM</th>
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<tbody>
<tr>
<td>50 bp</td>
<td>MgCl₂ Concentration</td>
<td>50 bp</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

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Optimizing a Gene Scanning Experiment

**Sample Material**

- Use consistent extraction protocols for all samples to be analyzed via High Resolution Melting.

- Quantify DNA samples using spectrophotometry. Adjust them to the same concentration prior to PCR.

- Use the same amount of template in each reaction (5 to 30 ng template DNA in a 20 µl reaction). Amplification plots should produce a crossing point value of < 30.

- Crossing points (aka C_T) should be within 5 cycles of each other.
Optimizing a Gene Scanning Experiment

**PCR Primers**

- Design PCR primers that have annealing temperatures around 60°C and produce short amplicons, ideally 100–250 bp.

- Use a software package to design primers
  - Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)
  - LightCycler® Probe Design Software 2.0.

- BLAST (http://www.ncbi.nlm.nih.gov/BLAST) the primer sequences to ensure they are specific for the target species and gene.

- Use primers that have been purified by HPLC.

- Use low primer concentrations (e.g., 200 nM each) to avoid primer-dimer formation.
Optimizing a Gene Scanning Experiment

**PCR Programs: Amplification**

Example:

**Touchdown PCR**
Optimizing a Gene Scanning Experiment

PCR Programs: High Resolution Melting

Example:
HRM program
Optimizing a Gene-Scanning Experiment

Controls

• Negative Controls – ensure PCR products not result of carryover
• Positive Controls – may be eliminated if known reference standards are used
• Known Reference Genotypes – „Melt Standards“
  – Especially useful when only a few samples are compared or when unlabeled probes are used and designed against a specific sequence variant
• Replicates?
  – Biological replicates can be used to provide an estimate of variation within a genotype
    • Replicates of individual samples not required
  – „experimental“ replicates used to confirm extraction / pipetting / PCR repeatability
Guidelines for successful HRM Assays

1. Analyze small DNA fragments
   There will be a bigger effect of a single base variation on a small amplicon.

2. Analyze a single pure product
   Primer-dimers and non-specific products make HRM difficult to interpret.

3. Use sufficient pre-amplification template
   Make sure the product has a $C_p$ ($C_T$) no more than 30 cycles. Samples that amplify later than this produce variable HRM results due to amplification artifacts.

4. Check for aberrant amplification plots
   Check the qPCR plots carefully for log-linear plots that are not steep, jagged, or reach a low signal plateau. This can indicate poor amplification, incorrect reaction setup, etc.
Guidelines for successful HRM Assays

5. Keep post-amplification sample concentrations similar
   The concentration of a DNA fragment affects its $T_M$. Try to keep DNA concentrations as similar as possible. Make sure every reaction reached a plateau.

6. Ensure sample-to-sample uniformity
   All samples must be of equal volume and should contain the same concentration of dye.
   DNA melting behavior is affected by salts in the reaction mix so make sure the buffer, Mg and other salts is the same in all samples. Use identical tubes or plates for all comparisons.

7. Allow sufficient data collection for pre-and post-melt phases
   Collect HRM data points over about a $10^\circ$ C window centered on the observed $T_M$. 
## Optimizing a Gene-Scanning Experiment

### Troubleshooting – Montgomery et al (2007)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reasons</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraneous melting transitions or poor curve clustering</td>
<td>Secondary PCR products</td>
<td>Optimize PCR conditions to obtain clean product</td>
</tr>
<tr>
<td></td>
<td>Low PCR yield</td>
<td>Optimize PCR to enhance product yield</td>
</tr>
<tr>
<td></td>
<td>Inconsistent genomic DNA preparation</td>
<td>Ensure that the genomic DNA concentration and buffer is consistent</td>
</tr>
<tr>
<td>Amplicon and probe melting transitions not visible or are very small</td>
<td>Probe $T_m$ too high, preventing PCR extension</td>
<td>Redesign probe with lower $T_m$, use and exonuclease-positive Taq or add the probe after PCR</td>
</tr>
<tr>
<td></td>
<td>Amplicon too long</td>
<td>Design primers for shorter amplicon length</td>
</tr>
<tr>
<td></td>
<td>Low PCR yield</td>
<td>Optimize PCR to enhance product yield</td>
</tr>
<tr>
<td>PCR product $T_m$ too high</td>
<td>High GC content</td>
<td>Add DMSO, betaine or glycerol to the PCR buffer</td>
</tr>
</tbody>
</table>
Optimizing a Gene-Scanning Experiment

LightCycler® 480 High Resolution Master

- Cat. No. 04 909 631 001 Kit for 5 x 100 reactions (20µL)
- Contents:
  - Master Mix 2 x conc. contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), and ResoLight
  - MgCl$_2$, 25 mM to adjust MgCl$_2$ concentration
  - H$_2$O, PCR-grade to adjust the final reaction volume
- Application
  For amplification and detection of a specific DNA sequence (with suitable primers) followed by high resolution melting curve analysis for detection of sequence variants among several samples.
Utility

• Optimization requirements
• Data and Results
• Possibilities
• References and Papers
HRM 2 - Sensitivity testing

*Dilution series of wild type/mutant mixes*

- 50% mutant
- 12.5% mutant
- 25% mutant
- 6.25% mutant
- 3.125% mutant
- 1.5% mutant
- 100% wildtype
HRM 3 - Sensitivity testing

Mutations identified in 650bp product (samples shown in replicates)

- 466 C>T
- 421 C>T
- 606 G>A
- 434 C>G
- 421 C>T, 606 C>G
- Wild type
HRM of grape varieties

HRM Data – 5
DNA Methylation

Normalized and Temp-Shifted Melting Curves

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HRM Data – 6

Utility

- Optimization requirements
- Data and Results
- **Possibilities**
- References and Papers
High Resolution Melting

**Key applications**

- Scan genes to discover SNPs and/or somatic mutations
- Genotyping of known SNPs
- Characterization of haplotype blocks – “hap maps”
- DNA methylation analysis
- DNA mapping
- Species identification/taxonomy
- HLA compatibility
- Screening for loss of heterozygosity
- Association (case/control) studies
- Allelic prevalence in a population
- Identification of candidate predisposition genes
Utility

- Optimization requirements
- Data and Results
- Possibilities
- References and Papers
HRM References


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**HRM References**


HRM References


History – High-Resolution Melting

Why High Resolution Melting?

Robust, non-destructive closed-tube method with many applications; highly informative and flexible. More convenient and cost-effective than current technologies, such as sequencing or dHPLC.

Why HRM on the LightCycler® 480 System?

Only plate-based Real-Time PCR HRM platform offering high-throughput HRM as a highly versatile, integrated system (hardware, software, reagents).

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Utility

- Optimization requirements
- Data and Results
- Possibilities
- References and Papers
- What this means for other real time Applications
Real Time PCR, HRM, and Quantification

• The technological and biochemical requirements for accurate and meaningful HRM studies are fulfilled by the LC 480 system.

• HRM Scanning is another software module that expands the capabilities of the LC 480, the premier real time PCR system on the market.

• The technologies that enable HRM also provide unsurpassed accuracy and consistency for the amplification, producing excellent quantitative data and results.
The LightCycler® 480 System

Data Homogeneity

Quantification analysis

“A Walk Around the Block”

Figure 2: Reproducibility and sensitivity of real-time PCR on the LightCycler® 480 Instrument. Serial dilutions of a viral target sequence (seven steps, $10^6$ to $10^0$ copies/20 μl) were amplified via PCR and detected with HybProbe probes. The whole dilution series was assayed in nine replicates positioned on different areas of the plate (e.g., wells A1 to A7 corresponding to one of the nine replicates). The graphic illustration of the amplification curves shows that the position of a sample in the plate has no significant influence on the results, thus demonstrating very high well-to-well homogeneity over the entire block (e.g., standard deviation for 100 copies: 0.11).

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LightCycler® 480 Instrument
Temperature Homogeneity

96-fold replicates of 3 genotypes

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<thead>
<tr>
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<th>Tm(2) / °C</th>
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<td>64.88</td>
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<tr>
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<tr>
<td>SD</td>
<td>0.1612</td>
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Prototype Software
Thermal Homogeneity

Demonstration by Melting Curve Analysis

SimpleProbe probes, FAM-label

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Thermal Homogeneity - Experimental Setup

Analysis of four 96-well Plate Subsets

Total - 384 samples

A1: 96 x Tm-low (52°C)  A2: 96 x Tm-high (78°C)
B1: 96 x Tm-med (66°C)  B2: 96 x negative control
Thermal Homogeneity – LightCycler 480

**Intra-Run Reproducibility of 96 Replicates**

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Data Uniformity

Dilution Series/Neighboring Wells – 165 bp target

Experiment:
- Serial 10-fold dilutions
- 3 replicates
- Target: Cyp2C9.2; 165 bp long fragment
- Fast & Standard protocol (Hydrolysis Probe Format)
- Samples in neighboring wells

LightCycler® 480 (96): 55 min
Data Uniformity
Two Copy Numbers/Spread Across Plate – 442 bp target

Experiment:
• Samples in checkerboard pattern
• 1000 & 100 copies
• 48 replicates
• Target: CycA; 442 bp long fragment
• Fast & Standard protocol (Hydrolysis Probe Format)
• Samples in neighboring wells

LightCycler® 480 (96): 55 min
Data Uniformity
Two Copy Numbers/Spread Across Plate – 442 bp target

 ABI 7900 (96): 90 min

 AB 7900 (96)  Fast: 44 min

Experiment:
- Samples in checkerboard pattern
- 1000 & 100 copies
- 48 replicates
- Target: CycA; 442 bp long fragment
- Fast & Standard protocol (Hydrolysis Probe Format)
- Samples in neighboring wells

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LightCycler® 480 System Applications

- **Gene Detection**: Detecting e.g., bacteria in sample material
- **Gene Expression**: Analyzing expression level of gene of interest
- **Genotyping**: Detecting known variants
- **Gene Scanning**: Finding new variants
Credits

• Special thanks for contributions for this presentation:
  – Natalie Barnes – RAS Australia Systems Account Representative
  – Dr. Michael Hoffman – RAS Global Marketing Manager
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    • Alex Pierson
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  – Dr. Oliver Geulen – RAS Global Training and Applications
  – Steve Hurwitz – RAS US LightCycler Manager
  – John Ogden, Ph.D – RAS US Genomics Marketing Manager
HRM Genotyping – History, Technology, and Utility

• Questions?

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