REAL-TIME PCR FOR NUCLEIC ACID AMPLIFICATION AND DETECTION

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Objectives of Talk

- Discuss the evolution of traditional end point PCR to real-time PCR
- Describe the principles of real-time PCR
- Identify the available technology for performing real-time PCR
- Define the advantages of real-time PCR for clinical testing
Polymerase Chain Reaction

- First in vitro DNA amplification method; currently the most mature technology
- Conceived by Dr. Kary Mullis in 1983
- Worked for Cetus Corporation in Emeryville, CA; one of the first molecular biotechnology companies
- Method relies on a heat-stable DNA polymerase and thermal cycling of repeated steps of heating and cooling the reaction
- Referred to as **target amplification** because it makes copies of a specific target sequence
Typical Polymerase Chain Reaction (PCR)

PCR happens in cycles with three basic steps:

1. **Denaturation**
   - The double-stranded DNA is separated into two single strands.

2. **Annealing**
   - Primers (short DNA sequences) bind to the single-stranded DNA.

3. **Extension**
   - DNA polymerase adds nucleotides to the primer, extending the strands.

PCR allows a small amount of the DNA molecule to be amplified exponentially....

40 cycles = $1 \times 10^{12}$ copies of target! (theoretically)
Old School (Traditional) End-Point PCR
Traditional End-Point PCR

Reagent Prep

Sample Prep

End-point PCR

Detection

Analysis
Traditional PCR Amplicon Detection

- Agarose
- Et Br

- Polyacrylamide
- $^{32}$P probes
Traditional PCR Assays

- Complex; labor-intensive
- Time-consuming
- Cumbersome; many steps and manipulations
- Low throughput; difficult to automate
- Highly variable; lack of standardization
- Less sensitive & specific
- Expensive to perform
- Hard to train & maintain skilled staff
- Quantification difficult
- Chance of amplicon contamination high
- Hard to commercialize
Real-Time PCR Technology

- Introduced in mid-1990s
- Resulted in rapidly evolving field with numerous technological advances
- Now the “gold-standard” for molecular testing
- Uses fluorogenic dyes to detect amplified product as it accumulates
- Increased sensitivity and specificity
- Easier to quantify over wide dynamic range
- Risk of contamination has been reduced
- Shortened turn-around time to generate results
- Lots of automation
- New innovative systems
PCR Amplification Plot

Fluorescence ($\Delta R_n$) vs. Cycle Number

- **Baseline**
- **Threshold**
- **Exponential Phase**
- **Linear Phase**
- **Plateau**
- **$C_T$**
With real-time PCR, the more copy numbers of nucleic acid present, the sooner an increase in fluorescence is detected.
Real-Time PCR Advances

- Simple and versatile technology
- Pre-optimized universal master mixes
- Universal conditions for amplification
- Multiple amplicon detection chemistries available
- Many choices of instrumentation
- Rapid and simplified assay development
Universal Master Mixes

- Available for most real-time platforms
- Premixed components
- Easy to use; generic for all assays
- Highly robust chemistry
- Provides sensitive and reproducible results in less time than traditional reagents
- Often work well with other chemistries and other instruments
Select Vendors: Universal Master Mixes

- Applied Biosystems
- Roche Molecular Biochemicals
- Qiagen
- Bio-Rad

- Invitrogen
- Epicentre
- ABgene
- Stratagene
- Eurogentec
Ready-To-Go PCR Beads

- Preformulated and predispensed
- Single-dose, temperature stable
- Minimizes master mix preparation and pipetting steps
- Simply reconstitute and add nucleic acid, primers and probe or intercalating dye
# Universal Amplification Conditions

<table>
<thead>
<tr>
<th>Viral Analyte</th>
<th>Cycle</th>
<th>Time</th>
<th>Temp (°C)</th>
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<tbody>
<tr>
<td>RNA</td>
<td>1</td>
<td>30 min</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10 min</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>15 sec</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>60</td>
</tr>
<tr>
<td>DNA</td>
<td>1</td>
<td>2 min</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10 min</td>
<td>95</td>
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<tr>
<td></td>
<td>45</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>60</td>
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</table>
Real-Time PCR Detection Chemistries

- DNA Binding Dyes
- Hydrolysis Probes (TaqMan)
- Hybridization Probes (FRET)
- Molecular Beacons
SYBR Green Fluorescent Dye

- DNA binding dye
- Binds dsDNA
- Generates fluorescent signal proportional to DNA concentration
- Lacks specificity; may detect dsDNA artifacts
- Measure $T_m$ to distinguish PCR products
Dissociation Curves for Adenovirus Real-Time PCR

- w primer dimers
- w/o primer dimers

Temperature
Taq-Man PCR

- Set of primers
- Hydrolysis probe
- Probe contains two fluorogenic dyes, a reporter and quencher
- Dyes control fluorescence during PCR
Common Fluorescent Dyes

- **Reporters**
  - FAM, TET, HEX, JOE, VIC
- **Quenchers**
  - TAMRA, DABCYL, Black Hole, MGB
- **Passive ROX**
  - Reference
Positive VZV DNA PCRs

Back Lesion

Left Cheek Lesion

+ Control
Hybridization Probes (FRET)

- Two hybridization probes
- Probe 1 - donor fluorophore on 3’ end
- Probe 2 - acceptor fluorophore on 5’ end
- When hybridize in proximity and exposed to light, donor emits light that excites acceptor
**Molecular Beacons**

- Hairpin oligonucleotide
- DNA probes with a stem-loop structure and two modified ends
- 20-25 loop sequence complementary to the target sequence for hybridization
- 6-7 base pair stem sequences
- Ends are labeled with fluorescent dye and quencher
- Different labels for different targets (wild type and calibrator)
Real-Time Detection with Beacons

Fluorescent signal (real-time)

Normalized fluorescence

Time (minutes)

0 10 20 30 40 50 60

WT IC
Scorpion Primers

- Variation of molecular beacons
- Similar in that the reporter and quencher are attached to a stem-loop structure
- Linked to 5’ end of a PCR primer via a blocker
- Blocker prevents polymerase from extending the PCR primer until thermal cycling begins
- Once primer is extended, the hairpin loop unfolds and the unfolded probe hybridizes to the newly synthesized target
- Fluorescence emission occurs

http://www.sigmaaldrich.com/life-science/custom-oligos
Real-Time PCR Instruments

- Small and large systems for 1 to 96 reactions
- Rapid turn-around times (20 min-2hrs)
- Closed systems
- Minimal hands-on
- Dynamic range of orders of magnitude
- Multicolor (multiplex) detection
Thermal Cycler Optics

- Fluorescence Reader
  - Lamp excites samples
  - Filter cube specific to each dye:
    - Excitation filter
    - Beamsplitter
    - Emission filter
  - Camera takes a picture of entire plate
Selected Real-Time PCR Instruments

- Abbott Molecular
  - m2000 rt
- Applied Biosystems/Life Technologies
  - 7500/7500 Fast/7500 Fast Dx
  - StepOne/Step One Plus
  - Viia 7
  - QuantStudio 12K Flex/Dx
- BioMerieux
  - NucliSens Easy Q
- Cepheid
  - Smart Cycler
  - GeneXpert Systems
- Qiagen
  - Roto-Gene Q
- Roche
  - LightCycler 1.5 & 2.0 Carousel-Based Systems
  - LightCycler 96
  - Light Cycler 480
  - Light Cycler 1536
  - Light Cycler Nano
  - COBAS TaqMan 48
  - COBAS TaqMan 96
- Agilent Stratagene
  - Mx 3000P/3005P
- Focus/3M
  - Integrated Cycler
ABI Real-Time PCR Instruments

7500/Fast/Fast Dx

ViiA 7

StepOne/StepOne Plus

QuantStudio 12K Flex/Dx
Thermal Cycler - RNA Protocol

- 45°C for 10 min RT Activation for cDNA
- 95°C for 10 min Taq Pol Activation
- 95°C for 15 sec Denature DNA
- 60°C for 1 min Primer & Probe Annealing + Target Extension
Thermal Cycler - DNA Protocol

- 95°C for 10 min Taq Pol Activation & AmpErase UNG Deactivation
- 95°C for 15 sec Denature DNA
- 60°C for 1 min Primer & Probe Annealing + Target Extension
- 50°C for 2 min AmpErase UNG Activation
- 50°C for 2 min AmpErase UNG Activation
Roche Real-Time PCR Instruments

Light Cyclers

- LC 1.5
- LC 2.0

COBAS TaqMan

- 48
- 96

Other Models

- Light Cycler 96
- Light Cycler 480
- Light Cycler 1536
- Light Cycler Nano
Other Selected Real-Time Instruments

- Cepheid SmartCycler
- Cepheid GeneXpert
- Abbott Molecular m2000 rt
- BioMerieux NucliSens Easy Q
- QIAGEN Rotor-Gene Q
- Stratagene Mx 3005P & Mx 3000P
- 3M Integrated Cycler
Things to Consider When Developing and Implementing Real-Time PCR

- Specimen collection and handling
- Nucleic acid isolation
- Target selection
- Design of primers and probes
- Optimization of PCR amplification conditions
- Method of amplified product detection
- Instrumentation, space
- Quality control/quality assurance
- Result interpretation and reporting
Select Primer/Probe Design Software

- Primer Express
  Life Technologies/Applied Biosystems, Foster City, CA.
  www.appliedbiosystems.com

- OLIGO
  Molecular Biology Insights, Cascade, CO.
  http://oligo.net

- Online oligo $T_m$ calculator
  http://alces.med.umn.edu/rawtm.html

- Primer 3
  www-genome.wi.mit.edu/cgi-bin/primer/primer3/www.cgi

- PremierBiosoft
  www.premierbiosoft.com/molecular_beacons/molecular_beacons.html

- IDT Oligo Design and Analysis Tools
  www.idtdna.com
Quantitation by Real-Time PCR
Quantitative Molecular Assays

- Associate infection with disease
- Monitor efficacy of therapy
- Predict treatment failure and emergence of drug-resistant viruses
- Assess disease progression
- Facilitate understanding of natural history and pathogenesis of organism
Multiplex/Multianalyte Capabilities

- Multiplex using one or more primer sets and multiple probes to amplify and detect multiple templates within a single reaction
- Multianalyte detection by running multiple different assays on a single plate
- Syndrome-specific test panels
- Reduces running costs, time and sample manipulation
- Important that amplification of one target does not dominate other
Why Use Real-Time PCR?

- Technique is fast and very flexible
- Highly sensitive, specific, reproducible
- Greatly simplifies the recognition and quantification of specific amplified products
- Decreases labor and supply costs; improves throughput and turn-around time
- More standardized; easier to train and monitor proficiency and competency
- Will result in an increase in the availability of commercial PCR kits
Evolution of Molecular Testing in the CHOP Virology Laboratory

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
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<tbody>
<tr>
<td>Extr</td>
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<tr>
<td>A/D</td>
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<tr>
<td>Ampl</td>
<td>Ampl</td>
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<tr>
<td>Detection</td>
<td>Detection</td>
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- **Extraction**: 9, 10, 11, 12, 13, 14, 15, 16
- **Amplification**: 9, 10, 11, 12, 13, 14, 15, 16
- **Detection**: 9, 10, 11, 12, 13, 14, 15, 16

- **Conventional**
- **Automated Extraction**
- **Fully Automated**
Other Amplification Technologies

- **Signal Amplification**
  - Hybrid Capture
  - Branched DNA (bDNA)

- **Target Amplification**
  - Nucleic Acid Sequence-Based Amplification (NASBA)
  - Transcription-Mediated Amplification (TMA)
  - Strand-Displacement Amplification (SDA)
  - Loop Mediated Isothermal Amplification (LAMP)
  - Helicase-Dependent Amplification (HAD)
Hot Diggity Dog!
Questions Anyone?