PCR Diagnostics: The bad news and the good news

Endpoint PCR versus Real Time qPCR

History

- The Polymerase Chain Reaction (PCR) was not a discovery, but rather an invention
- A special DNA polymerase (Tag) is used to make many copies of a short length of DNA (100-10,000 bp) defined by primers
- Kary Mullis, the inventor of PCR, was awarded the 1993 Nobel Prize in Chemistry

What PCR Can Do

- PCR can be used to make many copies of any DNA that is supplied as a template
- Starting with one original copy an almost infinite number of copies can be made using PCR
- "Amplified" fragments of DNA can be sequenced, cloned, probed or sized using electrophoresis
- Defective genes can be amplified to diagnose any number of illnesses
- Genes from pathogens can be amplified to identify them (e.g. VHSV)
PCR

**Melting**

94 °C

**Annealing**

Primers

50 °C

**Extension**

72 °C

**Temperature**

0

50

100

**Time**

30x

3'

5'

Fragments of defined length

DNA Between The Primers Doubles With Each Thermal Cycle

<table>
<thead>
<tr>
<th>Number</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

QuickTime™ and a Photo-JPEG decompressor are needed to see this picture.
**Theoretical Yield Of PCR**

Theoretical yield = $2^n \times y$

Where $y$ = the starting number of copies and $n$ = the number of thermal cycles

If you start with 100 copies, how many copies are made in 30 cycles?

$$2^n \times y = 2^{30} \times 100 = 1,073,741,824 \times 100 = 107,374,182,400$$

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**What is Wrong with Agarose Gels?**

- Poor precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non-automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide staining is not very quantitative

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**Real-Time PCR**

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection.

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**Real-Time Principles**

Consider two methods for the quantitative assays:
1. Hydrolysis probes (TaqMan®)
2. DNA-binding agents (SYBR Green)

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**Methods of fluorescence detection**

- SYBR Green
- Taqman
- Molecular Beacons
- Light Cycler

**FRET = Förster/fluorescence resonance energy transfer**

- Intact Probe
- Cleaved Probe
**TaqMan qPCR**

- Probe is hydrolyzed when it encounters Taq/primer displacement
- Fluorescence accumulates in proportion to generation of the PCR product
- Software back-calculates the original template copies by monitoring the increasing fluorescence

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**DNA Polymerase 5’ Exonuclease Activity**

- The five-fold dilution series seems to plateau at the same place even though the exponential phase clearly shows a difference between the points along the dilution series. This reinforces the fact that if measurements were taken at the plateau phase, the data would not truly represent the initial amounts of starting target material.

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**Real-time PCR is kinetic**

- Detection of “amplification-associated fluorescence” at each cycle during PCR
- No gel-based analysis at the end of the PCR reaction
- Computer-based analysis of the cycle-fluorescence time course

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**Absolute Quantitation vs. Relative Quantitation**

- **Absolute quantitation**
  - To quantitate unknown samples by interpolating their quantity from a standard curve
  - You can know the exact copy number of the target.

- **Relative quantitation**
  - To analyze changes in gene expression in a given sample relative to another reference samples (such as an untreated control sample)
Efficiency

The slope of the log-linear phase is a reflection of the amplification efficiency. The efficiency of the reaction can be calculated by the following equation: \( \text{Eff} = 10^{\left(-1/\text{slope}\right)} - 1 \). The efficiency of the PCR should be 90-100% (ideal slope = -3.3). A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, secondary structure, and primer design, to name a few.

Approximation vs Pfaffl method

(Effect of Amplification Efficiency)

Using the PCR Equation

\[ X_n = X_0(1 + E)^n \]

- \( X_n \) = PCR product after cycle \( n \)
- \( X_0 \) = initial copy number
- \( E \) = amplification efficiency
- \( n \) = cycle number

Effect of Amplification Efficiency

\[ X_n = X_0(1+E)^n \]

Case 1: \( E = 0.9 \)

\[ X_n = 100 (1+0.9)^n \]

\[ X_n = 2.3 \times 10^{10} \]

Case 2: \( E = 0.8 \)

\[ X_n = 100 (1+0.8)^n \]

\[ X_n = 4.6 \times 10^9 \]

Result

A difference of 0.1 in amplification efficiencies created a five-fold difference in the final ratio of PCR products after 30 cycles.
Real-time PCR advantages

- not influenced by non-specific amplification
- amplification can be monitored real-time
- no post-PCR processing of products (high throughput, low contamination risk)
- wider dynamic range of up to 10^{10}-fold
- requirement of 1000-fold less RNA than conventional assays (3 picogram = one genome equivalent)
- detection is capable down to a 2-fold change
- most specific, sensitive and reproducible

Real-time PCR disadvantages

- not ideal for multiplexing
- setting up requires high technical skill and support
- high equipment cost
- Sample preparation and quantification are critical

SYBR Green (double-stranded DNA binding dye)

- emits a strong fluorescent signal upon binding to double-stranded DNA
- nonspecific binding is a disadvantage
- requires extensive optimization
- requires melting point curve determination
- longer amplicons create a stronger signal
- may be multiplexed when coupled with melting curve analysis

SYBR Green

1. At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis.
2. After annealing of the primers, a few dye molecules can bind to the double stranded DNA. Binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation.
3. During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

Mapping Protein/DNA Interactions by Cross-Linking (NCBI Books)
**When to Choose SYBR Green**

- Assays that do not require specificity of probe based assays. Detection of 1000s of molecules
- General screening of transcripts prior to moving to probe based assays
- When the PCR system is fully optimized – no primer dimers or non-specific amplicons, e.g. from genomic DNA

**When Not to Choose SYBR Green**

- Allelic discrimination assays (not an absolute one)
- Multiplex reactions (not an absolute one)
  - Amplification of rare transcripts
  - Low level pathogen detection
Fish kill investigations 2006

<table>
<thead>
<tr>
<th>Date</th>
<th>Location</th>
<th>Species</th>
<th>Cell Culture</th>
<th>PCR results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/3/06</td>
<td>Cape Vincent, St. Lawrence</td>
<td>Round goby</td>
<td>5/5</td>
<td>5/5</td>
<td>Large die-off, confirmed PCR USGS, 1 pooled sample of 5 fish, 2 individual fish samples</td>
</tr>
<tr>
<td>5/5/06</td>
<td>Clayton, St. Lawrence</td>
<td>Round goby</td>
<td>1/1</td>
<td>1/1</td>
<td>Large die-off, confirmed PCR USGS</td>
</tr>
<tr>
<td>5/12/06</td>
<td>Clayton, St. Lawrence</td>
<td>Burbot</td>
<td>1/1</td>
<td>1/1</td>
<td>Continuing mortalities of multiple species, confirmed PCR USGS</td>
</tr>
<tr>
<td>5/15/06</td>
<td>Irondequoit Bay, Lake Ontario</td>
<td>Round goby</td>
<td>5/5</td>
<td>5/5</td>
<td>Large die-off, confirmed PCR USGS</td>
</tr>
<tr>
<td>5/15/06</td>
<td>Sodus Bay, Lake Ontario</td>
<td>Round goby</td>
<td>5/5</td>
<td>5/5</td>
<td>Large die-off, confirmed PCR USGS</td>
</tr>
<tr>
<td>5/23/06</td>
<td>Watertown, St. Lawrence</td>
<td>Muskellunge</td>
<td>0/1</td>
<td>0/1</td>
<td>Multiple large fish found dead</td>
</tr>
<tr>
<td>5/24/06</td>
<td>Clayton, St. Lawrence</td>
<td>Muskellunge</td>
<td>1/1</td>
<td>1/1</td>
<td>Multiple large fish found dead, confirmed PCR USGS</td>
</tr>
<tr>
<td>5/30/06</td>
<td>Clayton, St. Lawrence</td>
<td>Muskellunge</td>
<td>0/1</td>
<td>0/1</td>
<td>Multiple large fish found dead</td>
</tr>
<tr>
<td>6/1/06</td>
<td>Cayuga Lake</td>
<td>Pumpkinseed</td>
<td>N/A</td>
<td>0/8</td>
<td>Fish kill</td>
</tr>
<tr>
<td>6/1/06</td>
<td>Cayuga Lake</td>
<td>Bluegill</td>
<td>N/A</td>
<td>0/6</td>
<td>Fish kill</td>
</tr>
<tr>
<td>6/1/06</td>
<td>Cayuga Lake</td>
<td>Largemouth bass</td>
<td>N/A</td>
<td>0/5</td>
<td>Fish kill</td>
</tr>
</tbody>
</table>

Healthy Fish Survey 2005

<table>
<thead>
<tr>
<th>Location</th>
<th>Date</th>
<th>Species</th>
<th>Cell Culture</th>
<th>PCR results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Lake</td>
<td>10/27/06</td>
<td>Smallmouth bass</td>
<td>Pending</td>
<td>1/36</td>
<td>1/36</td>
</tr>
<tr>
<td>Red Lake</td>
<td>10/27/06</td>
<td>Yellow perch</td>
<td>Pending</td>
<td>2/36</td>
<td>2/36</td>
</tr>
<tr>
<td>Rondout Creek</td>
<td>11/27/06</td>
<td>Yellow perch</td>
<td>Pending</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Seneca Lake</td>
<td>10/27/06</td>
<td>Yellow perch</td>
<td>Pending</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>Seneca Lake</td>
<td>10/27/06</td>
<td>Smallmouth bass</td>
<td>Pending</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td>Seneca Lake</td>
<td>10/27/06</td>
<td>Chain pickerel</td>
<td>Pending</td>
<td>0/14</td>
<td>0/14</td>
</tr>
<tr>
<td>Seneca Lake</td>
<td>10/27/06</td>
<td>Northern pike</td>
<td>Pending</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>Seneca Lake</td>
<td>10/27/06</td>
<td>Landlocked salmon</td>
<td>Pending</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

qRT-PCR limits

- mRNA
- Virus
- Antibody

RT-PCR negative or positive if sampled from a carrier?

Forward transcribe

qRT-PCR

N Gene mRNA

N Gene cDNA

Reverse transcribe
**Baitfish Survey 2005**

<table>
<thead>
<tr>
<th>Location</th>
<th>Date</th>
<th>Species</th>
<th>Cell Culture</th>
<th>PCR results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chippewa Point, St. Lawrence</td>
<td>9/6/06</td>
<td>Bluntnose minnows</td>
<td>4/4</td>
<td>4/4</td>
<td>4 pooled samples of 5 fish each</td>
</tr>
<tr>
<td>Niagara River</td>
<td>8/5/06</td>
<td>Emerald shiners</td>
<td>1/4</td>
<td>3/4</td>
<td>4 pooled samples of 5 fish each</td>
</tr>
<tr>
<td>Lake Erie, Barcelona Harbour</td>
<td>8/7/06</td>
<td>Emerald shiners</td>
<td>4/4</td>
<td>4/4</td>
<td>4 pooled samples of 5 fish each</td>
</tr>
<tr>
<td>Van Camp Pond</td>
<td>10/24/06</td>
<td>Emerald shiners</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>Van Camp Pond</td>
<td>11/6/06</td>
<td>Emerald shiners</td>
<td>Pending</td>
<td>5/5</td>
<td>Pending</td>
</tr>
<tr>
<td>Van Camp Pond</td>
<td>11/6/06</td>
<td>Golden shiners</td>
<td>Pending</td>
<td>1/6</td>
<td>Pending</td>
</tr>
</tbody>
</table>

**COHO SALMON SURVEY**

- mRNA Copy #

**FISH CASE**

**FISH SURVEY, ONTARIO**

- Applications of qPCR in aquatic animal health
- Viral associated cancers in wildlife species

**Green Turtle Fibropapillomatosis**

**Prevalence of Fibropapillomatosis**
Leeches Do have an FPTHV DNA peak at 1.17 g/mL suggesting that some virus is expressed in tumors.

### Marine Leech

- **Ozobranchus**

### Barnacles

### Blood fluke

### Bladder fluke

**Average FPTHV pol copy number as measured by qPCR in several types of samples**

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Average FPTHV pol Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP tumors from Hawaiian green turtles*</td>
<td>2.3 x 10^6 copies per 100ng DNA</td>
</tr>
<tr>
<td>Tumoral tissue from FP(+) turtles*</td>
<td>39 copies per 100ng DNA</td>
</tr>
<tr>
<td>Hawaiian green turtle**</td>
<td>11 copies per 100ng DNA</td>
</tr>
<tr>
<td>FP tumors from Australian green turtles*</td>
<td>1.8 x 10^6 copies per 100ng DNA</td>
</tr>
<tr>
<td>Tumoral tissue from FP(+) turtles*</td>
<td>39 copies per cell</td>
</tr>
<tr>
<td>Australian green turtle**</td>
<td>7.8 x 10^6 copies per 100ng DNA</td>
</tr>
<tr>
<td>Water from the Kaneohe Bay, Oahu Island, Hawaii**</td>
<td>4 x 10^5 copies per Liter</td>
</tr>
</tbody>
</table>

*Quackenbush et al, 2001

**Our unpublished data
Applications of qPCR

Detection of Largemouth Bass Virus (LMBV)

Largemouth Bass Virus (LMBV)
- Virus that causes lethal disease in largemouth bass
- Genus Ranavirus
- Family Iridovirus, contains many viruses of invertebrate and vertebrate animals.
- Difficult to diagnose based on clinical signs.

Discovery of LMBV in USA
- Virus first isolated from largemouth bass collected in 1991 from Lake Weir, Florida.
- First fish kill recognized in 1995 in the Santee Cooper Reservoir, South Carolina.
- Has been detected in some hatchery stocks.
- Although mass mortality events associated with LMBV have been relatively few, the virus has been found throughout Eastern United States.
- 2002: LMBV reported in Lake Champlain by Vermont Fish and Wildlife Department.

Diagnosis of LMBV
- Difficult to distinguish by clinical signs.
- Sensitive testing via PCR.
  - Quantitative PCR technique developed at Cornell.
  - Confirmation of virus in cell culture.
- LMBV grows well on a variety of cell lines at varying temperatures in vitro.
- Common protocol used Bluegill Fry or Fathead Minnow cell lines incubated at 25°C – 30°C.

Quantitative PCR
- Protocol developed by R. Getchell at Cornell.
- Amplification of the major capsid protein, a highly conserved region in ranaviruses.
- Detection of low-levels of viral particles.
- Very sensitive procedure, more sensitive than standard cell culture.

2005 results
- 148 samples tested to date.
  - 131 negative.
  - 17 positive by PCR.
- 4 positive by cell culture.
- 4 pending cell culture results.

Smallmouth Bass Samples:
- Seneca River: 5/7
  - 2 samples cell culture positive.
  - Union Falls: 0/1
  - No cell culture performed.

Example of PCR results for LMBV samples

Example of diagram:
- Diagram showing the classification of ranaviruses, including LMBV.
Conclusions

- qPCR methods are a more sensitive than cell culture for detection of LMBV in wild New York fish.
  - Fast, relatively cheap.
  - Will not determine whether there is active, viable virus in the sample.
- Cell culture remains gold standard.
  - Takes a long time (>1 month per sample), labor intensive, more expensive.
  - Detects virus capable of producing pathogenesis in cells.