AAFS Workshop #6

Human DNA Quantification Using Real-Time PCR Assays

February 18th, 2008
60th Annual American Academy of Forensic Sciences
Washington, D.C.

Workshop Goals

• Human DNA Quantification using Real-Time PCR Assays

• An overview of various qPCR methods in a forensic context

• Many of the speakers have been directly involved in the design, optimization, and implementation of qPCR methods in their labs

• An opportunity to interact within the forensic qPCR community

The Speakers

• Dr. Peter M. Vallone (NIST)  
  – Introduction and Fundamentals of qPCR

• Ms. Margaret Kline (NIST)  
  – qPCR Sources of Variability: How Can They Be Minimized?

• Dr. Eric Buel (State of Vermont Forensic Lab)
• Dr. Janice A. Nicklas  
  – Applying Real-Time PCR to Solve Forensic Problems

• Dr. Mark D. Timken (California Dept of Justice)  
  – Multiplex qPCR Assays at the California DOJ: Diagnosing DNA in Challenging Samples

http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_qPCRworkshop.htm
The Speakers

- Ms. Melanie L. Richard (Centre Forensic Science Toronto)
  - The CFS-humRT QPCR Assay: Developmental Validation, Casework Experience and Lessons Learned

- Dr. Marie L. Allen (Uppsala University, Sweden)
  - Quantification of Nuclear and Mitochondrial DNA

- Ms. Toni M. Diegoli (Armed Forces DNA Identification Laboratory)
  - qPCR at AFDIL: Our Experiences Quantitating mtDNA and More

- Dr. David R. Foran (Michigan State University)
  - Identifying Stains or Tissues as Human or Non-Human

NIST Disclaimer

Funding: Interagency Agreement 2003-IJ-R-029 between the National Institute of Justice and NIST Office of Law Enforcement Standards

Points of view are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

STRBase qPCR Webpage

Updated pdfs of all today’s talks
Potential to host information on workshop comments, validation materials from your lab
qPCR Literature References
qPCR Website Links
petev@nist.gov

http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_qPCRworkshop.htm
Introduction and Fundamentals of qPCR

- The Need to Quantitate DNA
- PCR Amplification
- qPCR Curve Analysis
- Detection Chemistry
- Instrumentation

Why Do We Care About Quantitating DNA?

- Forensic laboratories commonly use commercial STR typing kits
  - PowerPlex 16
  - Identifiler
  - Other kits (PPY, Yfiler, COifiler, ProfilerPlus, minifiler)
- These kits are optimized for multiplex PCR
  - DNA input range 0.5 to 2 ng
  - ~83 to 333 copies of the human genome
- Optimal amounts of input DNA result in quality electropherograms
- DNA Advisory Board (DAB) Standard 9.3 requires human-specific DNA quantitation so that appropriate levels of human DNA can be included in the subsequent PCR amplification

Multiplex PCR

Identifiler kit with 1 ng of input DNA

- Good balance between loci
- Good balance for heterozygous loci
- Signal in range
- Allele calls can easily be assigned
Why Do We Care About Quantitating DNA?

• Too little input DNA results in:
  – Allele drop out
  – High signal to noise ratio (noisy baseline)
  – Heterozygote allele imbalance
  – Signal below RFU thresholds

Stochastic effect when amplifying low levels of DNA produces allele dropout.

Why Do We Care About Quantitating DNA?

Too little input DNA

No signal in red channel

Why Do We Care About Quantitating DNA?

• Too much input DNA results in:
  – Pull up (spectral artifact)
  – Locus imbalance
  – Split peaks (+/-A)
  – Signal off scale
  – Stutter increases

http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_qPCRworkshop.htm
Why Do We Care About Quantitating DNA?

- Poor quality data means that...

- Samples will have to be re-run
  - Extraction process
  - PCR
  - CE

- Poor quality data will take longer for an analyst to review

- Cost: time + reagents + extract = $$$

Why Do We Care About Quantitating DNA?

- Not limited to 'conventional' STR markers

- With degraded or low amounts of nuclear DNA we may have an interest in the amount of mitochondrial DNA available

- In a male – female mixture we may want an estimate of the Y-chromosome component

- An estimate as to the degree of degradation (and degree of inhibition as well)
Why Do We Care About Quantitating DNA?

- When obtaining samples from an outside source (collaborator, other lab) it is a good QC measure to confirm the quantity and integrity of the materials
- If evaluating a new technique (DNA extraction) qPCR can help quantitate performance
- When developing a new assay it is important to know the optimal [DNA] range

Why Do We Care About Quantitating DNA?

- If we can confidently determine the amount of DNA in an extract we can then ask questions:
  - Will mitochondrial sequencing be required? (skip STR analysis)
  - Should we use a miniSTR assay?
  - Should we use low copy number (LCN) methods for STRs?
  - Re-extract the sample?
- If problems occur in the STR typing process we can have confidence that the DNA template is not the source (CE, cycler, kit)

PCR Nomenclature

- qPCR – quantitative PCR (usually implies using PCR for DNA quantitation in "real time", i.e., not at the end point)
- RT-PCR – Real-Time PCR, but often reverse transcription PCR (and often in conjunction with real-time PCR, too)
- Amplicon – product of PCR
- Calibrant DNA – DNA of a known concentration that is serially diluted to prepare a standard curve (can be called the Standard DNA)
PCR Nomenclature

- Baseline – a linear function subtracted from the data to eliminate background signal
- Threshold – a value selected when the PCR is in the exponential phase of growth
- $C_T$ – Cycle Threshold – the cycle number at which the amplification curve crosses the selected threshold value
- $E$ – Efficiency - measure relating to the rate of PCR amplification

Why Do We Care About Quantitating DNA??

- Other methods…..
  - UV (260 nm, 1 OD = 50 ng/$\mu$L)
  - Yield Gel
  - AluQuant
  - Quantiblot
  - Pico Green (fluorescence)
  - others
- Time consuming (multiple steps)
- Not connected to software analysis
- Limited dynamic range
- Some not human specific

qPCR

- qPCR is a recently developed technique
  - Developed by Higuchi in 1993
  - Used a modified thermal cycler with a UV detector and a CCD camera
  - Ethidium bromide was used as intercalating reporter: As [dsDNA] increased, fluorescence increased
- First paper on qPCR:
PCR/qPCR What is the Difference?

- **PCR**: the products are analyzed after the cycling is completed (static)
  - gel, CE, UV, fluorimeter
  - End point assay

- **qPCR**: the products are monitored as the PCR is occurring (dynamic)
  - Once per thermal cycle
  - Fluorescence is measured
  - Kinetics of the system

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Why Real-Time qPCR?

**Advantages**

- The availability of commercial qPCR kits (labs are switching over to this method)
- Higher throughput and reduced user intervention
  - Automated set up
  - Simple data analysis
  - Experimental data rapidly analyzed in software; interpolating into the calibration curve
- qPCR will be sensitive to the same inhibitors as faced in a traditional STR test (both PCR based)

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Why Real-Time qPCR?

**Advantages**

- No post PCR manipulation (reduced contamination issues)
- High sensitivity (down to a single copy number?)
- Large dynamic range: ~30 pg to 100 ng
- Assays are target specific (autosomal, mito, Y) and can be multiplexed – to a degree…
Why Real-Time qPCR?
Challenges

• qPCR is subject to inhibition
  – internal PCR controls (IPC) can help

• qPCR quantitation precision suffers at low copy numbers (below 30 pg by a factor of 2)

• When working below 100 pg qPCR is still subject to variability and uncertainty

Why Real-Time qPCR?
Challenges

• qPCR quantitates specific target sequences, it does not quantify “DNA”
  – In highly degraded samples, assays that amplify short target sequences will detect and measure more DNA than assays that amplify long target sequences (relevant to STR typing)

• Accurate qPCR quantitation assumes that each unknown sample is amplified at the same efficiency as the Calibrant sample in the dilution series

• Results are relative to the Calibrant (which can vary)

PCR Mechanism

• PCR amplification results in an exponential increase in PCR products

• The amount of DNA theoretically doubles with every cycle of PCR

• After 2 cycles of the PCR we have 2 x 2 more DNA; after 3 cycles 2 x 2 x 2 more DNA and so on…

• $2^N$, where $N$ is the number of cycles
PCR Mechanism

- The amount of DNA theoretically doubles with every cycle of PCR
  \[ 2^N \]
- This is true when the reaction is running at 100% efficiency

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<td>3</td>
<td>8</td>
</tr>
<tr>
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<td>16</td>
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- Typically PCR is run for 28-32 cycles (E=100%)
  - Starting with one copy:
    - After 28 cycles = 268,435,456
    - After 32 cycles = 4,294,967,296
  - Lower volume PCR may require fewer cycles
  - At >40 cycles non-template controls may start to give signal
  - Toward the end of the cycling: reagents are consumed and the PCR is less efficient

PCR Amplification

- 4 phases of qPCR amplification
  1. Lag (doubling, but not detected)
  2. Exponential (doubling)
  3. Linear (less than doubling)
  4. Plateau (little change)

- The exponential phase is where we make our qPCR measurements

Efficiency is dropping < 100%
qPCR Real Time Curves

Raw fluorescence signal versus Cycle Number

Linear plot

~10 fold increase in fluorescence signal

Y scale 0.5 to 5.5

Quantifiler Data

qPCR Real Time Curves

Raw fluorescence signal versus Cycle Number

Log plot (Log of fluorescence)

The Log plot is common when reviewing qPCR data. It will pull out data hidden in the linear plot.

Y scale 0.001 to 10

Quantifiler Data

Lag Phase (1)

In the lag phase the amount of DNA is doubling with every cycle, but not in sufficient amounts to give a corresponding signal increase.

Typically, the baseline is selected in the lag phase.

Cycles ~ 1 - 20

Quantifiler Data
**Exponential Phase (2)**

In the exponential phase, the amount of DNA is doubling with every cycle. This is evidenced by the almost linear portion of the curve. Plenty of reagents are available to the reaction (primers, dNTPs, fresh polymerase).

*Threshold is selected in this phase of PCR.*

- **Cycle Number**
  - Cycles ~ 20 - 27

**Log(Reporter fluorescence)**

**Cycles ~ 20 - 27**

**Quantifier Data**

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**qPCR Real Time Curves**

These plots are on the same X scale (Cycle Number).

- **Log**
  - The log plot is useful to see where the amount of DNA is doubling with cycle number.
  - This cannot be readily observed in the linear plot.

**Linear**

**Quantifier Data**

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**Linear Phase (3)**

In the linear phase, the amount of DNA is no longer doubling with every cycle; a drop off starts to occur. Limiting amount of reagents are available; the rate of amplification starts to vary. Different samples (even replicates) may exhibit different rates of growth in the linear/plateau phase.

*Cycles ~ 27 - 35*

**Quantifier Data**

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Plateau Phase (4)

It's over...
Little increase in signal/products with cycles

Reagents are depleted
This is the end point

Log(Reporter fluorescence)
Cycle Number
Cycles ~ 36 - end
Quantifier Data

PCR Efficiency

- How is the PCR progressing?
- Is the PCR running at maximum efficiency?
- Are there PCR inhibitors present in reaction?
- Are we at the optimal annealing-extension temperatures? (during assay development)
- Are the unknowns amplifying with the same E as the Calibrants?

PCR Efficiency

PCR inhibitors:
- Hemoglobin
- Urine, hair
- Organic or anisole compounds
- Cholesterol, Fat, C/P
- Tissue necrosis effects
- Lab management, DNA degradation

PCR enhancers:
- DMSO, Mercaptoethanol
- Forensic, PEG, THM, D2O etc.
- Special commercial enhancers
- Some 32 pCN: Public health, Ted Emmon, E. C. or other

http://www.gene-quantification.info/
Michael W. Pfaffl
pfaffl@wzw.tun.de
PCR Efficiency

- Taking our previous relationship $2^N$
- The efficiency of the PCR can be represented as:
  \[ X_N = X_0 (1 + E)^N \]
  - $X_N$: predicted copies
  - $X_0$: starting copy number
  - $E$: efficiency (0 to 1)
  - $N$: number of cycles

- Starting with 100 copies and 100% Efficiency and 28 cycles
  \[ X_N = 100(1 + 1)^{28} = 2.68 \times 10^{10} \text{ copies} \]

- 90%
  \[ X_N = 100(1 + 0.9)^{28} = 6.38 \times 10^{9} \text{ copies} \]

- 80%
  \[ X_N = 100(1 + 0.8)^{28} = 1.40 \times 10^{9} \text{ copies} \]
PCR Efficiency

- Plotting the log(copies of DNA) versus Cycles of PCR results in a straight line with a slope of 3.32.

\[ y = 3.3219x + 5 \times 10^{-15} \]

\[ R^2 = 1 \]

- We will explore line parameters later.

---

PCR Efficiency

- When applied to qPCR the relationship between cycles and log(copies) is the inverse.

\[ y = -3.3219x + 20 \]

\[ R^2 = 1 \]

- The signal at lower cycles indicates more DNA in the sample.

---

PCR Efficiency

- When applied to qPCR the relationship is the inverse (the signal at lower cycles indicates more DNA in the sample).

- The line has a negative slope.

We will discuss more about this type of plot later.
PCR Efficiency

- A optimal reaction is typically between 90% to 110% slope = -3.58 to -3.10
- The slope may exhibit greater variation when running more complex (multiplex) qPCR assays; multiplex probes, targets, copies etc

PCR Efficiency

- Taking the relationship between log (copies of DNA) and cycles of PCR one can rearrange the equation $X_n = X_0 (1 + E)^n$ in order to determine efficiency

$$\text{Reaction Efficiency} = \left[10^{(-1/m)}\right] - 1$$

A reaction efficiency of 1 is 100%
- We will see later that the slope from our qPCR data plots can be used to estimate the efficiency of the reaction

Quantitation Using PCR

- Visually inspect qPCR curves
- Set Baseline and Threshold values
- Construct and evaluate a Calibrant Curve
- Review estimated DNA concentrations

- This can be done rapidly in the instrument software package
- Estimated DNA concentrations can be easily manipulated in Excel
Fluorescence vs Cycle Number

The concentration of PCR products is always doubling, but is not readily detected until over 24 cycles (for this example).

Higher DNA concentrations 10 ng
Lower DNA concentrations 40 pg

Log View of Data

Range of interest 26-34 cycles
Exponential amplification

Data Measured in the Exponential Phase

In the exponential phase the amount of DNA is doubling with every cycle.

This Threshold is selected in the phase of PCR.

This is evidenced by the almost linear portion of the curve. Plenty of reagents are available to the reaction (primers, dNTPs, fresh polymerase).
Setting the Baseline

- A low and high value are set
- The baseline is set to eliminate the background signal found in the early cycles of amplification
- The baseline should not interfere with the exponential phase of the amplification
- The baseline is set to allow for accurate C\text{t} determination
- Many qPCR methods have a prescribed baseline

Log View of Data

- As can be observed below, baselines vary from sample to sample
- This is due to fluorescent noise/fluctuations (due to chemistry)
- A baseline range is selected and a line is subtracted from the curve
- This usually "tightens" replicates
- Typically choose a baseline range after the first few cycles and when the signal is linear (and not into the exponential region)

Setting the Baseline

- A range of 7 – 16 would probably be optimal
- A range of 15 – 21 would be too high
- A range of 1 – 6 would be too low
Setting the Baseline

An empirical way to set the Baseline would be to try different ranges and observe their effect on the C<sub>T</sub> values.

Log View of Data

The odd looking or missing data is due to taking the log of reporter fluorescence raw values less than 1 or barely greater than 1.

What is with the Confetti?
The $C_T$ Value

- $C_T$ is the simply the cycle number selected at a specific threshold value
- The threshold value is selected where all the data is undergoing exponential amplification
- The threshold value can be selected manually or by the software
- The threshold value for different methods may vary
- Selected in the log(signal) plot view

Selecting the Threshold Value

After selecting a threshold in the exponential phase the software will report the cycle number that corresponds to that point of the amplification curve (The $C_T$)

Quantifiler data
Selecting the Threshold Value

Log(Reporter fluorescence) vs Cycle Number

Light blue line represents an unknown

CT = 27.45

CT Value and the Standard Curve

- After a suitable threshold has been selected the data is analyzed and the CT values are determined
- The CT values of the serial dilution are plotted versus the log[DNA] – your serial dilution of a calibrant DNA
- The line is visually inspected and the parameters are reviewed
- If the standard curve is linear and the line parameters are acceptable, the unknown concentrations can then be estimated

Log [DNA] versus CT

This type of standard curve is automatically generated in the software

Represents the linear relationship between log[DNA] and CT

The estimated concentrations of the unknowns are extrapolated from the equation of the fit line (not the data points)
**Equation of a Straight Line**

- The equation $Y = mX + b$ defines a straight line
- $m$ is the slope
  - $(y_1-y_2)/(x_1-x_2)$
  - The “steepness” of the line
  - Relates to the efficiency of the PCR
- $b$ is the Y-intercept (where the line crosses the Y-axis)
- $X$ is your log[DNA] concentration (serial dilutions)
- $Y$ is the $C_T$ value

**Linear Least Squares Regression**

- The most widely used modeling method
  - "regression," "linear regression," or "least squares"
- Many processes in science and engineering are well-described by linear models
- Good results can be obtained with relatively small data sets
- Main disadvantages: limitations in the shapes that linear models can assume over long ranges, possibly poor extrapolation properties, and sensitivity to outliers

- Carried out by the instrument software
  - Can also be easily performed in Excel, Sigma Plot etc
  - Briefly, the method solves for $m$ and $b$ from the data points (remember $X$ and $Y$ are constants)
  - Finds numerical values for the parameters that minimize the sum of the squared deviations between the observed responses (your data!) and the functional portion of the model (the line!)
Calculating PCR Efficiency

• Taking the relationship between log(copies) and cycles of PCR one can rearrange the equation $X_N = X_0 (1 + E)^N$ in order to determine efficiency

$$\text{Rxn Efficiency} = \left[10^{\frac{1}{-m}}\right] - 1$$

$$= \left[10^{\left(-1/-3.317296\right)}\right] - 1$$

$$E = 2.0019 - 1$$

Just over 100% efficient

$$E = (2.0019 - 1) = 1.019$$

R² (R-squared)

• Coefficient of determination

• A statistic for a predictive model’s lack of fit using the data from which the model was derived

$$R^2\text{-squared} = 1 - \frac{\sum (Y_i - \hat{Y}_i)^2}{\sum (Y_i - \bar{Y})^2}$$

• A perfectly fitting model yields an $R^2$ of 1 (all points fall directly on the line)
R² (R-squared)

- For most log[DNA] versus Ct standard curves R² should be greater than 0.990
- Sometimes outliers can be removed to improve the R² values
- Outliers can be at low/high concentrations or outside the performance range of the qPCR assay (or just a bad point – pipet error, dirty well etc)

Removing An Outlier

CT value
Log [DNA]
Alu assay data

Removing An Outlier

CT value
Log [DNA]
Alu assay data
Solving for an Unknown

• From the data

• \( Y = mX + b \)

• \( C_T = m \log[\text{DNA}] + b \)

• Solving for [DNA]

\[
[C_T - b] = 10^{-\frac{27.45-28.71\text{ng}}{3.3712}}
\]

• The equation above is used to estimate the [DNA] of the unknowns

Solving for an Unknown

• From the data

• Solving for [DNA]

\[
[\text{DNA}] = 10^{-\frac{27.45-28.71\text{ng}}{3.3712}}
\]

• After solving for the equation when \( C_T = 27.45 \) this corresponds to a [DNA] of 2.39 ng

• The software will do this for you...

Data Report

An example of a data report from the 7500 collection software

Report can be exported and manipulated in a spreadsheet
Varying the Threshold Value

• What happens when we change the Threshold value?

• Of course the absolute C_T values will change
  – But it will be consistent for that data set

• You don’t want to compare C_T values from different methods or even runs

• What is the effect of varying Threshold on the standard curve and the estimated values for the unknowns?

Varying the Threshold Value

Log(Reporter fluorescence)

Cycle Number

Try 6 different Threshold values

High

Low

Quantifiler data

Varying the Threshold Value

• Selecting 6 Threshold values then estimating [DNA] for a sample run in duplicate

<table>
<thead>
<tr>
<th>Threshold</th>
<th>[A]</th>
<th>[B]</th>
<th>[Avg]</th>
<th>Stdev</th>
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<td>23.18</td>
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<td>0.71</td>
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<tr>
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<td>17.5</td>
<td>16.83</td>
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<tr>
<td>High</td>
<td>17.58</td>
<td>16.68</td>
<td>17.13</td>
<td>0.64</td>
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~6.8 ng/µL difference (max)

1.3 ng/µL
**Varying the Threshold Value**

- Selecting 6 Threshold values then estimating [DNA] for a sample run in duplicate

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<thead>
<tr>
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<td>1.7</td>
<td>0.993</td>
<td>-3.421</td>
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**Rxn efficiency**

- Est DNA concentration ng/µL

<table>
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<th>[Avg]</th>
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<td>Optimal</td>
<td>0.2</td>
<td>1.30</td>
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<td>1.7</td>
<td>1.22</td>
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</tr>
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~0.4 ng/µL difference (max)

- ~0.4 ng/µL difference (max)
**Importance of the Calibrant!**

• All qPCR results are relative to the standard curve

• Serial dilutions of the Calibrant DNA comprise the standard curve

• Any errors involving the Calibrant DNA directly affect the estimates of your unknown DNA concentrations
  – Pipetting errors
  – Miscalculation of concentrations
  – New lots or vendors of Calibrant DNA
  – Contamination of Calibrant
  – Evaporation of Calibrant DNA

**Detection Chemistry**

• Intercalation Dyes

• TaqMan Probes

• EraGen/Plexor
qPCR: Detection - Chemistry

- Two General Approaches for Detection
  - Fluorophore is not sequence-specific – detects any double-stranded PCR product at each cycle; specificity of detection and quantification is due to specificity of primers.
    - fluorophore typically SYBR Green
  - Fluorophore is sequence-specific – detects only specific double-stranded PCR product at each cycle; specificity of detection and quantification is due to specificity of primers AND to specificity of reporter fluorophore.
    - fluorophore commonly a “TaqMan” probe
    - many others

qPCR: Detection – SYBR Green

- What is SYBR Green (SG)?
  - Proprietary fluorophore (Molecular Probes)
  - Binds to dsDNA (in minor groove); binding is NOT sequence-dependent (binds to any dsDNA)
  - Upon binding to dsDNA, shows greatly enhanced fluorescence (>10x greater fluorescence)
    - Unbound SG = “dark”
    - dsDNA-bound SG = “FAM-like”
  - SYBR Green is typically a pre-added ingredient in so-called “SYBR Green Master Mixes”

qPCR: Detection – SYBR Green

SYBR Green Detection

Detection of specific and non-specific products

Typically detect fluorescence in real time at the end of each extension step in PCR
qPCR: Detection – SYBR Green

• Advantages of SYBR Green Detection
  – Simple to design – just need to find good, specific primers for the target sequence of interest
  – Sensitive - produces >1 reporter per amplicon
  – Inexpensive, relative to “TaqMan” detection, because dye-labeled oligo-nucleotides are not required
  – Can use melt curve to assess specificity of PCR

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: Detection – SYBR Green

• Disadvantages of SYBR Green Detection
  – SYBR Green detects ALL double-stranded DNA, so if PCR is poorly designed, “primer-dimer” product will be detected and quantified
  – Cannot multiplex SYBR Green qPCR assays

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: Detection – TaqMan

- TaqMan detection probe = a dual-labeled oligonucleotide
  – Complimentary to target sequence (anneals between primers)
  – Designed to anneal ~6-10 degrees higher than PCR primers
  – 5' end of probe = a Reporter fluorophore (e.g., FAM, VIC, NED, Cy5, etc.)
  – 3' end of probe = a Quencher a chemical group that will quench the fluorescence of the Reporter (e.g., TAMRA, “BHQ,” or “NFQ”)
  – Quenching occurs only if R and Q are sufficiently proximate so that excitation energy is transferred from R to Q
  – Ideally, an “intact” TaqMan probe is not fluorescent (“dark”)
qPCR: Detection – TaqMan

• Annealing/Extension Step
  – TaqMan probe hybridizes to denatured DNA (sequence specific)
  – Reporter fluorescence is quenched due to proximity to quencher (reporter starts ~dark)

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: Detection – TaqMan

• Annealing/Extension Step
  – Lengthening strand displaces 5’ end of probe

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: Detection – TaqMan

• Annealing/Extension Step
  – Taq polymerase mediates hydrolysis of probe from 5’ end (“5’ exo-nuclease activity”)
  – Reporter fluorophore is no longer quenched
  – Hydrolyzed TaqMan probe eventually dissociates

Slide courtesy of Dr. Mark Timken, CA DOJ
qPCR: Detection – TaqMan

• End of Annealing/Extension Step
  – Extension is completed
  – Fluorescence is detected by qPCR instrument
  – Ready for next cycle of PCR

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: Detection – TaqMan

• Advantages
  – Very specific, because combines specificity of primers and specificity of the TaqMan probe – typically do not detect non-specific PCR product
  – Can design multiplex qPCR assays to simultaneously amplify and detect different target sequences in the same tube
    e.g., use FAM-labeled probe for nuclear target sequence and VIC-labeled probe for mitochondrial target (or Y-specific target, or Internal PCR control target, etc.)

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: Detection – TaqMan

• Some Disadvantages (relative to SYBR Green)
  – More difficult to design because of need for efficient amplification AND efficient probe hydrolysis (and possibility that amplification and hydrolysis chemistries inhibit differently)
  – More difficult to design because some TaqMan probes do not quench efficiently => large background fluorescent and lower signal-to-noise
  – For some target sequences, AT-rich sequences make probe design difficult (see “MGB” probes)
  – More expensive, due to cost of dual-labeled oligonucleotide

Slide courtesy of Dr. Mark Timken, CA DOJ
qPCR- Other Detection Chemistries

- Fluorescence detection of amplicons in real time by any number of methods
  - FRET Hybrids (Roche)
  - Molecular Beacons (NJ Dept of Public Health)
  - Scorpions
  - Light Upon Extension (LUX) primer
  - EraGen, a.k.a., “Plexor” (licensed by Promega)

EraGen qPCR Detection Chemistry

Watson-Crick pairing of synthetic (non-natural) dNTPs

- one primer is labeled on 5’-end with fluorophore (e.g., FAM) linked to a terminal iso-CTP
- fluorophore is NOT quenched before PCR
- PCR is done with standard dNTPs AND iso-GTP linked to a quencher
EraGen qPCR Detection Chemistry

**Advantages**
- Can also probe multiple target sequences
- Proposed to give good sensitivity

**Disadvantages**
- Not as widely used as TaqMan or SYBR Green, so less experimental history to rely on

**Comments**
- Licensed to Promega (for many applications, not just forensic typing) see Plexor HY

qPCR Target Region

- Autosomal, Y chromosome, mitochondrial, IPC (synthetic)
- Species specific – source specific?
- Single Copy Locus (e.g. hTERT)
- Multi Copy Locus (e.g. Alu)
- Can be a STR locus (TH01)
- The PCR amplicon can vary in size
  - 50, 100, 150, 200 base pairs
qPCR Target Region

<table>
<thead>
<tr>
<th>Assay Marker</th>
<th>Chromosome</th>
<th>Copy</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantifier Y</td>
<td>5</td>
<td>Single</td>
<td>64</td>
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<tr>
<td>Quantifier Y</td>
<td>14</td>
<td>Single</td>
<td>140</td>
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<tr>
<td>Quantifier Y</td>
<td>7</td>
<td>Single</td>
<td>130</td>
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<tr>
<td>Quantifier Y</td>
<td>17</td>
<td>Multi</td>
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<tr>
<td>Quantifier Y</td>
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<tr>
<td>Plexor HY</td>
<td>Indel</td>
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</tr>
<tr>
<td>Quantifier Y</td>
<td>17</td>
<td>Multi</td>
<td>133</td>
</tr>
</tbody>
</table>

- Multi Copy Locus (e.g. Alu)
- Increased sensitivity due to the use of a multi-copy locus
  - One cell will still have ~2,500 copies of the target
- Limited dynamic range (on the high end)
- Is there any variance between the unknown and a Calibrant in terms of number of Alu copies/cell?

Real-Time PCR Instrumentation

Basics

- Light hits the tube/vessel containing the PCR (once per cycle)
- Fluorescent dye(s) emit light corresponding to their spectral characteristics
- The emitted light is focused onto a detector
- The computer-software interface interprets the detector signal
Real-Time PCR Instrumentation

• Excitation light source range
  – Visible range 330 – 1100 nm (bulb)
  – Laser 488 nm (Argon ion)
  – Light Emitting Diodes (specific wavelength)

• Emission (fluorescence) range
  – Common fluorescent dyes
  – 500 – 700 nm
  – Filters allow light of a specific wavelength onto detector

Real-Time PCR Instrumentation

• Source: laser, LED, tungsten-halogen lamp
  – Excite the fluorescent dye
• Detector: CCD (charge coupled device), PMT (photomultiplier tube)
  – Detect the light emitted from the excited dye
• Heating/Cycling
  – Traditional heat block (plate)
  – Convection (fan oven) (capillaries, single tubes)
• How many dyes can be detected?
  – Determines the level of multiplexing

Fluorescence Detection

• As the amount of amplified DNA in the PCR increases there is a change in the amount of fluorescence

• Organic dyes
  – Free in solution (SYBR Green I)
  – Attached to a probe

FAM
SYBR Green I

Zipper et al., Nucleic Acids Research 2004 32: e103
Dye Characteristics

- Absorption and Emission Spectra for 5-FAM

Fluorophore: 5-Carboxyfluorescein (5-FAM)

- Range of light that must be put into the system in order to cause fluorescence
- Range of light that is emitted from the dye molecule
- Your light source must fit this range


- Some fluorescent dyes commonly used in qPCR

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR</td>
<td>497</td>
<td>520</td>
</tr>
<tr>
<td>FAM</td>
<td>495</td>
<td>520</td>
</tr>
<tr>
<td>TET</td>
<td>521</td>
<td>536</td>
</tr>
<tr>
<td>JOE</td>
<td>520</td>
<td>548</td>
</tr>
<tr>
<td>VIC</td>
<td>~555</td>
<td></td>
</tr>
<tr>
<td>HEX</td>
<td>526</td>
<td>556</td>
</tr>
<tr>
<td>R6G</td>
<td>524</td>
<td>557</td>
</tr>
<tr>
<td>Cy3</td>
<td>550</td>
<td>570</td>
</tr>
<tr>
<td>TAMRA</td>
<td>555</td>
<td>~578</td>
</tr>
<tr>
<td>NED</td>
<td>~576</td>
<td></td>
</tr>
<tr>
<td>Cy3.5</td>
<td>581</td>
<td>596</td>
</tr>
<tr>
<td>ROX</td>
<td>575</td>
<td>602</td>
</tr>
<tr>
<td>Texas Red</td>
<td>583</td>
<td>603</td>
</tr>
<tr>
<td>Cy5</td>
<td>649</td>
<td>670</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>675</td>
<td>694</td>
</tr>
</tbody>
</table>

Detecting Multiple Dyes

- Multiplexing from an instrument perspective

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Multiplexing</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR</td>
<td>497</td>
<td>520</td>
<td></td>
</tr>
<tr>
<td>FAM</td>
<td>495</td>
<td>520</td>
<td>1</td>
</tr>
<tr>
<td>TET</td>
<td>521</td>
<td>536</td>
<td>2</td>
</tr>
<tr>
<td>JOE</td>
<td>520</td>
<td>548</td>
<td></td>
</tr>
<tr>
<td>VIC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HEX</td>
<td>535</td>
<td>556</td>
<td></td>
</tr>
<tr>
<td>R6G</td>
<td>524</td>
<td>557</td>
<td></td>
</tr>
<tr>
<td>Cy3</td>
<td>550</td>
<td>570</td>
<td>3</td>
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<tr>
<td>TAMRA</td>
<td>555</td>
<td>576</td>
<td></td>
</tr>
<tr>
<td>NED</td>
<td>~576</td>
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<td>581</td>
<td>596</td>
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<td>ROX</td>
<td>575</td>
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<tr>
<td>Texas Red</td>
<td>583</td>
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<tr>
<td>Cy5</td>
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<tr>
<td>Cy5.5</td>
<td>675</td>
<td>694</td>
<td>7</td>
</tr>
</tbody>
</table>
CCD Charge-Coupled Device

- A charge-coupled device (CCD) is a light-sensitive integrated circuit that stores and displays the data for an image in such a way that each pixel (picture element) in the image is converted into an electrical charge.

Photomultiplier Tube (PMT)

- A photomultiplier tube, useful for light detection of very weak signals
- The absorption of a photon results in the emission of an electron
- These detectors work by amplifying the electrons

General Schematic of Instrumentation
General Schematic of Instrumentation

AB 7500
- AB 7500 is the successor to the 7000
- 7500 can be fitted for 'high speed thermal cycling'
- 96 well format
- 5 color detection
- Peltier heating block

AB 7500
- Tungsten-halogen lamp
- Fluorescence emitted from dyes is focused onto a CCD (charge-coupled device)
  - Range 500 - 660 nm
  - Cycler similar to an AB 9700
Detecting Multiple Dyes

- Multiplexing from an instrument perspective
- Ability to detect different emission wavelengths

<table>
<thead>
<tr>
<th>AB 7500</th>
<th>AB 7000</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM/SYBR</td>
<td>FAM/SYBR</td>
</tr>
<tr>
<td>VIC/JOE</td>
<td>VIC/JOE</td>
</tr>
<tr>
<td>NED/TAMRA/Cy3</td>
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<tr>
<td>ROX/Texas Red</td>
<td>ROX</td>
</tr>
<tr>
<td>Cy5</td>
<td>ROX</td>
</tr>
</tbody>
</table>

ROX is typically used as passive reference on AB instruments to correct for variance between wells.

Other Instrumentation

- Other instrumentation exists!
  - Different methods of sample heating
  - Flexibility (heating – dye detection)
  - Portability
  - Speed of thermal cycling
  - Different light sources
  - Cost (initial and consumables)
  - Different calibration/maintenance requirements

qPCR Resources

- http://www.gene-quantification.info/
  - The Reference in qPCR - Academic & Industrial Information Platform
- http://pathmicro.med.sc.edu/pcr/realtime-home.htm
  - Margaret Hunt Univ. of South Carolina School of Med.
  - Promega Plexor HY Homepage
  - Applied Biosystems Quantifiler
qPCR Bibliography


Peter M. Vallone qPCR Workshop AAFS 2008