Human DNA Quantification
Using Real-Time PCR Assays

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Introduction and Fundamentals of qPCR

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

Example experiments & troubleshooting
Steps in Forensic DNA Analysis

Usually 1-2 day process (a minimum of ~8 hours)

- Sample Collection & Storage
  - Blood Stain
  - Buccal swab

- DNA Extraction

- DNA Quantitation

- Statistics Calculated
- DNA Database search
- Paternity test
- Reference sample

- Applied Use of Information

- Multiplex PCR Amplification

- DNA separation and sizing

- STR Typing

- Interpretation of Results
Why Do We Care About Quantitating DNA?

- Forensic laboratories commonly use commercial STR typing kits
  - PowerPlex 16
  - Identifiler
  - Other kits (PPY, Yfiler, COfiler, 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


Calculation of the Quantity of DNA in a Cell

- Molecular Weight of a DNA Basepair = 618 g/mol
  A =: 313 g/mol; T: 304 g/mol;                A-T base pairs = 617 g/mol  
  G = 329 g/mol;  C: 289 g/mol;               G-C base pairs = 618 g/mol

- Molecular Weight of DNA = 1.85 x10^{12} g/mol

There are 3 billion base pairs in a haploid cell ~3 x 10^9 bp
(~3 x 10^9 bp) x (618 g/mol/bp) = 1.85 x 10^{12} g/mol

- Quantity of DNA in a Haploid Cell = 3 picograms

1 mole = 6.02 x 10^{23} molecules
(1.85 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)
= 3.08 x 10^{-12} g = 3.08 picograms (pg)
A diploid human cell contains ~6 pg genomic DNA

- One ng of DNA contains the DNA from 167 diploid cells

1 ng genomic DNA (1000 pg)/6pg/cell = ~333 copies of each locus
(2 per 167 diploid genomes)
Multiplex PCR

Identifiler kit with 1 ng of input DNA

D8S1179  D21S11  D7S820  CSF1PO

D3S1358  TH01  D13S317  D16S539  D2S1338

D19S433  VWA  TPOX  D18S51

AMEL  D5S818  FGA

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

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**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

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**Why Do We Care About Quantitating DNA??**

- Other methods.....
  - UV (260 nm, 1 OD = 50 ng/µL)
  - 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
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)

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 $2 \times 2$ more DNA; after 3 cycles $2 \times 2 \times 2$ more DNA and so on...

• $2^N$; where $N$ is the number of cycles

### Cycle Copies

<table>
<thead>
<tr>
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<th>Copies</th>
</tr>
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<tr>
<td>0</td>
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</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
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<tr>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
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<td>6</td>
<td>64</td>
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<tr>
<td>7</td>
<td>128</td>
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<td>8</td>
<td>256</td>
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<td>18</td>
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</tr>
<tr>
<td>19</td>
<td>524288</td>
</tr>
<tr>
<td>20</td>
<td>1048576</td>
</tr>
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</table>

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

$2^N$

• This is true when the reaction is running at 100% efficiency
**PCR Mechanism**

94°C: Denature the genomic DNA template

60°C: PCR primers bind to the DNA template; this will define the size of amplicon

72°C: The Taq primer complex forms and the dNTPs are incorporated into the new strand(s)
PCR Mechanism

- 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
- Cycle Number
qPCR Real Time Curves

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

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
Exponential Phase (2)

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

Very precise - reproducible

The threshold is selected in this 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).

Cycle Number

Cycles ~ 20 - 27

qPCR Real Time Curves

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

The log plot is useful to see where the amount of DNA is doubling with cycle number.

This can not be readily observed in the linear plot.
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.

Plateau Phase (4)

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

Reagents are depleted This is the end point.

Cycles ~ 27 - 35

Cycles ~ 36 - end
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, Urea, Heparin
- Organic or phenolic compounds
- Glycogen, Fats, Ca²⁺
- Tissue matrix effects
- Laboratory items, powder, etc.

PCR enhancers:
- DMSO, Glycerol, BSA
- Formamide, PEG, TMAC, TMAC etc.
- Special commercial enhancers:
  - Gene 32 protein, Perfect Match, Taq Extender,
  - E. Coli ss DNA binding

real-time PCR efficiency

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

$X_N = 100(1 + 1)^{28}$
= $2.68 \times 10^{10}$ copies

- 90%
  $X_N = 100(1 + 0.9)^{28}$
  = $6.38 \times 10^9$ copies
- 80%
  $X_N = 100(1 + 0.8)^{28}$
  = $1.40 \times 10^9$ copies
PCR Efficiency

• PCR amplifying at 100% efficiency results in the doubling of the DNA concentration with each cycle

<table>
<thead>
<tr>
<th>Copies</th>
<th>log Copies</th>
<th>Cycle</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.30103</td>
<td>1</td>
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<tr>
<td>4</td>
<td>0.60206</td>
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<td>0.90309</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>1.20412</td>
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<td>32</td>
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<td>2.40824</td>
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</tr>
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<td>512</td>
<td>2.70927</td>
<td>9</td>
</tr>
<tr>
<td>1024</td>
<td>3.0103</td>
<td>10</td>
</tr>
</tbody>
</table>

Example data illustrating the doubling in concentration with each PCR cycle

PCR Efficiency

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

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.

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

![Reaction Efficiency formula]

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

- 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

Very precise - reproducible

The 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 $C_T$ 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

An empirical way to set the Baseline would be to try different ranges and observe their effect on the CT values

Quantifiler data
Log View of Data

What is with the Confetti?

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.
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 $C_T$)
Selecting the Threshold Value

When selecting a threshold value for a full data set try and ensure that the curves are in the exponential phase (lines parallel).

Dark blue lines represent the serial dilution of a calibrant DNA. Light blue line represents an unknown.

Quantifier data

Cycle Number

Selecting the Threshold Value

Light blue line represents an unknown. $C_T = 27.45$

Quantifier data

Cycle Number
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)

~27.45
**Equation of a Straight Line**

- The equation \( Y = mX + b \) defines a straight line
- **\( m \)** is the slope
  - \( \frac{(y_1-y_2)}{(x_1-x_2)} \)
  - 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
Linear Least Squares Regression

• 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!)

Log [DNA] versus C_T

Note: the line does not go through all the points, but best approximates a line from the given data

CT value

Log [DNA]
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.

\[
\text{Rxn Efficiency} = [10^{(-1/m)}] - 1
\]

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

\[ E = 2.0019 - 1 \]

\[ E = (2.0019 - 1) = 1.019 \]

Just over 100% efficient

---

R^2 (R-squared)

• Coefficient of determination

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

\[ R^2 - \text{squared} = 1 - \frac{\sum (Y_i - \bar{Y})^2}{\sum (Y_i - \bar{Y})^2} \]

• 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]
Removing An Outlier

Solving for an Unknown

- From the data

- \( Y = mX + b \)
- \( C_T = m \log [\text{DNA}] + b \)
- Solving for [DNA]

\[
[\text{DNA}] = 10^{\frac{CT - b}{m}}
\]

- The equation above is used to estimate the [DNA] of the unknowns
Solving for an Unknown

- From the data

- Solving for [DNA]

\[
\frac{27.45 - 28.71}{-3.3172} = 10
\]

- 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

• 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>24.48</td>
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<td>18.83</td>
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<tr>
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<td>0.2</td>
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<tr>
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</tr>
<tr>
<td>High</td>
<td>1.7</td>
<td>17.58</td>
<td>16.68</td>
<td>17.13</td>
</tr>
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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

<table>
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<tr>
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<td>High</td>
<td>0.993</td>
<td>-3.421</td>
<td>1.96</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Rxn efficiency

Amp efficiency
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 effect 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

Importance of the Calibrant!

• Things to keep in mind about Calibrants

• The Calibrant is usually a pristine well-characterized DNA sample
  – Not extracted the same as the unknown
  – Not subjected to the same environment as your unknown(s)
  – Will not contain inhibitors, Heme, Ca++ etc
  – May be from a cell line or mixed source sample
  – May exhibit lot-to-lot variation (monitor this)
SRM 2372 Now Available

• The NIST SRM Office began selling SRM 2372 Human DNA Quantitation Standard on October 5, 2007

• Cost is $316.00 per unit

SRM 2372
Human DNA Quantitation Standard

Components

A: Male/single donor/RNased/NIST
B: Female/multiple donors/NIST
C: Mixture/male & female/commercial

Quantities supplied:
110 µL of Human Genomic DNA ≈ 50ng/µL

Certification

Decadic Attenuance (Absorbance) by a US National Reference Spectrophotometer
Homogeneity by a Cary 100 Bio Spectrophotometer
Validation of conventional [DNA] by Interlaboratory Study and NIST qPCR studies
HAS II Certified Values of Decadic Attenuance for SRM 2372

<table>
<thead>
<tr>
<th>Component</th>
<th>260 nm</th>
<th>error at 260nm</th>
<th>Nominal [DNA], ng/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.049</td>
<td>± 0.025</td>
<td>52.5</td>
</tr>
<tr>
<td>B</td>
<td>1.073</td>
<td>± 0.030</td>
<td>53.6</td>
</tr>
<tr>
<td>C</td>
<td>1.086</td>
<td>± 0.028</td>
<td>54.3</td>
</tr>
</tbody>
</table>

The nominal DNA concentration was estimated Using 1 OD = 50 ng/µL double stranded DNA. We do not know the uncertainty in this conversion.

Information on SRM 2372 Now on STRBase

- **Lab Resources and Tools**
  - Addresses for scientists working with STRs
  - Training Materials
  - STR Allele Sequencing
  - Population data
  - Data from NIST U.S. Population Samples
  - NIST-Developed Software including AutoDamer, mixSTR, and Multiplex QA
  - NIST Standard Reference Material for PCR-Based Testing
  - New STR Markers under Development at NIST
  - Chromosomal Locations
  - DNA Advisory Board Quality Assurance Standards
  - Interlaboratory Studies
  - NIST Mixture 2005 Interlab Study MIX05 Data
  - Validation information
  - DNA Quantitation - SRM 2372 - Click here
  - Technology for resolving STR alleles
Additional Information

Supplemental data for SRM 2372 can be found on STRBase.

Includes information on the production and characterization of the materials:
- Homogeneity study
- Interlaboratory study
- Quantifier, Alu, CFS assays
- DNA standard calibration

Overview of SRM 2372 Values and Use

Attenuance ($\lambda_{260}$) 1 OD = 50 ng/µL, DNA Concentration (ng/µL)

Certified Values

SRM 2372 Components

- A
- B
- C

Informational Values

Quantifier
- Alu qPCR
- Other assays

Different Assays
- Different Calibrants

Adjust calibrant values for each lot

Forensic Labs

“Calibrated” NIST-Traceable Calibrant for Use in Daily Work

The workflow illustrates the intended use of the SRM 2372 components. The SRM materials should be used to calibrate existing qPCR standards. It is not intended for everyday qPCR use or to replace existing kit standards.

Measure Unknown DNA Samples
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

Slide courtesy of Dr. Mark Timken, CA DOJ
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”

SYBR Green Detection

1. Denaturation
2. Annealing
3. Extension

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

Buel/Nicklas Alu qPCR Assay

Post cycling melt of products
Temperature ramps from 60°C to 95°C

The data is better viewed by taking the first derivative of the curves…
Buel/Nicklas Alu qPCR Assay

- Post cycling melt of products
- Temperature ramps from 60°C to 95°C

Derivative of the melt curve

Melts may give an indication if multiple amplicons were produced

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
TaqMan detection probe = a dual-labeled oligonucleotide
- Complimentary to target sequence (anneals between primers)
- Designed to anneal ~8-10 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”)

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

• Annealing/Extension Step

– Lengthening strand displaces 5’ end of probe

```
      Forward Primer
      5’—R—Q—5’
      3’—3’

Reverse Primer
      5’—5’
      3’—R—Q—3’
```

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
### qPCR: Detection – TaqMan

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

![Diagram of PCR process](image)

**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

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

Slide courtesy of Dr. Mark Timken, CA DOJ
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

<table>
<thead>
<tr>
<th>Assay</th>
<th>Marker</th>
<th>Chromosome</th>
<th>Copy</th>
<th>Amplicon Size (bp)</th>
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<tbody>
<tr>
<td>Quantifiler Y</td>
<td>hTERT</td>
<td>5</td>
<td>Single</td>
<td>62</td>
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<tr>
<td>Quantifiler Duo</td>
<td>SRY</td>
<td>Y</td>
<td>Single</td>
<td>64</td>
</tr>
<tr>
<td>Plexor HY</td>
<td>SRY</td>
<td>Y</td>
<td>Single</td>
<td>130</td>
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<td>Quantifiler Y</td>
<td>RPH1</td>
<td>14</td>
<td>Single</td>
<td>140</td>
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<td>Quantifiler Duo</td>
<td>SRY</td>
<td>Y</td>
<td>Single</td>
<td>130</td>
</tr>
<tr>
<td>Plexor HY</td>
<td>RNU2</td>
<td>17</td>
<td>Multi</td>
<td>99</td>
</tr>
<tr>
<td>Quantifiler Y</td>
<td>TSPY/DYZ5</td>
<td>Y</td>
<td>Multi</td>
<td>133</td>
</tr>
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<td>Richard - Toronto</td>
<td>HUMTH01</td>
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<td></td>
<td>mito tRNA Lys Gene</td>
<td>1</td>
<td>Mitochondria</td>
<td>143</td>
</tr>
</tbody>
</table>
qPCR Target Region

- 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

![Chemical structure of SYBR Green I and FAM](image)

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
- Your light source must fit this range

- Range of light that is emitted from the dye molecule
- Your detection system must be tuned to this range (filters)

Dye Characteristics

- 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>535</td>
<td>~555</td>
</tr>
<tr>
<td>HEX</td>
<td>524</td>
<td>557</td>
</tr>
<tr>
<td>R6G</td>
<td>550</td>
<td>570</td>
</tr>
<tr>
<td>Cy3</td>
<td>555</td>
<td>576</td>
</tr>
<tr>
<td>TAMRA</td>
<td>581</td>
<td>596</td>
</tr>
<tr>
<td>NED</td>
<td>575</td>
<td>602</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>
</tr>
</thead>
<tbody>
<tr>
<td>SYBRI</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>
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<td>VIC</td>
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<td>~555</td>
</tr>
<tr>
<td>HEX</td>
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<tr>
<td>R6G</td>
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<td>570</td>
</tr>
<tr>
<td>Cy3</td>
<td>555</td>
<td>576</td>
</tr>
<tr>
<td>TAMRA</td>
<td>581</td>
<td>596</td>
</tr>
<tr>
<td>NED</td>
<td>575</td>
<td>602</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>

- Singleplex - FAM
- Duplex - FAM, VIC
- Triplex - FAM, VIC, NED
**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.

http://mcdonaldobservatory.org/research/instruments/instrument.php?id=3

**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.

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/photomultipliers.html
General Schematic of Instrumentation

AB 7000

General Schematic of Instrumentation

Excitation light

Optical filter

CCD

Thermal block

light
**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

![Picture courtesy of Michelle Shepherd at AB](image-url)

**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

![Picture courtesy of Michelle Shepherd at AB](image-url)
Detecting Multiple Dyes

• Multiplexing from an instrument perspective

• Ability to detect different emission wavelengths

<table>
<thead>
<tr>
<th></th>
<th>AB 7500</th>
<th>AB 7000</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM/SYBR</td>
<td>FAM/SYBR</td>
<td></td>
</tr>
<tr>
<td>VIC/JOE</td>
<td>VIC/JOE</td>
<td></td>
</tr>
<tr>
<td>NED/TAMRA/Cy3</td>
<td>TAMRA</td>
<td></td>
</tr>
<tr>
<td>ROX/Texas Red</td>
<td>ROX</td>
<td></td>
</tr>
<tr>
<td>Cy5</td>
<td></td>
<td></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
- Introduction_to_Quantitative_PCR_Stratagene.pdf
  - Download from http://www.stratagene.com
- http://pathmicro.med.sc.edu/pcr/realtime-home.htm
  - Margaret Hunt Univ. of South Carolina School of Med.
  - Flash PCR animations
- http://www.dna.iastate.edu/frame_qpcr.html
  - Iowa State University
  - Promega Plexor HY Homepage
  - Applied Biosystems Quantifiler

qPCR Bibliography

qPCR Bibliography


Applied Biosystems Quantifiler
Applied Biosystems Quantifiler

- Quantifiler™ Human DNA Quantification Kit
  - Commercial kit (reagent QC)
  - Contains an IPC for the detection of PCR inhibitors
  - hTERT probe FAM-MGB/NFQ
  - IPC probe VIC-MGB/NFQ
    - Duplex assay
  - Validation paper published

Applied Biosystems Quantifiler

- Quantifiler™ Human DNA Quantification Kit
  - Autosomal specific
  - Single copy target
  - hTERT gene (human telomerase reverse transcriptase gene)
    - Located on chromosome 5 (at 5p15.33)
    - 62 base pair amplicon
  - Dynamic range 23 pg to 50 ng
**Applied Biosystems Quantifiler**

- Thermal Cycling Conditions
- 95°C for 10 min
- 40 cycles of:
  - 95°C for 15 sec
  - 60°C for 1 min (read fluorescence)

- No melt curve

---

**Applied Biosystems Quantifiler**

- FAM – blue – serial dilution of calibrant
- VIC – green - IPC

**Threshold set at 0.2**

**Baseline 3 to 15**

**Cycle Number**
Quantifiler Calibrant Data

- 10 ng down to 41 pg
- ~25 to 33 cycles

Quantifiler IPC

- For the standard curve the IPC curves overlay at the threshold value
- IPC is a sequence not found in nature
- VIC labeled
- Size?
Indication of inhibition by IPC
Hair samples probably melanin

IPC of Calibrants

IPC of 2 different unknowns (extracted hairs)

Cycle Number
Designing an Experiment

- 7 samples need to be evaluated
- Experiments will be performed in duplicate
- The experiment will require 2 x 2 μL of extract
- An appropriate Calibrant will be serially diluted

The experiment plate may look something like:

<table>
<thead>
<tr>
<th></th>
<th>10 ng</th>
<th>10 ng</th>
<th>1a</th>
<th>1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>4</td>
<td>2a</td>
<td>2b</td>
</tr>
<tr>
<td>C</td>
<td>1.6</td>
<td>1.6</td>
<td>3a</td>
<td>3b</td>
</tr>
<tr>
<td>D</td>
<td>0.64</td>
<td>0.64</td>
<td>4a</td>
<td>4b</td>
</tr>
<tr>
<td>E</td>
<td>0.256</td>
<td>0.256</td>
<td>5a</td>
<td>5b</td>
</tr>
<tr>
<td>F</td>
<td>0.102</td>
<td>0.102</td>
<td>6a</td>
<td>6b</td>
</tr>
<tr>
<td>G</td>
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<td>0.041</td>
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<td>7b</td>
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<tr>
<td>H</td>
<td>NTC</td>
<td>NTC</td>
<td>NTC</td>
<td>NTC</td>
</tr>
</tbody>
</table>

Standards | Samples

May vary:
Range of dilutions
Spacing of dilutions
Designing an Experiment

- Or the experiment plate may look something like:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>A</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>B</td>
<td>10</td>
<td>4</td>
<td>1.6</td>
<td>0.64</td>
<td>0.256</td>
<td>0.102</td>
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<td>NTC</td>
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<tr>
<td>C</td>
<td>10</td>
<td>4</td>
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<td>0.64</td>
<td>0.256</td>
<td>0.102</td>
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<td>D</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standards

Samples

Exact plate setup may vary
Sometimes the perimeter of the plate is avoided (evaporation, variations in cycler block heating)

Designing an Experiment

- Preparing the serial dilution (7 dilutions)
  - Will need 4 μl to run dilution series in duplicate
  - Use volumes that are reasonable to pipette
  - Prepare fresh that day

<table>
<thead>
<tr>
<th>Stock (ng/μL)</th>
<th>200</th>
<th>Buffer</th>
<th>Total</th>
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<tr>
<td>1</td>
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<td>95</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Use Tris EDTA buffer
10 mM Tris-HCl (pH 8.0)
0.1 mM Na₂EDTA

Not Water
Designing an Experiment

- Quantifiler Kit example
- The kit comes with
  - PCR Reaction Mix (dNTPs, buffer, Taq Gold, ROX)
  - Human DNA Standard (200 ng/μL)
  - Primer mix (hTERT-FAM, IPC template, and IPC-VIC)

- Total reaction volume of 25 μL

Designing an Experiment

- 10.5 μL of Primer Mix
- 12.5 μL of PCR Reaction Mix
- 2.0 μL of extract/unknown

- Add 23 μL of the Master Mix to plate/tubes
- Add 2 μL of template
- Cover with clear plastic (centrifuge to remove air bubbles)
Data Analysis - Quantifiler

- Duplex assay
  - hTERT (FAM)
  - IPC (VIC)
- Open data
- Review curves
- Set Baseline and Thresholds
- Review Standard Curve
- Review and Export data

Data Analysis - Plate

- Open data file *.SDS file
- Click on “Setup” Tab
- 48 wells used
- Columns 1-2 Calibrant
- Columns 3-6 Unknowns
- Row H – NTC (non-template controls)

Key:
- Blue – hTERT (FAM)
- Green – IPC (VIC)
- S = standard
- U = Unknown
Data Analysis - Well

Wavelengths for FAM and VIC will be detected. The material in that well is a "Standard" and has a quantity of 10 ng/μL.

Data Analysis - Instrument

We can review the thermal cycling parameters in the Instrument view.
Data Analysis - Spectra

The spectra view allows us to see how fluorescence changes with each cycle:
- Filter “A” FAM
- Filter “B” VIC
- Filter “D” Rox

Note: clicking on well(s) below will select and show specific data.

Data Analysis - Component

The component view allows us to review the raw fluorescence in each well.
Data Analysis – Amp Plot

This is where we:
- Review curve(s)
- Select a Baseline range
- Set a Threshold
- Review IPC

The majority of data review/manipulation takes place in this view.

Data: Linear and Log views are selected
Detector: Color - FAM or VIC or both
Line Color: per well or per Detector
Data Analysis – Amp Plot

- The Threshold can be set by typing in a value (0.2) or by moving the horizontal bar.
- Baseline range can be typed in manually.
- This “play” button is used to apply the Threshold/Baseline Settings.
- Green – values need to be applied.
- Grayed out – values already applied.

Data Analysis – Std Curve

- Review line parameters:
  - Slope
  - R²

- How does the plot look?
- Any outliers?
- Any non-linear behaviour in the points?
Selecting wells will give the estimated concentration of that sample. For the Standards that value will be the concentration you set (no estimate made).

All data is exported to a *.CSV file (comma delimited file).
Troubleshooting

- $R^2 < 0.99$

- The low (or high) concentration point(s) of the dilution series can sometimes be removed to improve the $R^2$ value

- If your unknowns fall in this low range you may want to repeat the experiment
Troubleshooting

Remove both points

R^2 improves
Troubleshooting

• If a point(s) in the standard curve “looks off”

• Make sure the correct concentrations are put into the plate view
  – Example (6.4 ng vs 0.64 ng)

I put 6.4 ng instead 0.64 ng in the Well Inspector
Troubleshooting

- Replicates are inconsistent
  - Evaporation of wells?
  - Do you have different volumes in the wells?
  - Volumes should all be the same
    - Review wells post-run
- Very noisy curves (observed at all [DNA])
- Spikes in the signal
  - Lamp going bad
  - Optics misaligned
  - Some technical issue (mechanical, electronics)

Troubleshooting

- The manual for any Real Time PCR instrument should probably have a section on troubleshooting
- Commercial assays typically come with a manual and literature containing details/troubleshooting tips
- For an assay taken from the literature you may want to contact the authors or other labs that are running that qPCR method
Thanks!

Questions?

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