**AAFS Workshop #6**

# Human DNA Quanitifcation 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**

## 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**

*Peter M. Vallone qPCR Workshop AAFS 2008*

## 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.

*Peter M. Vallone qPCR Workshop AAFS 2008*

## STRBase qPCR Webpage


AAFS 2008 qPCR Workshop

qPCR: Human DNA Quantification using Real-Time PCR Assays

Morning Session

Afternoon Session

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



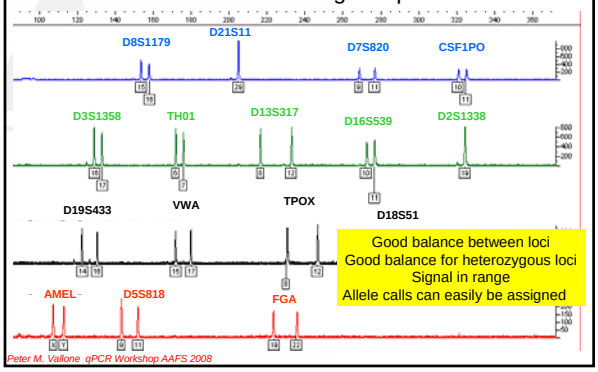
## Why Do We Care About Quantitating DNA?

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



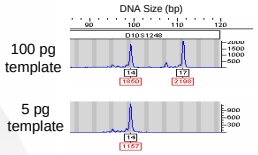
## Multiplex PCR

Identifiler kit with 1 ng of input DNA



### 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

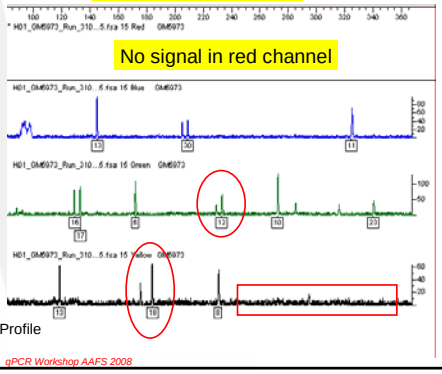



Stochastic effect when amplifying low levels of DNA produces allele dropout



### Why Do We Care About Quantitating DNA?

Too little input DNA

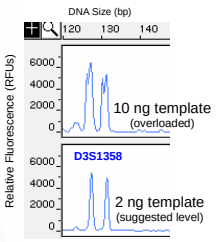



Identifiler Profile



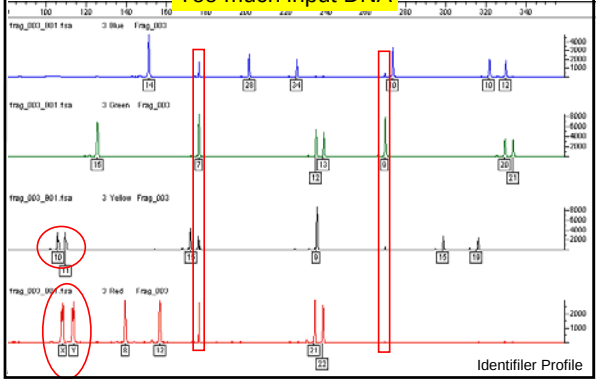
### Why Do We Care About Quantitating DNA?

- 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?**

Too much input DNA



Identifiler Profile

**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 = $$$

*Peter M. Vallone qPCR Workshop AAFS 2008*

**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)

*Peter M. Vallone qPCR Workshop AAFS 2008*

**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

Peter M. Vallone qPCR Workshop AAFS 2008

**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)

Peter M. Vallone qPCR Workshop AAFS 2008

**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)

Peter M. Vallone qPCR Workshop AAFS 2008

## 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/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

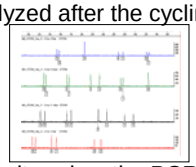


## qPCR

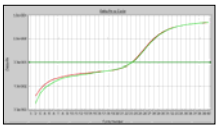
- qPCR is a recently developed technique
  - Developed by Higuchi in 1993
  - Used a modified thermal cycler with a UV detector and a CCD camera
  - Ethidium bromide was used as intercalating reporter: As [dsDNA] increased, fluorescence increased
- First paper on qPCR:
  - Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. “Kinetic PCR analysis: real-time monitoring of DNA amplification reactions” Biotechnology (N Y). 1993 Sep;11(9):1026-30

## 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



Peter M. Vallone qPCR Workshop AAFS 2008

## 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)

Peter M. Vallone qPCR Workshop AAFS 2008

## 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…

Peter M. Vallone qPCR Workshop AAFS 2008

## 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

| Cycle | Copies |
|---|---|
| 0 | 1 |
| 1 | 2 |
| 2 | 4 |
| 3 | 8 |
| 4 | 16 |
| 5 | 32 |
| 6 | 64 |
| 7 | 128 |
| 8 | 256 |
| 9 | 512 |
| 10 | 1024 |
| 11 | 2048 |
| 12 | 4096 |
| 13 | 8192 |
| 14 | 16384 |
| 15 | 32768 |
| 16 | 65536 |
| 17 | 131072 |
| 18 | 262144 |
| 19 | 524288 |
| 20 | 1048576 |

Peter M. Vallone qPCR Workshop AAFS 2008

## 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

Peter M. Vallone qPCR Workshop AAFS 2008

## PCR Amplification

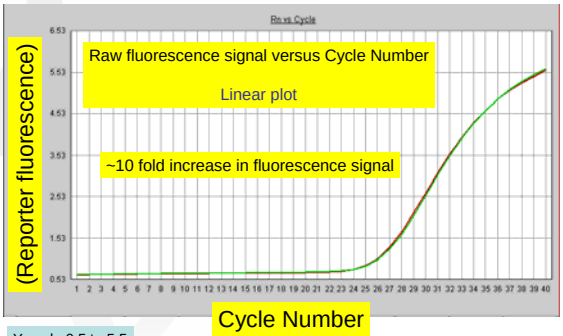
- 4 phases of qPCR amplification
  1. Lag (doubling, but not detected)
  2. Exponential (doubling)
  3. Linear (less than doubling)
  4. Plateau (little change)

Efficiency is dropping < 100%

- The exponential phase is where we make our qPCR measurements

Peter M. Vallone qPCR Workshop AAFS 2008

## qPCR Real Time Curves

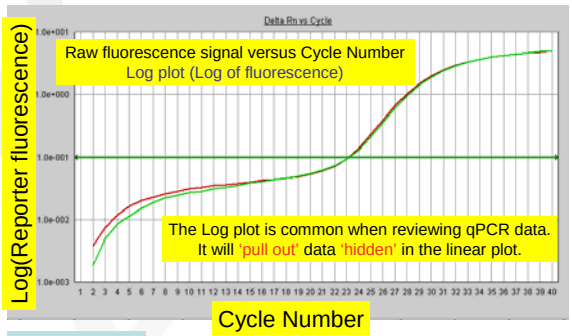



Y scale 0.5 to 5.5

Quantifiler Data



## qPCR Real Time Curves

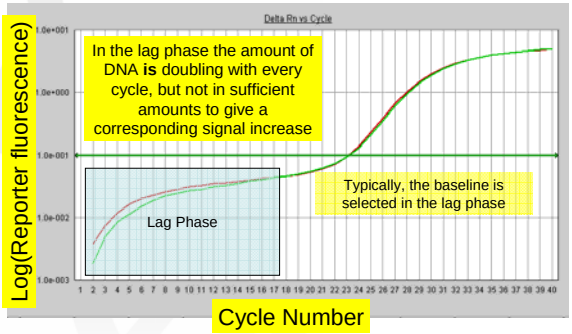



Y scale 0.001 to 10

Quantifiler Data



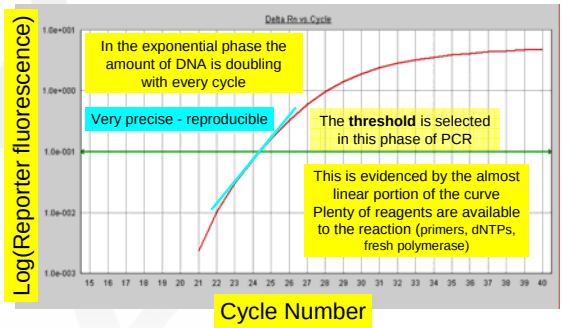
## Lag Phase (1)




Cycles ~ 1 - 20

Quantifiler Data

## Exponential Phase (2)

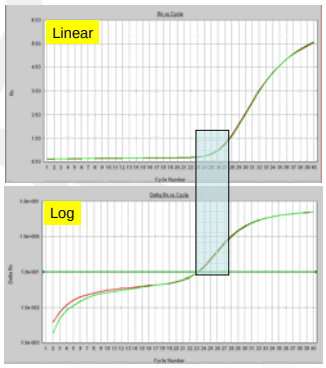



Cycles ~ 20 - 27

Quantifiler Data



## qPCR Real Time Curves




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

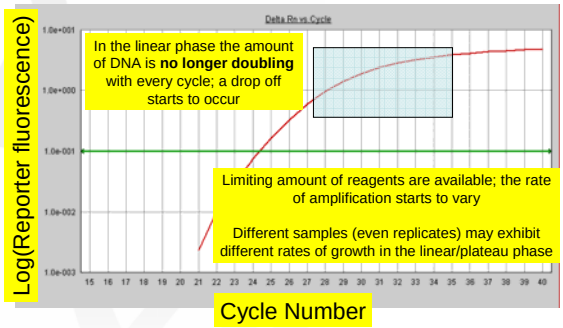
The log plot is useful to see where the amount of DNA is doubling with cycle number

This can not be readily observed in the linear plot

Quantifiler Data



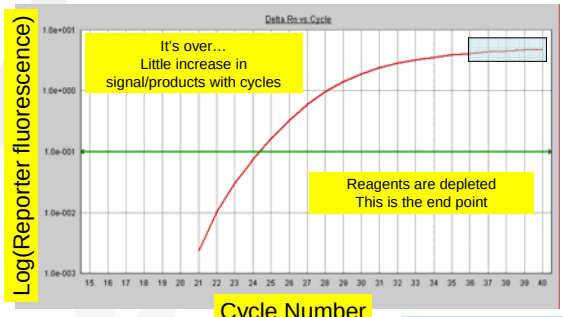
## Linear Phase (3)




Cycles ~ 27 - 35

Quantifiler Data

## Plateau Phase (4)




Cycles ~ 36 - end

Quantifiler Data

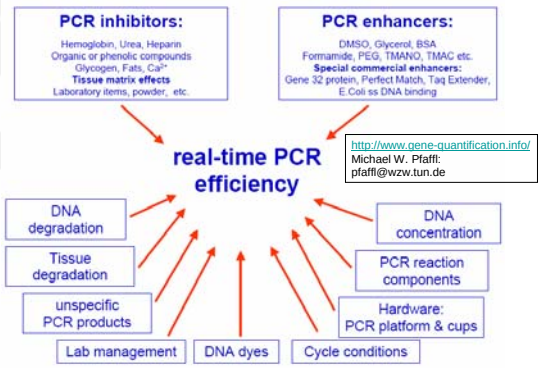


## PCR Efficiency

- 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 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



## PCR Efficiency

- 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

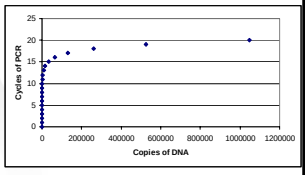


## PCR Efficiency

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

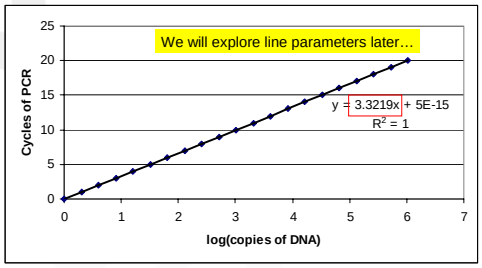
| Copies | log Copies | Cycle |
|---|---|---|
| 1 | 0 | 0 |
| 2 | 0.30103 | 1 |
| 4 | 0.60206 | 2 |
| 8 | 0.90309 | 3 |
| 16 | 1.20412 | 4 |
| 32 | 1.50515 | 5 |
| 64 | 1.80618 | 6 |
| 128 | 2.10721 | 7 |
| 256 | 2.40824 | 8 |
| 512 | 2.70927 | 9 |
| 1024 | 3.0103 | 10 |

Example data illustrating the doubling in concentration with each PCR cycle



## PCR Efficiency

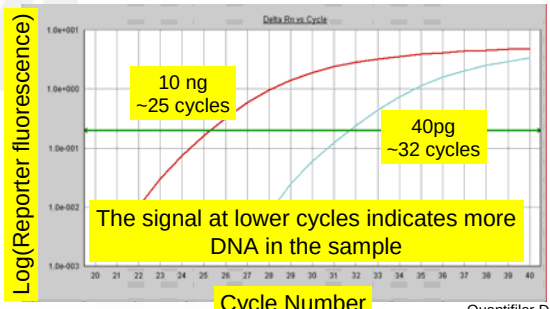
- Plotting the log(copies of DNA) versus Cycles of PCR results in a straight line with a slope of 3.32






## PCR Efficiency

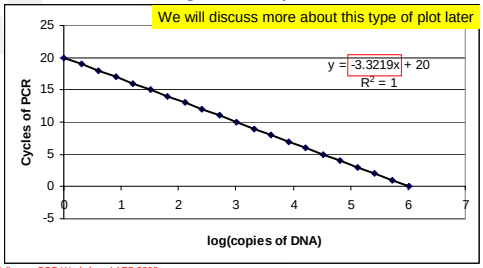
- When applied to qPCR the relationship between cycles and log(copies) is the inverse






## 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



## 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

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

A reaction efficiency of 1 is 100%

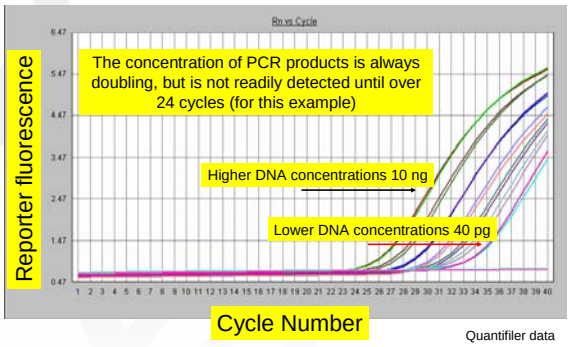
- We will see later that the slope from our qPCR data plots can be used to estimate the efficiency of the reaction



## 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

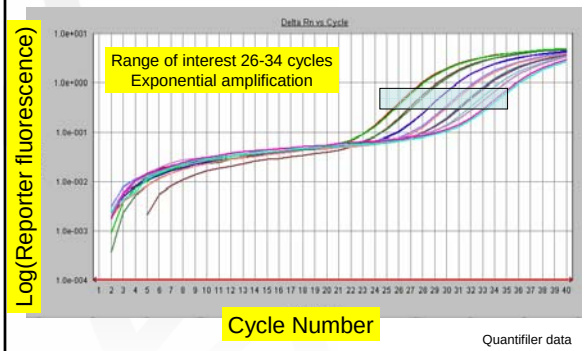
## Fluorescence vs Cycle Number




Quantifiler data



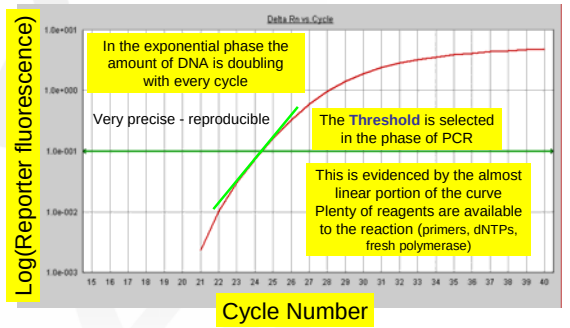
## Log View of Data




Quantifiler data



## Data Measured in the Exponential Phase




Quantifiler data

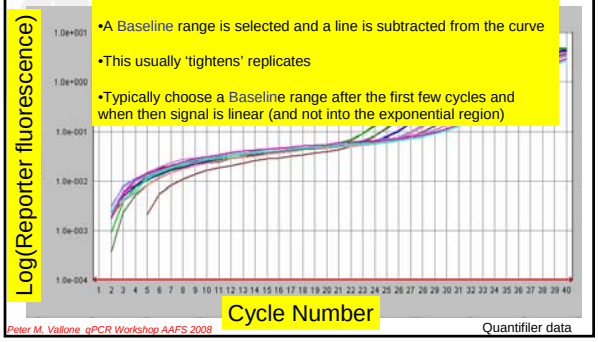
## Setting the Baseline

- 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

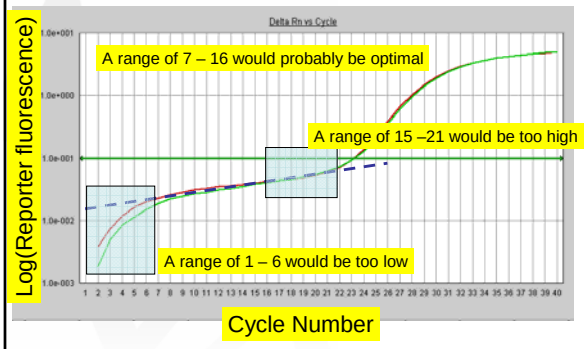
- As can be observed below, Baselines vary from sample to sample
- This is due to fluorescent noise/fluctuations (due to chemistry)
- A Baseline range is selected and a line is subtracted from the curve
- This usually 'tightens' replicates
- Typically choose a Baseline range after the first few cycles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





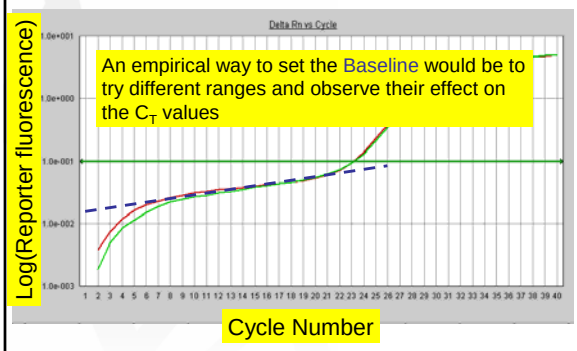

Quantifiler data

## Setting the Baseline




Quantifiler data

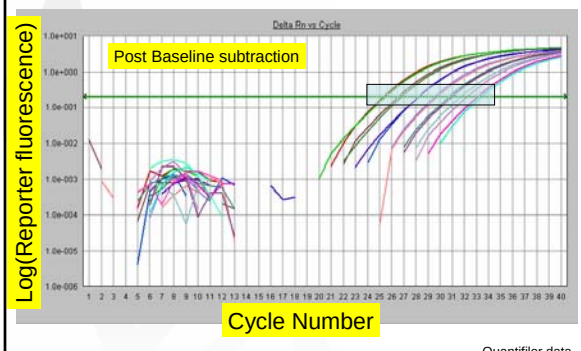
## Setting the Baseline




Quantifiler data



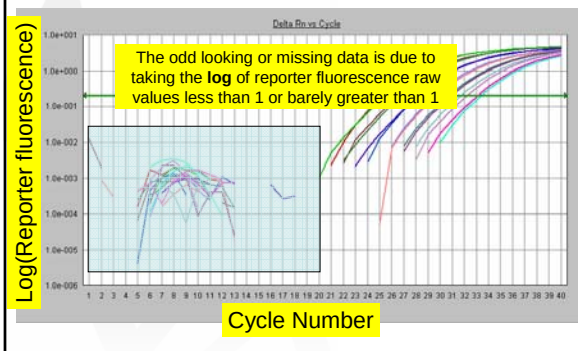
## Log View of Data




Quantifiler data



## What is with the Confetti?



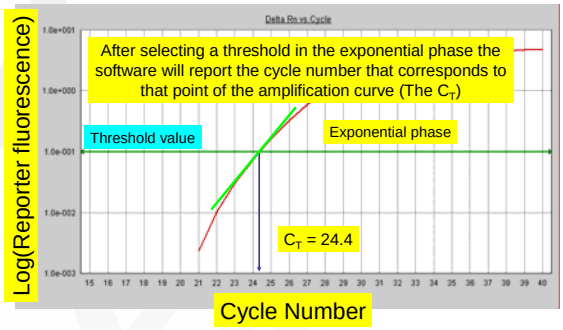

Quantifiler data

## 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



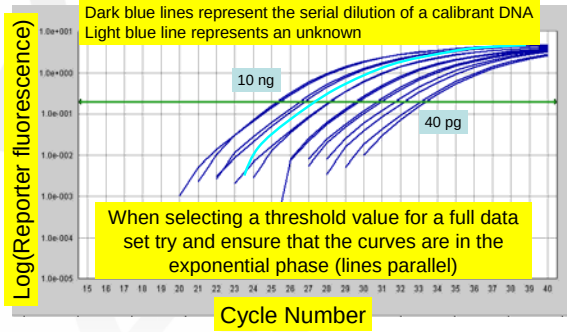
## Selecting the Threshold Value




Quantifiler data

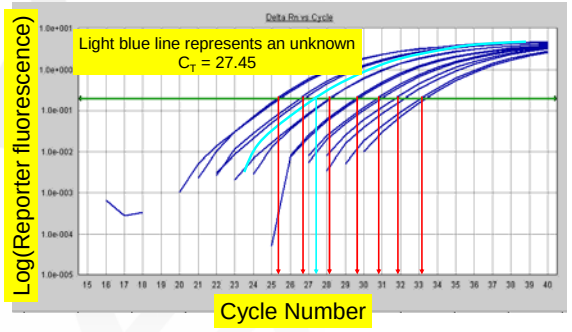


## Selecting the Threshold Value




Quantifiler data

## Selecting the Threshold Value




Quantifiler data

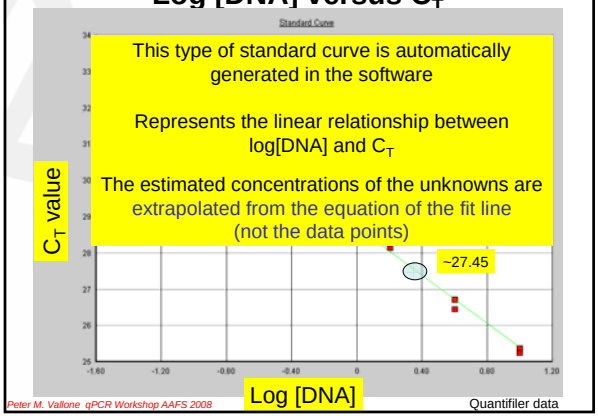


## $C_T$ Value and the Standard Curve

- After a suitable threshold has been selected the data is analyzed and the $C_T$ values are determined
- The $C_T$ 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 $C_T$






Quantifiler data

## 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

Peter M. Vallone qPCR Workshop AAFS 2008

## 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

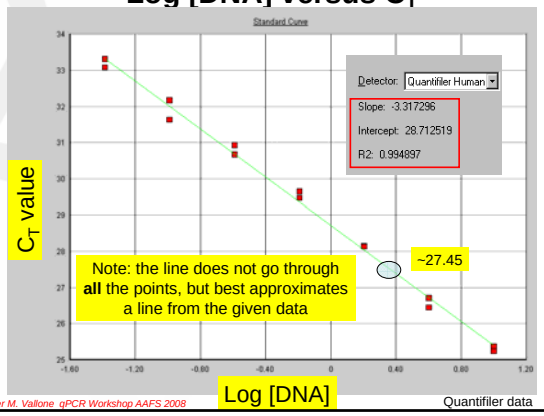
Peter M. Vallone qPCR Workshop AAFS 2008

## 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!)

Peter M. Vallone qPCR Workshop AAFS 2008

## Log [DNA] versus $C_T$






## 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

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

slope(m) = -3.317296

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

E = 2.0019-1

Just over 100 % efficient

E = (2.0019 – 1) = 1.019



## $R^2$ (R-squared)

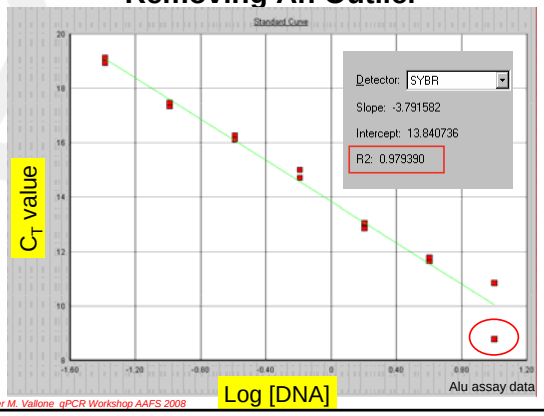
- Coefficient of determination
- A statistic for a predictive model's lack of fit using the data from which the model was derived
- $R^2$-squared $= 1 - \frac{\sum (Y_i - \hat{Y}_i)^2}{\sum (Y_i - \bar{Y}_i)^2}$
- A perfectly fitting model yields an $R^2$ of 1 (all points fall directly on the line)

## $R^2$ (R-squared)

- For most log[DNA] versus $C_T$ standard curves $R^2$ should be greater than 0.990
- Sometimes outliers can be removed to improve the $R^2$ 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)

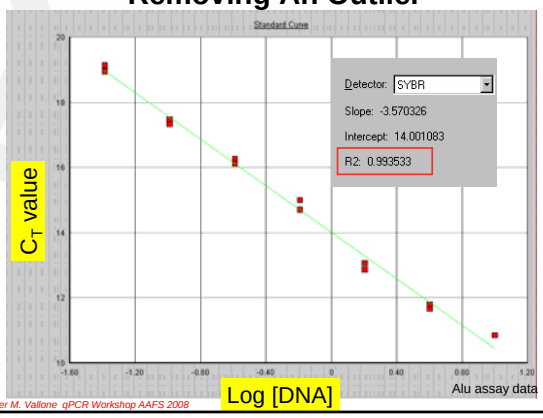


## Removing An Outlier



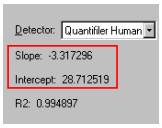



## Removing An Outlier



## Solving for an Unknown

- From the data




- Y = mX + b
- $C_T$ = m*log[DNA] + b
- Solving for [DNA]

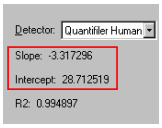
$$[\text{DNA}] = 10^{\frac{CT-b}{m}}$$

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

Peter M. Vallone qPCR Workshop AAFS 2008

## Solving for an Unknown

- From the data



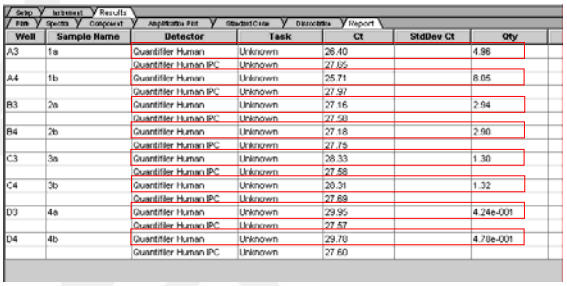

- Solving for [DNA]

$$[\text{DNA}] = 10^{\frac{27.45-28.71}{-3.3172}}$$

- 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…

Peter M. Vallone qPCR Workshop AAFS 2008

## Data Report



| Well | Sample Name | Detector | Task | Ct | StdDev Ct | Qty |
|---|---|---|---|---|---|---|
| A3 | 1a | Quantifiler Human | Unknown | 26.40 | | 4.96 |
| | | Quantifiler Human IPC | Unknown | 27.05 | | |
| A4 | 1b | Quantifiler Human | Unknown | 25.71 | | 8.05 |
| | | Quantifiler Human IPC | Unknown | 27.97 | | |
| B3 | 2a | Quantifiler Human | Unknown | 27.16 | | 2.64 |
| | | Quantifiler Human IPC | Unknown | 27.58 | | |
| B4 | 2b | Quantifiler Human | Unknown | 27.18 | | 2.60 |
| | | Quantifiler Human IPC | Unknown | 27.75 | | |
| C3 | 3a | Quantifiler Human | Unknown | 28.33 | | 1.30 |
| | | Quantifiler Human IPC | Unknown | 27.58 | | |
| C4 | 3b | Quantifiler Human | Unknown | 28.31 | | 1.32 |
| | | Quantifiler Human IPC | Unknown | 27.69 | | |
| D3 | 4a | Quantifiler Human | Unknown | 29.95 | | 4.24e-001 |
| | | Quantifiler Human IPC | Unknown | 27.57 | | |
| D4 | 4b | Quantifiler Human | Unknown | 29.78 | | 4.78e-001 |
| | | Quantifiler Human IPC | Unknown | 27.60 | | |

An example of a data report from the 7500 collection software
Report can be exported and manipulated in a spreadsheet

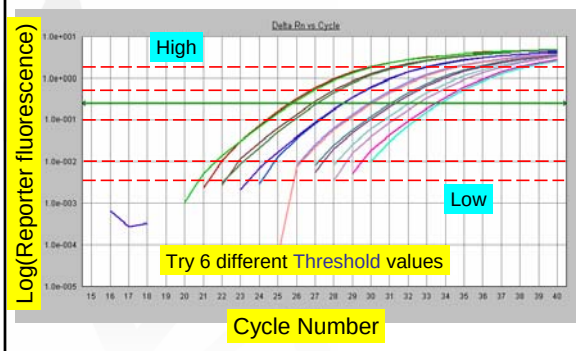
Peter M. Vallone qPCR Workshop AAFS 2008

## 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




Quantifiler data



## Varying the Threshold Value

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

Est DNA concentration ng/µL

| | Threshold | [A] | [B] | [Avg] | Stdev |
|---|---|---|---|---|---|
| Low | 0.004 | 23.51 | 24.48 | 24.00 | 0.69 |
| Low | 0.01 | 23.18 | 21.12 | 22.15 | 1.46 |
| Below Opt | 0.1 | 18.83 | 18.1 | 18.47 | 0.52 |
| Optimal | 0.2 | 17.13 | 18.13 | 17.63 | 0.71 |
| Above Opt | 0.25 | 17.5 | 16.83 | 17.17 | 0.47 |
| High | 1.7 | 17.58 | 16.68 | 17.13 | 0.64 |

1.3 ng/µL

~6.8 ng/µL difference (max)

## Varying the Threshold Value

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

Rxn efficiency

| | Threshold | R2 | slope | E | E -1 |
|---|---|---|---|---|---|
| Low | 0.004 | 0.989 | -3.474 | 1.94 | 0.94 |
| Low | 0.01 | 0.991 | -3.336 | 1.99 | 0.99 |
| Below Opt | 0.1 | 0.994 | -3.289 | 2.01 | 1.01 |
| Optimal | 0.2 | 0.994 | -3.317 | 2.00 | 1.00 |
| Above Opt | 0.25 | 0.995 | -3.322 | 2.00 | 1.00 |
| High | 1.7 | 0.993 | -3.421 | 1.96 | 0.96 |

Amp efficiency



## Varying the Threshold Value

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

Est DNA concentration ng/µL

| | Threshold | [A] | [B] | [Avg] | Stdev |
|---|---|---|---|---|---|
| **Low** | 0.004 | 1.49 | 1.67 | 1.58 | 0.127 |
| **Low** | 0.01 | 1.44 | 1.53 | 1.49 | 0.064 |
| **Below Opt** | 0.1 | 1.31 | 1.33 | 1.32 | 0.014 |
| **Optimal** | 0.2 | 1.30 | 1.32 | 1.31 | 0.014 |
| **Above Opt** | 0.25 | 1.30 | 1.32 | 1.31 | 0.014 |
| **High** | 1.7 | 1.22 | 1.18 | 1.20 | 0.028 |

~0.4 ng/µL difference (max)



## Varying the Threshold Value

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

Rxn efficiency

| | Threshold | R2 | slope | E | E -1 |
|---|---|---|---|---|---|
| **Low** | 0.004 | 0.9889 | -3.475 | 1.94 | 0.94 |
| **Low** | 0.01 | 0.9912 | -3.336 | 1.99 | 0.99 |
| **Below Opt** | 0.1 | 0.9948 | -3.290 | 2.01 | 1.01 |
| **Optimal** | 0.2 | 0.9949 | -3.317 | 2.00 | 1.00 |
| **Above Opt** | 0.25 | 0.9950 | -3.322 | 2.00 | 1.00 |
| **High** | 1.7 | 0.9931 | -3.421 | 1.96 | 0.96 |

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

Peter M. Vallone qPCR Workshop AAFS 2008

## 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)

Peter M. Vallone qPCR Workshop AAFS 2008

## Detection Chemistry

- Intercalation Dyes
- TaqMan Probes
- EraGen/Plexor

Peter M. Vallone qPCR Workshop AAFS 2008

## 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

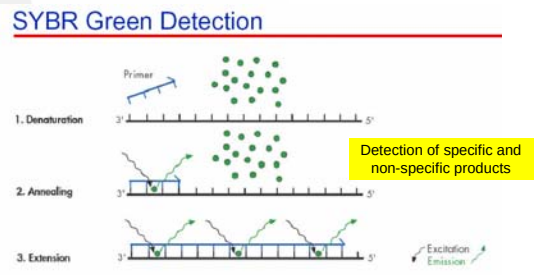
Peter M. Vallone qPCR Workshop AAFS 2008 — Slide courtesy of Dr. Mark Timken, CA DOJ

## 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"

Peter M. Vallone qPCR Workshop AAFS 2008 — Slide courtesy of Dr. Mark Timken, CA DOJ

## qPCR: **Detection – SYBR Green**




Typically detect fluorescence in real time at the end of each extension step in PCR

Peter M. Vallone qPCR Workshop AAFS 2008 — Slide courtesy of Dr. Mark Timken, CA DOJ

## 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

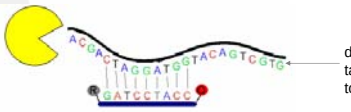
 *Slide courtesy of Dr. Mark Timken, CA DOJ*

## 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**



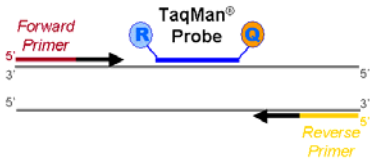

denatured target sequence to be detected

- 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")

 *Slide courtesy of Dr. Mark Timken, CA DOJ*

## 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)

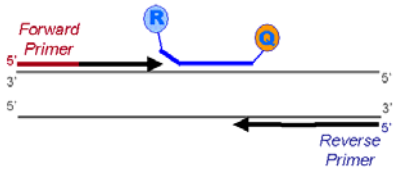




Slide courtesy of Dr. Mark Timken, CA DOJ

## qPCR: **Detection – TaqMan**

- **Annealing/Extension Step**
  - Lengthening strand displaces 5' end of probe

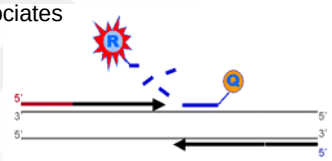




Slide courtesy of Dr. Mark Timken, CA DOJ

## 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

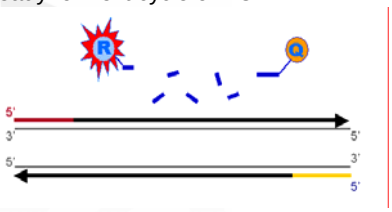




Slide courtesy of Dr. Mark Timken, CA DOJ

## 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

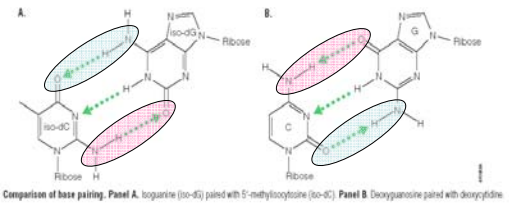
- **Fluorescence detection of amplicons in real time by any number of methods**
  - FRET Hybrids (Roche)
  - Molecular Beacons (NJ Dept of Public Health)
  - Scorpions
  - Light Upon Extension (LUX) primer
  - EraGen, a.k.a., "Plexor" (licensed by Promega)

 *Slide courtesy of Dr. Mark Timken, CA DOJ*

## EraGen qPCR Detection Chemistry

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

**(J.Am.Chem.Soc., 2004, v.126, 4550-6)**




**Comparison of base pairing. Panel A.** Isoguanine (iso-dG) paired with 5'-methylisocytosine (iso-dC). **Panel B.** Deoxyguanosine paired with deoxycytidine.

 *Slide courtesy of Dr. Mark Timken, CA DOJ*

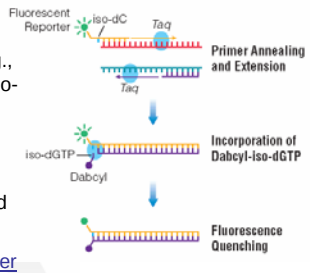
## EraGen qPCR Detection Chemistry

- one primer is labeled on 5'-end with fluorophore (e.g., FAM) linked to a terminal iso-CTP

- fluorophore is NOT quenched before PCR

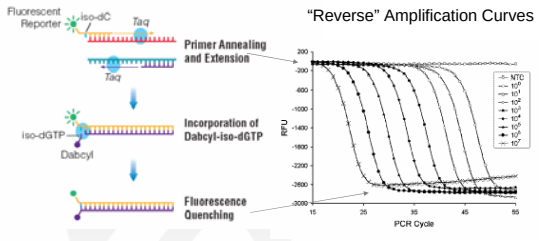
- PCR is done with standard dNTPs AND

iso-GTP linked to a quencher




 *Slide courtesy of Dr. Mark Timken, CA DOJ*

## EraGen qPCR Detection Chemistry






*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



*Slide courtesy of Dr. Mark Timken, CA DOJ*

## 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

| Assay | Marker | Chromosome | Copy | Amplicon Size (bp) |
|---|---|---|---|---|
| Quantifiler | hTERT | 5 | Single | 62 |
| Quantifiler Y | SRY | Y | Single | 64 |
| Quantifiler Duo | RPPH1 | 14 | Single | 140 |
| | SRY | Y | Single | 130 |
| Plexor HY | RNU2 | 17 | Multi | 99 |
| | TSPY/DYZ5 | Y | Multi | 133 |
| Richard - Toronto | HUMTH01 | 11 | Single | 62 |
| Timken - CA DOJ | CSF-1 | 5 | Single | 67 |
| | HUMTH01 | 11 | Single | ~180 |
| Buel - Vermont | Alu | "-" | Multi | 124 |
| | DYZ5 | Y | Multi | 137 |
| Allen - Uppsala | Retinoblastoma 1 | 13 | Single | 79 |
| | mito tRNA Lys Gene | Mitochondria | Single | 143 |

Peter M. Vallone qPCR Workshop AAFS 2008

## 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?

Peter M. Vallone qPCR Workshop AAFS 2008

## 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

Peter M. Vallone qPCR Workshop AAFS 2008

## 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



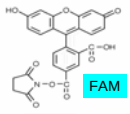
## Real-Time PCR Instrumentation

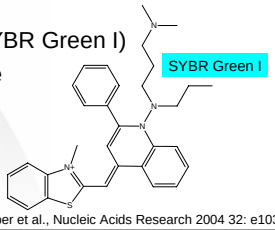
- Source: laser, LED, tungsten-halogen lamp
  - Excite the fluorescent dye
- Detector: CCD (charge coupled device), PMT (photomultiplier tube)
  - Detect the light emitted from the excited dye
- Heating/Cycling
  - Traditional heat block (plate)
  - Convection (fan oven) (capillaries, single tubes)
- 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



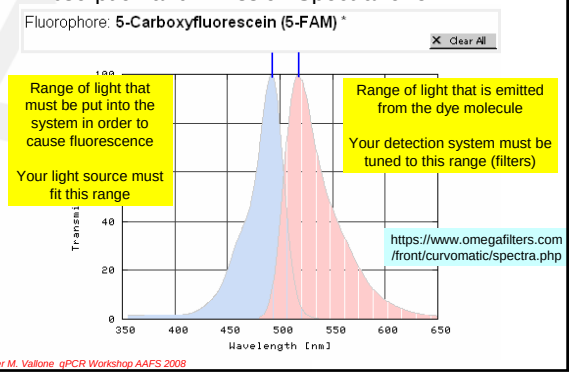






Zipper et al., Nucleic Acids Research 2004 32: e103

## Dye Characteristics

- Absorption and Emission Spectra for 5-FAM




Peter M. Vallone qPCR Workshop AAFS 2008

## Dye Characteristics

- Some fluorescent dyes commonly used in qPCR

| Dye | Excitation (nm) | Emission (nm) |
|---|---|---|
| SYBR | 497 | 520 |
| FAM | 495 | 520 |
| TET | 521 | 536 |
| JOE | 520 | 548 |
| VIC | | ~555 |
| HEX | 535 | 556 |
| R6G | 524 | 557 |
| Cy3 | 550 | 570 |
| TAMRA | 555 | 576 |
| NED | | ~576 |
| Cy3.5 | 581 | 596 |
| ROX | 575 | 602 |
| Texas Red | 583 | 603 |
| Cy5 | 649 | 670 |
| Cy5.5 | 675 | 694 |

Peter M. Vallone qPCR Workshop AAFS 2008

## Detecting Multiple Dyes

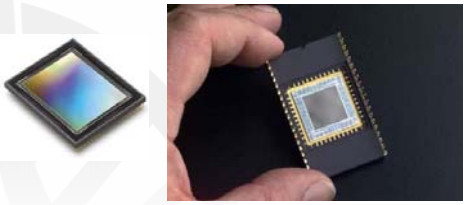
- Multiplexing from an instrument perspective

| Dye | Excitation (nm) | Emission (nm) | |
|---|---|---|---|
| SYBRI | 497 | 520 | |
| FAM | 495 | 520 | 1 |
| TET | 521 | 536 | 2 |
| JOE | 520 | 548 | |
| VIC | | ~555 | Singleplex - FAM |
| HEX | 535 | 556 | Duplex - FAM,VIC |
| R6G | 524 | 557 | Triplex - FAM,VIC,NED |
| Cy3 | 550 | 570 | 3 |
| TAMRA | 555 | 576 | |
| NED | | ~576 | |
| Cy3.5 | 581 | 596 | 4 |
| ROX | 575 | 602 | 5 |
| Texas Red | 583 | 603 | |
| Cy5 | 649 | 670 | 6 |
| Cy5.5 | 675 | 694 | 7 |

Peter M. Vallone qPCR Workshop AAFS 2008

## 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?i_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

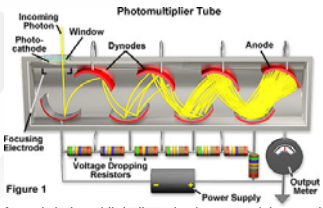
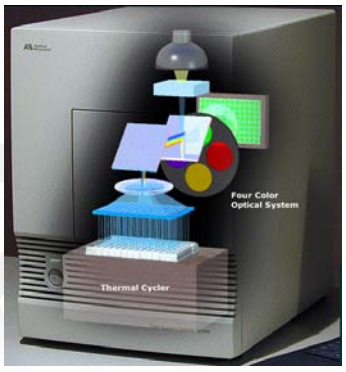



Figure 1

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/photomultipliers.html

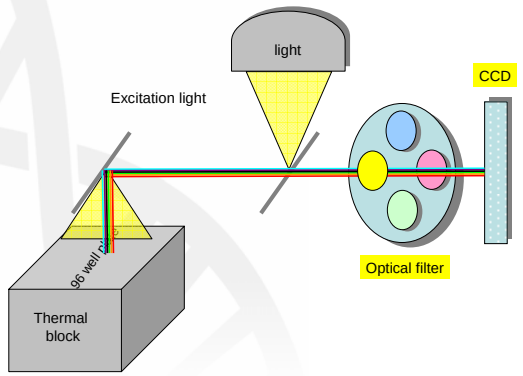


## General Schematic of Instrumentation




AB 7000

## General Schematic of Instrumentation




Peter M. Vallone qPCR Workshop AAFS 2008

## 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

Peter M. Vallone qPCR Workshop AAFS 2008

## 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

Peter M. Vallone qPCR Workshop AAFS 2008

## Detecting Multiple Dyes

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

| AB 7500 | AB 7000 |
|---|---|
| FAM/SYBRI | FAM/SYBRI |
| VIC/JOE | VIC/JOE |
| NED/TAMRA/Cy3 | TAMRA |
| ROX/Texas Red | ROX |
| Cy5 | |

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

Peter M. Vallone qPCR Workshop AAFS 2008

## 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






Peter M. Vallone qPCR Workshop AAFS 2008

## 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.
- http://www.dnalc.org/ddnalc/resources/shockwave/pcranwhole.html
  - Flash PCR animations
- http://www.dna.iastate.edu/frame_qpcr.html
  - Iowa State University
- http://www.promega.com/plexorhy/default.htm
  - Promega Plexor HY Homepage
- https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601641
  - Applied Biosystems Quantifiler

Peter M. Vallone qPCR Workshop AAFS 2008

## qPCR Bibliography


1. Andreasson, H. and Allen, M. (2003) Rapid quantification and sex determination of forensic evidence materials, *J. Forensic Sci. 48*, 1280-1287.
2. Andreasson, H., Nilsson, M., Budowle, B., Lundberg, H., and Allen, M. (2006) Nuclear and mitochondrial DNA quantification of various forensic materials, *Forensic Sci. Int. 164*, 56-64.
3. Green, R. L., Roinestad, I. C., Boland, C., and Hennessy, L. K. (2005) Developmental validation of the quantifiler real-time PCR kits for the quantification of human nuclear DNA samples, *J. Forensic Sci. 50*, 809-825.
4. Hudlow, W., Chong, M., Swango, K., Timken, M., and Buoncristiani, M. (2008) A quadruplex real-time qPCR assay for the simultaneous assessment of total human DNA, human male DNA, DNA degradation and the presence of PCR inhibitors in forensic samples: A diagnostic tool for STR typing. *Forensic Science International: Genetics 2*, 108-125.
5. Kline, M. C., Duewer, D. L., Redman, J. W., and Butler, J. M. (2005) Results from the NIST 2004 DNA Quantitation Study, *J. Forensic Sci. 50*, 570-578.
6. Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., Sindelka, R., Sjoback, R., Sjogreen, B., Strombom, L., Stahlberg, A., and Zoric, N. (2006) The real-time polymerase chain reaction, *Mol. Aspects Med. 27*, 95-125.
7. Nicklas, J. A. and Buel, E. (2003) Development of an Alu-based, real-time PCR method for quantitation of human DNA in forensic samples, *J. Forensic Sci. 48*, 936-944.
8. Nicklas, J. A. and Buel, E. (2003) Quantification of DNA in forensic samples, *Anal. Bioanal. Chem. 376*, 1160-1167.
9. Nicklas, J. A. and Buel, E. (2003) Development of an Alu-based, QSY 7-labeled primer PCR method for quantitation of human DNA in forensic samples, *J. Forensic Sci. 48*, 282-291.
10. Nicklas, J. A. and Buel, E. (2005) An Alu-based, MGB Eclipse real-time PCR method for quantitation of human DNA in forensic samples, *J. Forensic Sci. 50*, 1081-1090.
11. Nicklas, J. A. and Buel, E. (2006) Simultaneous determination of total human and male DNA using a duplex real-time PCR assay, *J. Forensic Sci. 51*, 1005-1015.




## qPCR Bibliography


12. Richard, M. L., Frappier, R. H., and Newman, J. C. (2003) Developmental validation of a real-time quantitative PCR assay for automated quantification of human DNA, *J. Forensic Sci. 48*, 1041-1046.
13. Shewale, J. G., Schneida, E., Wilson, J., Walker, J. A., Batzer, M. A., and Sinha, S. K. (2007) Human genomic DNA quantitation system, H-Quant: development and validation for use in forensic casework, *J. Forensic Sci. 52*, 364-370.
14. Swango, K. L., Timken, M. D., Chong, M. D., and Buoncristiani, M. R. (2006) A quantitative PCR assay for the assessment of DNA degradation in forensic samples, *Forensic Sci. Int. 158*, 14-26.
15. Swango, K. L., Hudlow, W. R., Timken, M. D., and Buoncristiani, M. R. (2007) Developmental validation of a multiplex qPCR assay for assessing the quantity and quality of nuclear DNA in forensic samples, *Forensic Sci. Int. 170*, 35-45.
16. Timken, M. D., Swango, K. L., Orrego, C., and Buoncristiani, M. R. (2005) A duplex real-time qPCR assay for the quantification of human nuclear and mitochondrial DNA in forensic samples: implications for quantifying DNA in degraded samples, *J. Forensic Sci. 50*, 1044-1060.
17. Walker, J. A., Hughes, D. A., Hedges, D. J., Anders, B. A., Laborde, M. E., Shewale, J., Sinha, S. K., and Batzer, M. A. (2004) Quantitative PCR for DNA identification based on genome-specific interspersed repetitive elements, *Genomics 83*, 518-527.
18. Walker, J. A., Hedges, D. J., Perodeau, B. P., Landry, K. E., Stoilova, N., Laborde, M. E., Shewale, J., Sinha, S. K., and Batzer, M. A. (2005) Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial, and male Y-chromosome DNA: application in human identification, *Anal.Biochem. 337*, 89-97.
19. Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions, *Biotechnology (N. Y. ) 11*, 1026-1030.