Human DNA Quanitifcation Using Real-Time PCR Assays

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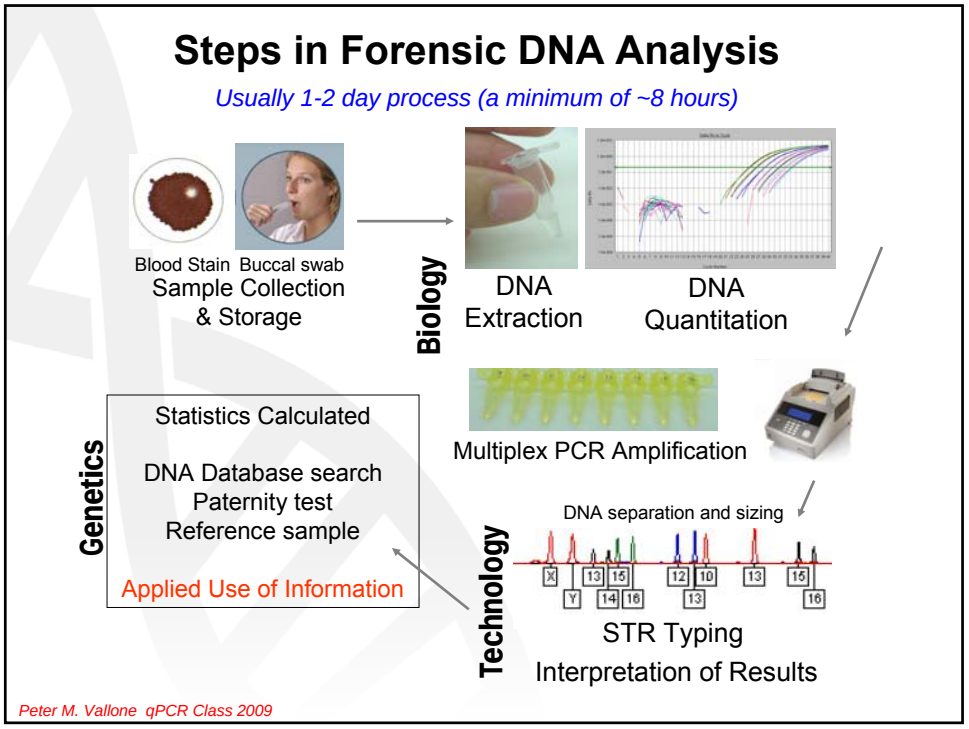
Introduction and Fundamentals of qPCR

- 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)
Blood Stain
Buccal swab
Sample Collection
& Storage
Biology
DNA
Extraction
DNA
Quantitation
Multiplex PCR Amplification
Genetics
Statistics Calculated
DNA Database search
Paternity test
Reference sample
Applied Use of Information
Technology
DNA separation and sizing
STR Typing
Interpretation of Results
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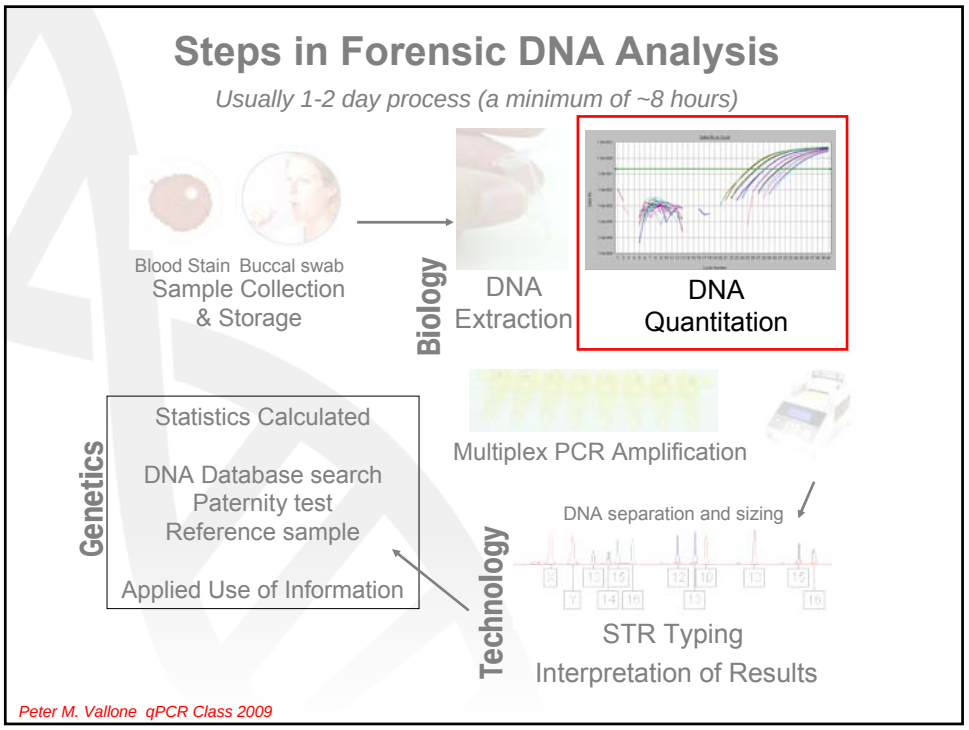
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Why Do We Care About Quantitating DNA?

- 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
  http://www.fbi.gov/hq/lab/fsc/backissu/july2000/codispre.htm

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Calculation of the Quantity of DNA in a Cell

- Molecular Weight of a DNA Basepair = 618g/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)

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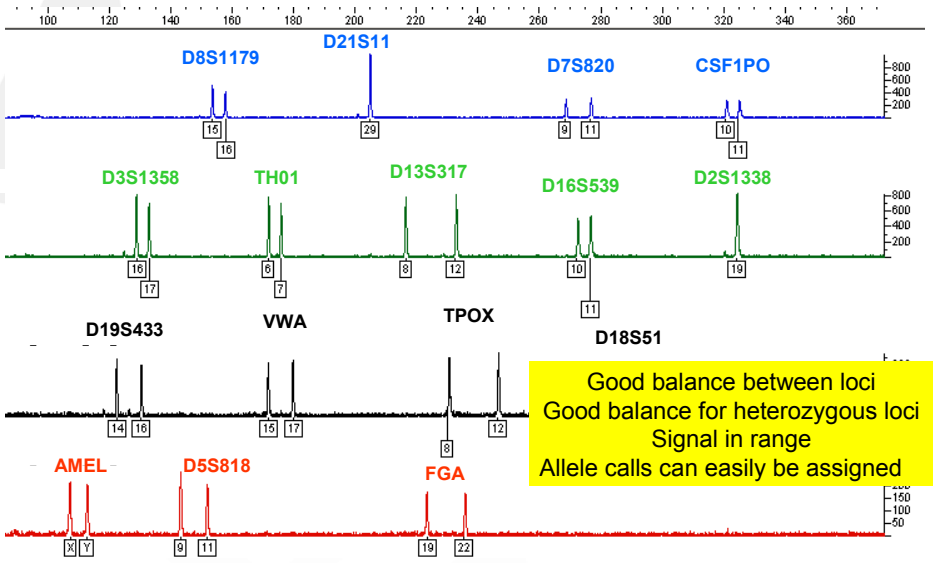
Multiplex PCR

Identifiler kit with 1 ng of input DNA



Good balance between loci
Good balance for heterozygous loci
Signal in range
Allele calls can easily be assigned

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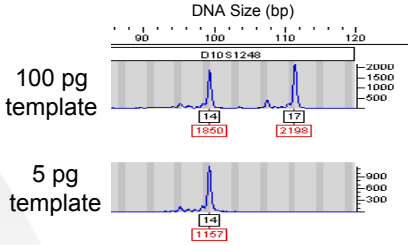
Why Do We Care About Quantitating DNA?

- 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

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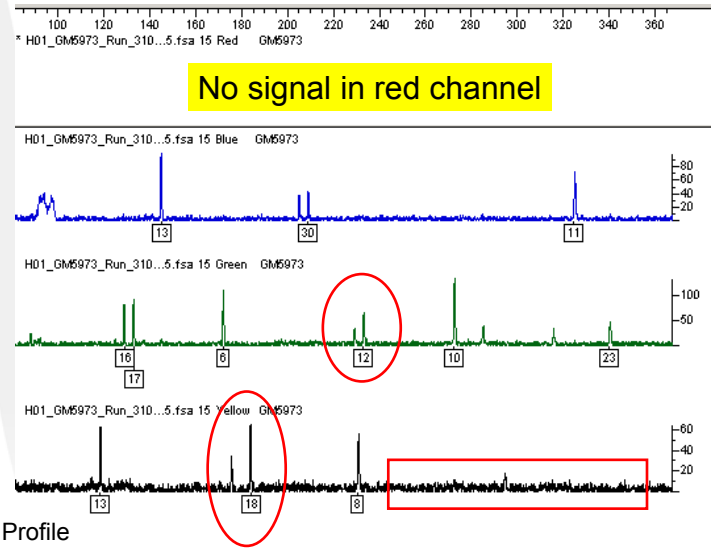
Why Do We Care About Quantitating DNA?

Too little input DNA



No signal in red channel

Identifiler Profile

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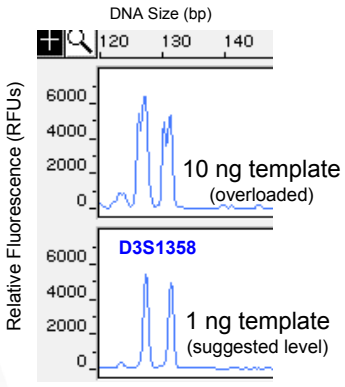
Why Do We Care About Quantitating DNA?

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



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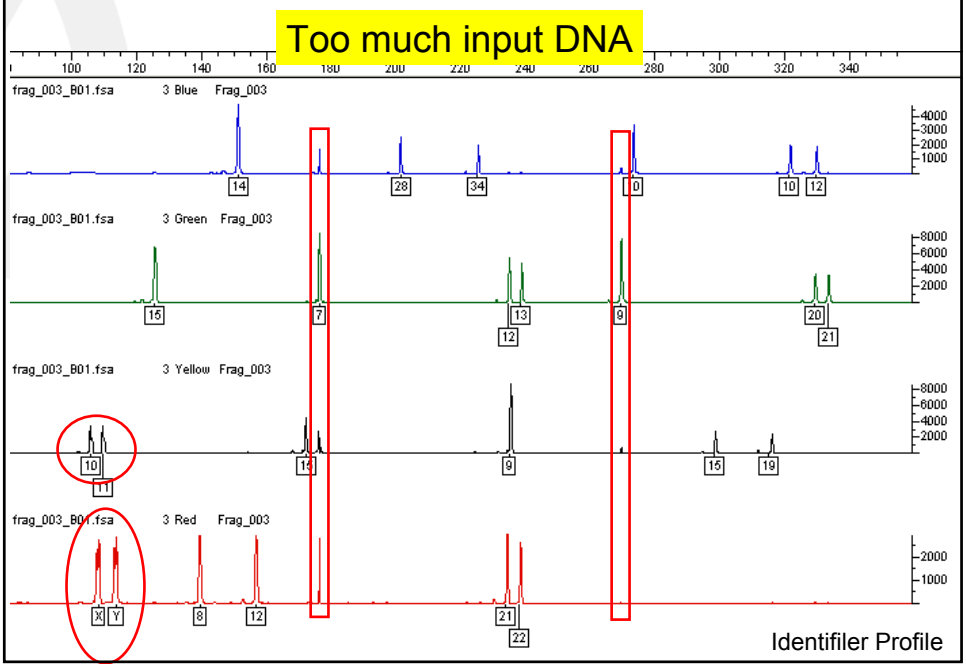
Why Do We Care About Quantitating DNA?



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)

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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

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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)

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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

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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

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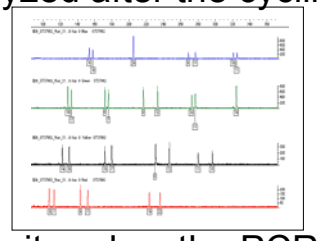
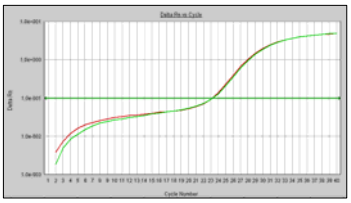
PCR/qPCR What is the Difference?

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



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



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Why Real-Time qPCR?

Advantages

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

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Why Real-Time qPCR?

Advantages

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

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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

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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)

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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

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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

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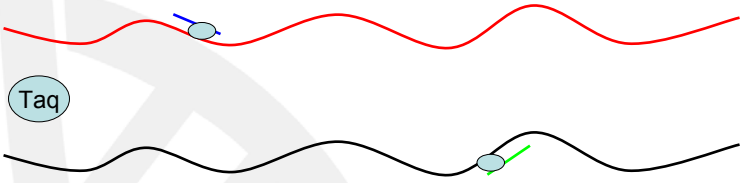
PCR Mechanism

94°C: Denature the genomic DNA template



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

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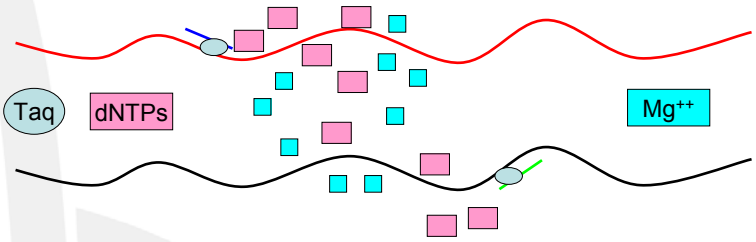
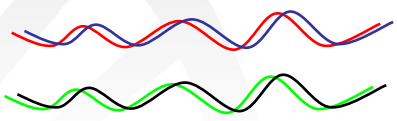
PCR Mechanism



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



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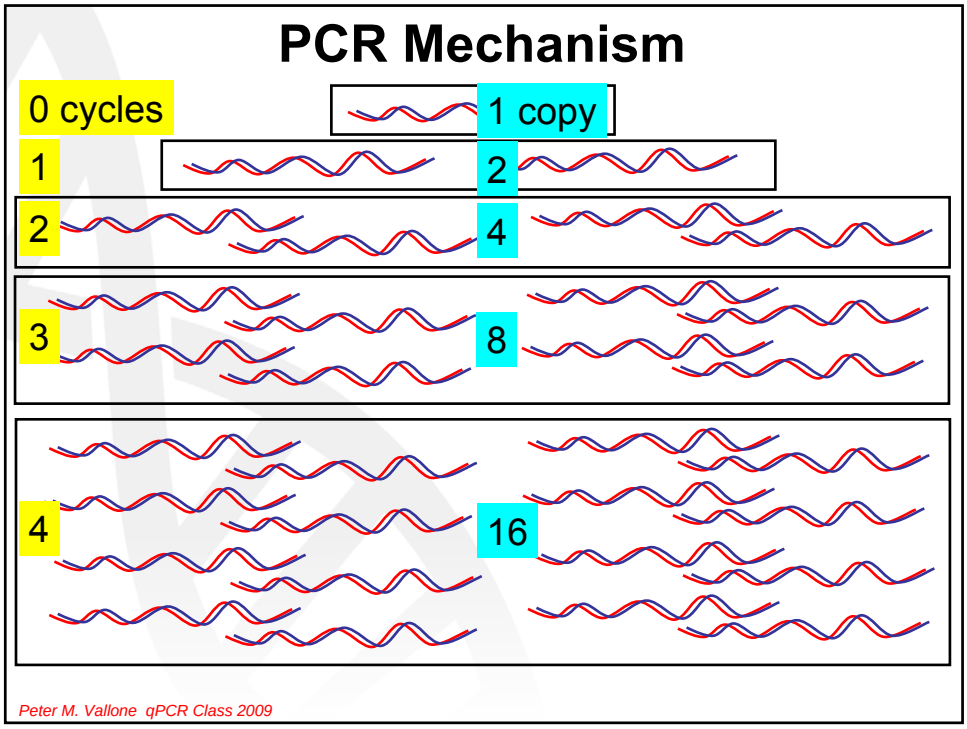

PCR Mechanism

- 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

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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

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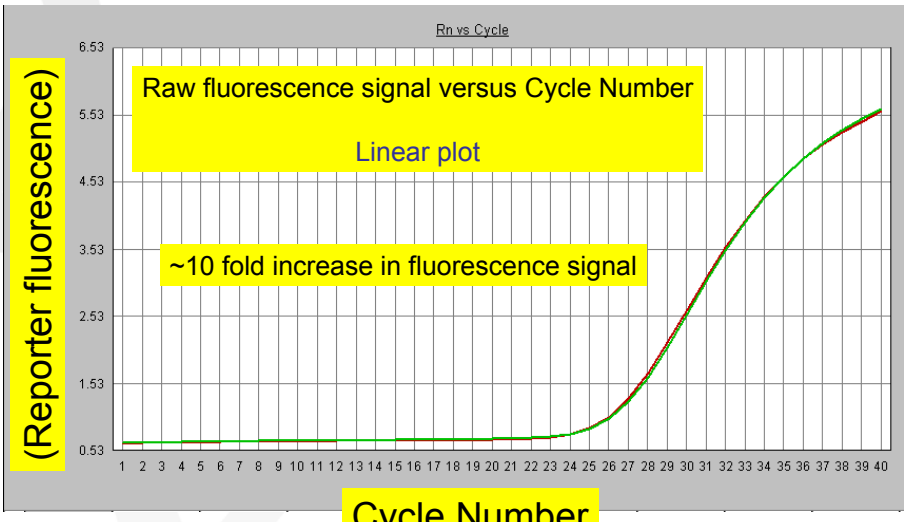
qPCR Real Time Curves



Y scale 0.5 to 5.5

Quantifiler Data

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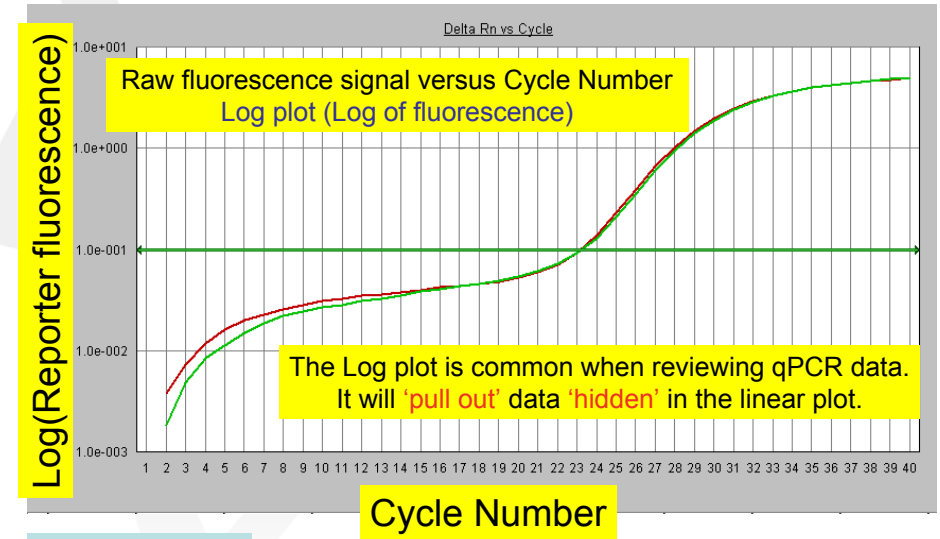
qPCR Real Time Curves



Y scale 0.001 to 10

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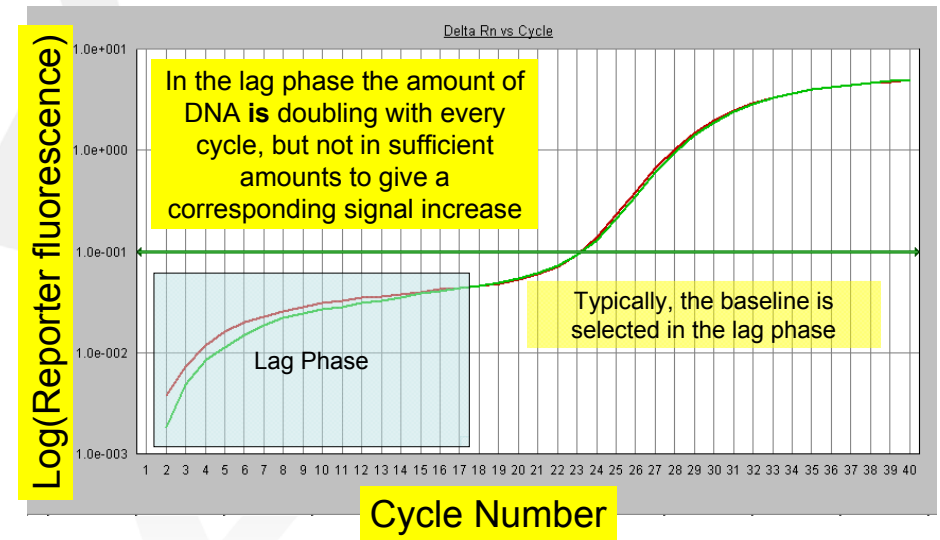
Quantifiler Data

Lag Phase (1)



Cycles ~ 1 - 20

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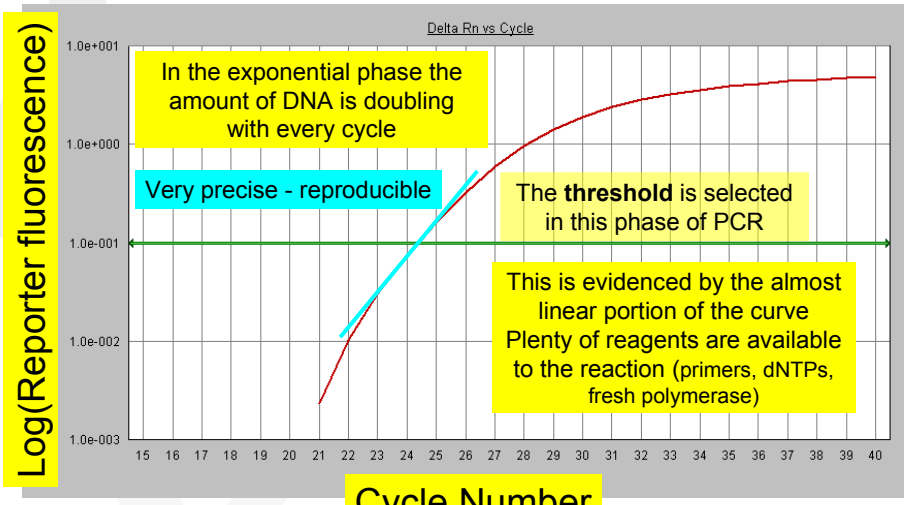
Quantifiler Data

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)

Cycles ~ 20 - 27

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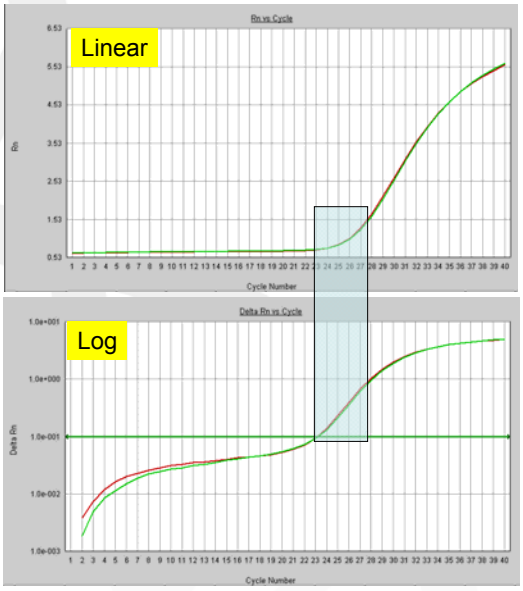
Quantifiler Data

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

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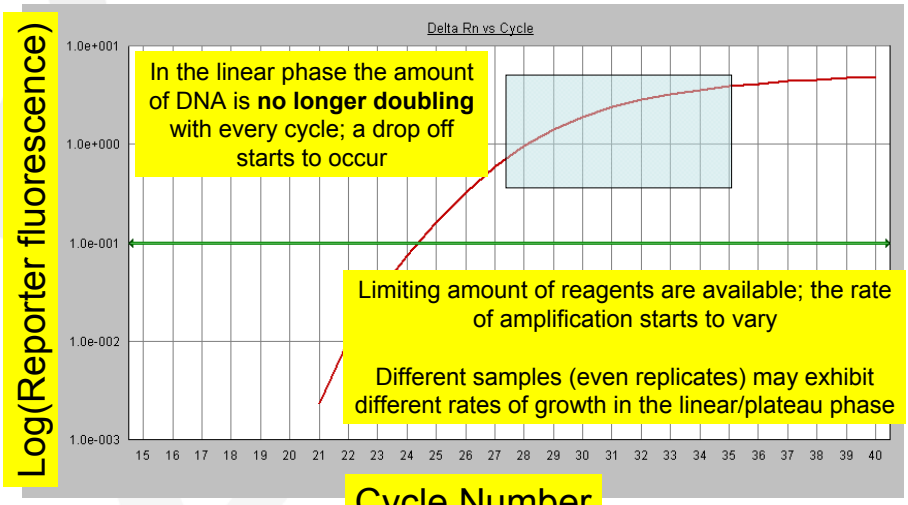
Quantifiler Data

Linear Phase (3)



Cycles ~ 27 - 35

Quantifiler Data

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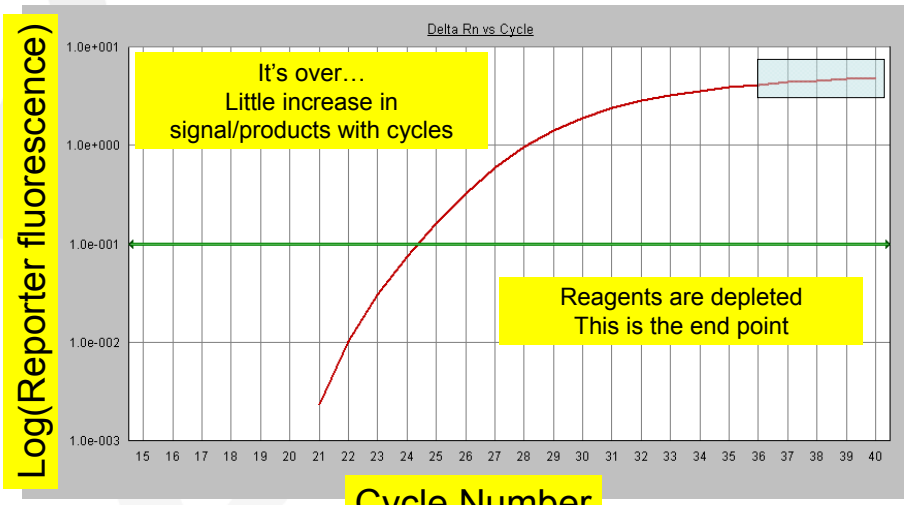
Plateau Phase (4)



Cycles ~ 36 - end

Quantifiler Data

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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?

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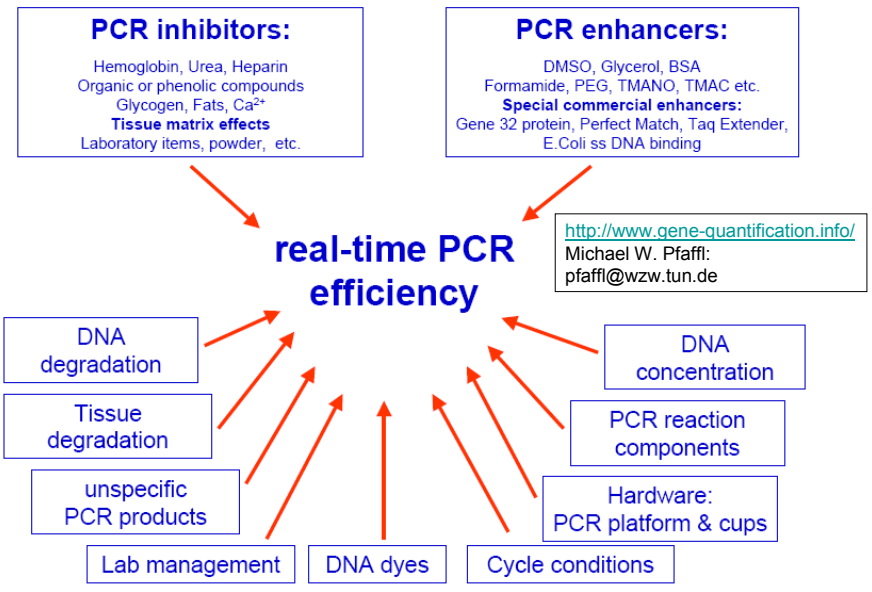
PCR Efficiency



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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%

  $X_N = 100(1 + 0.8)^{28}$

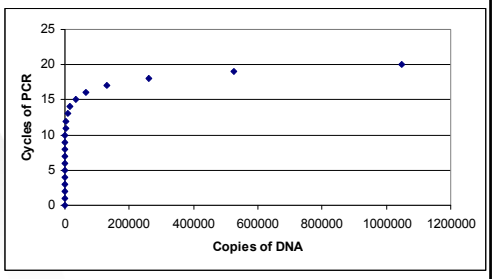
  $= 1.40 \times 10^{9}$ copies

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



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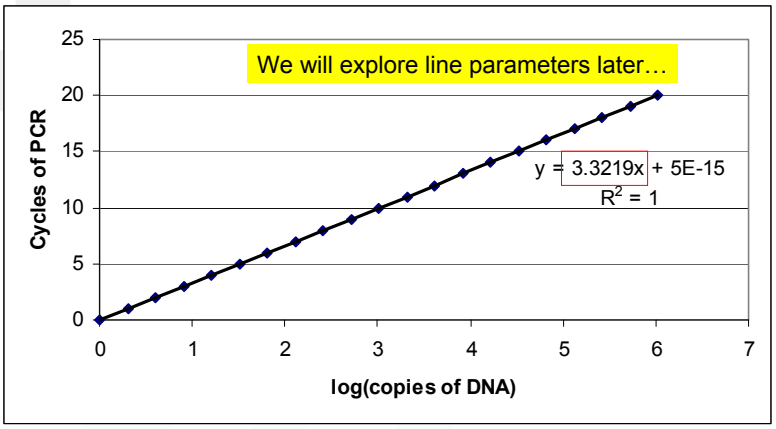
PCR Efficiency

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



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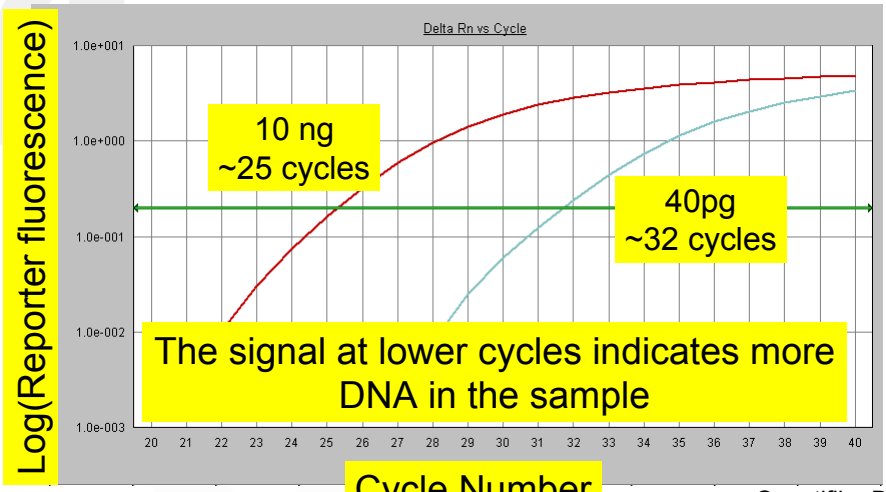
PCR Efficiency

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



Quantifiler Data

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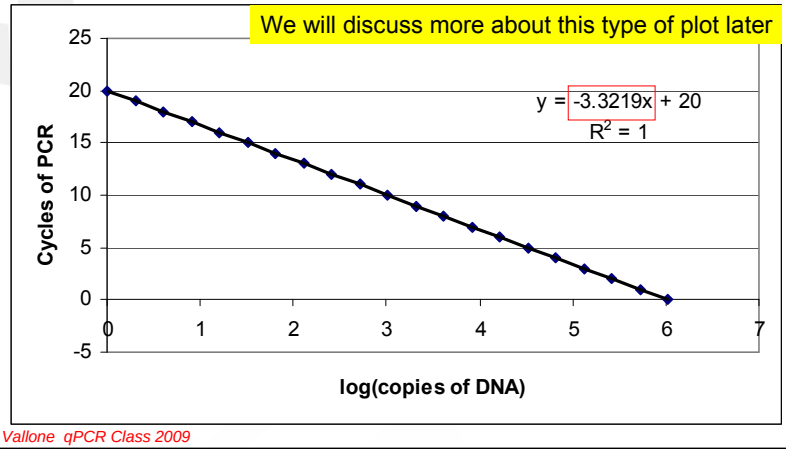
PCR Efficiency

- 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



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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

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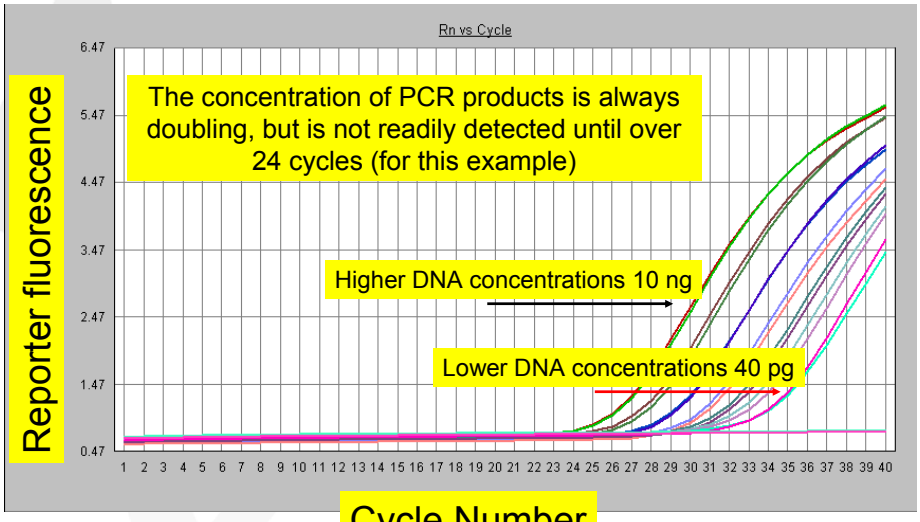
Fluorescence vs Cycle Number



Quantifiler data

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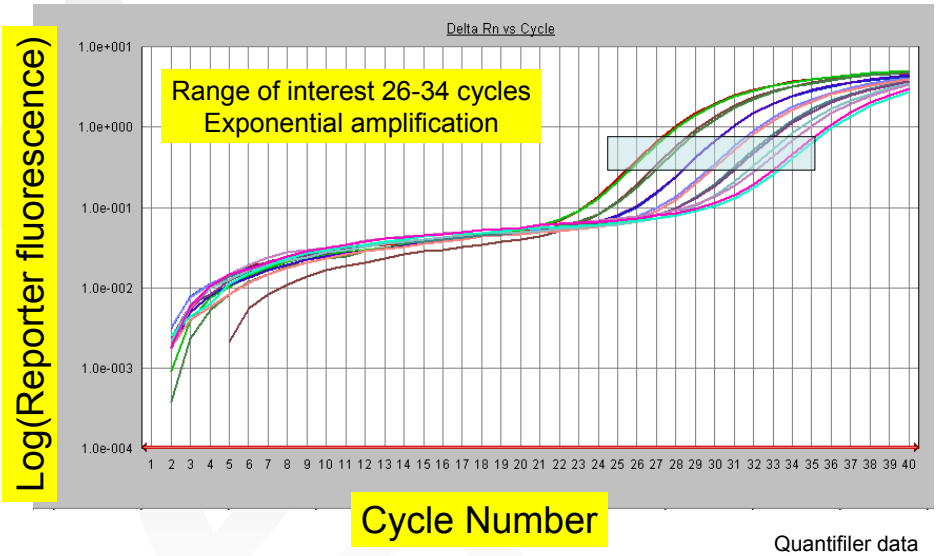
Log View of Data



Quantifiler data

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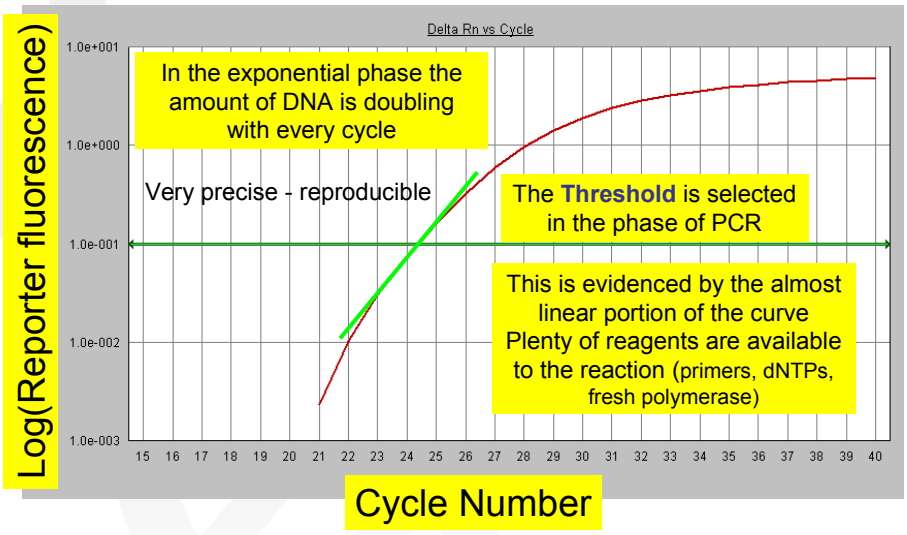
Data Measured in the Exponential Phase



Quantifiler data

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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

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Log View of Data

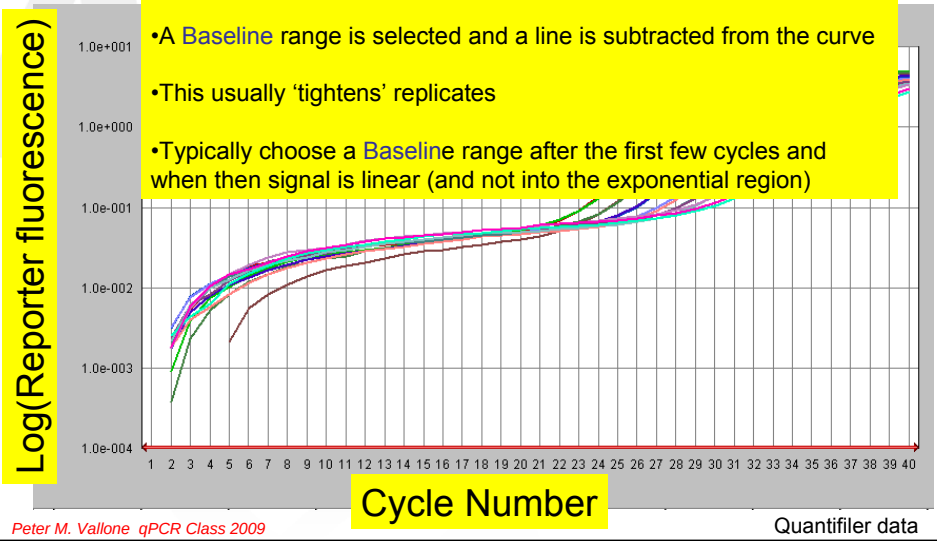
•As can be observed below, Baselines vary from sample to sample

•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)



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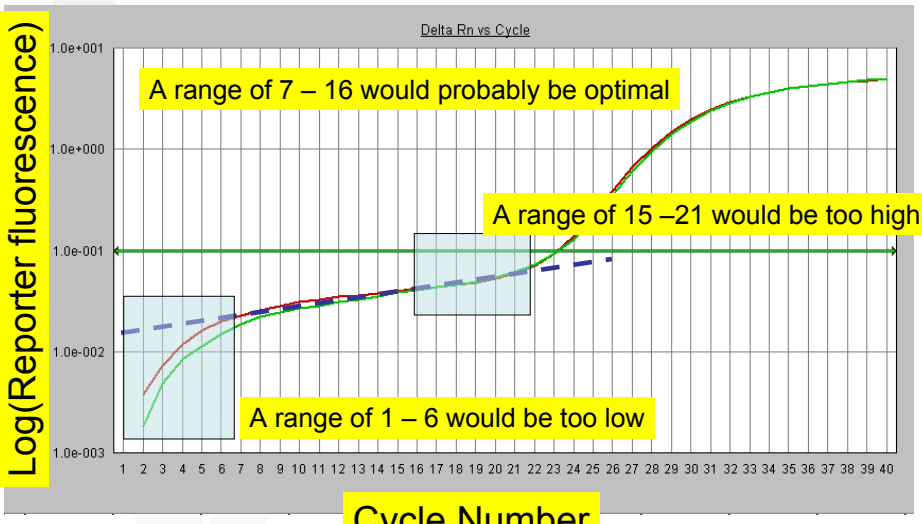
Setting the Baseline



Quantifiler data

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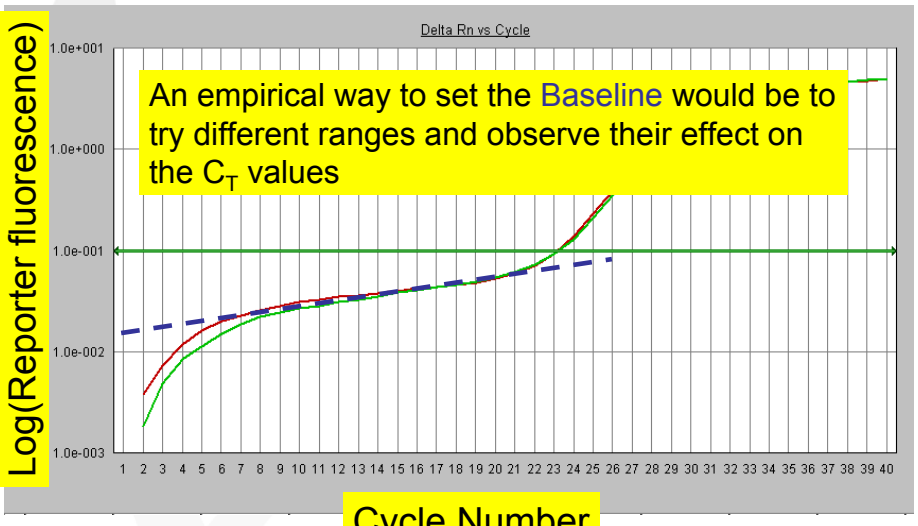
Setting the Baseline



Quantifiler data

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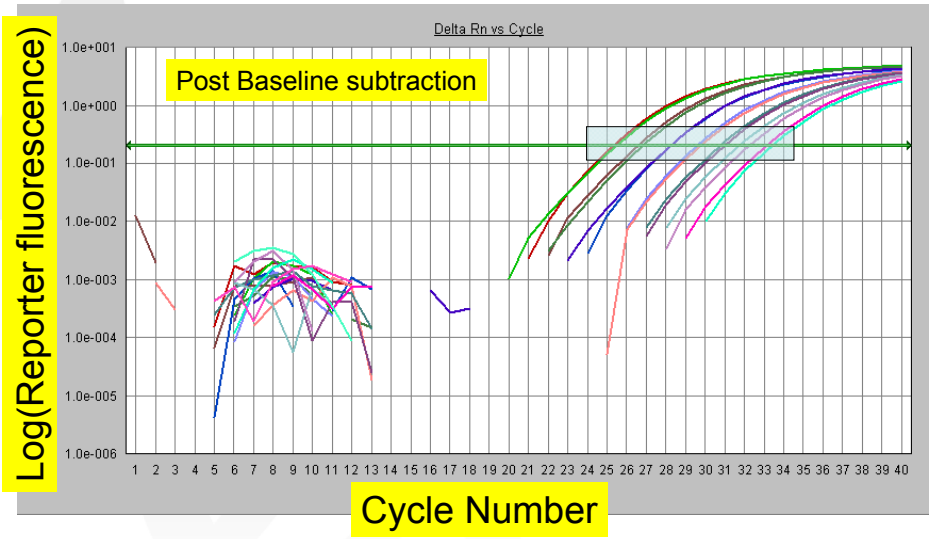
Log View of Data



Quantifiler data

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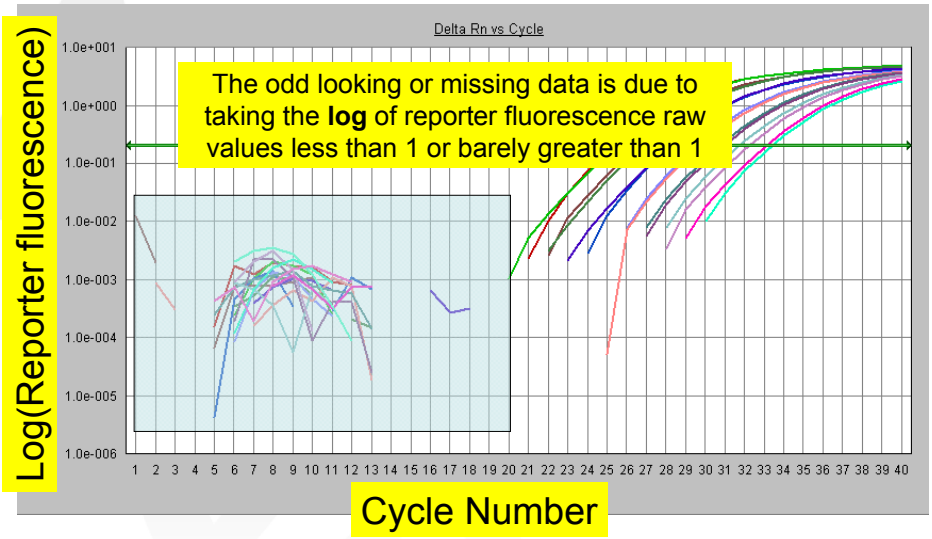
What is with the Confetti?



Quantifiler data

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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

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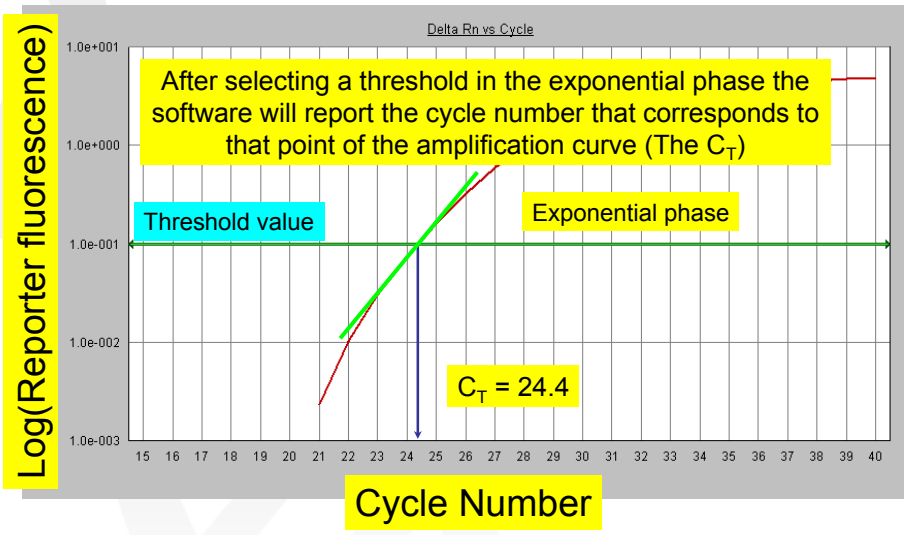
Selecting the Threshold Value



Quantifiler data

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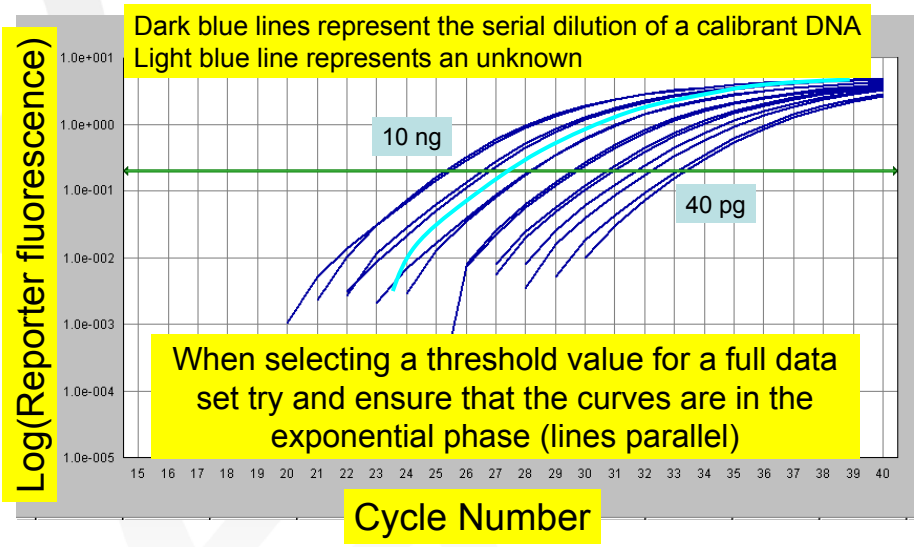
Selecting the Threshold Value



Quantifiler data

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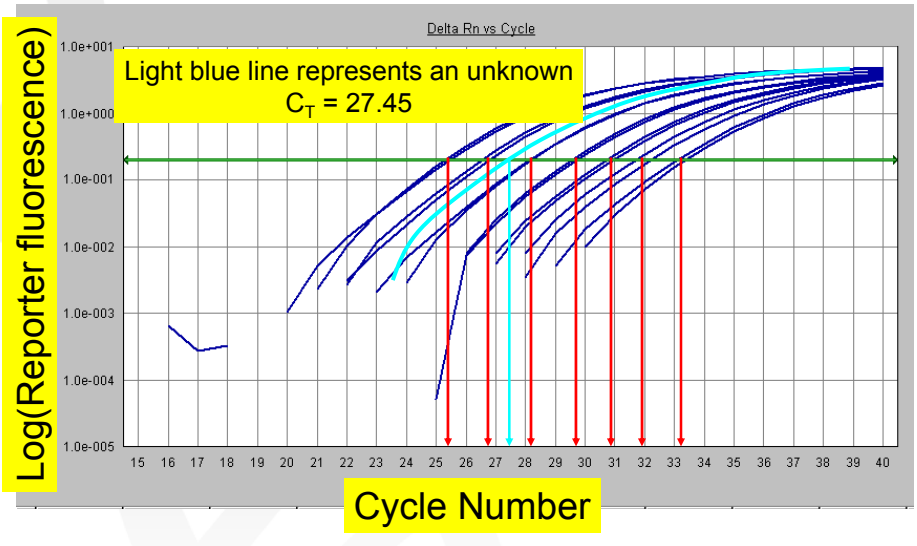
Selecting the Threshold Value



Quantifiler data

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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

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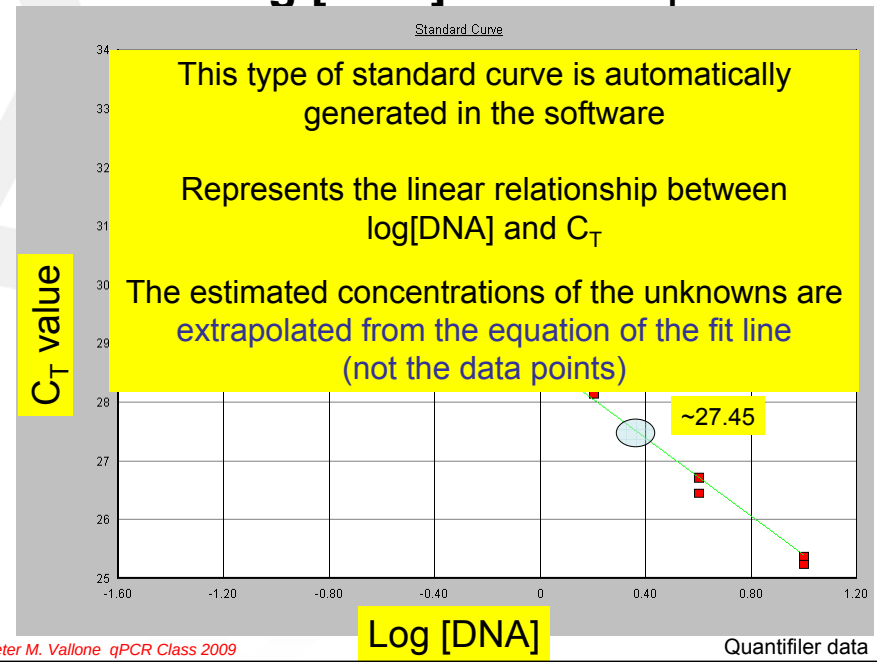
Log [DNA] versus C_T



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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

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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

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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!)

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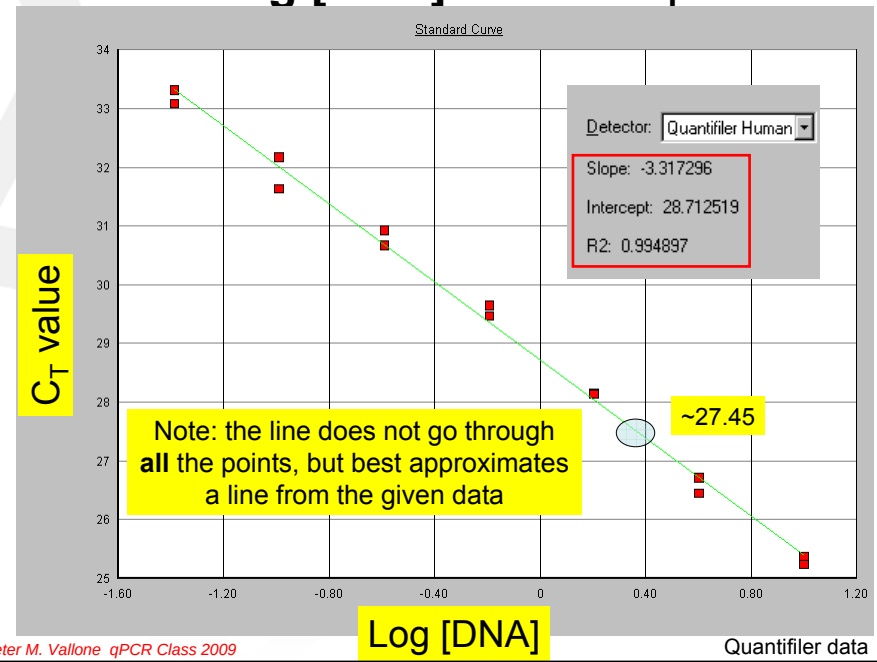
Log [DNA] versus C_T



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Quantifiler data

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

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

slope(m) = -3.317296

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

$$E = 2.0019\text{-}1$$

Just over 100% efficient

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

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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
- $R^2\text{-squared} = 1 - \frac{\sum (Y_i - \hat{Y}_i)^2}{\sum (Y_i - \bar{Y}_i)^2}$
- A perfectly fitting model yields an R^2 of 1 (all points fall directly on the line)

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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)

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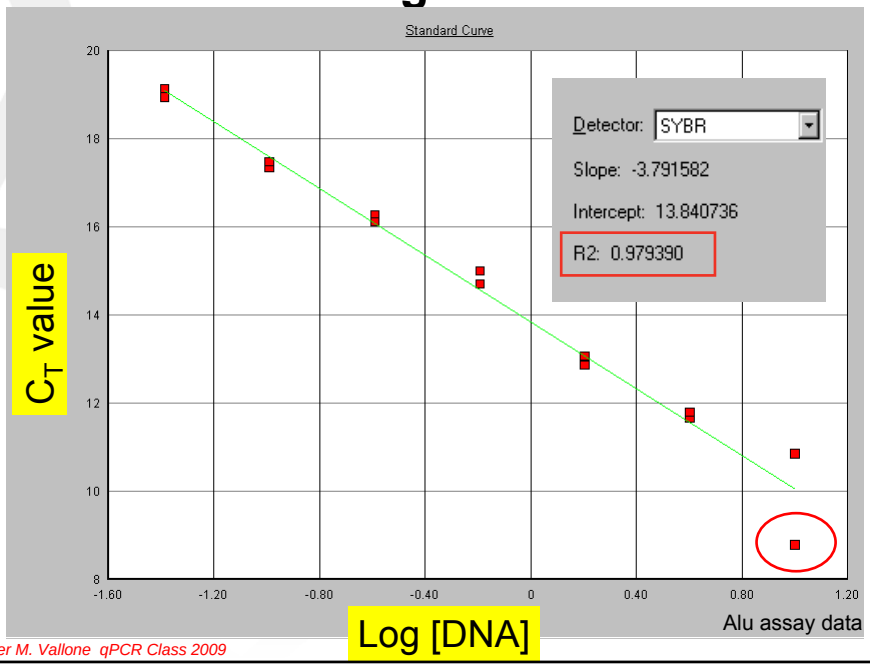
Removing An Outlier



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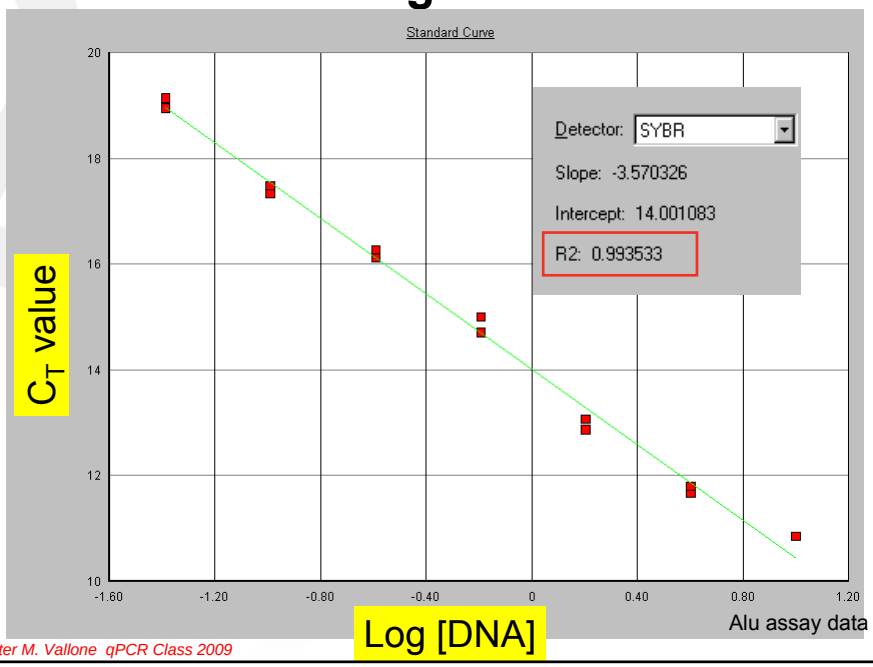
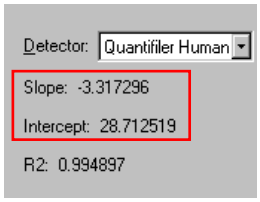
Removing An Outlier



Solving for an Unknown

- From the data



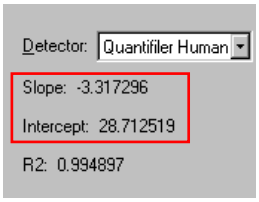
- Y = mX + b
- C_T = m*log[DNA] + b
- Solving for [DNA]

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

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

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…

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Data Report

Setup | Instrument | Results
Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty
A3	1a	Quantifiler Human	Unknown	26.40		4.96
		Quantifiler Human IPC	Unknown	27.65		
A4	1b	Quantifiler Human	Unknown	25.71		8.05
		Quantifiler Human IPC	Unknown	27.97		
B3	2a	Quantifiler Human	Unknown	27.16		2.94
		Quantifiler Human IPC	Unknown	27.58		
B4	2b	Quantifiler Human	Unknown	27.18		2.90
		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

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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?

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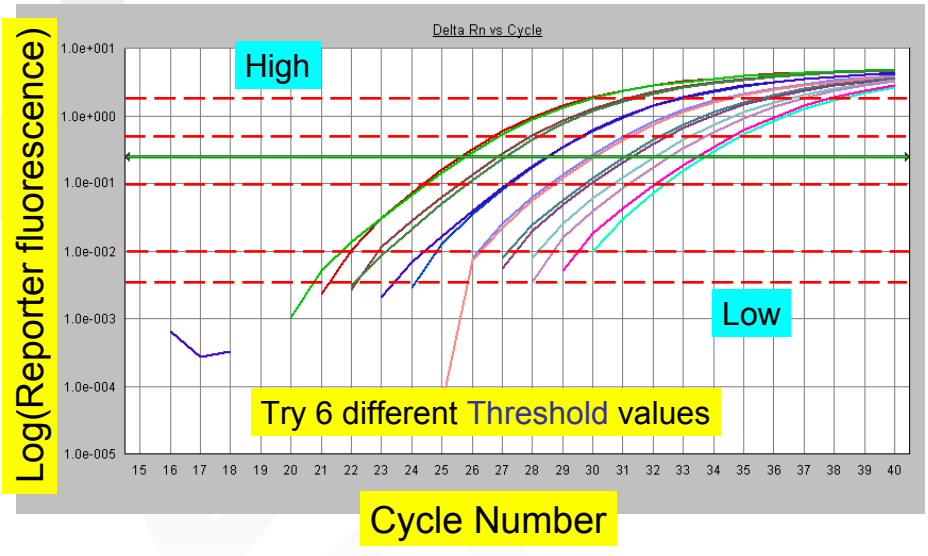
Varying the Threshold Value



Quantifiler data

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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)

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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

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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

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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)

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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

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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

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HAS II Certified Values of Decadic Attenuance for SRM 2372

Component	260 nm	error at 260nm	Nominal [DNA], ng/μL
A	1.049	± 0.025	52.5
B	1.073	± 0.030	53.6
C	1.086	± 0.028	54.3

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

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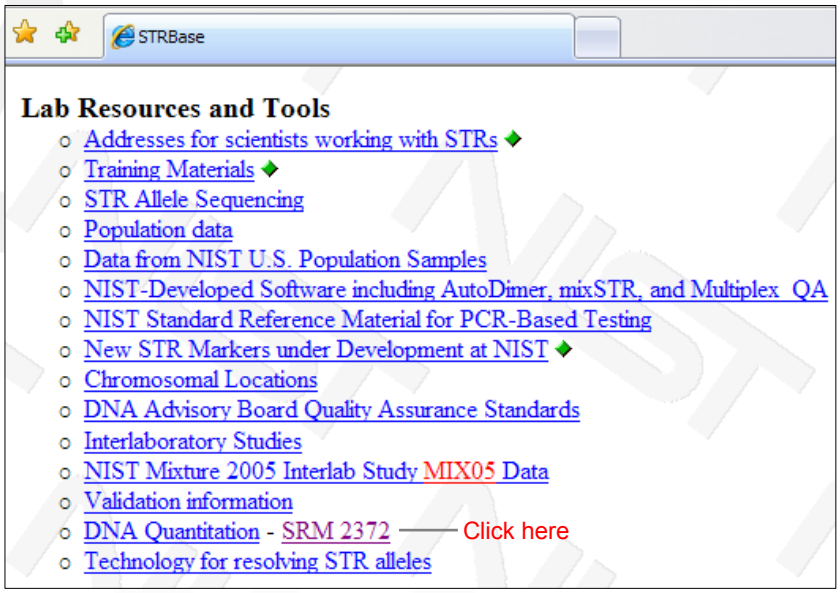
Information on SRM 2372 Now on STRBase



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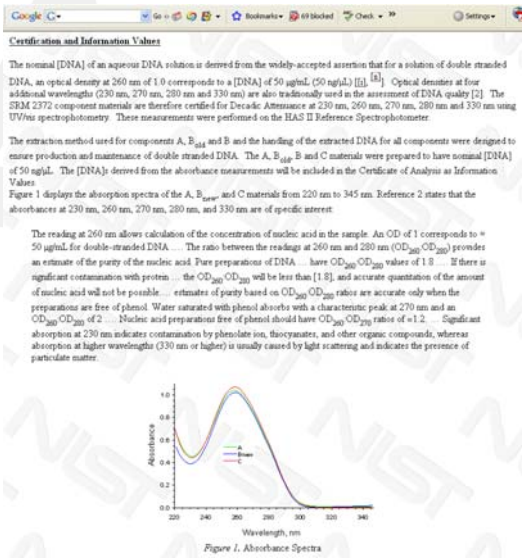
Additional Information



Certification and Information Values

The nominal [DNA] of an aqueous DNA solution is derived from the widely-accepted assertion that for a solution of double stranded DNA, an optical density at 260 nm of 1.0 corresponds to a [DNA] of 50 µg/mL (50 ng/µL) [1]. [2] Optical densities at four additional wavelengths (230 nm, 270 nm, 280 nm and 330 nm) are also traditionally used in the assessment of DNA quality [2]. The SRM 2372 component materials are therefore certified for Decadic Attenuance at 230 nm, 260 nm, 270 nm, 280 nm and 330 nm using UV/vis spectrophotometry. These measurements were performed on the HAS II Reference Spectrophotometer.

The extraction method used for components A, B_{old} and B and the handling of the extracted DNA for all components were designed to ensure production and maintenance of double stranded DNA. The A, B_{old}, B and C materials were prepared to have nominal [DNA] of 50 ng/µL. The [DNA]t derived from the absorbance measurements will be included in the Certificate of Analysis as Information Values.

Figure 1 displays the absorption spectra of the A, B_{new}, and C materials from 220 nm to 345 nm. Reference 2 states that the absorbances at 230 nm, 260 nm, 270 nm, 280 nm, and 330 nm are of specific interest:

The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to ≈ 50 µg/mL for double-stranded DNA. . . . The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA . . . have OD_{260}/OD_{280} values of 1.8 . . . . If there is significant contamination with protein . . . the OD_{260}/OD_{280} will be less than [1.8], and accurate quantitation of the amount of nucleic acid will not be possible. . . . estimates of purity based on OD_{260}/OD_{280} ratios are accurate only when the preparations are free of phenol. Water saturated with phenol absorbs with a characteristic peak at 270 nm and an OD_{260}/OD_{280} of 2 . . . . Nucleic acid preparations free of phenol should have OD_{260}/OD_{270} ratios of ≈1.2. . . . Significant absorption at 230 nm indicates contamination by phenolate ion, thiocyanates, and other organic compounds, whereas absorption at higher wavelengths (330 nm or higher) is usually caused by light scattering and indicates the presence of particulate matter.

Figure 1. Absorbance Spectra

Supplemental data for SRM 2372 can be found on STRBase

Includes information on the production and characterization of the materials:

Homogeneity study

Interlaboratory study

Quantifiler, Alu, CFS assays

DNA standard calibration

http://www.cstl.nist.gov/biotech/strbase/srm2372.htm

Overview of SRM 2372 Values and Use

Attenuance (λ_{260}) → **Certified Values** → **SRM 2372 Components** (A, B, C)

1 OD = 50 ng/µL, DNA Concentration (ng/µL) → **Informational Values**

Quantifiler, **Alu qPCR**, **Other assays**

Different Assays
Different Calibrants

Adjust calibrant values for each lot

Forensic Labs

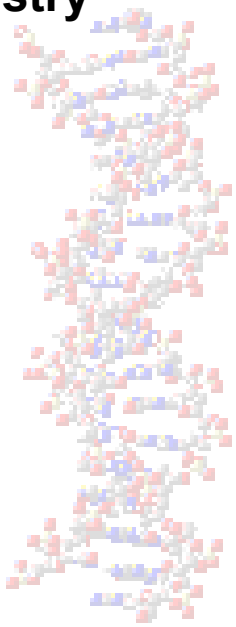
"Calibrated" NIST-Traceable Calibrant for Use in Daily Work

Measure Unknown DNA Samples

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.

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

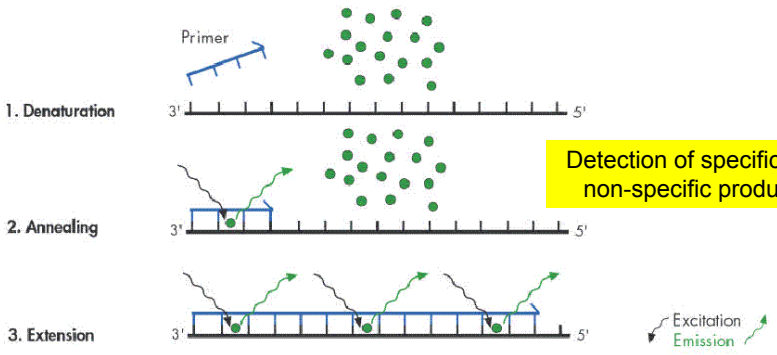
qPCR: **Detection – SYBR Green**

- **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"

Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: **Detection – SYBR Green**

SYBR Green Detection



Detection of specific and non-specific products

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

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

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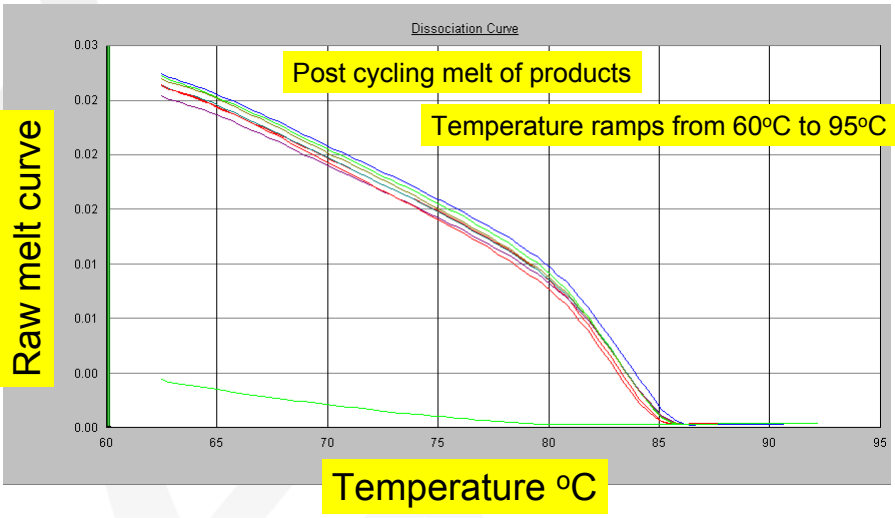
Buel/Nicklas Alu qPCR Assay



The data is better viewed by taking the first derivative of the curves…

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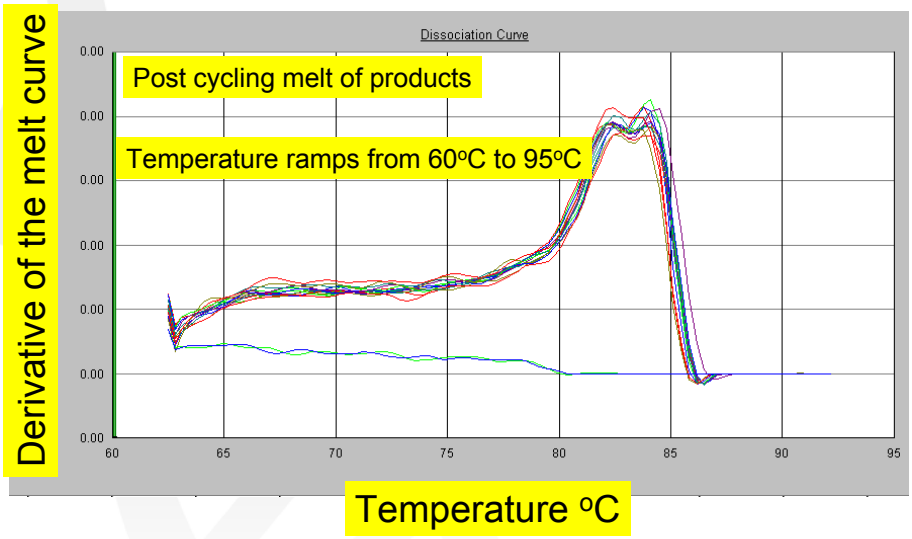
Buel/Nicklas Alu qPCR Assay



Melts **may** give an indication if multiple amplicons were produced

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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

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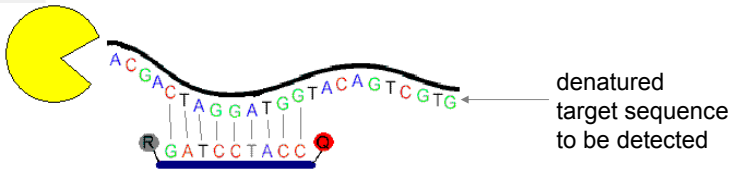
Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: **Detection – TaqMan**

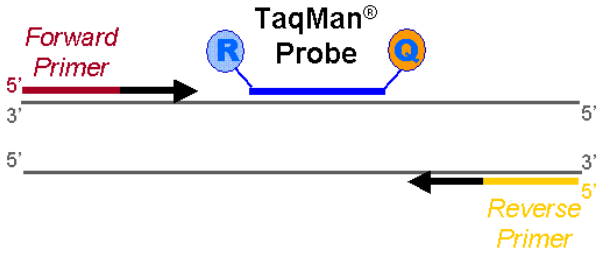


- TaqMan detection probe = a dual-labeled oligonucleotide
  - Complimentary to target sequence (anneals between primers)
  - Designed to anneal ~8-10 degrees higher than PCR primers
  - 5' end of probe = a Reporter fluorophore (e.g., FAM, VIC, NED, Cy5, etc.)
  - 3' end of probe = a Quencher a chemical 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")

Peter M. Vallone qPCR Class 2009 *Slide courtesy of Dr. Mark Timken, CA DOJ*

qPCR: **Detection – TaqMan**

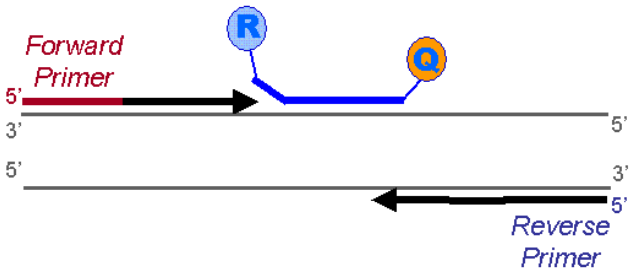
- **Annealing/Extension Step**
  - TaqMan probe hybridizes to denatured DNA (sequence specific)
  - Reporter fluorescence is quenched due to proximity to quencher (reporter starts ~dark)



Peter M. Vallone qPCR Class 2009 *Slide courtesy of Dr. Mark Timken, CA DOJ*

qPCR: **Detection – TaqMan**

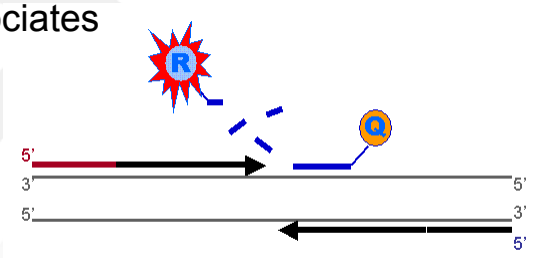
- **Annealing/Extension Step**
  - Lengthening strand displaces 5’ end of probe



Slide courtesy of Dr. Mark Timken, CA DOJ

qPCR: **Detection – TaqMan**

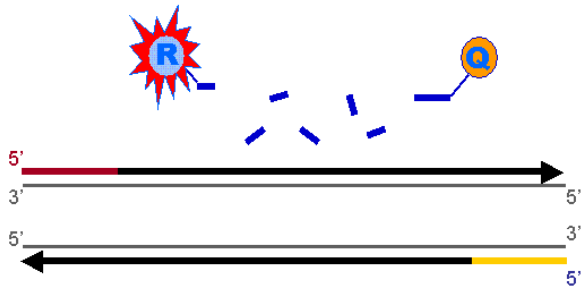
- **Annealing/Extension Step**
  - *Taq* polymerase mediates hydrolysis of probe from 5’ end (“5’ exo-nuclease activity”)
  - Reporter fluorophore is no longer quenched
  - Hydrolyzed TaqMan probe eventually dissociates



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

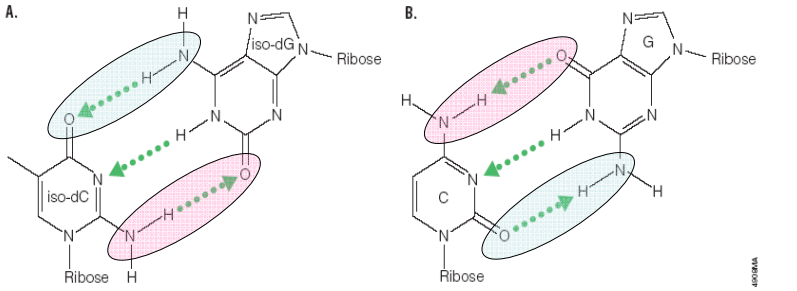
- **Fluorescence detection of amplicons in real time by any number of methods**
  - FRET Hybrids (Roche)
  - Molecular Beacons (NJ Dept of Public Health)
  - Scorpions
  - Light Upon Extension (LUX) primer
  - EraGen, a.k.a., “Plexor” (licensed by Promega)

 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

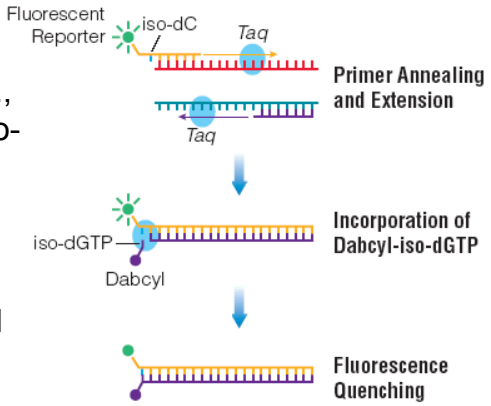
EraGen qPCR Detection Chemistry

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

- fluorophore is NOT quenched before PCR

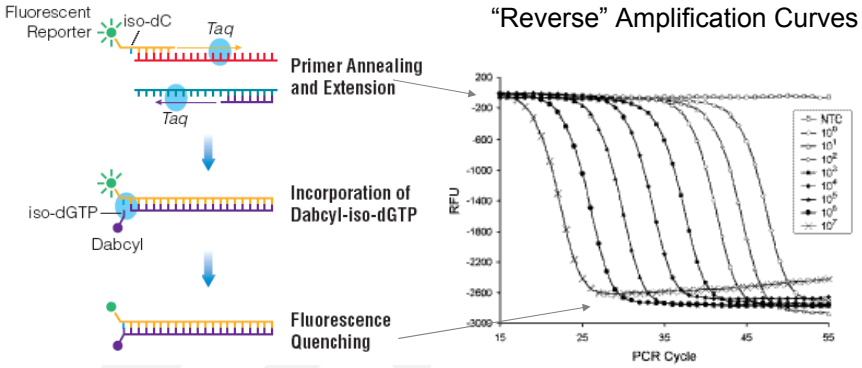
- PCR is done with standard dNTPs AND

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

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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

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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

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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

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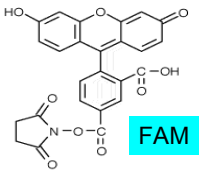
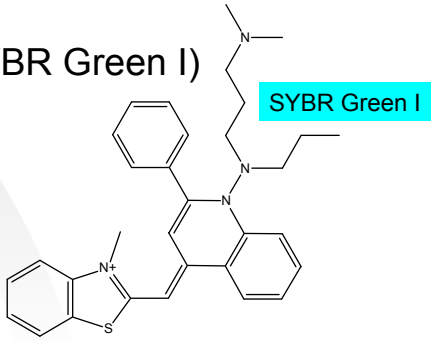
Fluorescence Detection

- As the amount of amplified DNA in the PCR increases there is a change in the amount of fluorescence
- Organic dyes
  - Free in solution (SYBR Green I)
  - Attached to a probe



FAM

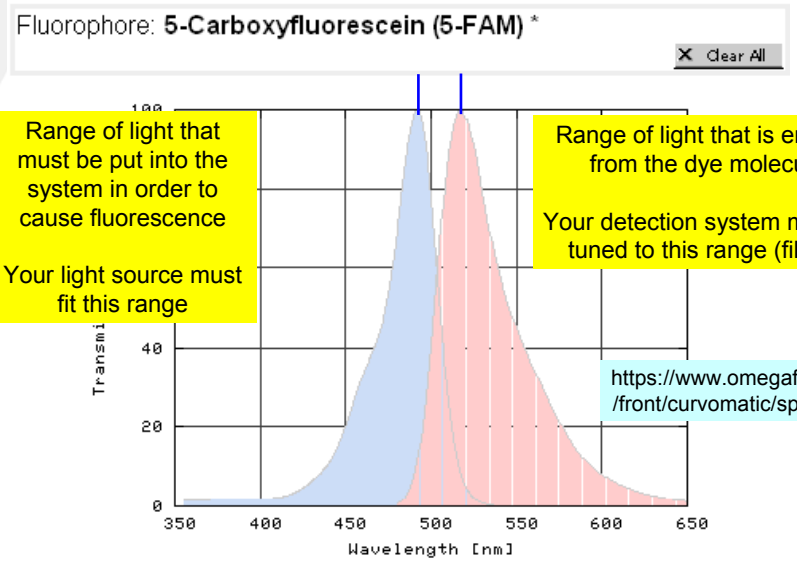


SYBR Green I

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

Dye Characteristics

- Absorption and Emission Spectra for 5-FAM



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)

https://www.omegafilters.com/front/curvomatic/spectra.php

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

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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	
HEX	535	556	
R6G	524	557	
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**

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

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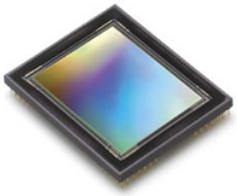
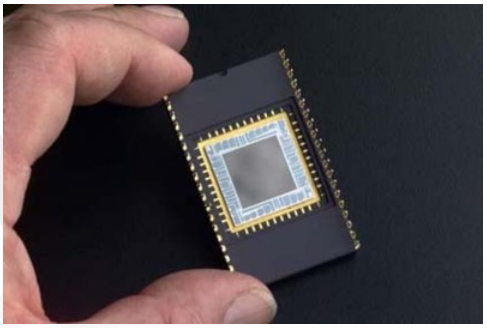
CCD Charge-Coupled Device

- 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

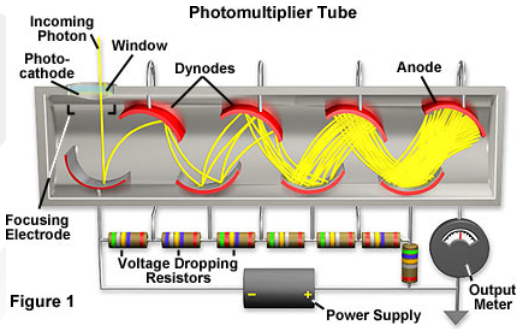
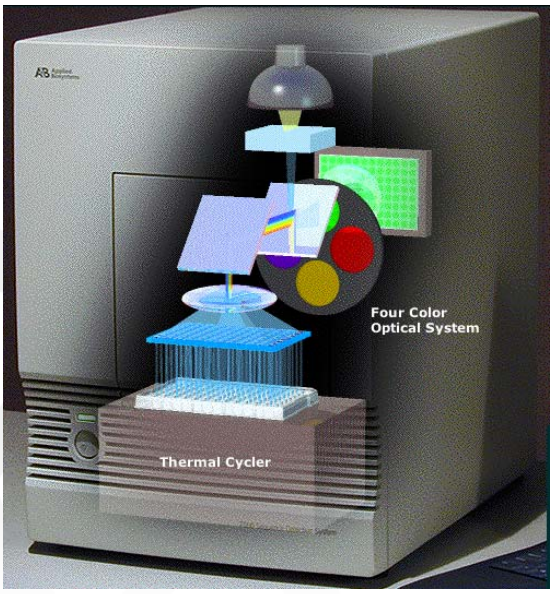


Figure 1

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

General Schematic of Instrumentation



AB 7000

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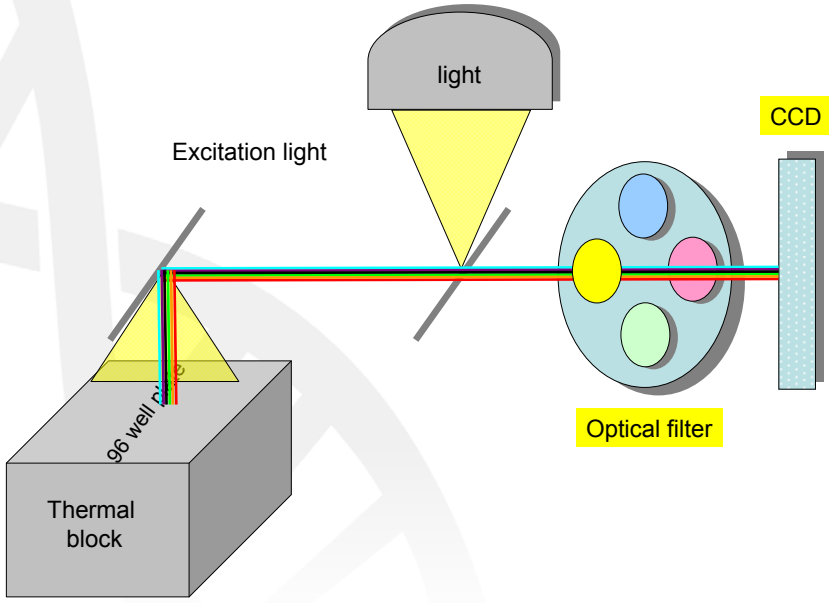
General Schematic of Instrumentation



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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

AB 7500

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

Detecting Multiple Dyes

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

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

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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

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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

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qPCR Bibliography

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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.
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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
  - Green et al J Forensic Sci 2005; 50: 809-825

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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

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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

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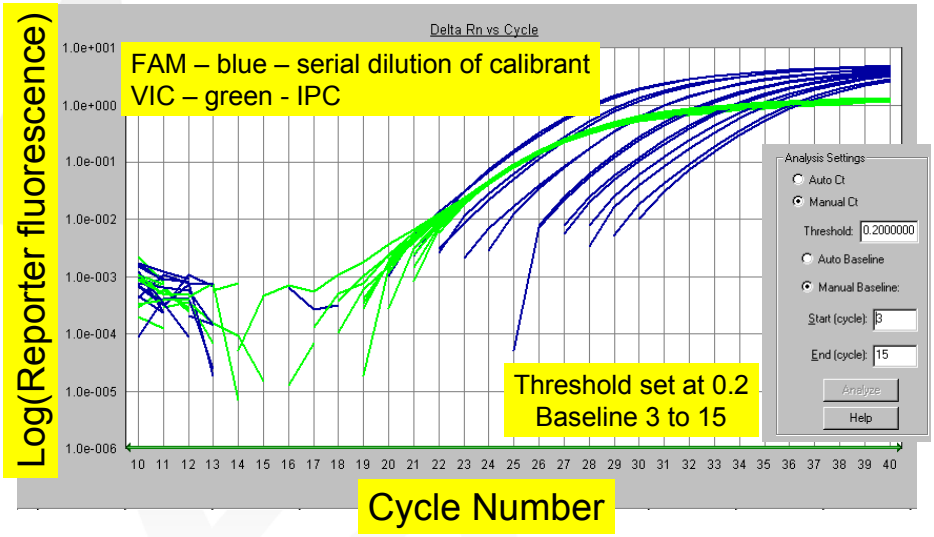
Applied Biosystems Quantifiler



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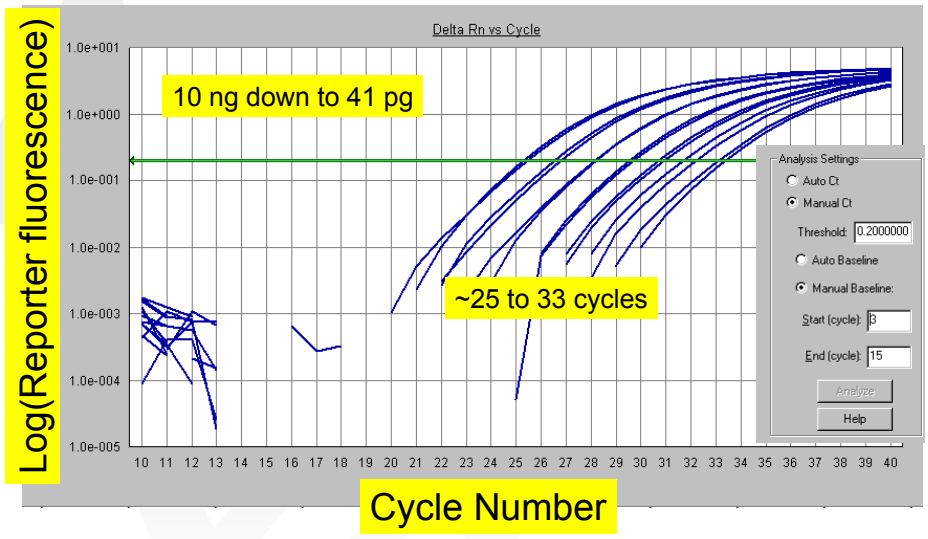
Quantifiler Calibrant Data



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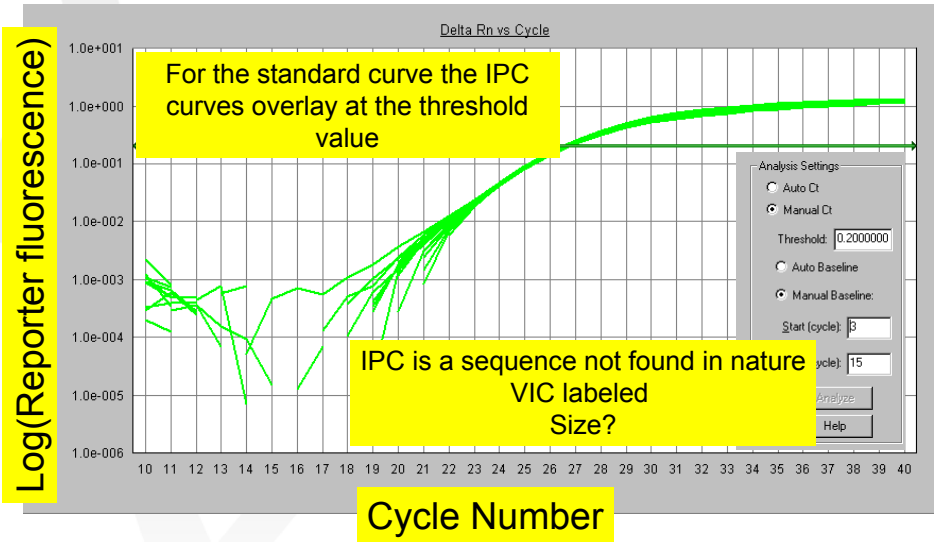
Quantifiler IPC



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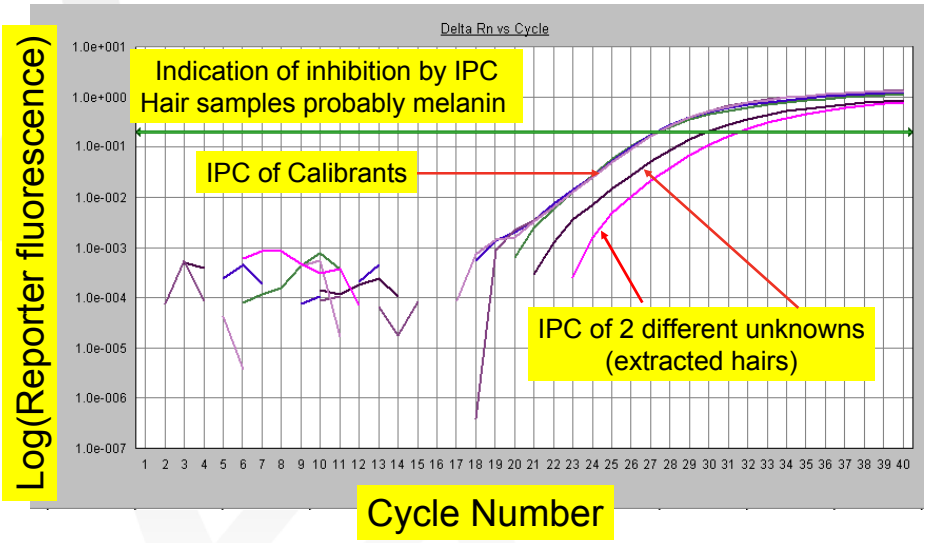
Quantifiler IPC



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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

Designing an Experiment

- The experiment plate may look something like:

A	10 ng	10 ng	1a	1b
B	4	4	2a	2b
C	1.6	1.6	3a	3b
D	0.64	0.64	4a	4b
E	0.256	0.256	5a	5b
F	0.102	0.102	6a	6b
G	0.041	0.041	7a	7b
H	NTC	NTC	NTC	NTC

Standards Samples

May vary:
Range of dilutions
Spacing of dilutions

Designing an Experiment

- Or the experiment plate may look something like:

	1	2	3	4	5	6	7	8	9
A									
B		10	4	1.6	0.64	0.256	0.102	0	NTC
C		10	4	1.6	0.64	0.256	0.102	0	NTC
D									
E			1a	2b	3c	etc			
F			1a	2b	3c				
G									
H									

Standards

Samples

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

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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

	Stock (ng/uL)	200		Buffer	Total
1		10	5	95	100
2		4	4	6	10
		1.6	4	6	10
		0.64	4	6	10
		0.256	4	6	10
		0.102	4	6	10
		0.041	4	6	10

Use Tris EDTA bufffer
10 mM Tris-HCl (pH 8.0)
0.1 mM Na_2EDTA

Not Water

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Designing an Experiment

- Quantifiler Kit example
- The kit comes with
  - PCR Reaction Mix (dNTPs, buffer, Taq Gold, ROX)
  - Human DNA Standard (200 ng/uL)
  - Primer mix (hTERT-FAM, IPC template, and IPC-VIC)
- Total reaction volume of 25 μL

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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)

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Data Analysis - Quantifiler

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

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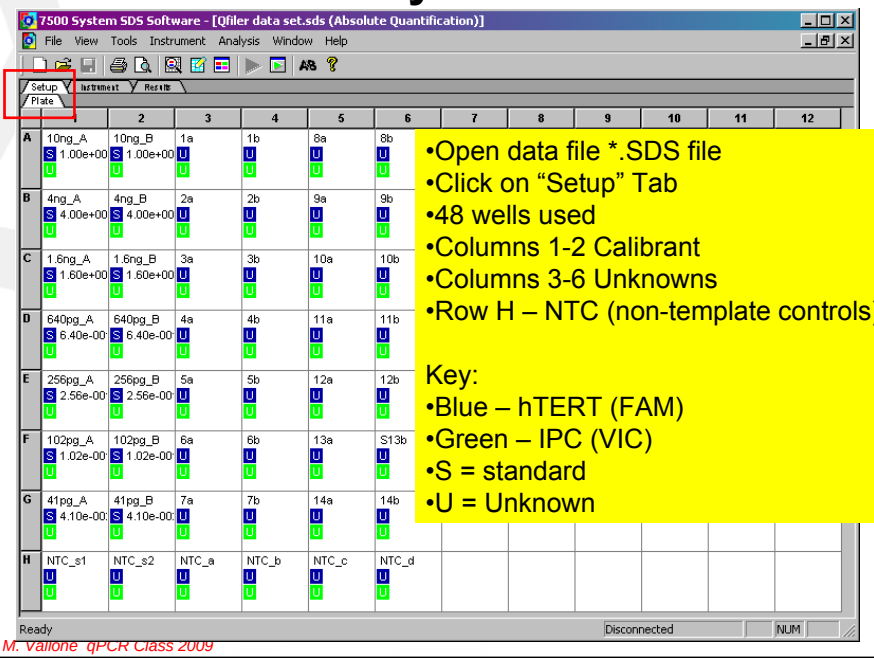
Data Analysis - Plate



- 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

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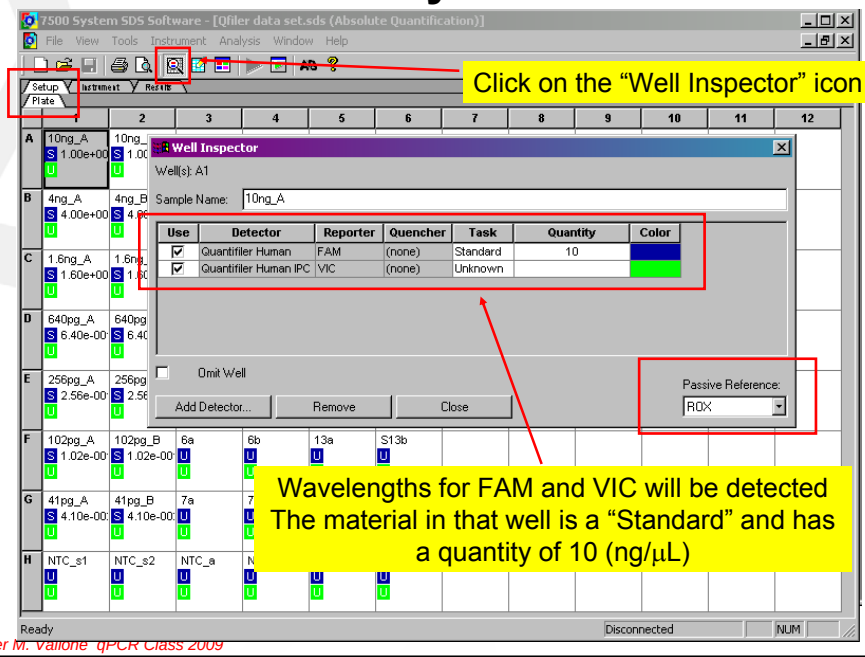
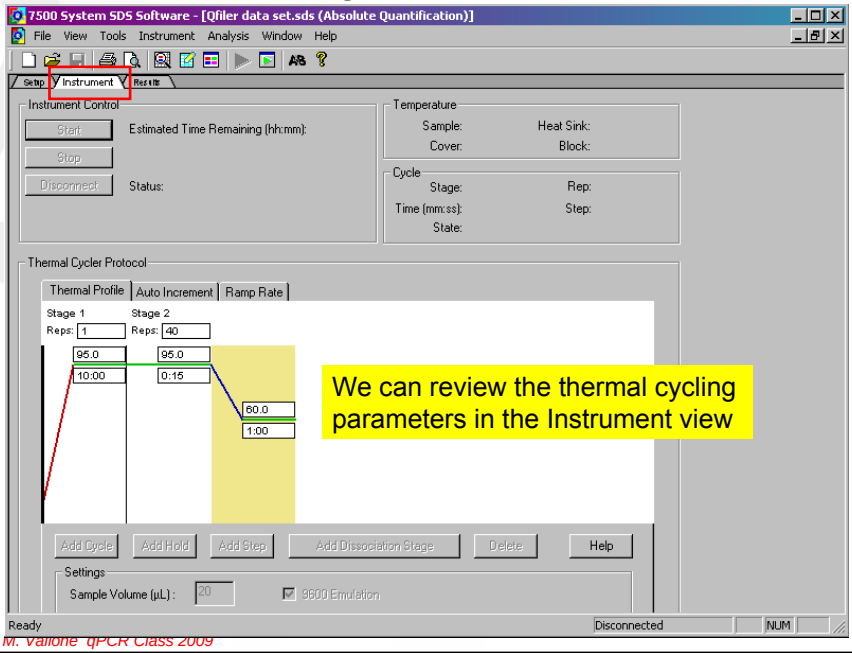
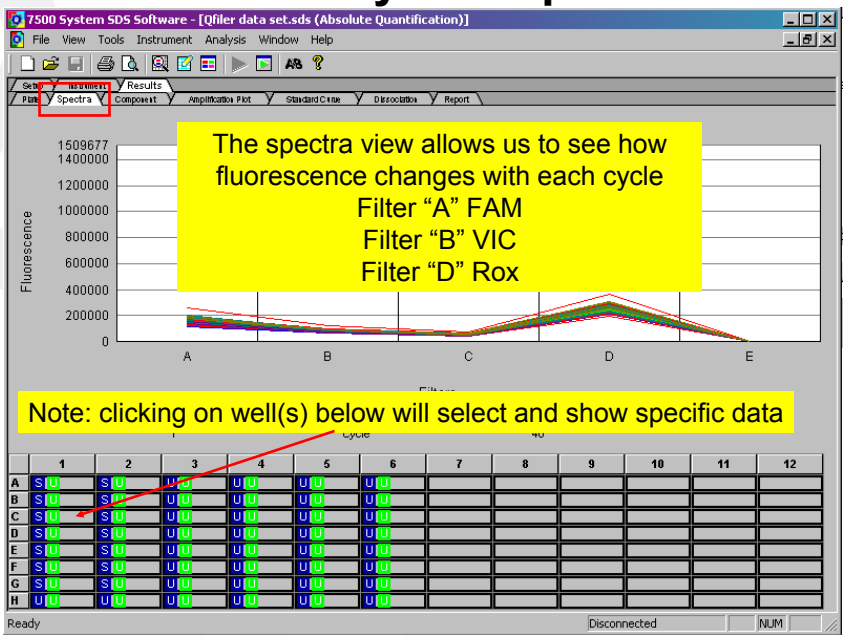
Data Analysis - Well



Data Analysis - Instrument



Data Analysis - Spectra



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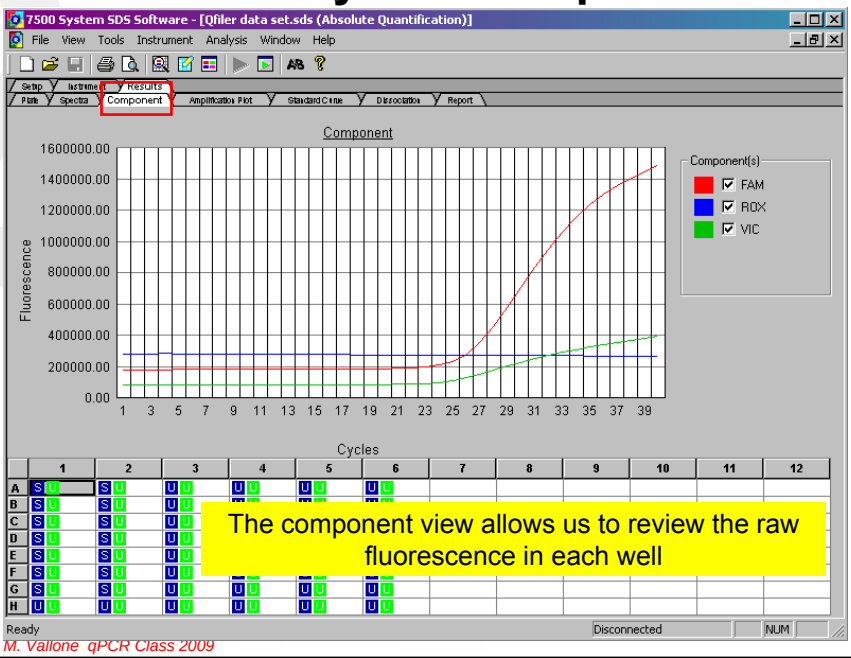
Data Analysis - Component



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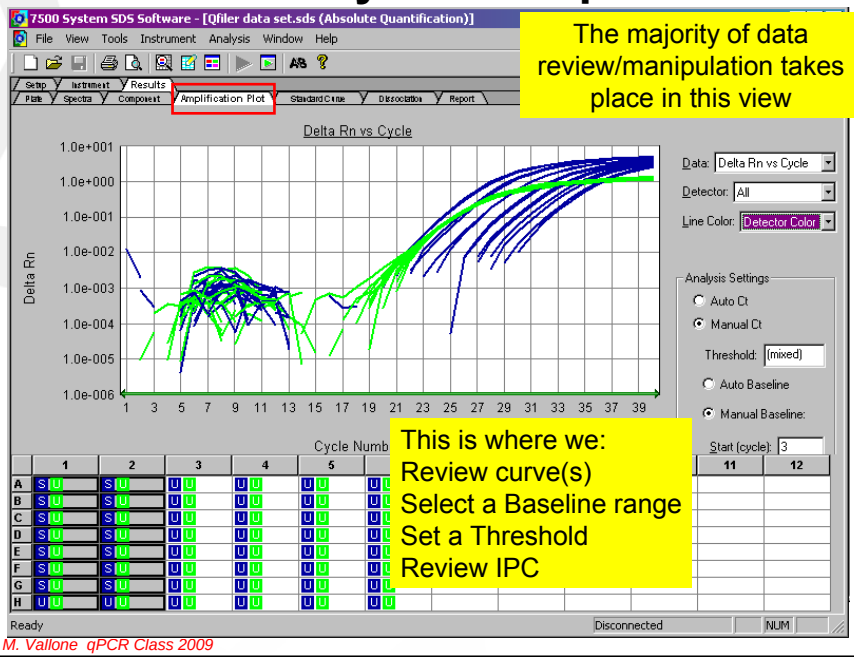
Data Analysis – Amp Plot



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

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

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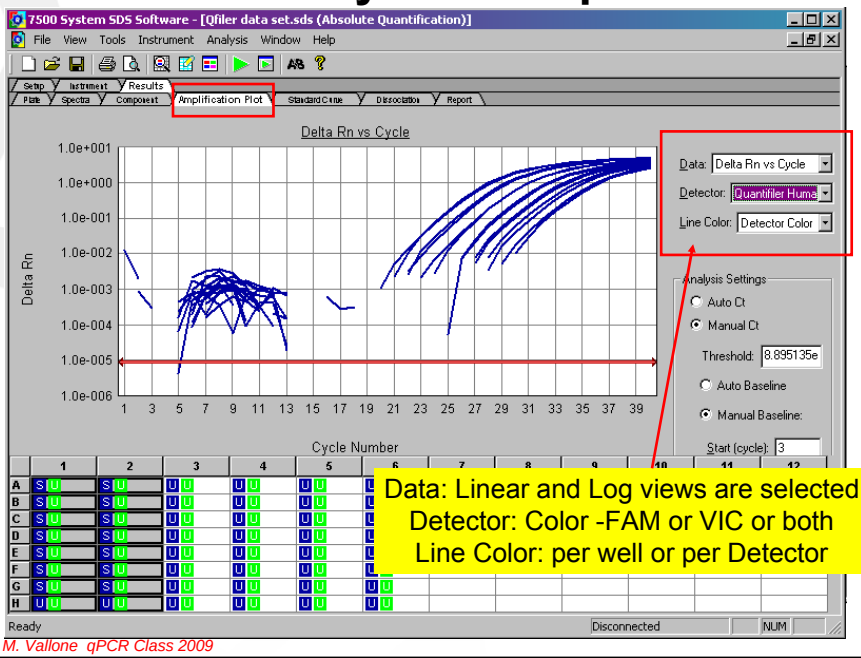
Data Analysis – Amp Plot



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

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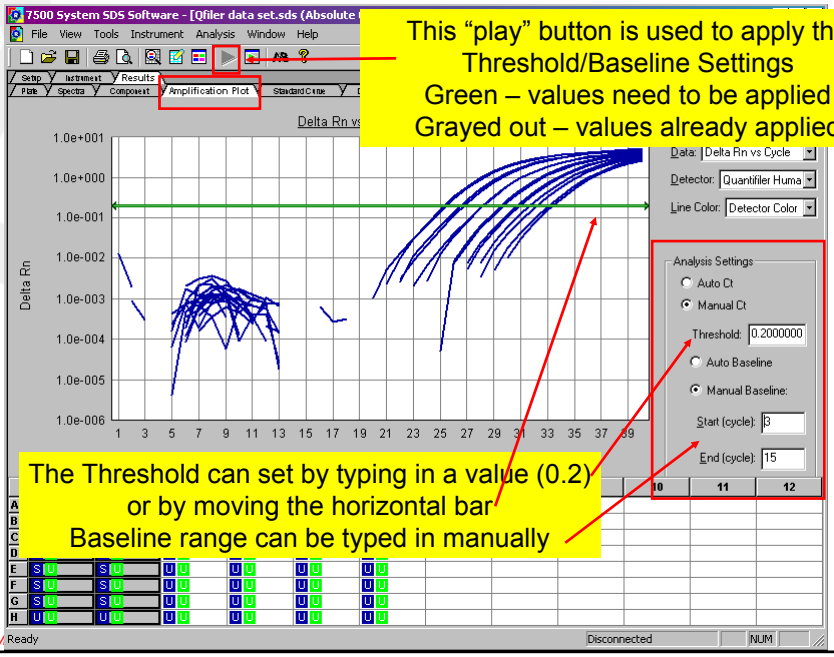
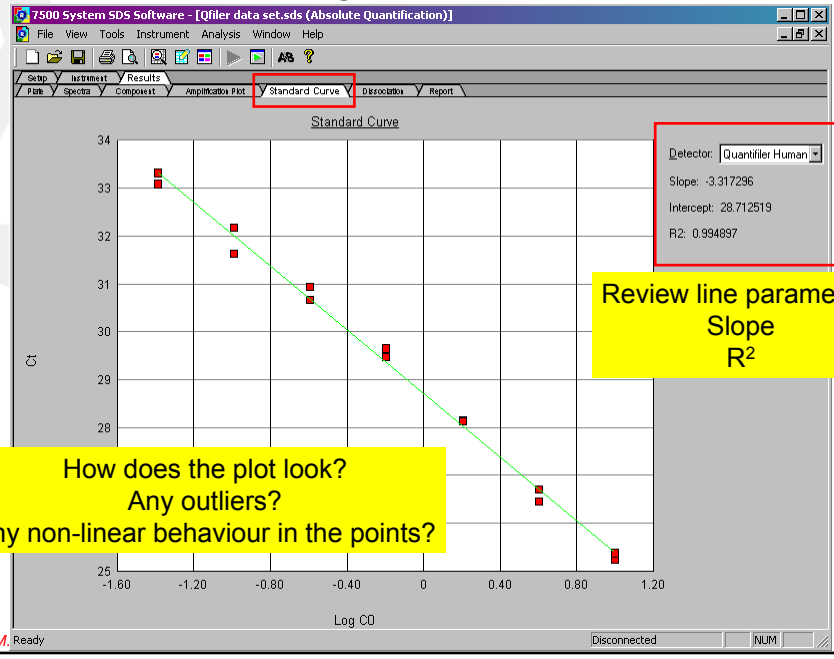
Data Analysis – Amp Plot



This "play" button is used to apply the Threshold/Baseline Settings
Green – values need to be applied
Grayed out – values already applied

The Threshold can set by typing in a value (0.2) or by moving the horizontal bar
Baseline range can be typed in manually

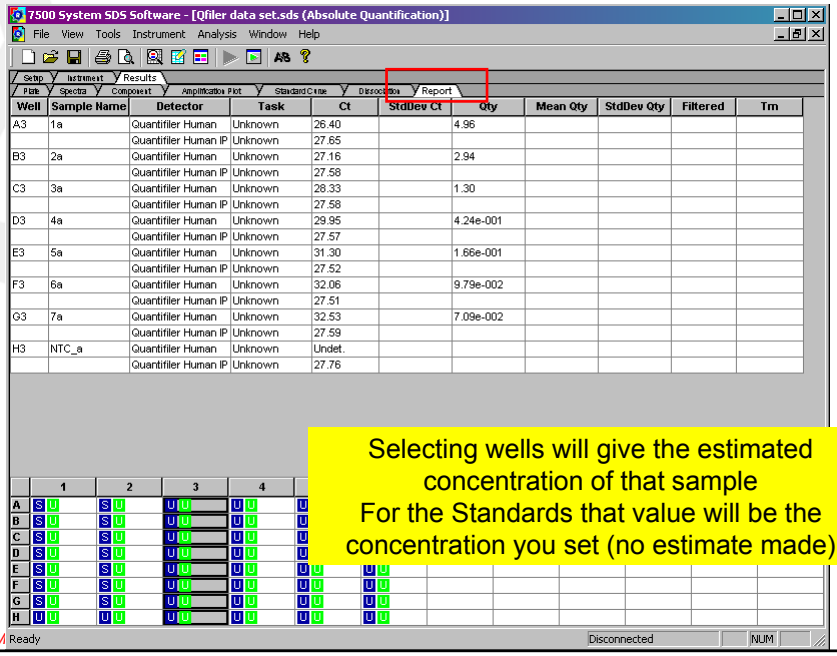
Data Analysis – Std Curve



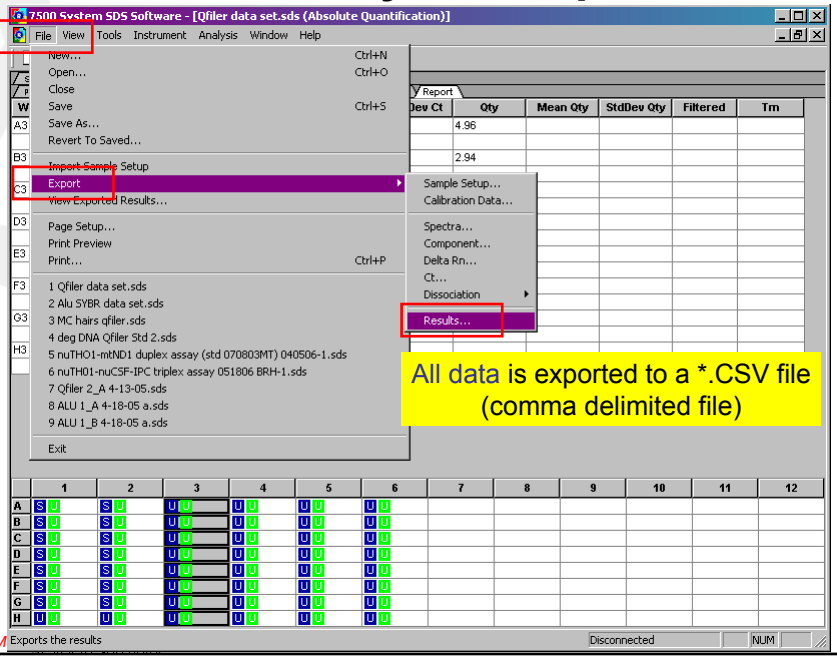
Review line parameters
Slope
R^2

How does the plot look?
Any outliers?
Any non-linear behaviour in the points?

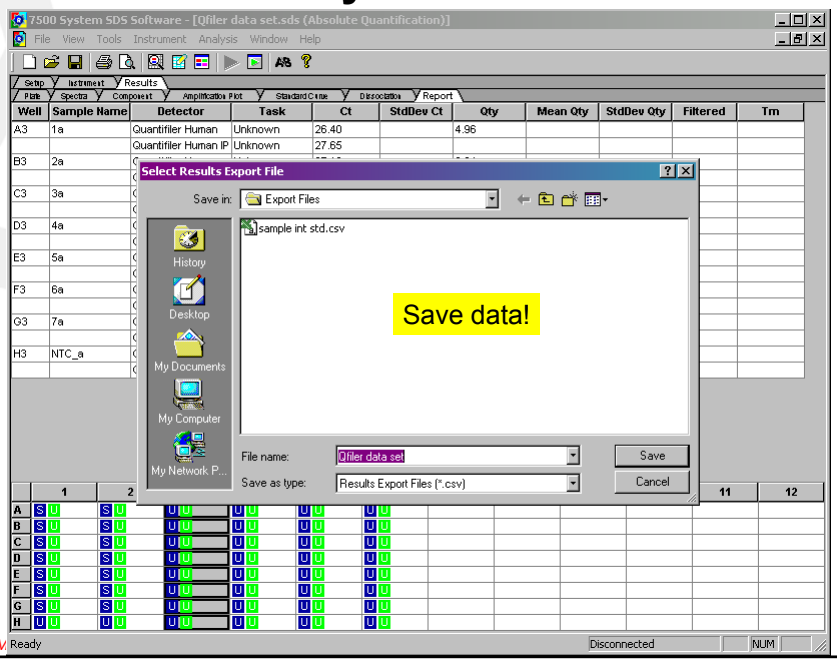
Data Analysis - Report



Data Analysis - Export



Data Analysis – Save CSV



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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

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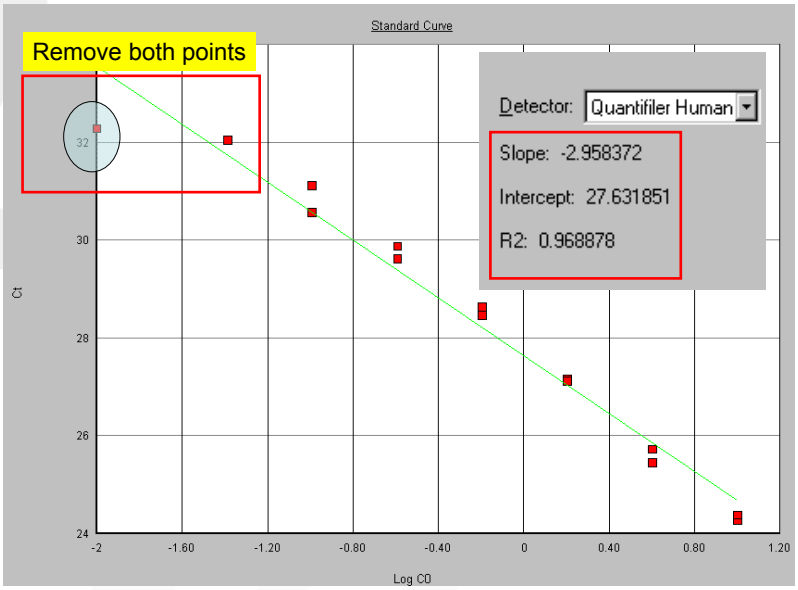
Troubleshooting



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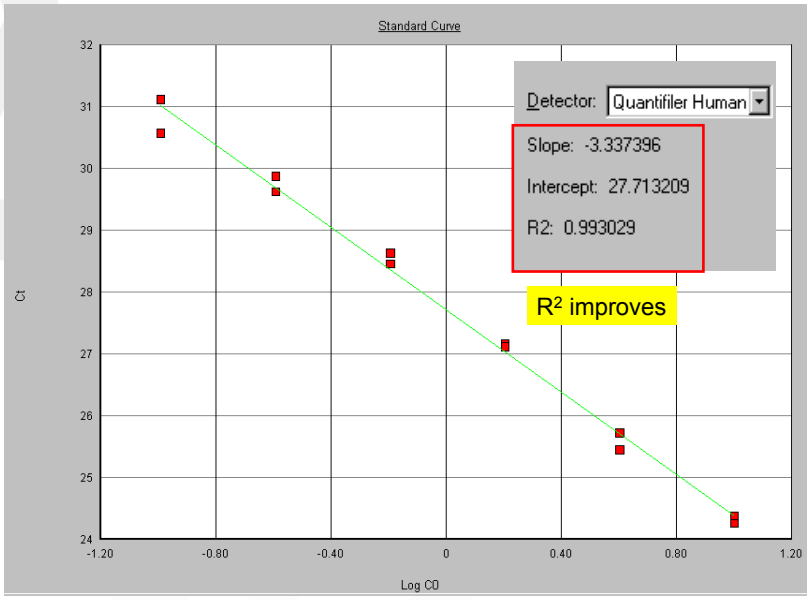
Troubleshooting



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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)

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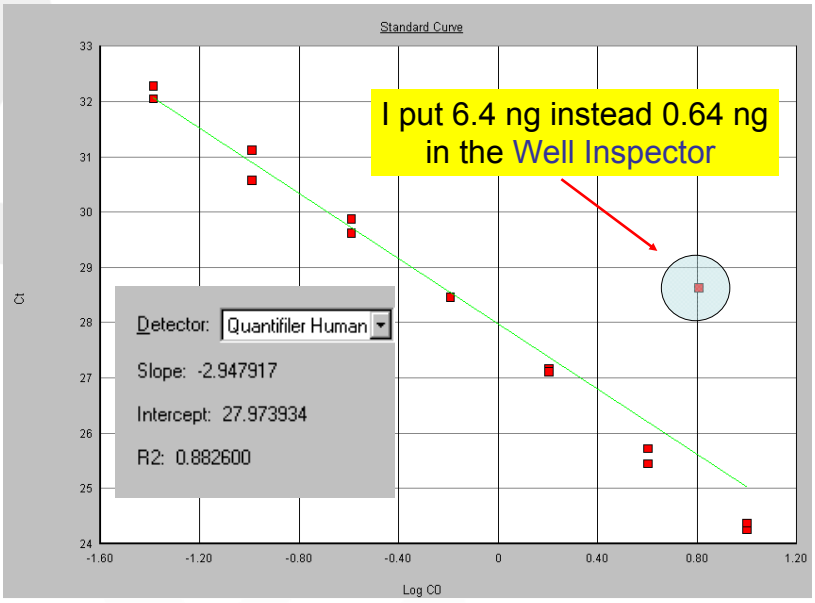
Troubleshooting



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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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