Session 1

Dr. Peter M. Vallone

Staff Scientist
NIST National Institute of Standards and Technology
100 Bureau Drive
Gaithersburg, MD 20899
petev@nist.gov
301-975-4872

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.

Why do we need to quantitate DNA?

PCR Mechanism

Phases of PCR amplification

PCR efficiency

Why Do We Care About Quantitating DNA?

Forensic laboratories commonly use commercial STR typing kits
- PowerPlex 16
- Identifiler
- Other kits (Y STRs, mini-STRs)

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

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 x1012 g/mol
There are 3 billion base pairs in a haploid cell ~3 x 109 bp
(~3 x 109 bp) x (618 g/mol/bp) = 1.85 x 1012 g/mol

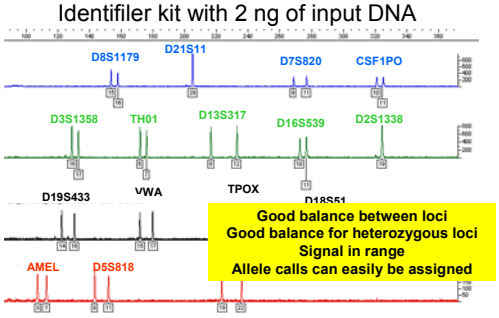
Calculation of the Quantity of DNA in a Cell

Quantity of DNA in a Haploid Cell = 3 picograms
1 mole = 6.02 x 10^{23} molecules
(1.85 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)
= 3.08 x 10^{-12} g = 3.08 picograms (pg)
A diploid human cell contains ~6 pg genomic DNA

One ng of DNA contains the DNA from 167 diploid cells
1 ng genomic DNA (1000 pg)/6pg/cell = ~333 copies of each locus (2 per 167 diploid genomes)

Multiplex PCR

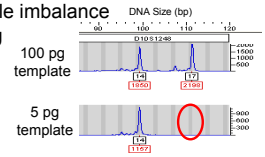
Identifiler kit with 2 ng of input DNA



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

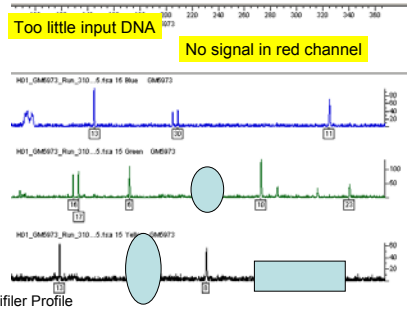


Stochastic effect when amplifying low levels of DNA produces allele dropout

Why Do We Care About Quantitating DNA?

Too little input DNA

No signal in red channel

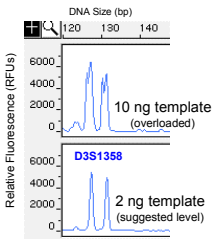


Identifiler Profile

Why Do We Care About Quantitating DNA?

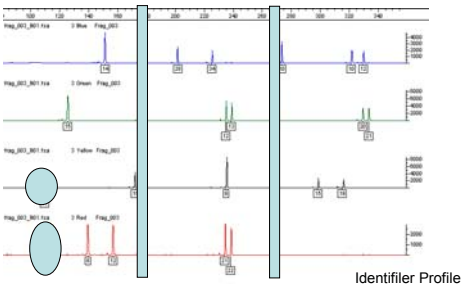
Too much input DNA results in:

- 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
- PCR
- CE

Poor quality data will take longer for an analyst to review

Cost: time + reagents + extract = $$$

Why Do We Care About Quantitating DNA?

Not limited to 'conventional' STR markers

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

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

An estimate as to the degree of degradation (and degree of inhibition as well)

Why Do We Care About Quantitating DNA?

When obtaining samples from an outside source (collaborator, other lab) it is a good QC measure to confirm the quantity and integrity of the materials

If evaluating a new technique (DNA extraction) qPCR can help quantitate performance

When developing a new assay it is important to know the optimal [DNA] range

Why Do We Care About Quantitating DNA?

If we can confidently determine the amount of DNA in an extract we can then ask questions:

- Will mitochondrial sequencing be required? (skip STR analysis)
- Should we use a miniSTR assay?
- Should we use low copy number (LCN) methods for STRs?
- Re-extract the sample?

If problems occur in the STR typing process we can have confidence that the DNA template is not the source (CE, cycler, kit)

Human Specific DNA Assays

All sources of DNA are extracted when biological evidence from a crime scene is processed to isolate the DNA present

Thus, non-human DNA such as bacterial, fungal, plant, or animal material may also be present in the total DNA recovered from the sample along with the relevant human DNA of interest

For this reason, the 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.cstl.nist.gov/biotech/strbase/pub_pres/AAFS2006_qPCR_LCN.pdf

PCR Nomenclature

qPCR – quantitative PCR (usually implies using PCR for DNA quantitation in "real time", i.e., not at the end point)

RT-PCR – Real-Time PCR, but often reverse transcription PCR (and often in conjunction with real-time PCR, too)

Amplicon – product of PCR

Calibrant DNA – DNA of a known concentration that is serially diluted to prepare a standard curve (can be called the Standard DNA)

PCR Nomenclature

Baseline – a linear function subtracted from the data to eliminate background signal

Threshold – a value selected when the PCR is in the exponential phase of growth

We will cover these in more detail in the next section

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

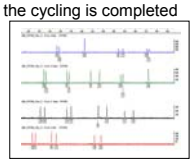
qPCR

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

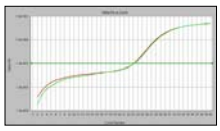
First paper on qPCR:
- Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. "Kinetic PCR analysis: real-time monitoring of DNA amplification reactions" Biotechnology (N Y). 1993 Sep;11(9):1026-30

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



Why Real-Time qPCR?

Advantages

The availability of commercial qPCR kits (labs are beginning to switch over to this method)

Higher throughput and reduced user intervention
- Automated set up
- Simple data analysis
- Experimental data rapidly analyzed in software; interpolating into the calibration curve

qPCR will be sensitive to the same inhibitors as faced in a traditional STR test (both PCR based)

Why Real-Time qPCR

Advantages

No post PCR manipulation (reduced contamination issues)

High sensitivity (down to a single copy number?)

Large dynamic range: ~30 pg to 100 ng

Assays are target specific (autosomal, mito, Y) and can be multiplexed – to a degree…

Why Real-Time qPCR

Challenges

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

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

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

Why Real-Time qPCR

Challenges

qPCR quantitates specific target sequences, it does not quantify "DNA"

- In highly degraded samples, assays that amplify short target sequences will detect and measure more DNA than assays that amplify long target sequences (relevant to STR typing)

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

Results are relative to the Calibrant (which can vary)

PCR Mechanism

Singleplex PCR

- A single locus is amplified
- Forward and reverse primer (~20 nt)
- dNTP (dATP, dCTP, dGTP, dTTP)
- Mg^{++} (~1-2 mM)
- PCR buffer
- Taq Polymerase
- Genomic DNA Template (0.25 ng – 100ng?)

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

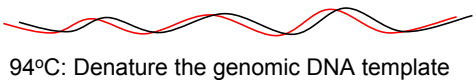
The amount of DNA theoretically doubles with every cycle of PCR

2^N

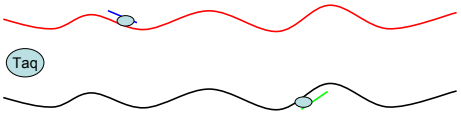
This is true when the reaction is running at 100% efficiency

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

PCR Mechanism

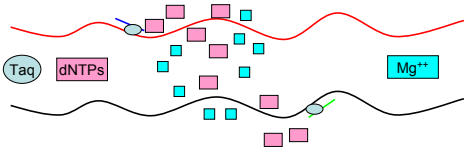


94°C: Denature the genomic DNA template

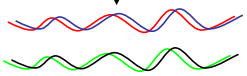


60°C: PCR primers bind to the DNA template; this will define the size of amplicon

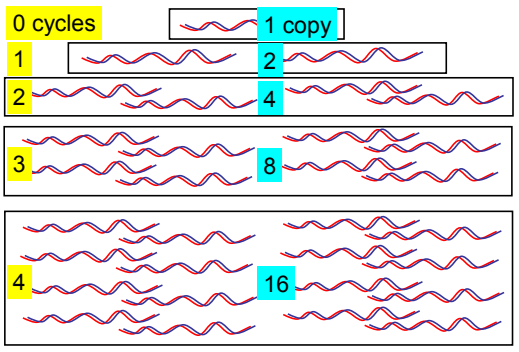
PCR Mechanism



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



PCR Mechanism



PCR Mechanism

Typically PCR is run for 28-32 cycles (E=100%)
Starting with one copy:
- After 28 cycles = 268,435,456
- After 32 cycles = 4,294,967,296

Lower volume PCR may require fewer cycles

At >40 cycles non-template controls may start to give signal

Toward the end of the cycling: reagents are consumed and the PCR is less efficient

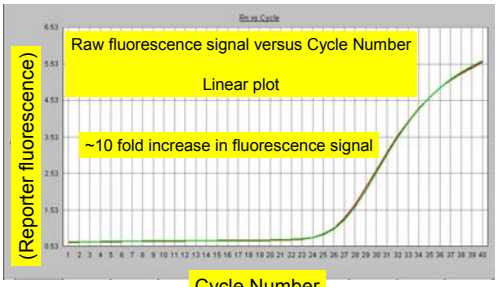
PCR Amplification

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

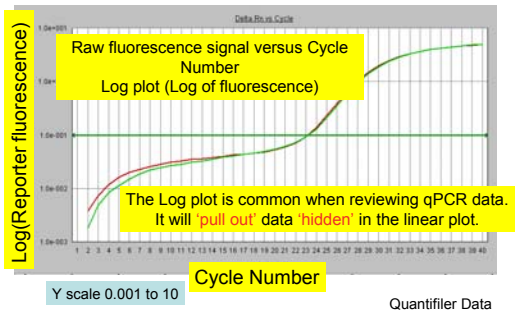
Efficiency is dropping < 100%

The exponential phase is where we make our qPCR measurements

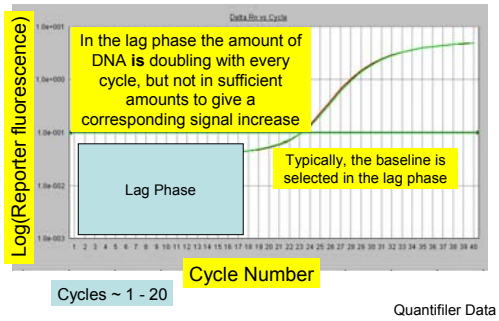
qPCR Real Time Curves



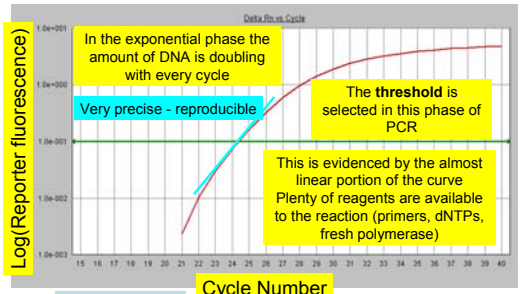
qPCR Real Time Curves



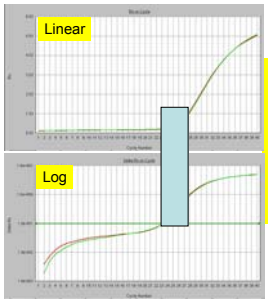
Lag Phase



Exponential Phase



qPCR Real Time Curves

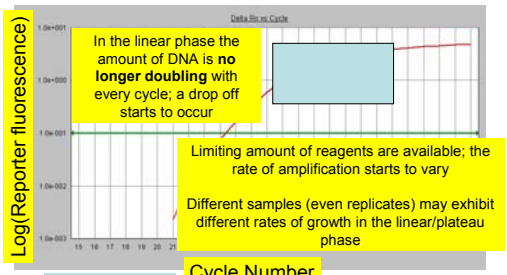


These plots are on the same X scale (Cycle Number)

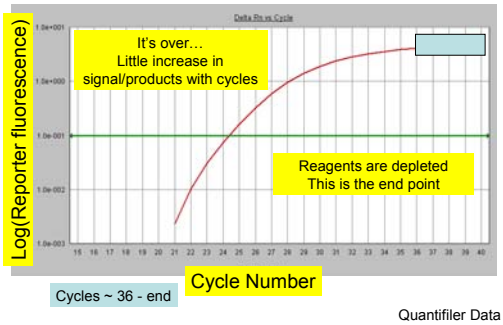
The log plot is useful to see where the amount of DNA is doubling with cycle number

This can not be readily observed in the linear plot

Linear Phase



Plateau Phase



PCR Efficiency

How is the PCR progressing?

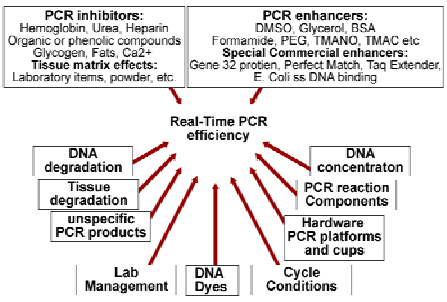
Is the PCR running at maximum efficiency?

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



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

PCR Efficiency

Taking our previous relationship 2^N
The efficiency of the PCR can be represented as:
$X_N = X_0 (1 + E)^N$

- X_N predicted copies
- X_0 starting copy number
- E efficiency (0 to 1)
- N number of cycles

PCR Efficiency

Starting with 100 copies and 100% Efficiency and N = 28
$X_N = 100(1 + 1)^{28}$
$= 2.68 \times 10^{10}$ copies

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

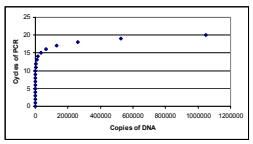
80%
$X_N = 100(1 + 0.8)^{28}$
$= 1.40 \times 10^{9}$ copies

PCR Efficiency

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

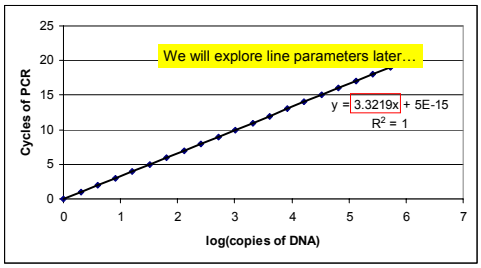
Copies	log Copies	Cycle
1	0	0
2	0.30103	1
4	0.60206	2
8	0.90309	3
16	1.20412	4
32	1.50515	5
64	1.80618	6
128	2.10721	7
256	2.40824	8
512	2.70927	9
1024	3.0103	10

Example data illustrating the doubling in concentration with each PCR cycle



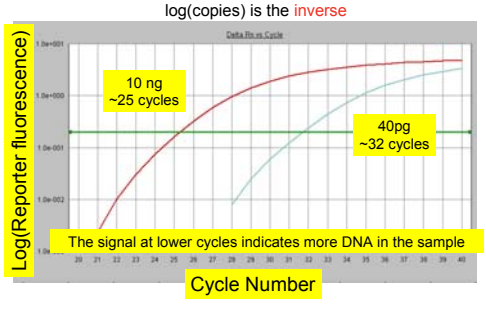
PCR Efficiency

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



PCR Efficiency

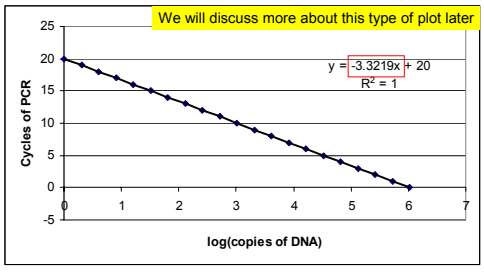
When applied to qPCR the relationship between cycles and log(copies) is the inverse



Quantifiler Data

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 should be 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

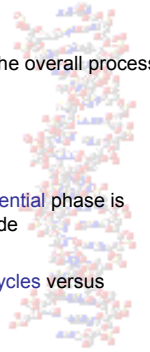
Taking the relationship between log (copies of DNA) and cycles of PCR one can rearrange the equation $X_N = X_0 (1 + E)^N$ in order to determine efficiency

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

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

Summary



Quantitation is an important step in the overall process of DNA typing

PCR is an exponential process; 2^N

Of the 4 phases of qPCR, the exponential phase is where qPCR measurements are made

We can determine E from a plot of cycles versus amplified copies of target DNA

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