Issues Behind Real-Time qPCR for Human DNA Quantitation

National Institute of Standards and Technology

amy.decker@nist.gov

Amy E. Decker, Margaret C. Kline, Peter M. Vallone and John M. Butler

U.S. National Institute of Standards and Technology, Gaithersburg, MD

Real-time quantitative PCR (qPCR) has generated a great deal of interest in the forensic DNA typing community in the past two years as this technique can rapidly detect low levels of DNA with minimum hands-on time and minor sample consumption. The ability to utilize human specific assays in U.S. crime laboratories is important in order to meet federally mandated requirements (DAB/FBI Standard 9.3) to assess the quantity of human DNA in casework samples, particularly where bacterial contamination may exist.

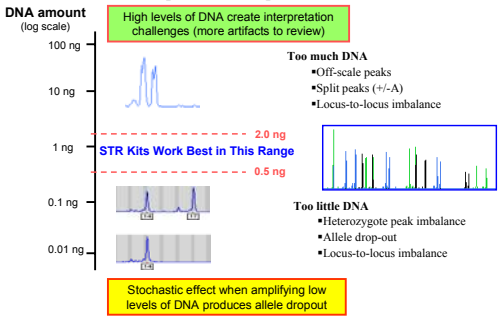
If reliable multiplex assays can be developed to assess the content of a forensic sample in terms of its total human DNA content along with the amount of mitochondrial or Y-chromosomal DNA material, laboratories would be able to evaluate the particular assays that need to be run from a limited amount of evidentiary material.

ABI 7500 Real-Time PCR System

- 96-well format thermal cycler
- five-color detection system with CCD camera
- Real-time monitoring of amplification growth curves enabling viewing of runs in progress

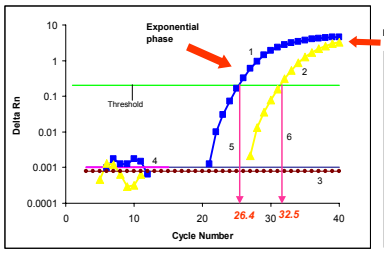
Importance of DNA Quantitation

(prior to multiplex PCR)



- Real-time qPCR chemistries allow for the detection of PCR amplification products during the early phases of the reaction, also known as the exponential phase. In the exponential phase, the amount of material is doubling for each cycle which is the optimal region for analyzing data.
- As reagents are consumed, the reactions begin to slow down or plateau, which is where traditional PCR is measured (end-point analysis). If measurements were taken at the plateau phase for Real-Time PCR, the data would not truly represent the initial amounts of starting target material. This is due to the PCR product no longer being doubled at each cycle.
- Quantitation of DNA is based on the number of cycles required to reach a threshold intensity, C_T.
- The greater the amount of starting DNA, the sooner this threshold value is reached.

Example Reference Plot



1 = high concentration of sample (ie: 10 ng)

2 = lower concentration of sample (ie: 41 pg)

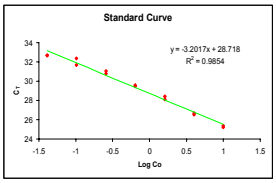
3 = Non-template control (NTC) - burgundy diamonds

4 = Baseline (3-15 cycles) – purple line

5-6 = Cycle threshold (C_T)

- Normalized reporter (R_n) = the reporter signal normalized to the passive reference for a given reaction (ie: FAM, VIC)
- Passive Reference = an internal fluorescence reference dye to which the reporter dye signal can be normalized during data analysis (ie: ROX)
- Delta R_n = R_n – baseline
- Threshold cycle (C_T) = cycle at which sample crosses threshold

Standard Dilution



Log Co = log of DNA concentration

- Calibration based on standard dilution with assumptions as to the initial starting value
- Forms a line of best fit through the standard dilutions, which is in turn used to back-calculate concentration of an unknown sample

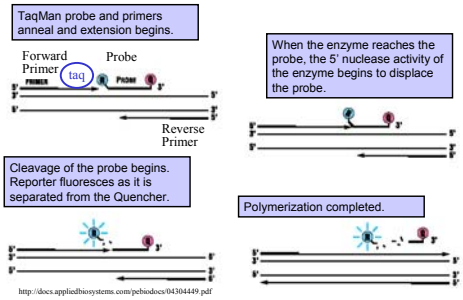
Quantifiler Human Nuclear DNA

Sample	C_T Value (unitless)	Quant Value (ng/µL)
Dilution 1a	25.04	14.12
Dilution 1b	25.02	14.26
Dilution 2a	29.05	0.79
Dilution 2b	29.03	0.80
Dilution 3a	32.41	0.07
Dilution 3b	33.10	0.04

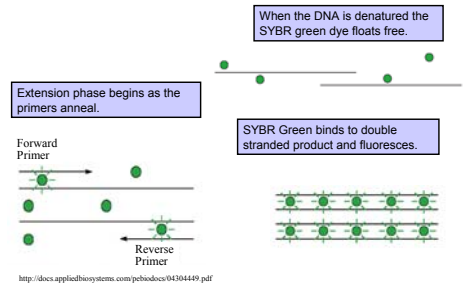
Nuclear DNA data displays reproducible C_T values for the samples

Real-Time Detection Chemistries

5'-nuclease assay using TaqMan probe



SYBR green assay



Assays Studied

Assay	amplicon	GeneTarget	Chemistry	Reference
CFS	62 bp	Flanking region of TH01, 11p15.5	TaqMan MGB	1
Qfiler Human	62 bp	Human telomerase reverse transcriptase gene (hTERT), 5p15.33	TaqMan MGB	2
Qfiler Y Male	64 bp	Sex determining region Y gene (SRY)	TaqMan MGB	3
Alu	124 bp	Alu , Ya5 Subfamily (multi-copy)	SYBR Green	4
CA DOJ nDNA	170-190 bp	TH01, 11p15.5	TaqManMGB	5
CA DOJ mtDNA	69 bp	NADH dehydrogenase (ND1) gene nt 3484-3552	TaqManMGB	5

MGB- minor groove binder

References

1. Richard et al. (2003) J Forensic Sci 48(5):1041-1046
2. Quantifiler™ Human DNA Quantification Kit
3. Quantifiler™ Y Human Male Quantification Kit PN4344790
4. Nicklas & Buel (2003) J Forensic Sci 48 (5):936-944
5. Timken, et al., *in press.*

Variability of DNA Standards

Quantitation difference between two commercial standards using Quantifiler Human assay

Sample n = 4	Standard Lot 1 (ng/mL)	Standard Lot 2 (ng/mL)
1	4*	2.91 ± 0.04
2	7.26 ± 0.79	4*
3	2.93 ± 0.27	1.88 ± 0.09
4	3.46 ± 0.30	2.22 ± 0.08
5	2.99 ± 0.28	1.91 ± 0.08
6	2.62 ± 0.22	1.70 ± 0.03

* - indicates standard value based on starting material provided by the manufacturer

Samples 1-3 = commercially available kit standards

Samples 4-6 = in-house standards based on UV absorbance

Variability Between Assays

Assay	Qfiler Human	Qfiler Y	Alu	CFS
DNA Conc. (ng/mL) (n = 4)	4.49 ± 0.38	3.85 ± 0.15	4.18 ± 0.32	3.91 ± 0.21

This chart displays nuclear quantitation variations in data presumed to be 4ng/ul. Data collected according to provided protocols.

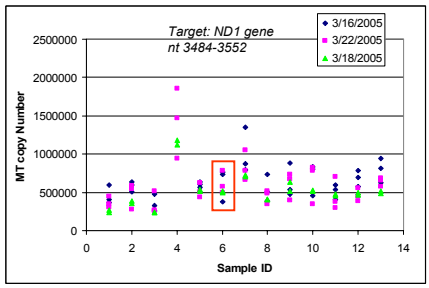
Why Multiplex a Real-Time PCR Assay?

- Multiplexing would provide information on nuclear, mitochondrial and Y chromosome DNA in the same assay

2 copies per cell	nDNA	→ STR Profiling
100s of copies per cell ?	mtDNA	→ Little or no nuclear available; such as hairs or bones
1 copy per cell	YDNA	→ Mixtures of victim and assailant in sexual-assault cases

Multiplexed qPCR assays are difficult to optimize due to compromises in amplification conditions leading to reduced efficiency compared to singleplex assays.

Variability of Mitochondrial DNA Copy Number



There is a large spread among and between samples. For example, sample 6 could result in quant values between 350,000-800,000 copies in triplicate runs.

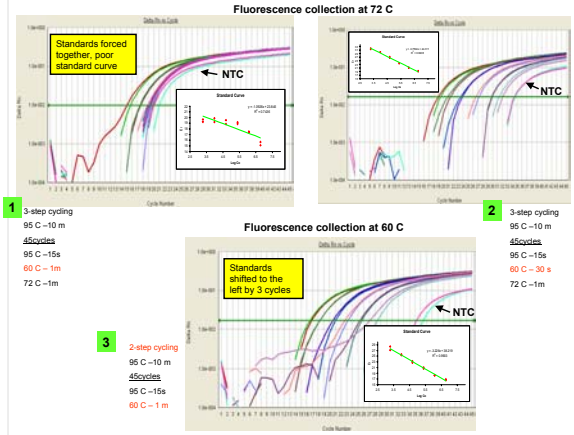
X-axis = sample number (1-13)

Chart displays the reproducibility of mtDNA for three experiments run in triplicate under the exact same conditions over three different days using an oligonucleotide (quantified using UV) as the mtDNA standard.

Possible Issues Contributing to mtDNA Copy Number Variability

- Various tissues in the human body have different amounts of mtDNA
- Various mitochondrion within a cell may have different numbers of copies of the mtDNA genome
- An appropriate mitochondrial quantitative material needs to be defined in order to provide reliable results

Effect of Changes in Time and Annealing Temperature on Assay Performance



Conclusions/Future Work

1. Real-time qPCR has become a widely used technique for quantifying low levels of DNA.
2. We have observed variability in commercial standards which will affect the relative concentration of DNA measurements.
3. Assay optimization is important to generate consistent results.
4. There is inherent variability when determining mitochondrial copy number for a sample that must be taken into account when using real-time qPCR for forensic purposes. This will affect nDNA/mtDNA ratios.
5. Future studies will involve evaluation of a plasmid standard versus oligonucleotide standards, additional examination of duplex/triplex assays, and further testing of mtDNA to determine copy number.
6. We hope to produce a quantitative reference material for human DNA (SRM 2372) in the near future.

Acknowledgments

Funding from the National Institute of Justice through the NIST Office of Law Enforcement Standards interagency agreement 2003-IJ-R-029; David Duewer for suggestions regarding data analysis.