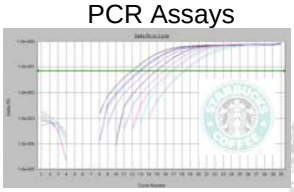
Examining Candidate DNA Quantitation Standards with Real-Time Quantitative PCR Assays



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February 23,2006
58th Annual AAFS Meeting
Seattle,WA

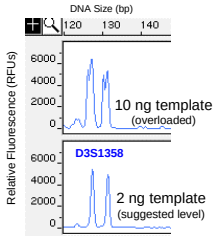
Why are we developing a DNA Quantitative Standard?

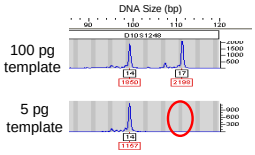
- Most DNA quantitation methods are relative (meaning that they rely on a "Calibrant")
- Provide an independent means to validate a DNA Calibrant (commercial or otherwise)
- Results from NIST Interlaboratory DNA Quantitation Study 2004 (QS04) indicated that there may be a Calibrant/Method bias

Impact of DNA Amount into PCR

Reason that DNA Quantitation is Important Prior to Multiplex Amplification

- Too much DNA
  - Off-scale peaks
  - Split peaks (+/-A)
  - Locus-to-locus imbalance
- Too little DNA
  - Heterozygote peak imbalance
  - Allele drop-out
  - Locus-to-locus imbalance





Stochastic effect when amplifying low levels of DNA produces allele dropout

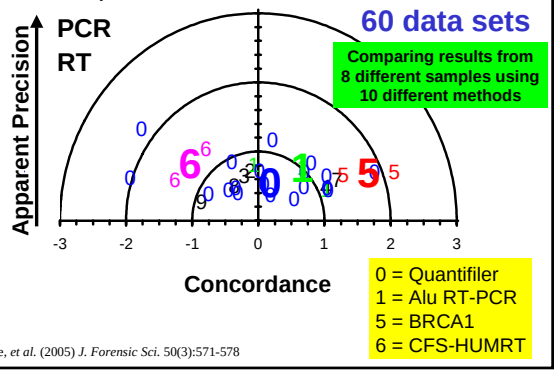
QS 04 Indicators

- Ten different qPCR methods were used to evaluate DNA samples distributed in the NIST Interlaboratory DNA Quantitation Study 2004 (QS04).
- These methods appeared to have some bias relative to each other.
- Is the bias method- or calibrant-based?

Kline, M.C., Duewer, D.L., Redman, J.W., Butler, J.M. (2005) Results from the NIST 2004 DNA Quantitation Study. *J. Forensic Sci.*, 50: 571-578.

Interlaboratory Comparisons

Laboratory Performances with Real-Time PCR Methods



Kline, *et al.* (2005) *J. Forensic Sci.* 50(3):571-578

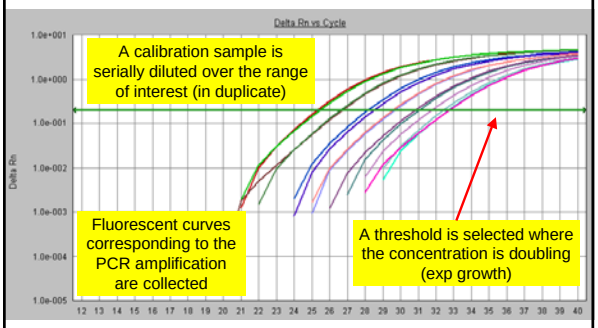
Why Real Time qPCR?

- Labs are beginning to switch over to this method
- Higher throughput and reduced user intervention
- 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)
- Inquiries from the community

General qPCR Comments from the Forensic Community

- "I feel that the calibrant may exhibit a two-fold difference from the 'true' value"
- "In practice we have found that utilizing a target range of 1-2 ng based on a method X result often times yields STR data below our rfu threshold"
- "There appears to be an obvious difference between the two lots of a calibrant"
- "We have not had any problems with the lot_X calibrant and our results have been relatively stable"

qPCR is a Relative Technique



Data collected: cycle number or C_T (cycle value at a selected threshold)
Unknowns are run on the same plate (same master mix etc)

Developing a Calibrant DNA

- Some sources of genomic DNA
  - Single source
  - Multiple source
  - Cell line
- How is the concentration of the Calibrant determined?
  - UV, fluorescence, phosphorus, others
- Since qPCR is relative to the DNA calibrant used, different calibrants may give different results
  - Are these within error?
  - Can this be controlled?
  - Is the error acceptable for our purpose?

Things to Consider with Calibrants

- Will the calibrant have inherent characteristics that may bias results?
- If probing a multi copy locus (Alu) will different calibrants have significantly different numbers of copies (cell line vs single source)?
- If using UV spectroscopy for quantitation: do the OD measurements correlate with qPCR results? (1 OD = 50 ng/µL double stranded DNA)

Methods

- qPCR methods will vary
  - Master mix
  - Annealing temp
  - Type of probe
  - Genetic marker (multi copy)
  - Instrument
  - PCR efficiency
  - Amplicon size
- How will different Methods perform with various Calibrants?

qPCR Method Evaluation Protocol

- 6 different calibrants:
  - 3 commercial (2 cell lines, one multiple source)
  - 3 purified at NIST (single source; one female, two males)
- Where possible, [DNA] was assigned from UV absorption at 260 nm; otherwise used manufacturer's values.
- Stocks of the candidates were diluted to:
  - 10.0, 4.0, 1.6, 0.64, 0.26, 0.1, and 0.04 ng/µL daily.
- Each candidate sample was run in duplicate on duplicate plates with each of the 5 qPCR methods.

Samples run on ABI 7500

qPCR Methods Evaluated at NIST

- Quantifiler Human (TaqMan MGB)
- Quantifiler Y Male (TaqMan MGB)
- Alu (SYBR Green)
- CA DOJ nDNA (TaqMan BHQ)
- CFS HumTH01 (TaqMan MGB)

1. Quantifiler™ Human DNA Quantification Kit PN4343895
2. Quantifiler™ Y Human Male Quantification Kit PN4343906
3. Nicklas J, Buel E. J Forensic Sci 2003; 48:936-944.
4. Timken M, Swango K, Orrego C, Buoncristiani M. J Forensic Sci 2005; 50:1044-60
5. Richard ML, Frappier RH, Newman JC. J Forensic Sci 2003;48:1041-1046.

qPCR Methods

Method	Amplicon (bp)	Target
Quantifiler Human	62	Human telomerase reverse transcriptase gene (hTERT), 5p15.33
Quantifiler Y Male	64	Sex determining region Y gene (SRY)
Alu	124	Alu , Ya5 Subfamily (**multi copy**)
CA DOJ	170-190	TH01, 11p15.5
CFS HumTH01	62	Flanking region of TH01, 11p15.5

6 DNA Calibrants were evaluated
5 qPCR methods were evaluated

Calibrant 1		Calibrant 2		Calibrant 3		Calibrant 4		Calibrant 5		Calibrant 6	
10	10	10	10	10	10	10	10	10	10	10	10
4	4	4	4	4	4	4	4	4	4	4	4
1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64
0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256	0.256
0.102	0.102	0.102	0.102	0.102	0.102	0.102	0.102	0.102	0.102	0.102	0.102
0.041	0.041	0.041	0.041	0.041	0.041	0.041	0.041	0.041	0.041	0.041	0.041
NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

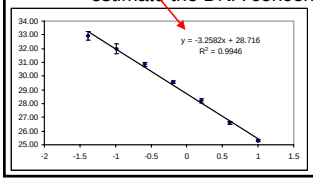
The above plate format was run twice for each of the 5 qPCR methods (10 plates total) 960 data points (C_T values)

Serial dilution of 10 ng to 41 pg were prepared fresh for each Cailbrant DNA material – based on UV or supplier information

Data Analysis

Calibrant 1		Calibrant 2		Calibrant 3		Calibrant 4		Calibrant 5		Calibrant 6	
10	10	25.90	25.93	24.54	24.72	25.04	25.02	24.84	24.70	24.68	24.84
4	4	27.22	27.26	26.16	26.24	26.43	26.31	26.20	26.14	26.13	25.96
1.6	1.6	28.54	28.53	27.53	27.58	27.72	27.89	27.58	27.55	27.35	27.31
0.64	0.64	30.06	30.07	29.01	29.05	29.05	29.03	29.14	28.70	28.66	28.64
0.256	0.256	31.09	31.03	30.08	29.77	30.32	30.07	29.74	29.97	29.66	29.74
0.102	0.102	31.83	32.21	31.05	31.05	31.07	31.19	31.04	31.08	30.76	31.27
0.041	0.041	32.71	32.56	31.71	32.06	32.41	33.10	32.41	32.66	32.94	32.36
NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

Each calibrant was used to generate a calibrant curve and estimate the DNA concentration of the other five



Raw C_T values are converted to concentration using C_T = m*log[DNA] + b

Data Analysis

Calibrant 1		Calibrant 2		Calibrant 3		Calibrant 4		Calibrant 5		Calibrant 6	
10	10	23.8	21.2	8.5	7.7	9.6	8.6	8.1	7.3	7.2	6.5
4	4	7.2	8.1	2.8	3.1	3.3	3.6	2.8	3.1	2.5	2.8
1.6	1.6	2.3	1.7	0.9	0.7	1.2	0.9	1.0	0.8	0.9	0.7
0.64	0.64	0.68	0.73	0.31	0.33	0.39	0.42	0.34	0.36	0.31	0.34
0.256	0.256	0.215	0.253	0.104	0.121	0.137	0.159	0.120	0.139	0.113	0.131
0.102	0.102	0.074	0.128	0.038	0.064	0.052	0.086	0.046	0.075	0.044	0.072
0.041	0.041	0.060	0.055	0.031	0.029	0.043	0.040	0.038	0.035	0.037	0.034
NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

96 concentrations estimated per calibrant on plate
Minus 12 NTC = 564
6 calibrants *96 = 504 values per plate
504*10 = 5040 values

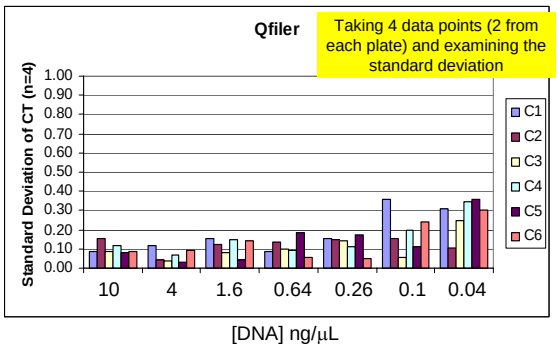
Questions that can be answered with the large data set

- Is there plate to plate bias for a single qPCR method?
- Is there a bias between qPCR methods?
- What effect do different DNA Calibrants have on results?

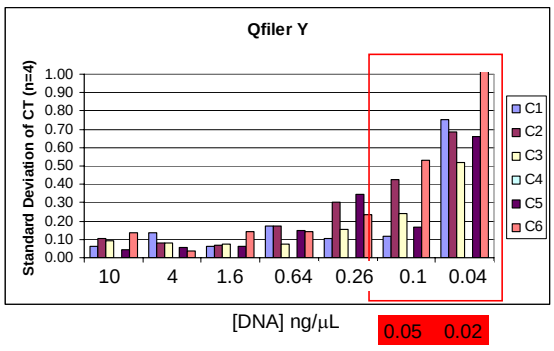
Evaluating Raw C_T Values

- Taking 4 data points (2 from each plate) and examining the standard deviation:
- All assays except Alu have higher error at the 2 lowest concentrations – this is to be expected
- Alu (multi copy locus) performs well at low [DNA]
- Qfiler Y exhibits higher deviation at low [DNA] possibly due to the haploid nature of the Y CHR
- Uncertainty is ~0.1 to 0.2 C_T units

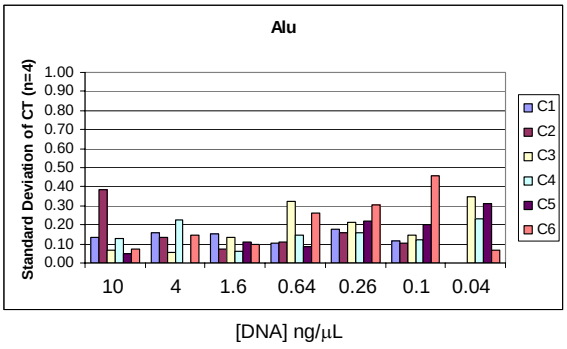
Raw C_T Values

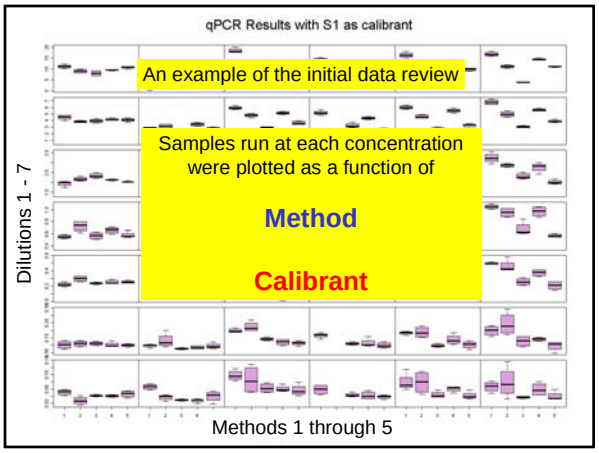


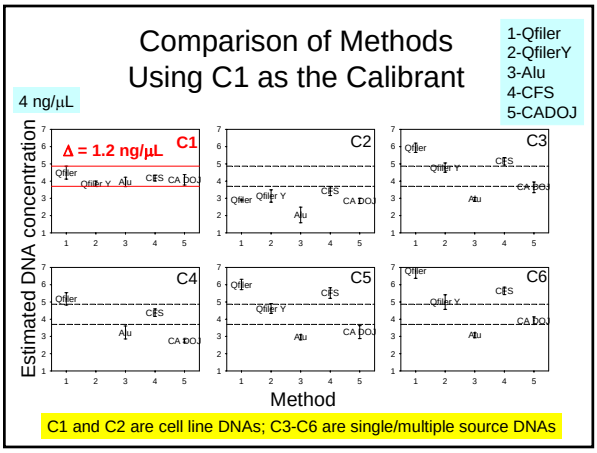
Raw C_T Values



Raw C_T Values



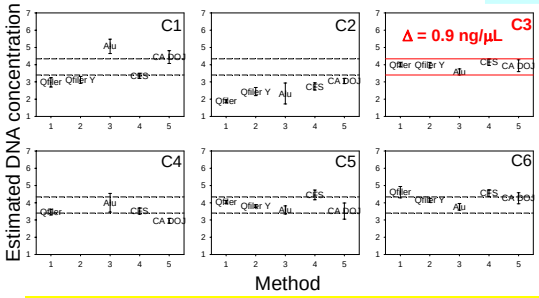




Comparison of Methods using C3 as the Calibrant

1-Qfiler
2-QfilerY
3-Alu
4-CFS
5-CADOJ

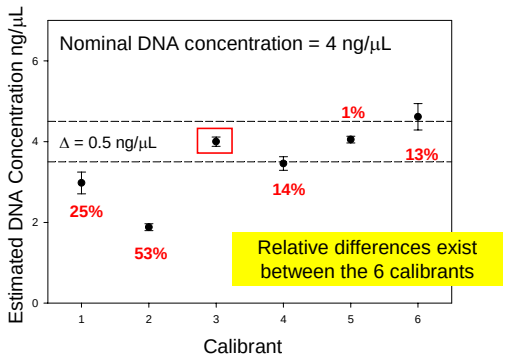
4 ng/µL



C1 and C2 are cell line DNAs; C3-C6 are single/multiple source DNAs

Six Calibrants quantitated using Quantifiler

(Calibrant Curve was generated with C3)



Relative differences exist between the 6 calibrants

Conclusions

- When the Calibrant DNA is 'different' than the 'unknown', a bias between the methods is observed
- When the Calibrant DNA and 'unknown' are similar, there is less Method Bias observed
- This makes cross comparisons of Methods difficult when using different calibrants
- For practical purposes: a consistent method/calibrant may be optimized to give acceptable STR results **(within a lab)**
- However new calibrants (new lots?) should be performance checked for consistency (correction factor?)

Practical Example

Different Calibrants

Amount of DNA added to reaction

½x C_1	"True" C_2	2x C_3
0.35	0.7	1.4
0.50	1.0	2.0
0.8	1.6	3.2
1	2	4

The "True" value is relative to the Calibrant
It could be a "True" value in an absolute sense or the "True" value determined to give the proper RFU range

SRM 2372
Human DNA Quantitation Standard

Anticipated 2006 issue

Component A: Male (blood)
Component B: Female (blood)
Component C: Mixture (placenta)

- Genomic DNA isolated by Salt out procedure
- Treated with RNAse and re-precipitated
- UV spectroscopy 340-220 nm on a NIST calibrated spectrophotometer
- Assume $A^{260} = OD^{260} = 1$ for a 50 µg/mL solution
- Planned Amounts: Each component 50 µL of Human Genomic DNA with a concentration targeted @ 50 ng/µL.

Stability of the DNA Standard Tube Study

Five different tubes were evaluated at :
3 different storage temperatures
3 different [DNA]
Duplicate tubes, duplicate qPCR runs
Duration 12 months : Averaged results for 4 time points

[DNA] ng/µL	A	B	C	D	E
0.2	1.0	1.05	0.81	1.06	0.87
1.0	1.0	1.14	1.31	1.01	0.23
5.0	1.0	1.31	1.33	1.21	0.86

B_ snap cap; C_ ambient tubes evaporated; E_ lids cracked

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

Acknowledgements

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Funding:

Interagency Agreement between National Institute of Justice and NIST Office of Law Enforcement Standards

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Thanks to Marc Salit for data analysis

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