Testing Candidate DNA Quantitation Standards with Several Real-Time Quantitative PCR Methods

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Preamble

- There is a set of questions that must be answered in the proposal for production of a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM).
- In the pursuit to answer these questions many studies were performed, some of which will be described in this talk.

Disclaimers

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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.

Our publications and presentations are made available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

Questions concerning a DNA Quantitative Standard

- **Do we really need a DNA Quantitative Standard?**
  - **There must be a demonstrated need for SRM development**
- **How good are we at quantifying DNA?**
  - **Determine NIST capabilities**
  - **Determine the community's capabilities through interlaboratory studies**
- **Are current Quantitation methods yielding answers that are "fit for purpose"?**
  - **How good do we really have to be?**

We have been working in this area for sometime now.

NIST Quantitation Interlaboratory Studies

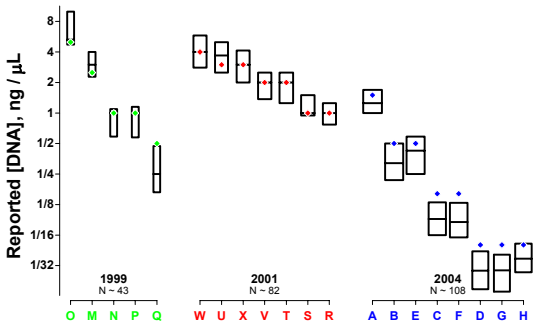
http://www.cstl.nist.gov./biotech/strbase/interlab.htm

1999 Duewer DL, Kline MC, Redman JW, Newall PJ, Reeder DJ. NIST Mixed Stain Studies #1 and #2: Interlaboratory Comparison of DNA Quantification Practice and Short Tandem Repeat Multiplex Performance with Multiple-Source Samples. J Forensic Sci 2001;46(5):1199-1210.

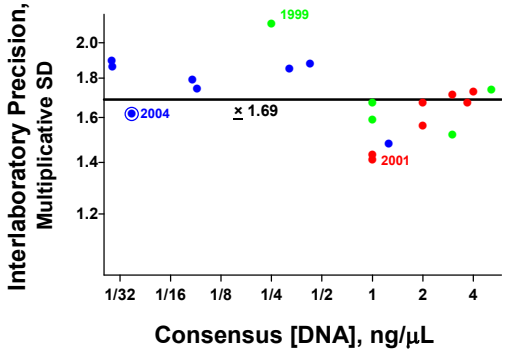
2001 Kline MC, Duewer DL, Redman JW, Butler JM. NIST Mixed Stain Study #3: DNA Quantification Practice and its Influence on Short Tandem Repeat Multiplex Performance. Anal Chem 2003;75(10):2463-2469.

2004 Kline MC, Duewer DL, Redman JW, Butler JM. Results from the NIST 2004 DNA Quantitation Study. J Forensic Sci, 50(3): 571-578.

Interlaboratory Performance



"Reproducibility"

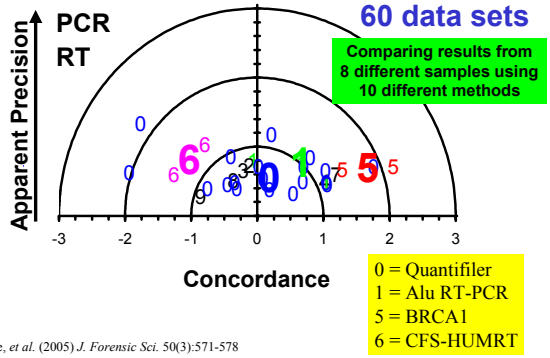


QS 04 Indicators

- 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 standard-based?

Interlaboratory Comparisons

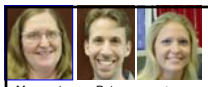
Laboratory Performances with Real-Time PCR Methods



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

qPCR Facts

- qPCR is **RELATIVE** to the standards used to generate a calibration curve.
- qPCR instruments use a selected Cycle Theshold (C_T) for calculations.
- The premise is that at 100% PCR efficiency you have a doubling of the PCR product.
- Therefore a ±1 difference C_T = [DNA] ×1/2 or ×2.
- Quantifiler Human and Y have an Internal PCR Control (IPC) to assist in evaluation of sample inhibition.



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qPCR Methods Evaluated at NIST

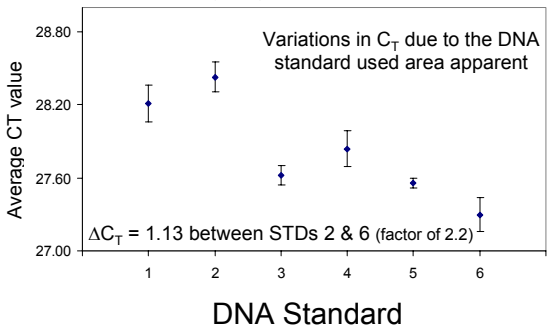
- Quantifiler Human (TaqMan MGB)
- Quantifiler Y Male (TaqMan MGB)
- Alu (SYBR Green)
- CA DOJ nDNA (TaqMan BHQ)
- CFS HumTH01 (TaqMan MGB)

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, et al.. J Forensic Sci 2005,50:1044-1060.
5. Richard ML, et al. J Forensic Sci 2003;48:1041-1046.

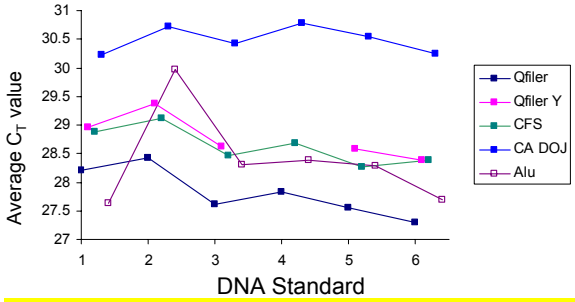
qPCR Method Evaluation Protocol

- 6 different Human Genomic samples were used.
  - 3 commercial
  - 3 purified at NIST
- 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.

Quantifiler Results
1.6 ng of genomic DNA; n=4



5 qPCR Methods run on 6 DNA Standards

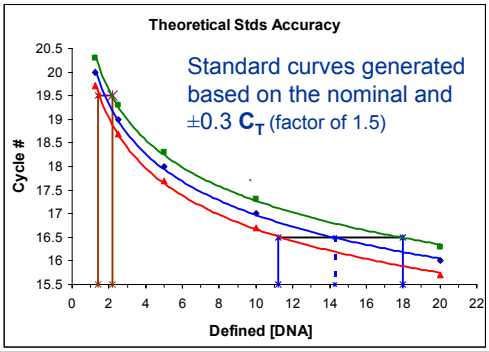


Absolute CT values vary for each qPCR method
The CT trends observed for 6 DNA standards are similar indicating no/minimal qPCR method based bias

Method Variability ?

- Results indicate there is little method-to-method bias on sample results.
- There do exist slight differences in relative sample performance that are consistent among the methods.
- 4 of the samples appear to be within 0.5 C_Ts of one another (factor of 1.4).
- The community in general is quantifying samples within a factor of 1.7 (QS04).
- QS04 qPCR method bias was probably Standard based.
- So a SRM Quantitation Material may help (a little)!

Does Variability (Consistency) of a Standard Matter?



Stability of the DNA Standard Tube Study

Can the end user get out what was put in?

Five different tubes were evaluated at :
3 different storage temperatures
3 different [DNA]
Quantifiler used to evaluate the [DNA]
Duplicate tubes, duplicate qPCR runs
Duration 7 months : Averaged results for 5 time points

[DNA]	A	B	C	D	E
0.20	1.00	0.74	1.14	0.72	0.69
1.00	1.00	0.88	0.98	0.86	0.88
5.00	1.00	0.99	0.91	0.94	0.72

30 data points / tube type / [DNA]

[DNA] in ng/µL

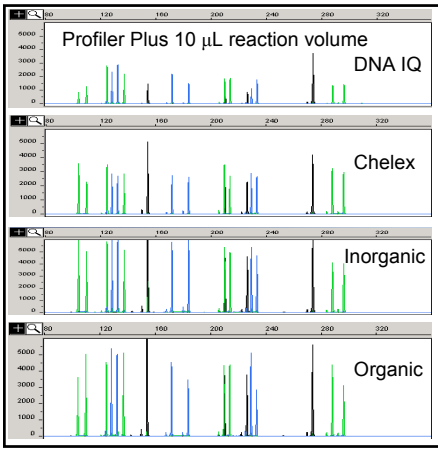
Extraction Method Affect on qPCR

- Question is the observed difference in the candidate samples a factor of extraction technique?
- For the 3 commercial samples, we do not know the extraction techniques used.
- For the 3 NIST samples, extraction was Inorganic salt-out.
- What about other extraction techniques?

Extraction Methods Study

1. Chelex
2. DNA IQ
3. Organic (Chloroform/Phenol)
4. Inorganic (saltout)

1. Walsh et al. (1991) BioTechniques, 10, 506-513.
2. Promega Corporation Part # TB296
3. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, Vol. 2. Cold Spring Harbor Press pp. E10 – E14
4. Miller et al. (1988) Nucleic Acids Research, 16, 1215.



Extraction method rfu's

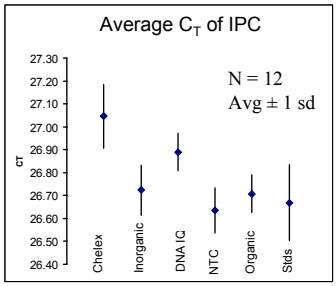
"1 ng" of all extracts were amplified.

Inorganic and organic extracts amplify equally.

DNA IQ and Chelex extracts amplified with lower rfu values, 40% and 60% respectively.

All extracts typed correctly.

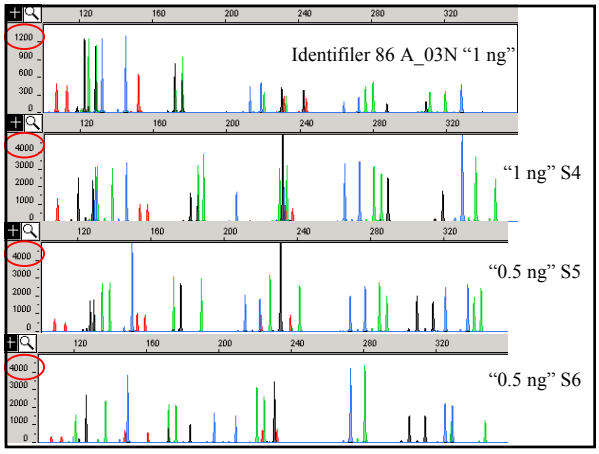
IPC of the Extracts



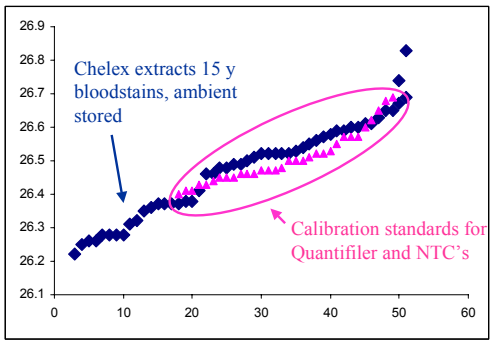
Chelex and DNA IQ extracts appear to have different IPC values from the Standards, NTC, organic, and inorganic extracts. This could be the reason they amplify less robustly.

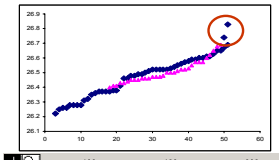
All samples were diluted to 0.5 ng/µL prior to amplification. Chelex samples were diluted 4-fold more than the DNA IQ extracts

Aged Stain extracts versus Candidate Standard Peak Heights

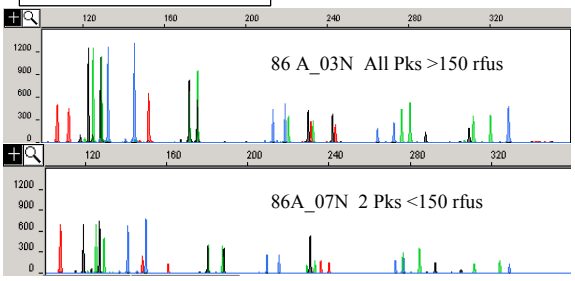


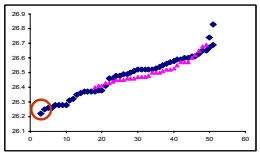
IPC's of 51 Aged Bloodstain extracts





Amplifying "1 ng" of these 2 extracts results in one complete profile and one partial profile given 150 rfu cut off.





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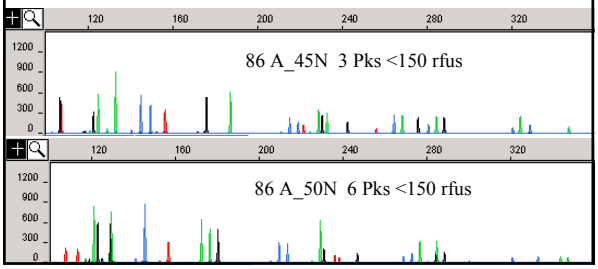
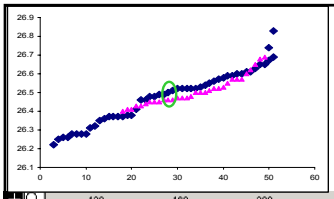
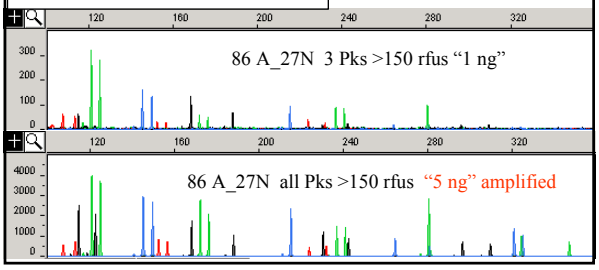




Amplifying "1 ng" of this extract resulted in partial profiles given 150 rfu cut off. Only 3 pks > 150 rfus.

"5 ng" yields complete profile.



Requirements for NIST SRM 2372 Human DNA Quantitation Standard

Material must be a reliable standard:

- **Homogeneity**
  - All tubes are the same
- **Stability**
  - Will withstand shipping and normal storage
- **Recoverability**
  - What went in the tubes comes out
- **Traceability**
  - Values assigned are traceable to the designated certification method.

SRM 2372
Human DNA Quantitation Standard

Anticipated 2006 issue

Component A: Male
Component B: Female
Component C: Mixture

Planned Amounts: Each component 50 µL of Human Genomic DNA with a concentration targeted @ 50 ng/µL. The [DNA] for each component will be list in the materials Certificate of Analysis.

Acknowledgements

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NIST Project Team:

John Butler, Pete Vallone, Margaret Kline, Jan Redman, Amy Decker, Mike Coble, Becky Hill, Dave Duewer

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