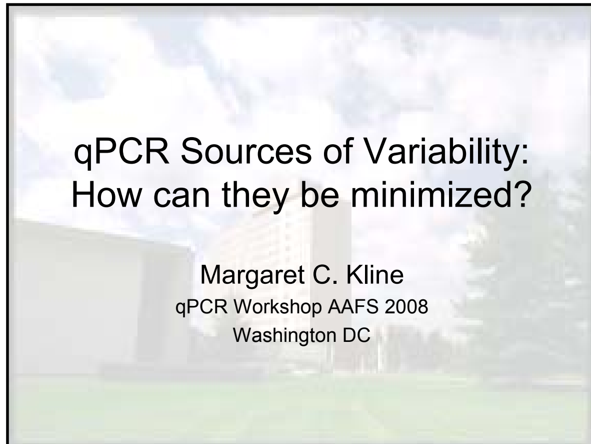


Disclaimers

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.

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

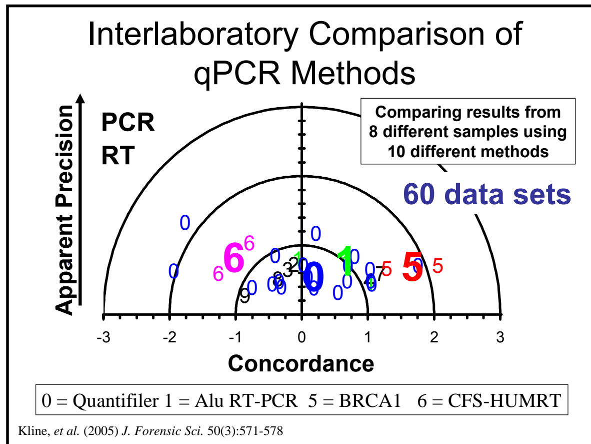
Discussion Points

- Instrumentation
  - Well to well /tube to tube
- Pipetting
  - Accuracy
  - Precision
- qPCR assay target
  - Single copy
  - Multiple copies
  - Range of linearity
- Calibration Material
  - Characterization
  - Similarity to samples
  - Range of dilution
- Trouble shooting
  - Poor calibration
    - Dye
    - Optical
    - Background
- Take home lessons

DNA Quantification Method Wish List

- Accurate — A nanogram is a nanogram
- Precise — Reproducible
- Sensitive — Appropriate dynamic range
- Robust — Results from different sample types are comparable
- Transferable — Analyst to Analyst - Lab to Lab

Interlaboratory Comparison of qPCR Methods



0 = Quantifiler 1 = Alu RT-PCR 5 = BRCA1 6 = CFS-HUMRT

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

Instrument Within Run Reproducibility

- Ideally we want to be able to “trust” that no matter where in a qPCR instrument block a sample is located you will get the same answer
- So calibrate the instrument
- Then perform the ultimate test
- A whole plate with the same sample

Experimental Design

Create a master-mix of qPCR reagents and template
Distribute across the entire plate.
Run the assay, determine Ct for each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	■	■	■	■	■	■	■	■	■	■	■	■
B	■	■	■	■	■	■	■	■	■	■	■	■
C	■	■	■	■	■	■	■	■	■	■	■	■
D	■	■	■	■	■	■	■	■	■	■	■	■
E	■	■	■	■	■	■	■	■	■	■	■	■
F	■	■	■	■	■	■	■	■	■	■	■	■
G	■	■	■	■	■	■	■	■	■	■	■	■
H	■	■	■	■	■	■	■	■	■	■	■	■

Adding the template to the master-mix reduces pipetting errors.
[DNA] 2.5 ng/µL
qPCR kit - Qfiler

Plate test results

	1	2	3	4	5	6	7	8	9	10	11	12	avg	sd
A	28.38	28.49	28.47	28.44	28.48	28.61	28.60	28.52	28.58	28.56	28.53	28.12	28.48	0.13
B	28.46	28.54	28.55	28.59	28.77	29.14	29.15	29.04	28.61	28.58	28.54	28.33	28.69	0.27
C	28.56	28.85	28.79	29.11	29	29.45	29.43	29.3	28.88	28.74	28.61	28.49	28.93	0.33
D	29.12	29.26	29.34	29.49	29.67	29.88	29.71	29.58	29.12	28.86	28.65	28.37	29.25	0.46
E	28.82	29.23	29.23	29.37	29.39	29.75	29.67	29.12	28.91	28.65	28.65	28.66	29.12	0.39
F	28.45	28.8	29.01	29.07	28.78	29.08	29.44	28.74	28.68	28.58	28.55	28.39	28.80	0.31
G	28.51	28.59	28.58	28.64	28.66	28.95	28.77	28.68	28.6	28.6	28.56	28.53	28.64	0.12
H	28.37	28.43	28.4	28.53	28.48	28.56	28.58	28.66	28.45	28.5	28.49	28.55	28.50	0.08
avg	28.58	28.77	28.80	28.91	28.90	29.18	29.17	28.96	28.73	28.63	28.57	28.43	**28.80**	**0.38**
sd	0.26	0.32	0.36	0.41	0.43	0.49	0.47	0.37	0.22	0.12	0.06	0.17		

The grand mean of the plate is 28.8 Cts with **sd of 0.38**

But look at the trends going on by column and by row.
Minimum = 28.12 Cts Maximum = 29.88 Cts
That's a maximum difference of **1.76 Cts** !

If we plot these results in a 3D plot >>>

Plate test plot

This is the result of the wrong lamp being installed in the instrument. Instrument calibration could not compensate.

Ct
30.00
29.50
29.00
28.50
28.00
row
11 9 7 5 3 1
Column
G D A
29.50-30.00
29.00-29.50
28.50-29.00
28.00-28.50

Correct lamp installed results

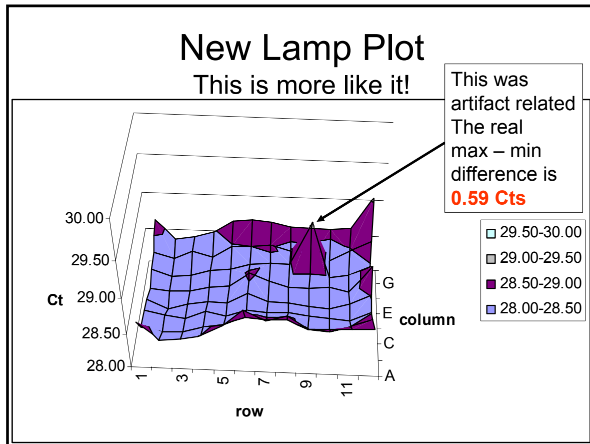
	1	2	3	4	5	6	7	8	9	10	11	12	avg	sd
A	28.68	28.43	28.46	28.51	28.67	28.91	28.77	28.84	28.67	28.68	28.70	28.70	28.67	0.14
B	28.36	28.30	28.32	28.40	28.45	28.57	28.55	28.56	28.43	28.40	28.49	28.61	28.45	0.10
C	28.38	28.31	28.27	28.40	28.34	28.44	28.41	28.42	28.39	28.37	28.33	28.47	28.38	0.06
D	28.31	28.18	28.26	28.30	28.30	28.45	28.34	28.34	28.29	28.28	28.36	28.51	28.33	0.09
E	28.25	28.22	28.24	28.29	28.32	28.53	28.42	28.35	28.33	28.36	28.30	28.68	28.36	0.13
F	28.31	28.22	28.25	28.33	28.36	28.44	28.47	28.46	29.06	28.29	28.33	28.48	28.42	0.22
G	28.36	28.28	28.39	28.32	28.49	28.50	28.52	28.44	28.38	28.33	28.48	28.63	28.43	0.10
H	28.63	28.36	28.40	28.48	28.64	28.67	28.65	28.59	28.59	28.57	28.60	29.03	28.60	0.17
avg	28.41	28.29	28.32	28.38	28.45	28.56	28.52	28.50	28.52	28.41	28.45	28.64	**28.45**	**0.17**
sd	0.157	0.08	0.08	0.08	0.14	0.16	0.14	0.16	0.25	0.14	0.14	0.18		

The grand mean of the plate is 28.45 Cts with **sd of 0.17**
Minimum = 28.18 Cts Maximum = 29.06 Cts
That’s a difference maximum difference of **0.88 Cts** !

If we plot these results in a 3D plot >>>

New Lamp Plot

This is more like it!



This was artifact related The real max – min difference is **0.59 Cts**

Pipetting Issues

- You are using small volumes of the DNAs
  - 2 µL to 5 µL normally
- Pipetting technique and pipettes must be at the highest level of reproducibility.
  - Check the tips for small amounts of sample remaining, every time!
  - Do not be in a hurry when you are pipetting
- Test the pipettes & analysts out! >>>

Pipette Verification

Electronic Pipette **E0000798E** **2 uL**
Reagent Lot Code 55091

Vial 1	Vol	Vial 2	Vol	Vial 3	Vol
1	1.94	1	1.96	1	1.97
2	1.95	2	1.98	2	1.96
3	1.92	3	2.00	3	1.92
4	1.96	4	1.95	4	1.95
5	1.91	5	1.96	5	1.94
6	1.93	6	1.96	6	1.96
7	1.92	7	1.95	7	1.95
8	2.00	8	2.01	8	1.95
9	2.00	9	1.95	9	1.96
10	1.99	10	1.96	10	1.93
11	1.95	11	1.95	11	1.97
12	1.99	12	1.94	12	1.96
13	1.94	13	1.94	13	1.97
14	1.92	14	1.94	14	1.97
15	1.96	15	1.94	15	1.97
16	1.93	16	1.99	16	1.99
17	1.94	17	2.02	17	1.95
18	1.98	18	1.93	18	1.93
19	1.97	19	1.97	19	1.88
20	1.94	20	1.93	20	1.96
21	1.95	21	1.95	21	1.97
22	2.20	22	1.93	22	1.96
Avg	**1.96**		**1.96**		**1.95**
sd	**0.06**		**0.02**		**0.02**

Grand Mean	SD	CV
1.96	**0.04**	**2.0 %**

← Precision

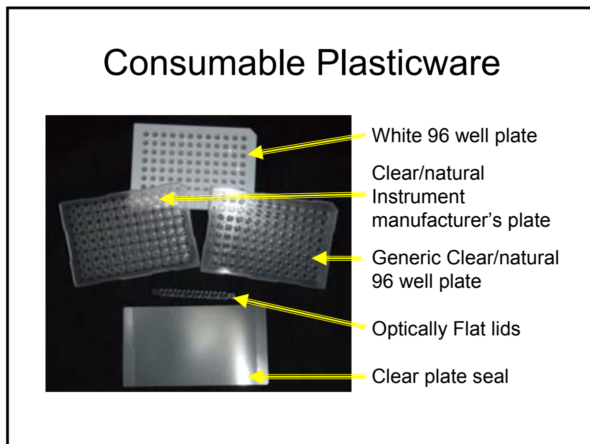
These results are from an electronic pipette. Good analysts can achieve the same results, or better, with manual pipetting.

Use of replicates of each sample per qPCR run is an automatic check of pipetting reproducibility, unless the sample is stochastically challenged.

Difference from Set point
-1.8 %

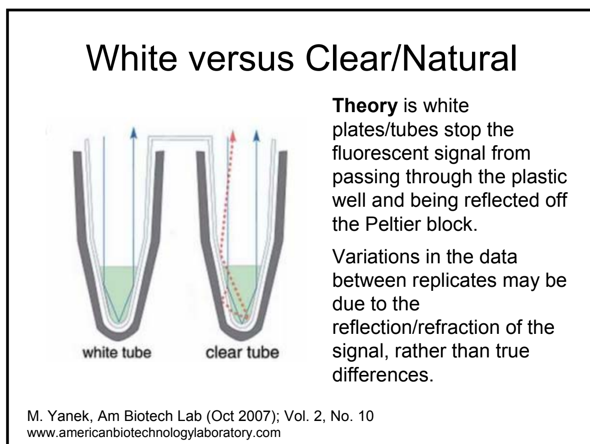
← Accuracy

Consumable Plasticware



- White 96 well plate
- Clear/natural Instrument manufacturer's plate
- Generic Clear/natural 96 well plate
- Optically Flat lids
- Clear plate seal

White versus Clear/Natural



Theory is white plates/tubes stop the fluorescent signal from passing through the plastic well and being reflected off the Peltier block.

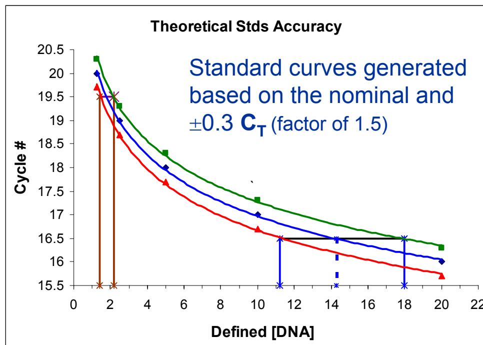
Variations in the data between replicates may be due to the reflection/refraction of the signal, rather than true differences.

M. Yanek, Am Biotech Lab (Oct 2007); Vol. 2, No. 10
www.americanbiotechnologylaboratory.com

Plasticware Variability

- White plate was **17%** less variable than the clear plate when using a **7900**. (based on the difference between the minimum and maximum Cts across a 96 well plate)
- There was **no apparent difference** in plate type used in the **7500**.
- The Flat lids gave no apparent difference from the clear film.

Does Variability (Consistency) of a Standard Matter?



Developing a Calibrant

- 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?

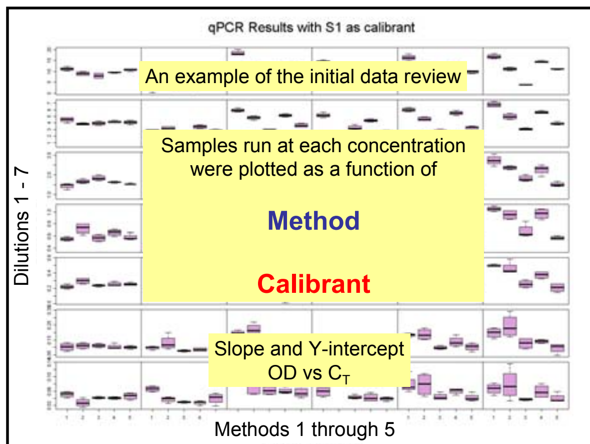
Things to Consider with Calibrants

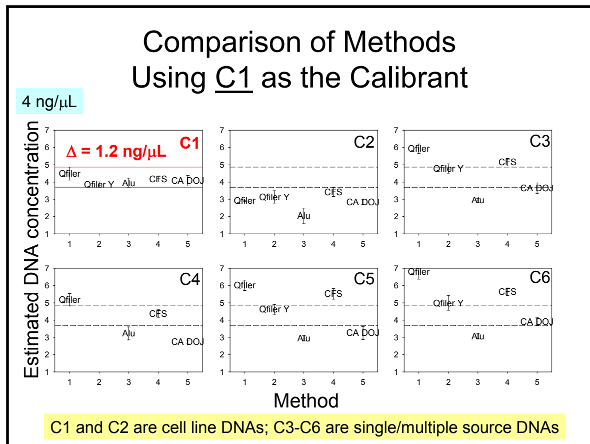
- Will the calibrant have inherent characteristics that may bias results?
- If probing a multi copy locus (Alu) will different calibrants have significantly different numbers of copies (cell line vs single source)?

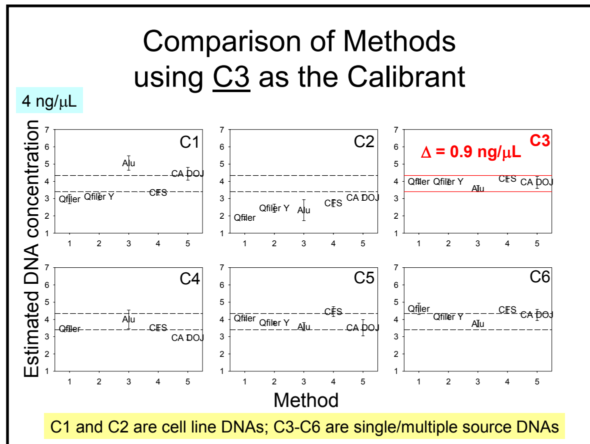
qPCR Method Evaluation Protocol

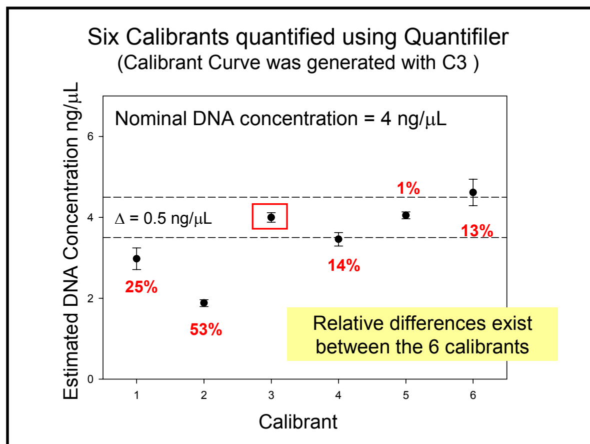
- 6 different calibrants:
  - 3 commercial (2 cell lines, one multiple source)
  - 3 purified at NIST (single source; one female, two males)
- Where possible, [DNA] was assigned from UV absorption at 260 nm; otherwise used manufacturer's values.
- Stocks of the candidates were diluted to:
  - 10.0, 4.0, 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



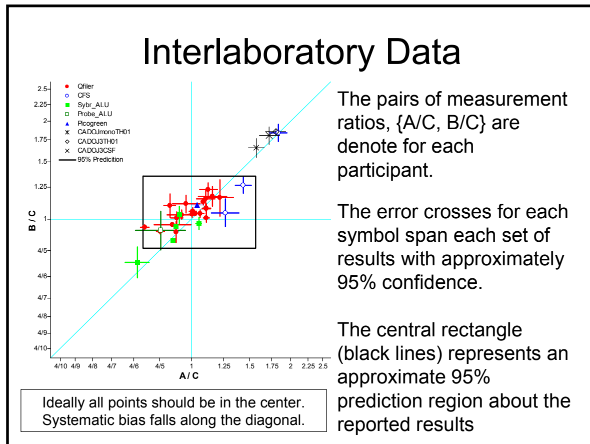

Comparison of Methods
Using C1 as the Calibrant
4 ng/μL
Δ = 1.2 ng/μL
C1
C2
C3
C4
C5
C6
Qfiler
Qfiler Y
Alu
CFS
CA DOJ
Estimated DNA concentration
Method
C1 and C2 are cell line DNAs; C3-C6 are single/multiple source DNAs


Comparison of Methods
using C3 as the Calibrant
4 ng/μL
Δ = 0.9 ng/μL
C1
C2
C3
C4
C5
C6
Qfiler
Qfiler Y
Alu
CFS
CA DOJ
Estimated DNA concentration
Method
C1 and C2 are cell line DNAs; C3-C6 are single/multiple source DNAs


Six Calibrants quantified using Quantifiler
(Calibrant Curve was generated with C3)
Nominal DNA concentration = 4 ng/μL
Δ = 0.5 ng/μL
1%
13%
14%
25%
53%
Relative differences exist between the 6 calibrants
Estimated DNA Concentration ng/μL
Calibrant

Evaluation of Standard Reference Materials

- **Interlaboratory comparison**
  - **Three different materials distributed (A, B, and C).**
  - **32 laboratories participated**
- **One material was assigned a [DNA].**
  - **a pre-determined dilution scheme to assign values to the other samples (generally 8 dilutions).**



Results from previous Interlaboratory Study

- Given the same materials to analyze
- Using the same material for calibration
- Within Method results cluster
- Most methods were within 1.5-fold region about the center of the reported results.
- Could still see some method dependent results.
  - How can these be explained?
    - Method dependent dynamic range was not appropriate?
    - Pipetting?

How “good” can within laboratory results be?

NEW!

Homogeneity Testing of SRM 2372

Finding out how good qPCR results can be.

Plate 1 set up

	1	2	3	4	5	6	7	8	9	10	11	12
A	A_1p	A_1p	C_1p	A_box_1	A_box_1	B_box_1	B_box_1	C_box_1	C_box_1	A_box_5	C_box_5	A_1p
B	A_2p	A_2p	C_2p	A_box_17	A_box_17	B_box_17	B_box_17	C_box_17	C_box_17	A_box_13	C_box_13	A_2p
C	A_3p	A_3p	C_3p	A_box_2	A_box_2	B_box_2	B_box_2	C_box_2	C_box_2	A_box_5	C_box_5	A_3p
D	A_4p	A_4p	C_4p	A_box_16	A_box_16	B_box_16	B_box_16	C_box_16	C_box_16	A_box_13	C_box_13	A_4p
E	B_1p	B_1p	C_1p	A_box_3	A_box_3	B_box_3	B_box_3	C_box_3	C_box_3	B_box_5	C_1p	B_1p
F	B_2p	B_2p	C_2p	A_box_15	A_box_15	B_box_15	B_box_15	C_box_15	C_box_15	B_box_13	C_2p	B_2p
G	B_3p	B_3p	C_3p	A_box_4	A_box_4	B_box_4	B_box_4	C_box_4	C_box_4	B_box_5	C_3p	B_3p
H	B_4p	B_4p	C_4p	A_box_14	A_box_14	B_box_14	B_box_14	C_box_14	C_box_14	B_box_13	C_4p	B_4p

Plate 2 set up

	1	2	3	4	5	6	7	8	9	10	11	12
A	A_1p	A_1p	C_1p	A_box_6	A_box_6	B_box_6	B_box_6	C_box_6	C_box_6	A_1p	A_1p	C_1p
B	A_2p	A_2p	C_2p	A_box_12	A_box_12	B_box_12	B_box_12	C_box_12	C_box_12	A_2p	A_2p	C_2p
C	A_3p	A_3p	C_3p	A_box_7	A_box_7	B_box_7	B_box_7	C_box_7	C_box_7	A_3p	A_3p	C_3p
D	A_4p	A_4p	C_4p	A_box_11	A_box_11	B_box_11	B_box_11	C_box_11	C_box_11	A_4p	A_4p	C_4p
E	B_1p	B_1p	C_1p	A_box_8	A_box_8	B_box_8	B_box_8	C_box_8	C_box_8	B_1p	B_1p	C_1p
F	B_2p	B_2p	C_2p	A_box_10	A_box_10	B_box_10	B_box_10	C_box_10	C_box_10	B_2p	B_2p	C_2p
G	B_3p	B_3p	C_3p	A_box_9	A_box_9	B_box_9	B_box_9	C_box_9	C_box_9	B_3p	B_3p	C_3p
H	B_4p	B_4p	C_4p	Blank	Blank	Blank	Blank	Blank	Blank	B_4p	B_4p	C_4p

revised

Reproducibility

$y = -1.34 \, Ln(x) + 29.125$
$R^2 = 0.996$
1:20
1:10
1:5
SRM 2372 dilutions
Neat
ct
[DNA]

Quantifiler Human Kit was used in conjunction with a 7900 instrument.

Results from two runs were combined to produce the following results.

n = 34	Ct	sd	%
A_boxes	26.95	**0.09**	0.3
B_boxes	27.02	**0.10**	0.4
C_boxes	26.90	**0.10**	0.4

Everything is under control!

1:10 dilutions of these materials were made prior to analysis

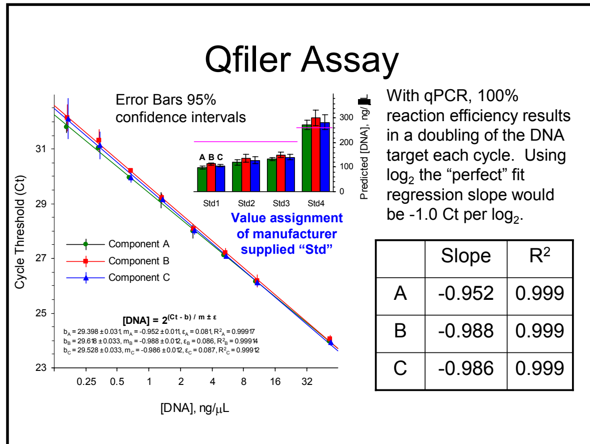
Assigning [DNA] to in-house calibration materials

	1	2	3	4	5	6	7	8	9	10	11	12
A		A_1	B_1	C_1	SP_1A	SP_1A	SP_1C	SP_1C	A_1	B_1	C_1	Blank
B		A_2	B_2	C_2	SP_2A	SP_2A	SP_2C	SP_2C	A_2	B_2	C_2	
C		A_3	B_3	C_3	SP_3A	SP_3A	SP_3C	SP_3C	A_3	B_3	C_3	
D		A_4	B_4	C_4	SP_4A	SP_4A	SP_4C	SP_4C	A_4	B_4	C_4	
E		A_5	B_5	C_5	SP_1B	SP_1B	SP_1D	SP_1D	A_5	B_5	C_5	
F		A_6	B_6	C_6	SP_2B	SP_2B	SP_2D	SP_2D	A_6	B_6	C_6	
G		A_7	B_7	C_7	SP_3B	SP_3B	SP_3D	SP_3D	A_7	B_7	C_7	
H		A_8	B_8	C_8	SP_4B	SP_4B	SP_4D	SP_4D	A_8	B_8	C_8	

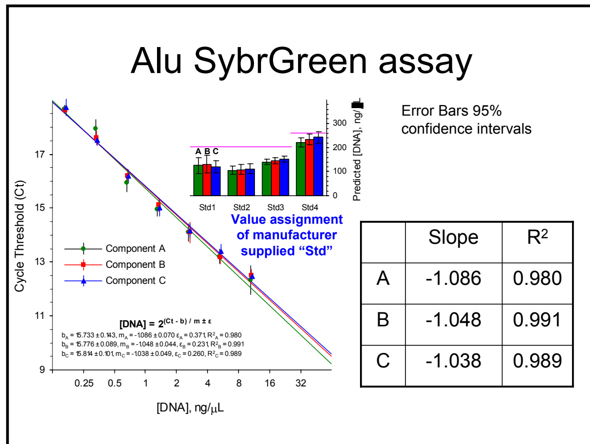
In-house materials designated SP_A, B, C, & D
4 dilutions of each in-house material were made and analyzed in duplicate

Three different standard materials were used to produce calibration curves eight dilutions of each applied in duplicate

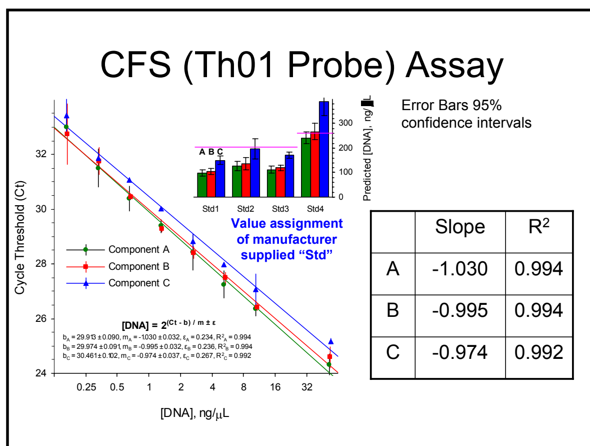
Results from three different qPCR methods are shown



	Slope	R^2
A	-0.952	0.999
B	-0.988	0.999
C	-0.986	0.999



	Slope	R^2
A	-1.086	0.980
B	-1.048	0.991
C	-1.038	0.989

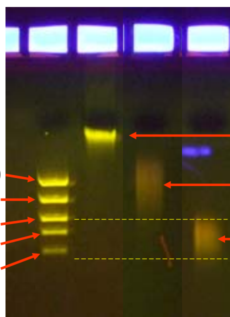


	Slope	R^2
A	-1.030	0.994
B	-0.995	0.994
C	-0.974	0.992

Degraded DNA Issue

- What is the difference in the qPCR results of **"good"** quality DNA verses **"degraded"** DNA?
- The Experiment:
  - Aliquot Control DNA into several different tubes
  - Hold one tube as a control.
  - Sonicate the remaining tubes for various lengths of time.
  - Quantify the samples and amplify based on quant values (Quantifiler – 62bp target).
  - Check the results!

Gel image of degraded DNA



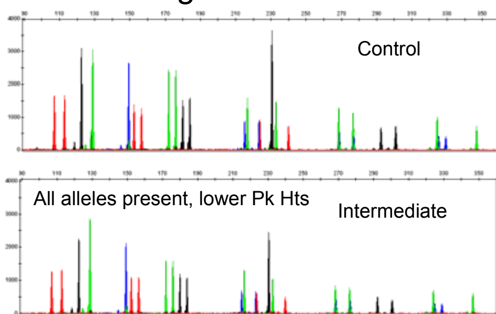
Control DNA was pipetted into several tubes. The tubes were sonicated until various levels of degradation was observed

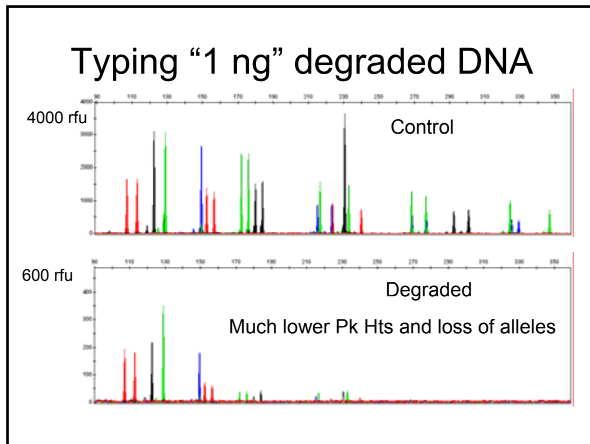
bp
1500
800
400
250
100

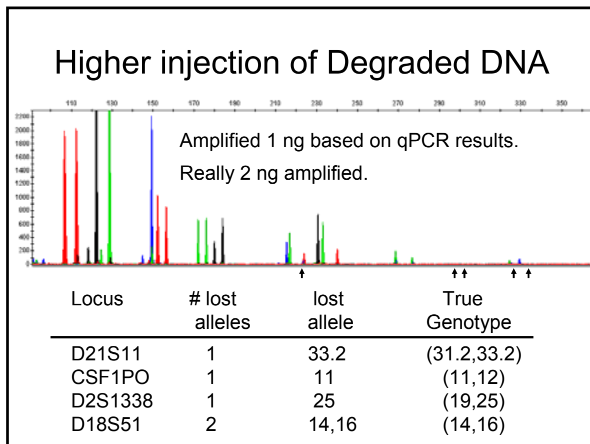
Control DNA
Intermediate Degradation
Degraded DNA

40 ng of DNA used for visualization, based on original [DNA].

"1 ng" Control / intermediate degraded DNA







Locus	# lost alleles	lost allele	True Genotype
D21S11	1	33.2	(31.2,33.2)
CSF1PO	1	11	(11,12)
D2S1338	1	25	(19,25)
D18S51	2	14,16	(14,16)

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 issues (mechanical, electronics)

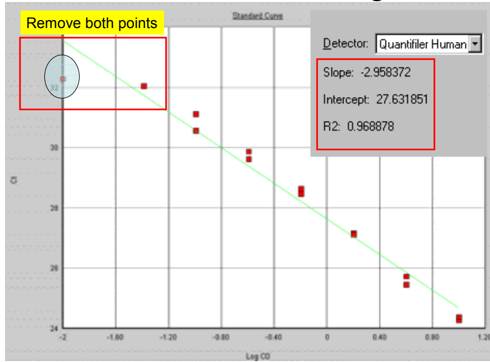
Troubleshooting

- 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

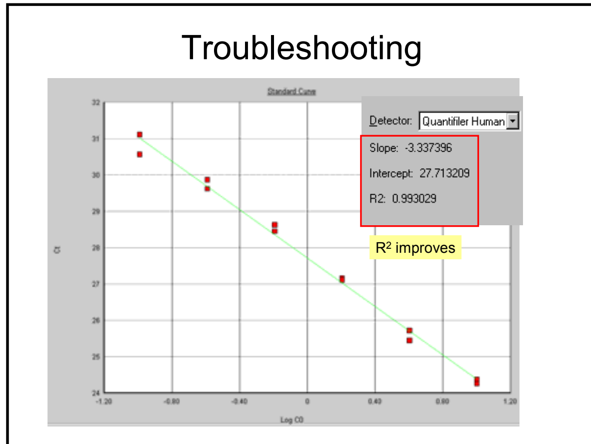
Troubleshooting

- $R^2 < 0.99$
- 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

Troubleshooting



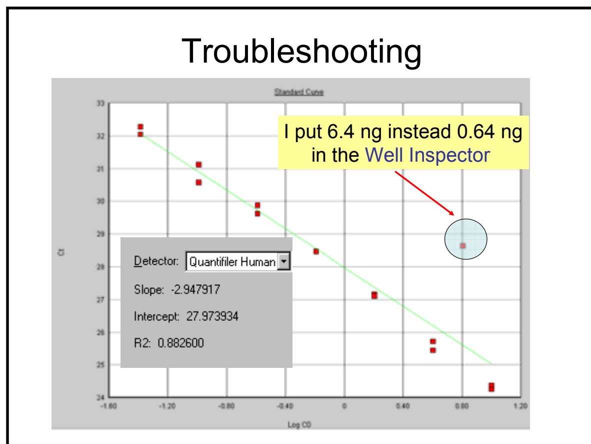
Troubleshooting



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)

Troubleshooting



Troubleshooting

- Make sure that the proper dye/detector is selected for the appropriate target
- When running multiple Calibrant curves on the same plate these will have to be analyzed separately

Take Home Lessons

- Check qPCR instrument calibration
- Check pipette and analyst reproducibility
- Know the limits of your qPCR assay
- Make sure you have a reliable Calibrant
- Degraded DNA does not perform like intact DNA in some qPCR assays
- Double check your analysis parameters before and after qPCR runs.

Thank you for your Attention!!

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