Nucleic Acids Research Group Presentation

ABRF 2005

February 6, 2005

4-5 PM

Chatham Ballroom B

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Agenda

2004/2005 Real-Time PCR Study
Scottie Adams

How to Interpret Your Data: A Platform Comparison
Brian Holloway

Open Discussion on Quality Control
Greg Shipley
Stephen Bustin

Nucleic Acids Research Group

- Gregory L. Shipley (Chair)
- Pamela “Scottie” Adams
- Yongde Bao
- Stephen A. Bustin
- Deborah S. Grove
- Brian P Holloway
- Anthony T Yeung
- Susan Hardin (Ad hoc)

- UT Health Science Ctr - Houston
- Trudeau Institute
- U. of Virginia Med School
- U. of London
- Penn State U
- CDC
- Fox Chase Cancer Center
- U. of Houston

Nucleic Acids Research Group 2005 Study

Validate your Real -Time PCR Technique

AND

A Comparison of Real-Time RT-PCR Technique, Chemistries and Hardware in Laboratories Utilizing the Same Assay

NARG 05 Study Goals

- Education/Self Evaluation
- Comparison of Platforms (Instruments)
- Comparison of Chemistries
- Are Researchers Analyzing Their Data Properly? (Setting Proper Baselines, Thresholds)
- What Are Researchers Using/Doing?

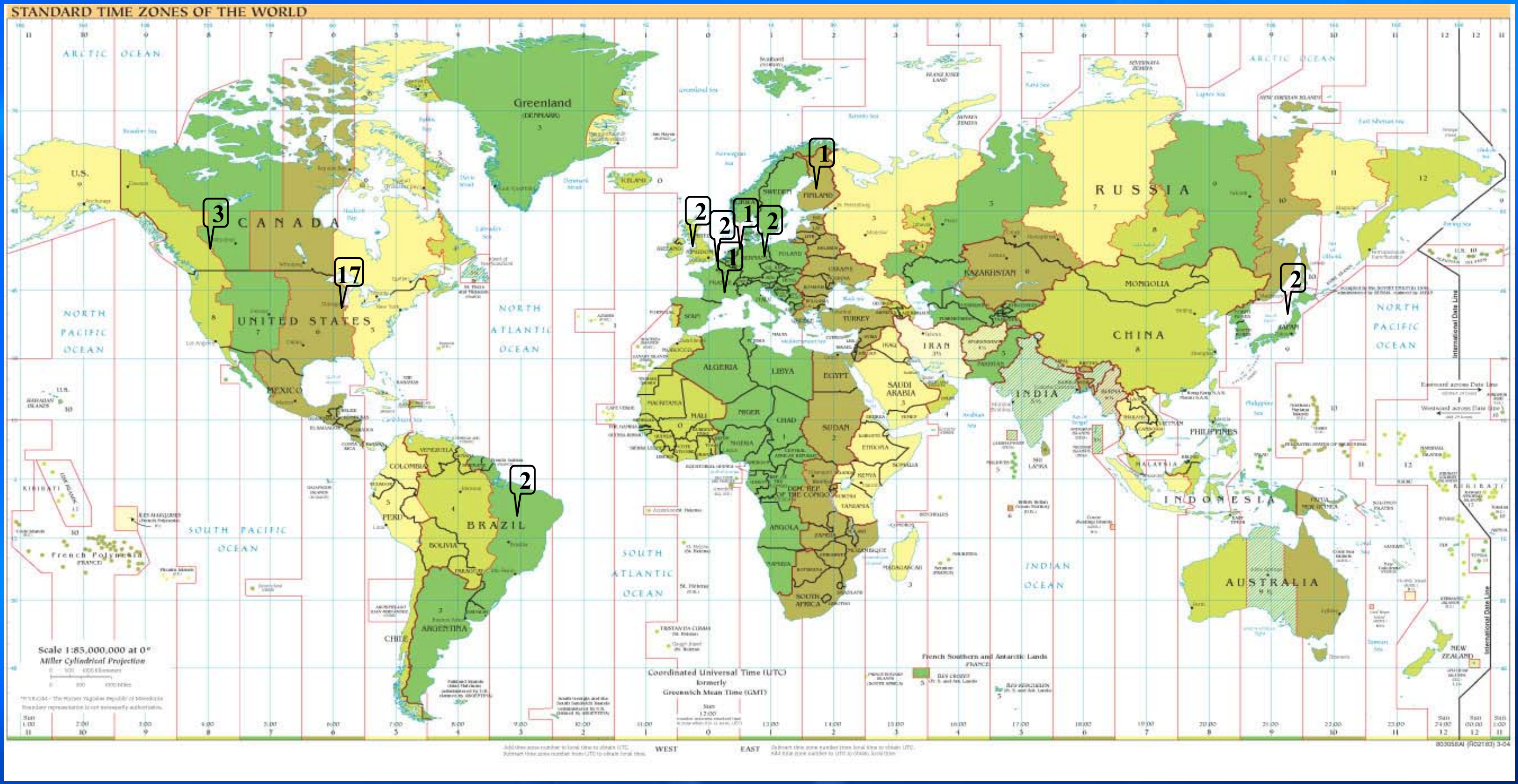
What Was Provided?

- 1 nmole Forward Primer: hβ-Actin-997(+) (CCCTGGCACCCAGCAC)
- 1 nmole Reverse Primer: hβ-Actin-1067(-) (GCCGATCCACACGGAGTAC)
- 0.4 nmoles Taqman® probe: hβ-Actin-1020(+) (FAM-ATCAAGATCATTGCTCCTCCTGAGCGC-BHQ1)
- 400 pg synthetic DNA oligo template for the hβ-Actin assay
- 400 pg *in vitro* transcribed RNA template for the hβ-Actin assay
- 500 µl 100 ng/µl yeast tRNA in nuclease free water as a diluent
- Examples of how to run the assays

Study Design

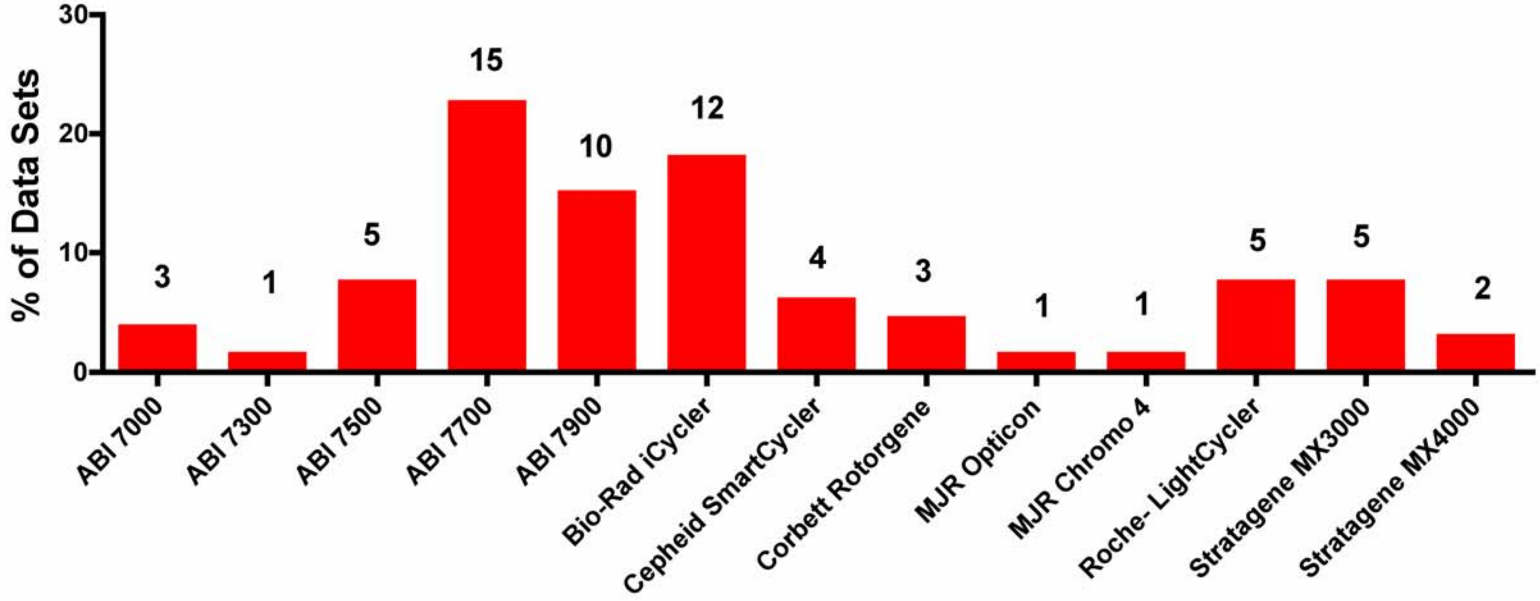
- Perform Reverse Transcription (RT) on BOTH the sDNA and the sRNA template using the reverse primer provided and RT method normally used, and
- Run a standard curve for each template over a 6-log range using Taqman® and/or SYBR Green I® chemistry and the instrument(s) and reagents used in their laboratory, and
- Answer questions on how they performed the experiment, report lowest Ct, slope, y- intercept and r^2 values, and
- Send jpg files of amplification curves, standard curves and raw data exports.

Participation by Country



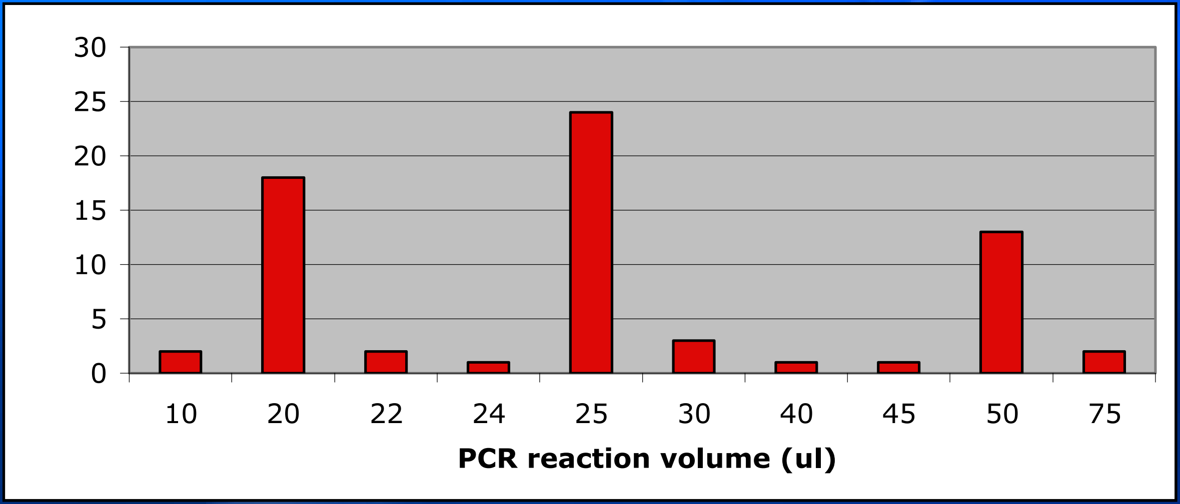
Requests	41
Particpants	33
Data Sets	67

Participation by Platform



How Were the Assays Run?

- Taqman® vs SYBR Green I® (71% vs 29%)
- One step vs Two step chemistry? (44% vs 56%)
- Robotics used? 4% yes
- ROX used? 70% yes
- UNG used? 18% yes
- PCR Volume?



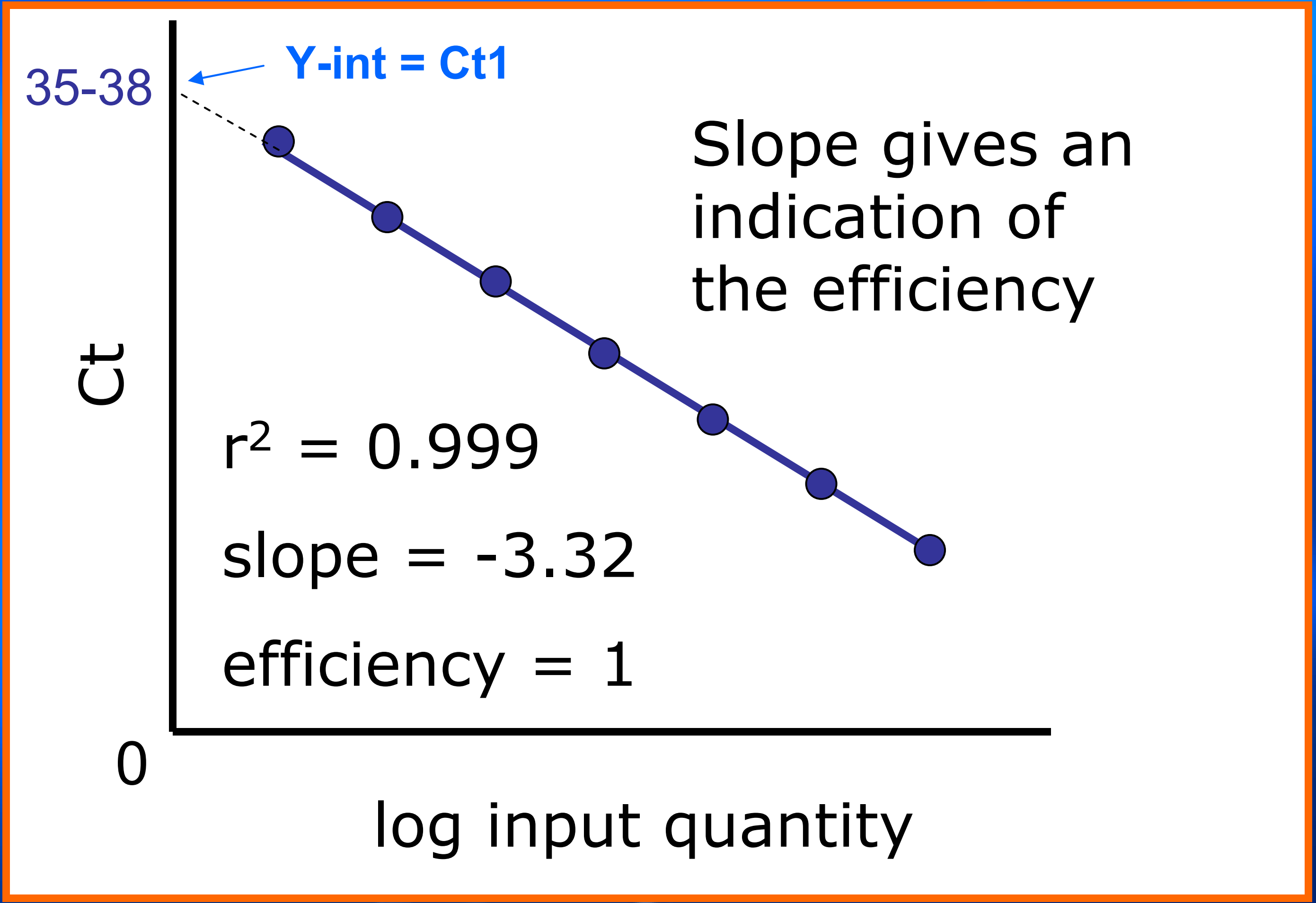
Analysis

- Sensitivity (Lowest or initial) C_t Value
- Efficiency of PCR for each template (slope)
- Reproducibility (r^2)
- Y-intercept = C_t1

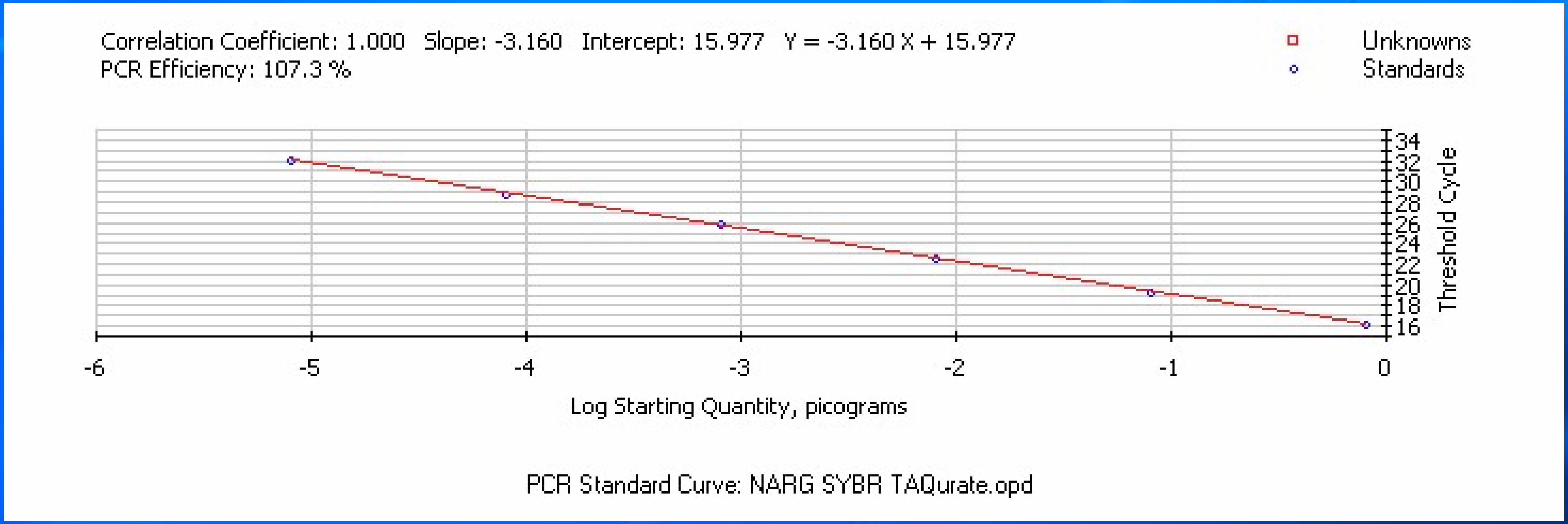
  Least squares method

  Y (C_t)= m(slope) X (molecules) + b (y- intercept)
- Δ DNA C_t1- RNA C_t1

Ideal Standard Curve



Y intercept ≠ C_t1



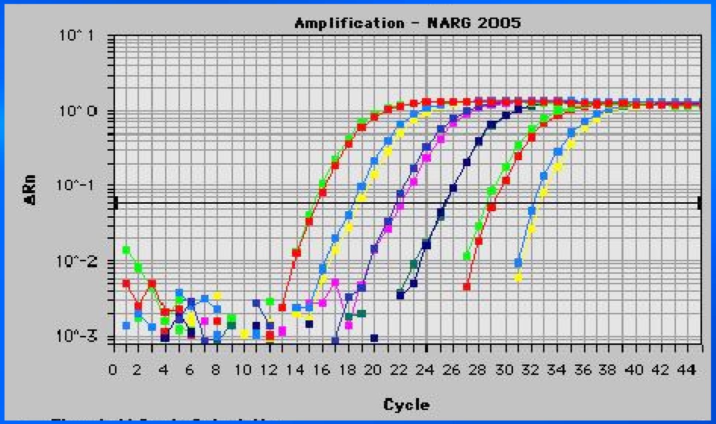
How to Convert Mass to Molecules

$$\frac{\text{Mass (in grams) x Avogadro's Number}}{\text{(average mol wt of a base x template length)}}$$

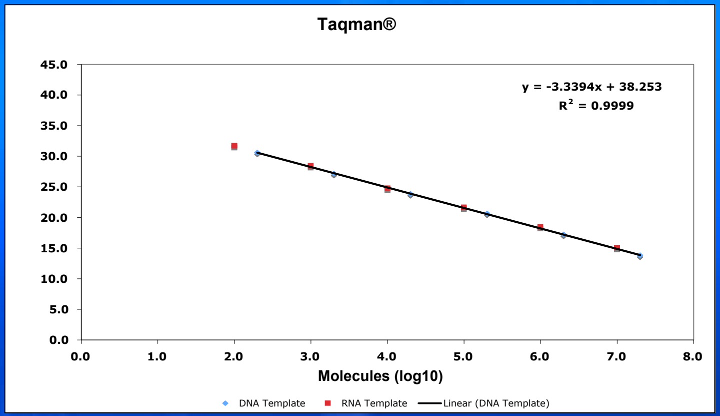
For sDNA

$$\frac{0.82 \times 10^{-12} \text{ gm} \times 6.023 \times 10^{23} \text{ molecules/mole}}{(330 \text{ gm/mole/base} \times 75 \text{ bases})} = 2.0 \times 10^{7}$$

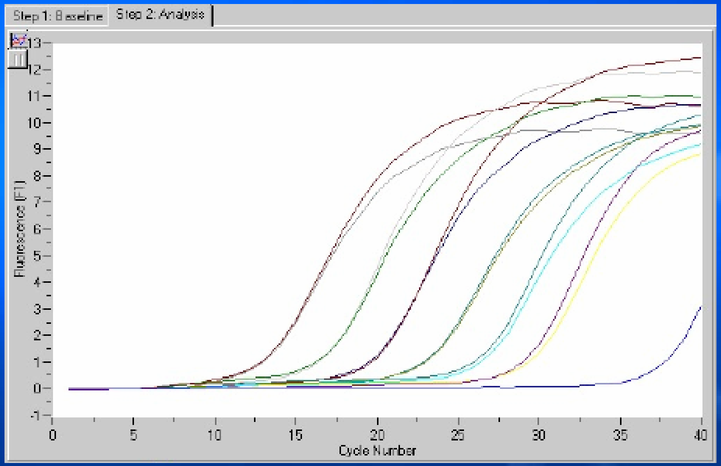
Good Assays



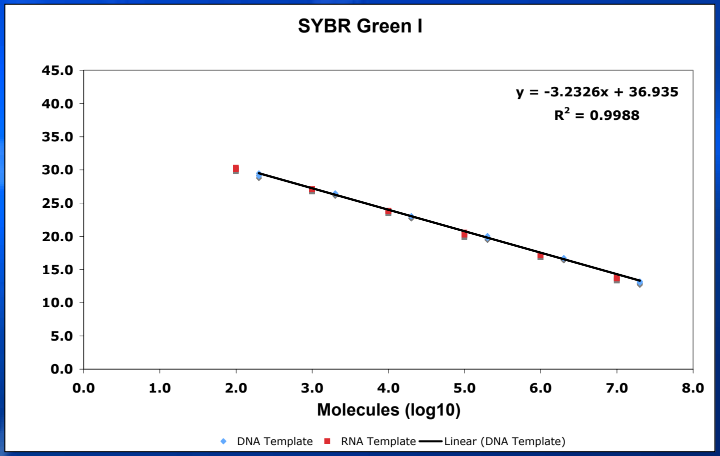
Amplification - NARG 2005
ΔRn
Cycle



Taqman®
y = -3.3394x + 38.253
R^2 = 0.9999
Molecules (log10)
DNA Template
RNA Template
Linear (DNA Template)

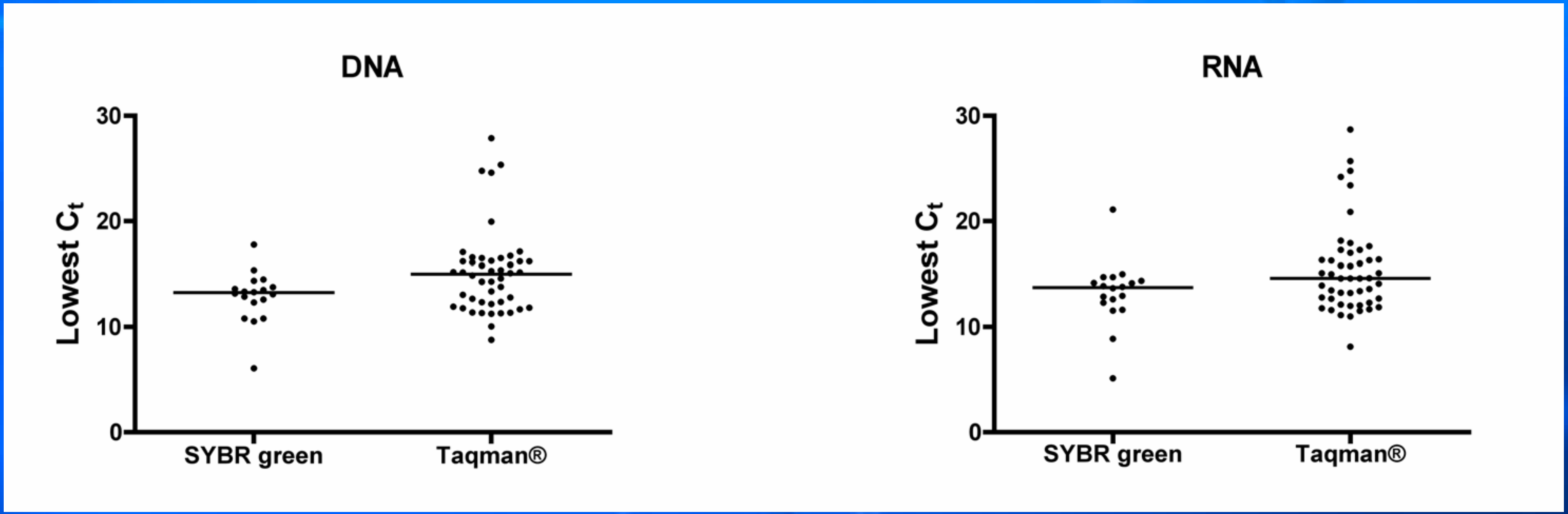


Step 1: Baseline
Step 2: Analysis
Fluorescence (F1)
Cycle Number



SYBR Green I
y = -3.2326x + 36.935
R^2 = 0.9988
Molecules (log10)
DNA Template
RNA Template
Linear (DNA Template)

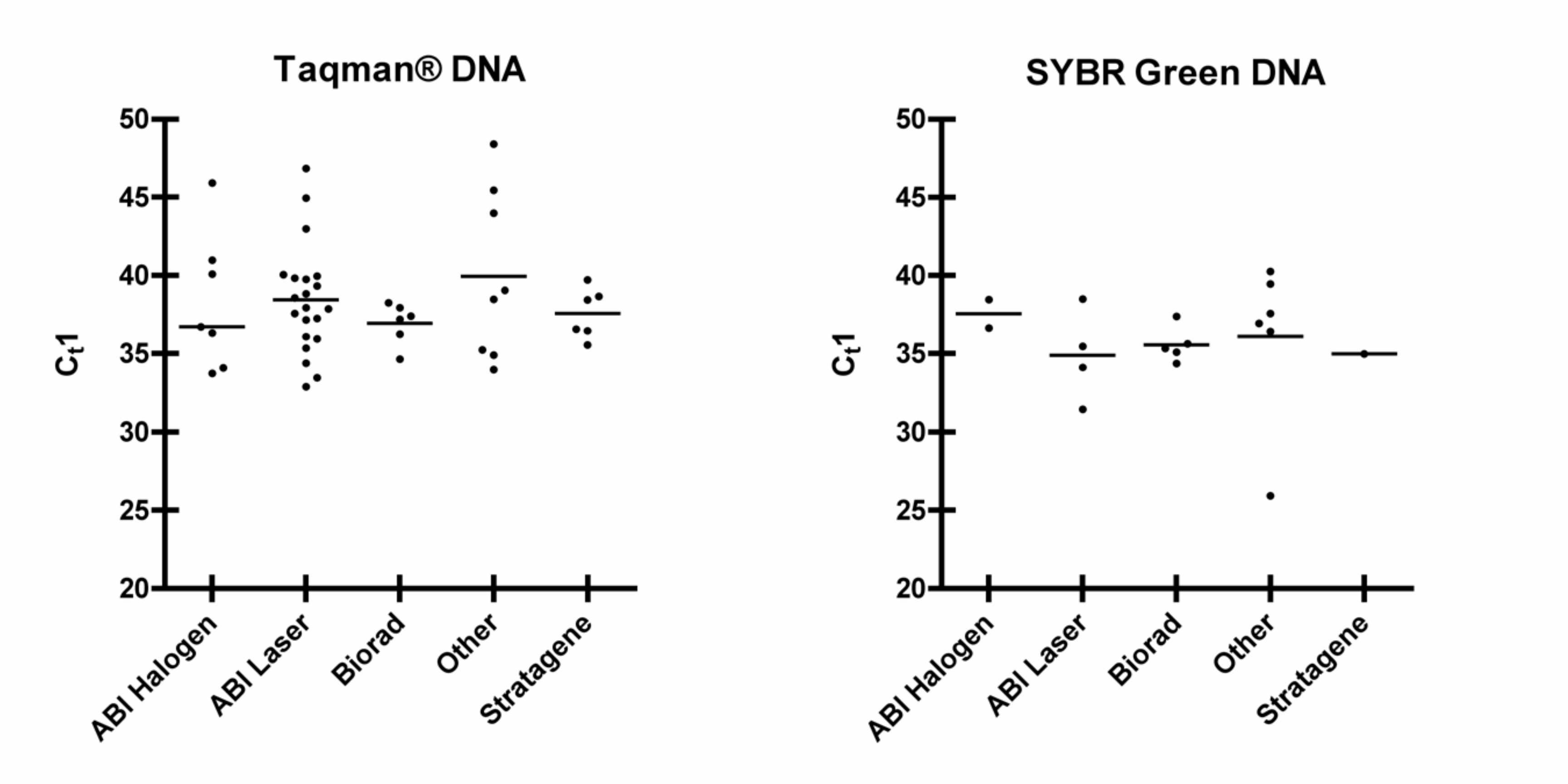
Assay Type Comparison by Lowest Ct



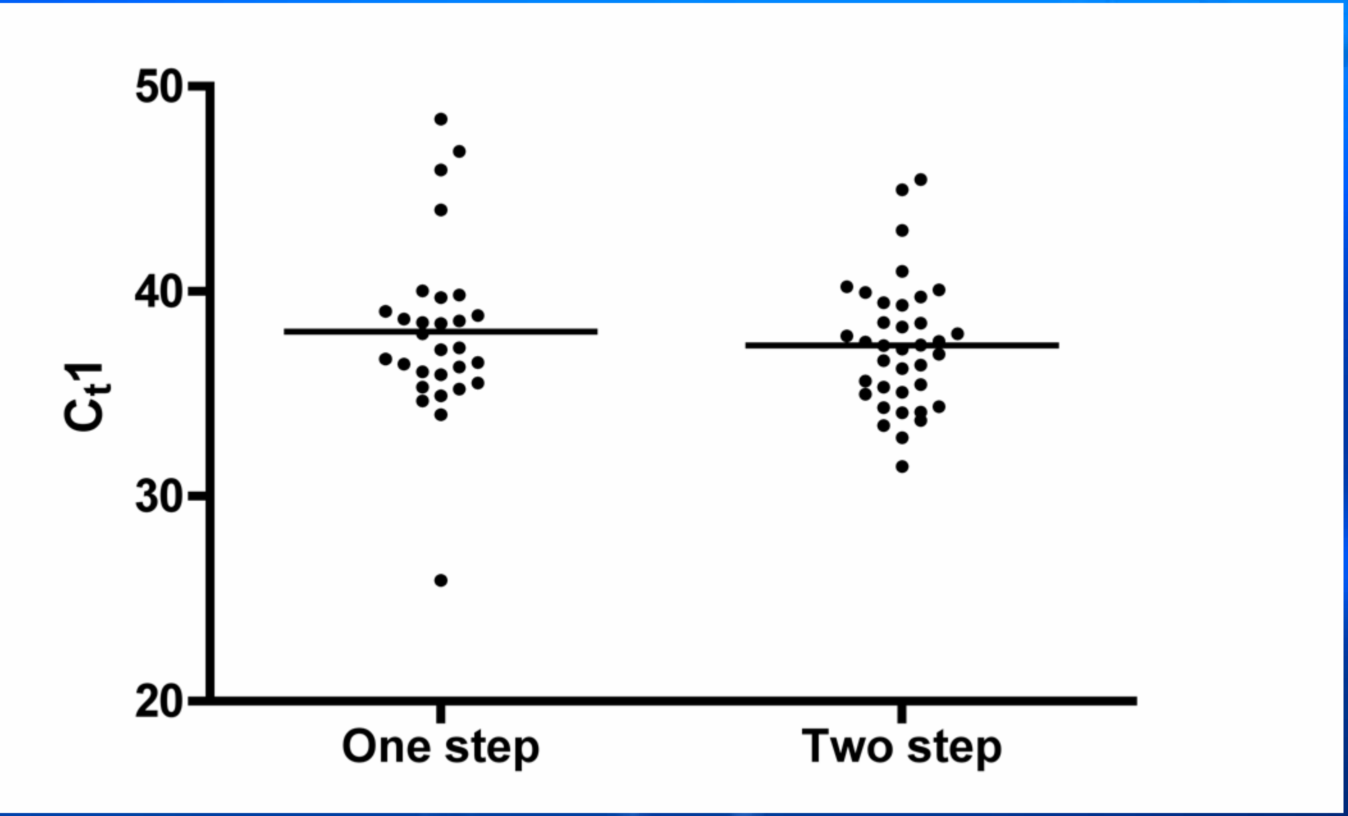
Mean Lowest Ct SYBR Green I® = 12.89
Mean Lowest Ct Taqman® = 15.11

Mean Lowest Ct SYBR Green I® = 13.21
Mean Lowest Ct Taqman® = 15.42

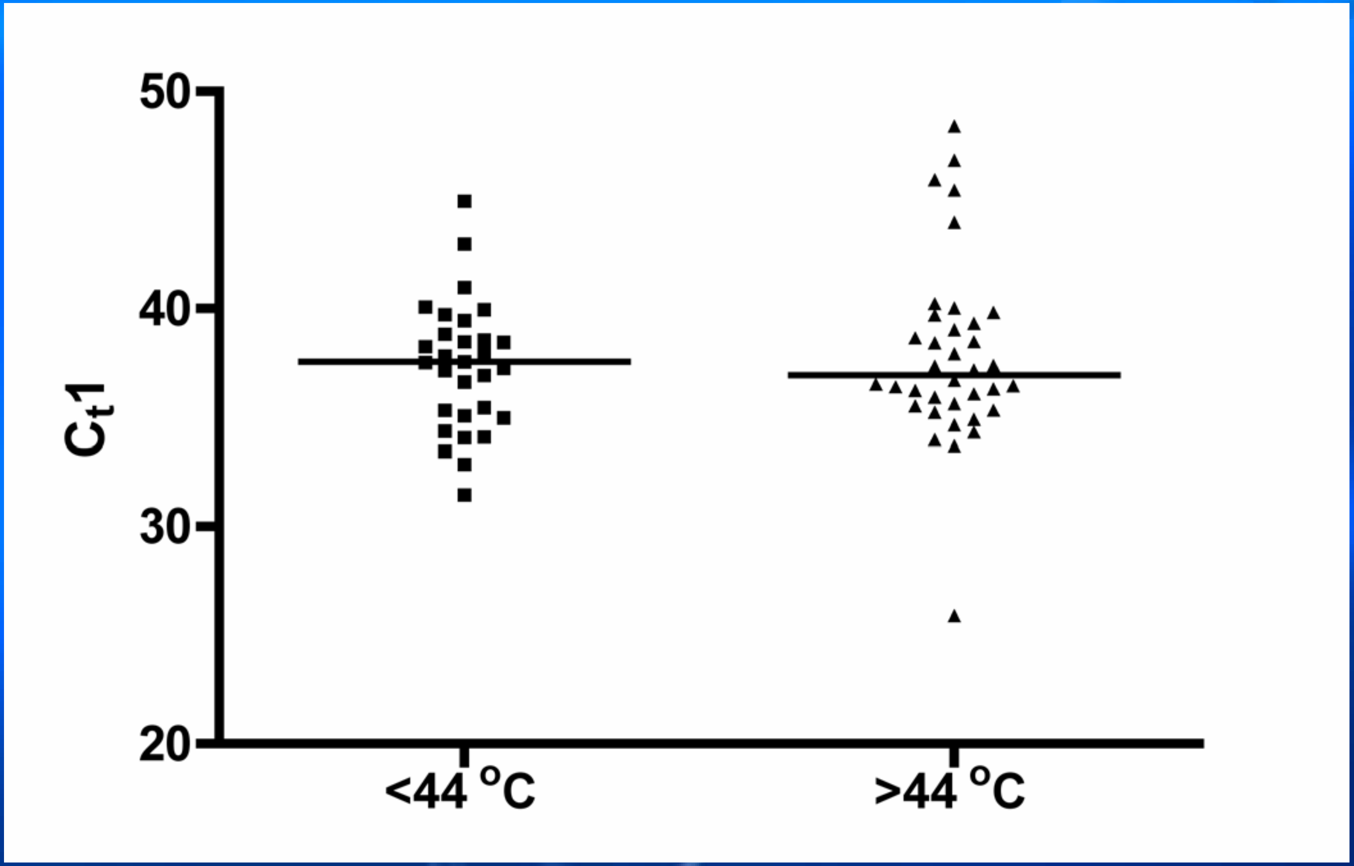
Comparison by Instrument



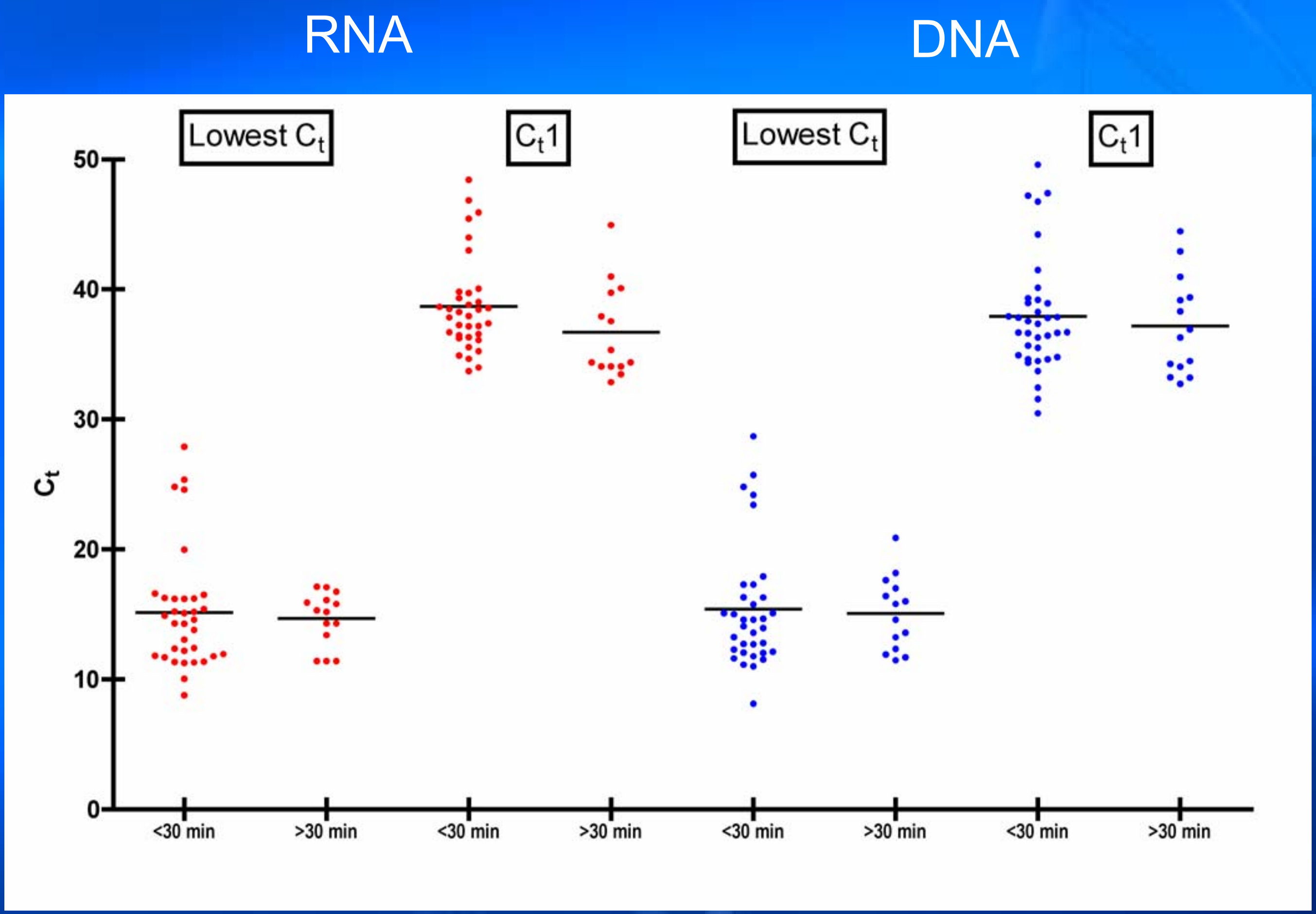
One-step vs Two-Step Assay Chemistry



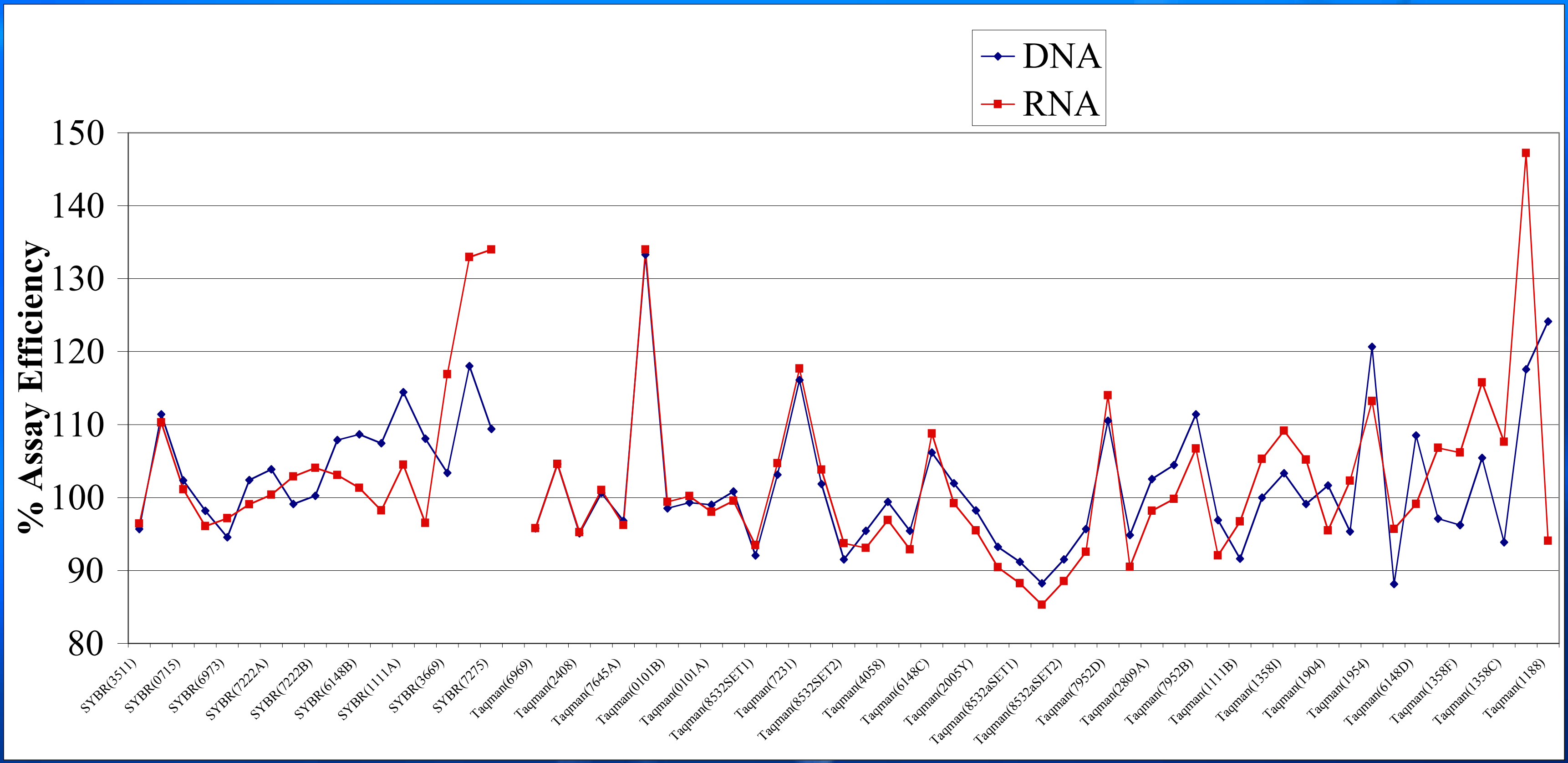
Effect of RT Temperature on C_t1



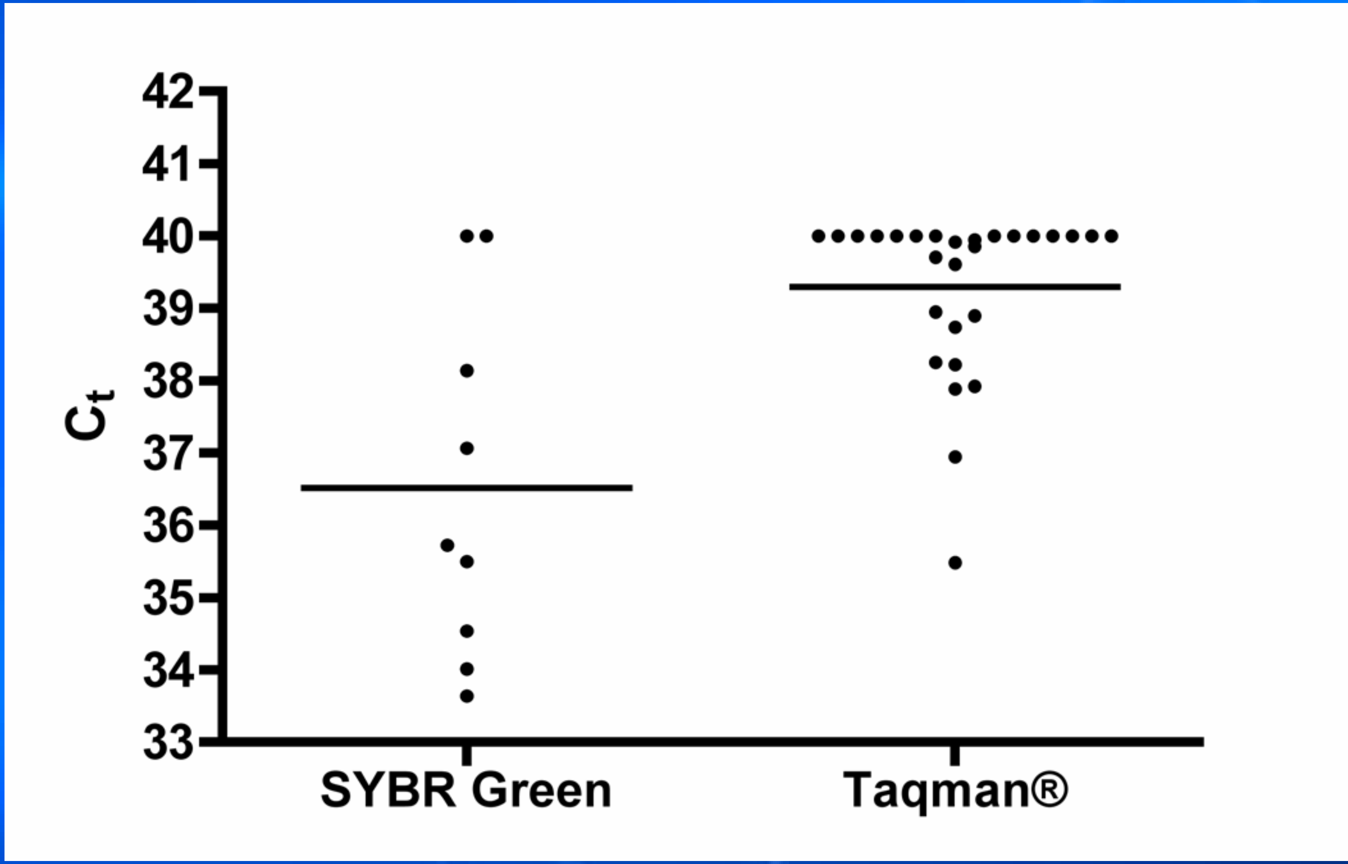
Effect of RT Time



Comparison of DNA vs RNA Efficiency

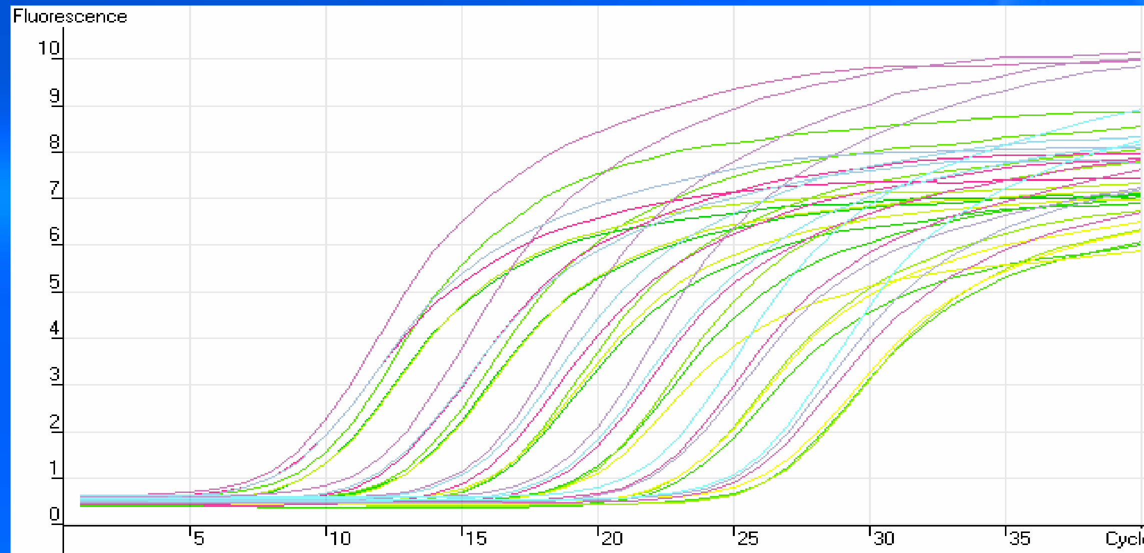


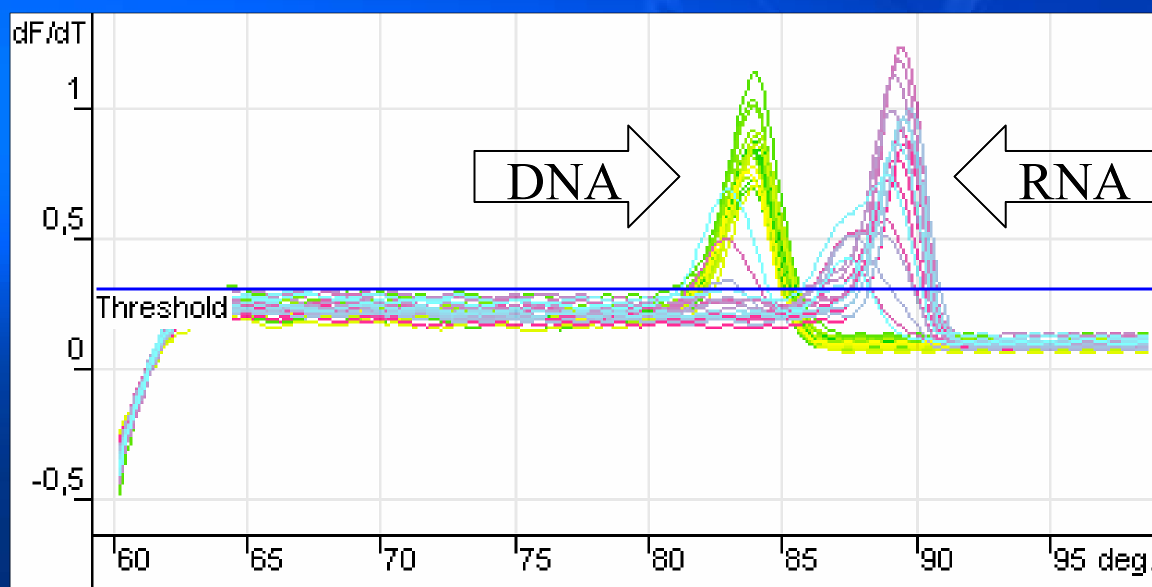
No Template Control



Not Done	>40	<40	Total
30	16	21	67
45%	24%	31%	100%

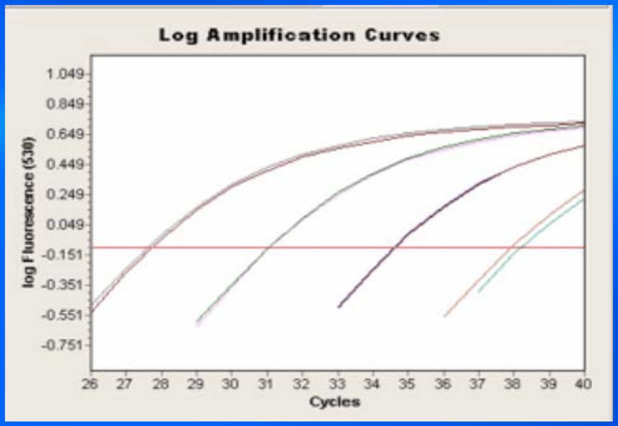
SYBR Green I® Melt Curve



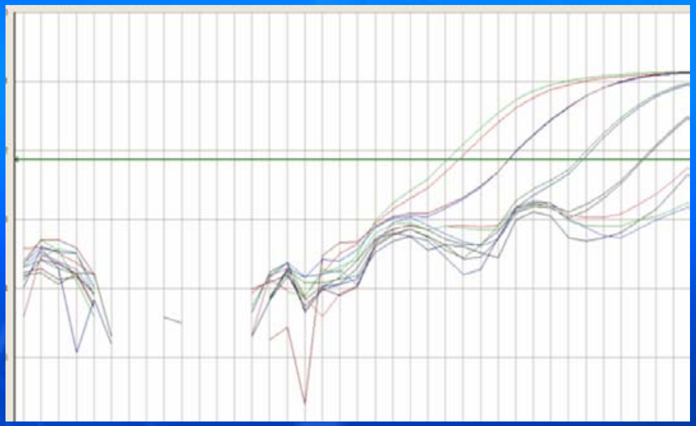


Extended Dilutions

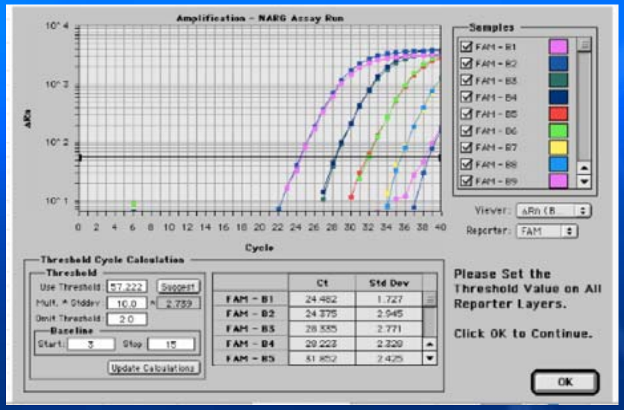
LightCycler $C_t1 = 48.42$



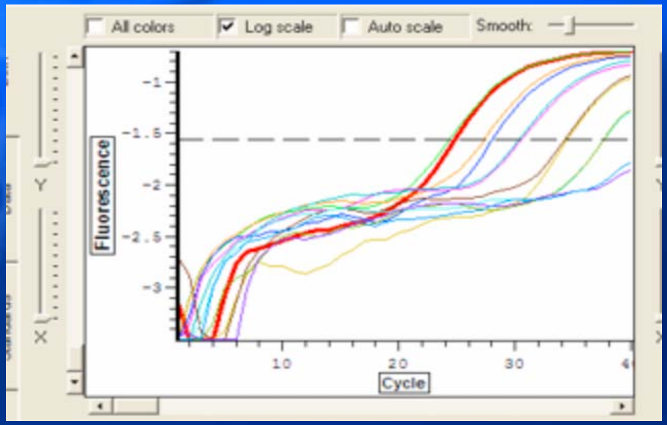
ABI 7500 $C_t1 = 45.93$



ABI 7700 $C_t1 = 46.85$



MJR Opticon $C_t1 = 43.99$



Report Card - www.abrf.org

DNA efficiency
RNA efficiency

DNA C_t1
RNA C_t1

DNA r^2
RNA r^2

ID	INSTRUMENT	ASSAY_TYPE	STEPS	Effic_DNA	Effic_RNA	DNA Y-int ca	RNA Y-int ca	R2 DNA ca	R2 RNA ca	Composite Sco
0000B	Bio-Rad iCycler	TaqmanØ	Two Step	99.3	100.2	38.25	38.27	0.9997	0.9989	95.61
0101A	ABI 7700	TaqmanØ	One Step	99.0	98.0	37.94	37.83	0.9997	0.9997	94.69
3413	ABI 7000	TaqmanØ	One Step	100.6	101.0	36.32	34.79	0.9999	0.9999	92.96
7645A	ABI 7700	TaqmanØ	One Step	96.8	96.2	40.05	40.14	0.9995	0.9999	92.96
6969	ABI 7700	TaqmanØ	Two Step	95.8	95.8	39.33	39.33	0.9992	0.9992	91.85
4058	ABI 7900	TaqmanØ	Two Step	99.4	96.9	35.34	36.31	1	0.9997	91.16
6932T	ABI 7900	TaqmanØ	Two Step	102.0	99.2	39.74	39.38	0.9998	1	90.33
0715	ABI 7500	SYBR green I	Two Step	102.3	101.1	38.45	37.82	0.9977	0.9995	89.88
7645B	Corbett RotorGene	TaqmanØ	One Step	100.8	99.5	39.04	37.81	0.9993	0.9984	89.7
0101B	ABI 7900	TaqmanØ	One Step	98.5	99.4	39.83	37.93	0.9996	0.9997	89.37
3611	ABI 7500	TaqmanØ	Two Step	95.5	92.9	40.98	40.97	0.9998	0.9998	88.36
1358H	Stratagene Mx4000	TaqmanØ	One Step	101.9	103.8	36.46	35.68	0.9985	0.9969	88.18
1358m	Stratagene MX3000	TaqmanØ	One Step	104.6	104.6	38.66	37.55	1	0.9999	87.79
7222B	Cepheid SmartCycler	SYBR green I	Two Step	100.2	104.0	37.57	36.62	0.9982	0.9992	87.1
3511	Corbett RotorGene	SYBR green I	One Step	95.7	96.4	25.92	24.96	0.9998	0.9993	86.84
1358G	Stratagene Mx3000	TaqmanØ	One Step	103.1	104.7	35.55	34.62	0.9995	0.9968	86.75
7222A	Roche Light Cycler	SYBR green I	Two Step	103.8	100.4	36.94	36.96	0.9988	0.9996	86.24
1358B	Bio-Rad iCycler	TaqmanØ	One Step	100.0	105.3	34.65	33.71	0.9988	0.9989	86.24
2408	ABI 7900	TaqmanØ	Two Step	95.1	95.2	37.55	38.31	0.9976	0.9993	86.12
2005Y	ABI 7900	TaqmanØ	Two Step	98.2	95.5	37.85	36.68	0.999	0.9981	86.03
3011	Roche Light Cycler	SYBR green I	Two Step	98.2	96.1	39.46	37.81	0.996	0.9995	85.97
1904	ABI 7700	TaqmanØ	Two Step	101.6	95.5	39.954	40.29	0.9886	0.9997	85.34
2809A	MJR Chromo4	TaqmanØ	Two Step	102.5	98.2	43.99	44.22	0.9955	0.9985	85.13
0000A	Bio-Rad iCycler	SYBR green I	Two Step	99.1	102.9	35.09	36.66	0.993	0.9997	84.36
8532SET1	ABI 7700	TaqmanØ	One Step	92.1	93.5	38.57	37.87	0.9998	0.9998	83.94
3303A	ABI 7700	TaqmanØ	Two Step	96.9	92.1	33.46	34.05	0.9986	0.9987	83.46
1358	Stratagene MX3000	TaqmanØ	One Step	104.4	99.8	39.71	38.95	0.9999	0.9999	82.99
2809C	Roche Light Cycler	TaqmanØ	One Step	95.4	93.1	48.42	49.6	0.9996	0.9993	82.72
2005Z	ABI 7900	SYBR green I	Two Step	102.4	99.0	35.46	33.76	0.9985	0.9993	82.6
5801	ABI 7000	TaqmanØ	Two Step	95.7	92.6	40.1	39.17	0.999	0.9995	82.57
6148C	Bio-Rad iCycler	TaqmanØ	Two Step	106.1	108.7	37.39	36.65	1	0.9932	82.03
0358	Stratagene MX3000	TaqmanØ	One Step	99.1	105.2	38.44	36.45	0.9989	0.9991	82
8532aSET2	ABI 7700	TaqmanØ	One Step	91.5	88.5	37.25	37.35	0.9998	0.9985	80.81
1358F	Roche LightCycler II	TaqmanØ	One Step	96.2	106.1	33.98	34.49	0.9987	0.9996	80.81
8532aSET1	ABI 7700	TaqmanØ	One Step	91.1	88.2	38.83	38.93	0.9998	0.9985	80.69
2005B	ABI 7900	SYBR green I	Two Step	111.4	110.3	34.11	34.43	0.9966	0.9962	80.54
8532SET2	ABI 7700	TaqmanØ	One Step	91.5	93.7	37.16	36.3	0.9997	0.9997	80.54
1358I	ABI 7700	TaqmanØ	One Step	103.3	109.1	35.93	34.63	0.9993	0.9994	80.27
6973	MJR Opticon 2	SYBR green I	Two Step	94.5	97.2	36.42	34.88	0.9993	0.9992	79.7
2005A	ABI 7900	TaqmanØ	Two Step	93.2	90.5	42.99	41.48	0.9997	0.9996	79.13
2809B	ABI 7500	TaqmanØ	One Step	94.8	90.5	45.93	46.75	0.9972	0.9973	78.96
6932S	ABI 7900	SYBR green I	Two Step	107.4	98.2	38.49	38.62	0.9995	0.9999	78.75
1644	Bio-Rad iCycler	TaqmanØ	Two Step	133.3	134.0	36.24	36.63	0.9926	0.9882	78.51
1358A	ABI 7500	TaqmanØ	One Step	97.1	106.8	36.7	34.37	0.9987	0.9995	77.73
3303B	ABI 7700	SYBR green I	Two Step	107.9	103.1	31.47	32.22	0.9988	0.9989	77.58
2809D	ABI 7700	TaqmanØ	One Step	88.2	85.3	46.85	47.41	0.9973	0.9995	77.34
7231	ABI 7700	TaqmanØ	One Step	116.1	117.7	36.09	34.93	0.9983	0.9973	75.94
7952D	ABI 7300	TaqmanØ	Two Step	110.5	114.0	34.08	33.24	0.9984	0.9963	75.37
1358E	Roche- LightCycler I	TaqmanØ	One Step	95.3	102.3	35.24	31.55	0.9999	0.9984	74.69
23932	ABI 7900	TaqmanØ	Two Step	173.0	179.0	32.86	32.74	0.9997	0.9991	73.73
3669	Bio-Rad iCycler	SYBR green I	One Step	103.4	116.9	35.33	32.65	0.9761	0.9943	73.46
1954	Stratagene MX4000	TaqmanØ	One Step	120.7	113.2	36.55	36.7	0.9983	0.9993	72.84
6148B	Bio-Rad iCycler	SYBR green I	Two Step	108.6	101.3	34.35	35.75	0.9991	0.9993	72.33
7456	ABI 7700	TaqmanØ	Two Step	88.1	95.7	44.95	44.47	0.9967	0.9949	72.24
6148D	Bio-Rad iCycler	TaqmanØ	Two Step	108.5	99.1	37.18	39.2	0.9997	0.9998	72.06
1111B	Cepheid SmartCycler	TaqmanØ	Two Step	91.6	96.7	45.45	47.21	0.9922	0.9849	71.82
6148A	Bio-Rad iCycler	SYBR green I	Two Step	108.0	96.5	37.36	36.1	0.9998	0.991	71.76
1358D	Corbett Rotorgene	TaqmanØ	One Step	105.4	115.8	34.9	32.44	0.993	0.9954	71.22
7952B	ABI 7700	TaqmanØ	Two Step	111.4	106.7	34.38	36.92	0.9984	0.9822	69.97
1358C	Cepheid SmartCycler	TaqmanØ	One Step	93.9	107.6	38.48	35.51	0.9939	0.9924	69.52
1644	Bio-Rad iCycler	SYBR green I	Two Step	118.0	132.9	35.64	35.06	0.9891	0.9664	68.51
0441	ABI 7000	SYBR green I	Two Step	105.5		36.64		0.9984		68.15
7275	Stratagene MX3000	SYBR green I	Two Step	109.4	134.0	34.99	31.11	0.9986	0.9943	66.18
1111A	Cepheid SmartCycler	SYBR green I	Two Step	114.5	104.5	40.24	43.82	0.965	0.9797	65.04
2625	ABI 7500	TaqmanØ	Two Step	117.6	147.2	33.72	30.47	0.9894	0.9977	64.06
1188	Bio-Rad iCycler	TaqmanØ	Two Step	124.1	94.1	37.93	42.93	0.9966	0.6273	62.36
1188	Bio-Rad iCycler	SYBR green I	Two Step		123.4		36.53		0.313	60.75

Conclusions

Excellent results using sDNA and/or sRNA can be obtained using

- Both Taqman® and SYBR green I® assays
- All instrumentation, past and current
- One-step or Two-step assays
- Using these templates is an excellent way to assess your technique

"It's not what you've got - it's what you do with it"

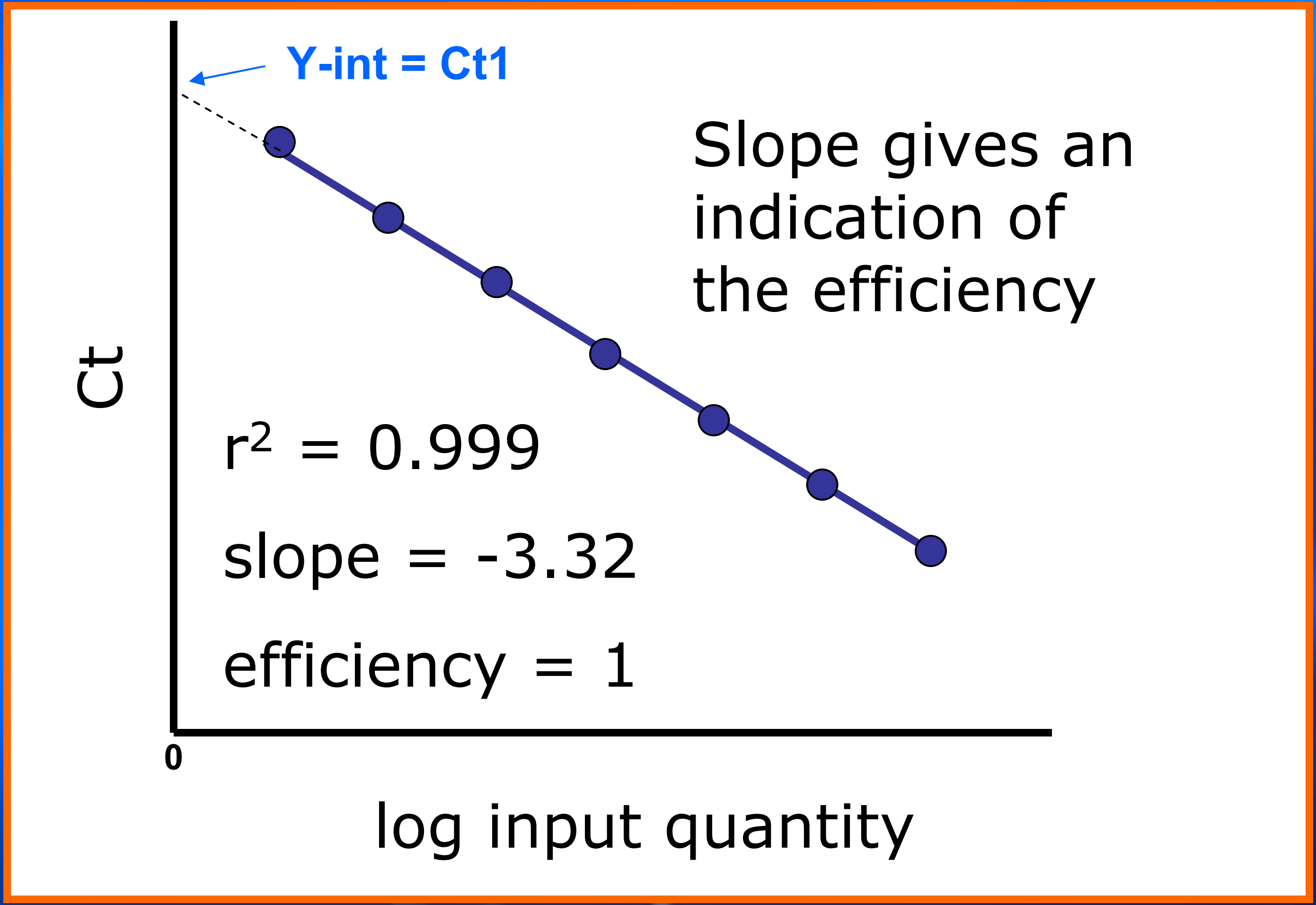
Agenda

2004/2005 Real-Time PCR Study
Scottie Adams

How to Interpret Your Data: A Platform Comparison
Brian Holloway

Open Discussion on Quality Control
Greg Shipley
Stephen Bustin

Standard Curve

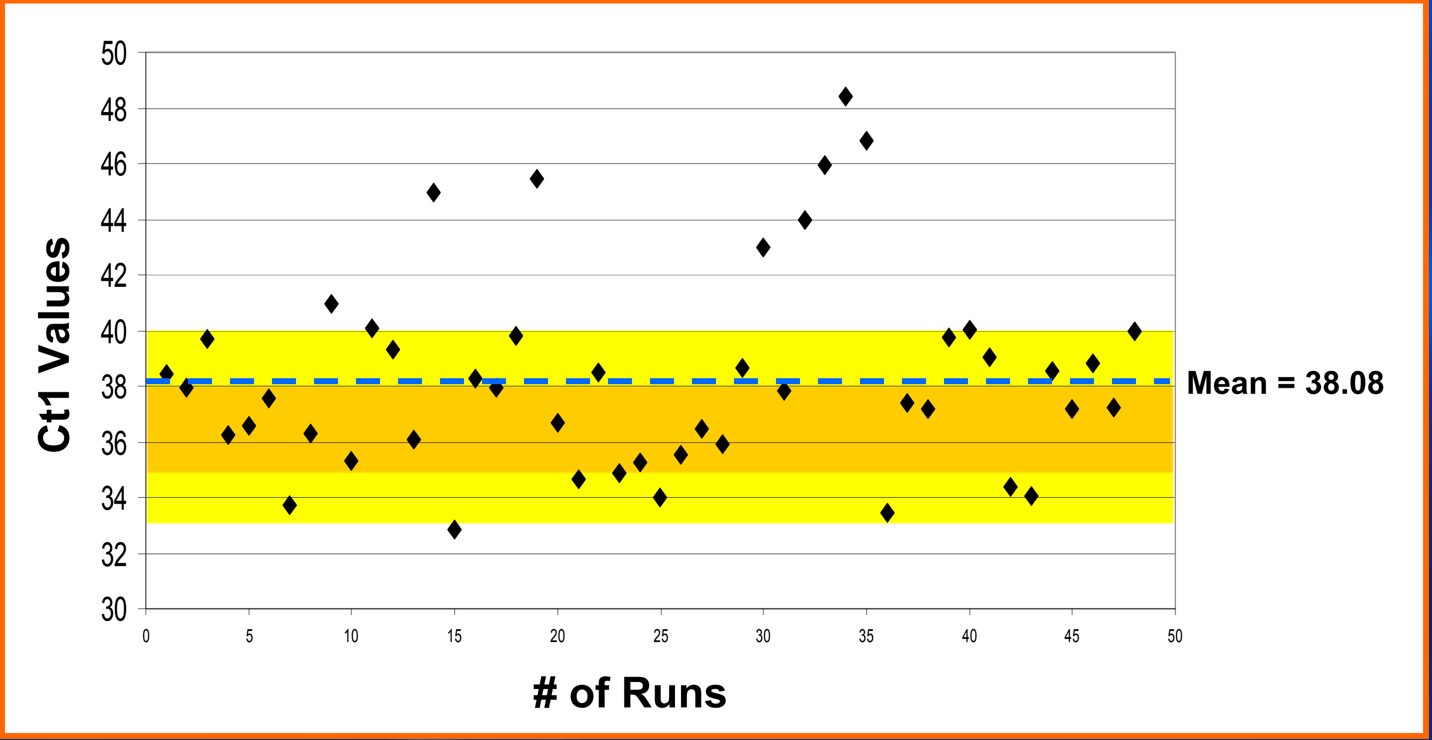


Theoretical Perfect Ct1 Value

- Signal detection limit of free FAM is $10^{10} – 10^{11}$ molecules on most platforms
- At 100% PCR efficiency, a single copy of template should therefore be detected between 35 – 38 cycles

NARG 2005 Study
Ct1 Values for DNA TaqMan Assays

PCR Efficiency	Theoretical Perfect Ct1 Values	# of Assays (n=48)	% of Overall
100% ± 0%	35 < Ct1 < 38	18	37.5%
100% ± 5%	33 < Ct1 < 40	37	77.1%



Factors which can Affect the Theoretical Ideal Ct1 Value

- Quality of Template
- Reagents
  - Master mix
  - Enzyme
  - Primers and Probe
- Operator Error
  - Pipetting errors
  - Inaccurate calculation of standards
- Platform
  - Thermocycler precision
  - Analysis settings

Platforms used for Testing at CDC

Real-Time Instruments

- ABI 7500
- ABI 7700
- BioRad Icycler
- Cepheid Smartcycler
- Corbett Rotor-Gene
- Roche Lightcycler 1.2
- Roche Lightcycler 2.0
- Stratagene Mx3000p
- Stratagene Mx4000

ABI 7500

ABI PRISM 7700

BioRad icycler

Cepheid Smartcycler

Corbett Rotor-Gene

Roche Lightcycler 1.2

Roche Lightcycler 2.0

Stratagene Mx3000p

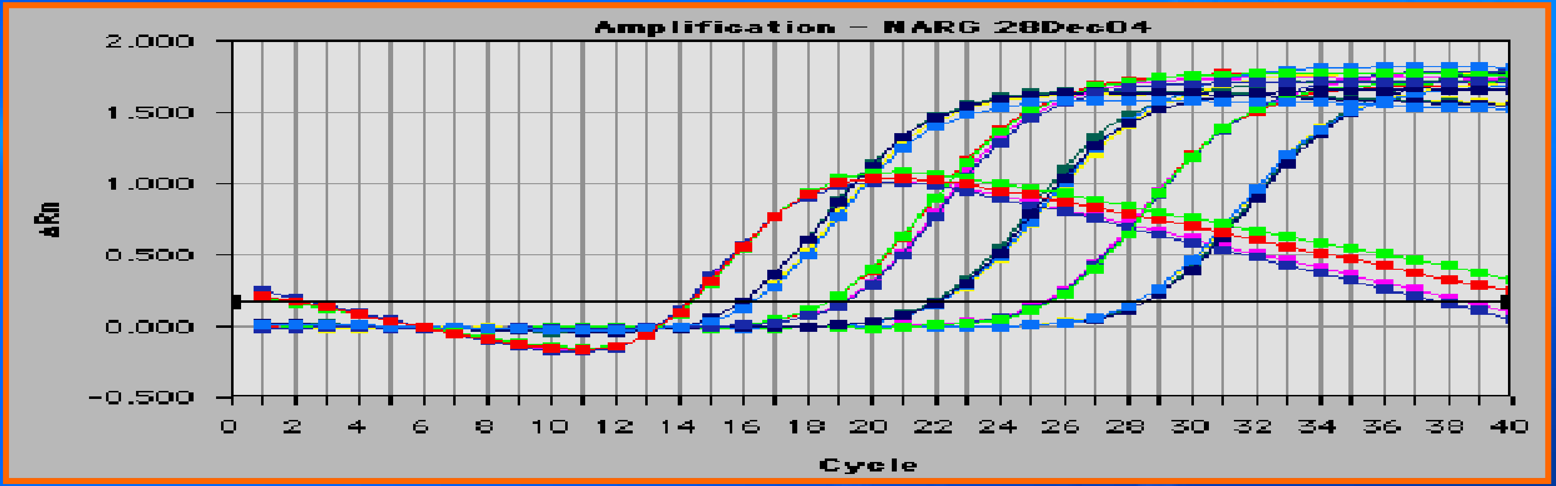
Stratagene Mx4000

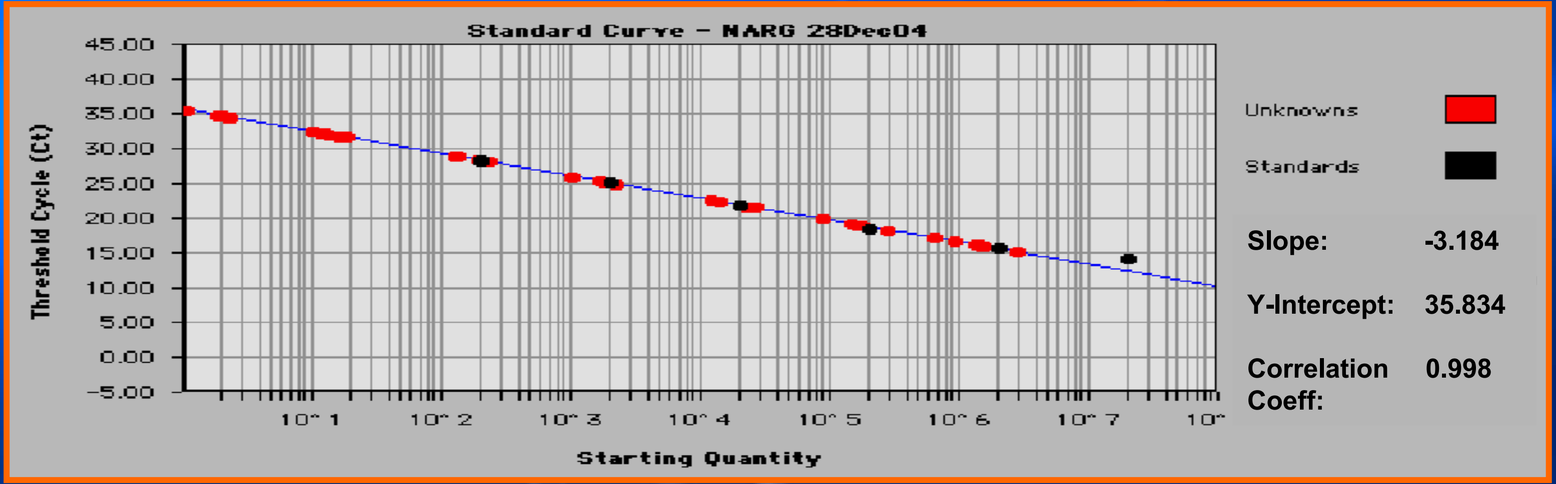
Ct1, Slope, and r^2 values for 9 Platforms using the same Reagent Kit and “Default" Analysis Parameters

Instrument	# copies	avg Ct	Slope	r^2	Y-int	Analysis Settings
ABI 7500	1.91E+02	26.88	-2.80	0.982	33.26	manual baseline 3-15, manual set for the threshold
ABI 7700	2.34E+02	28.29	-3.18	0.997	35.83	baseline default 3-15, threshold 10x SD
BioRad iCycler	2.00E+02	27.13	-3.33	0.999	34.79	auto set baseline & threshold, baseline subtracted curve fit
Cepheid smartcycler I	3.41E+02	29.78	-3.48	0.994	38.48	background subtraction
Corbitt Rotorgene	3.41E+02	27.96	-3.31	1.000	36.33	dynamic tube normalization, noise slope correction (amp based similar to Mx)
Roche LC 1.2	3.14E+02	28.15	-3.46	0.999	36.79	second derivative maximum
Roche LC 2	3.27E+02	26.15	-3.34	0.998	34.52	second derivative maximum
Stratagene Mx3000p	2.35E+02	29.99	-3.27	1.000	37.75	amplification based threshold, adaptive baseline, moving average of 3pts
Stratagene Mx4000	1.74E+02	29.52	-3.29	0.998	36.87	amplification based threshold, adaptive baseline, moving average of 3pts
Mean	**2.65E+02**	**28.21**	**-3.27**	**0.997**	**36.07**	
Range	**1.74+E02-3.84+E02**	**26.88-29.99**	**-2.80-3.48**	**0.982-1.000**	**33.26-38.48**	

NARG Assay Run On 7700 with Default Settings

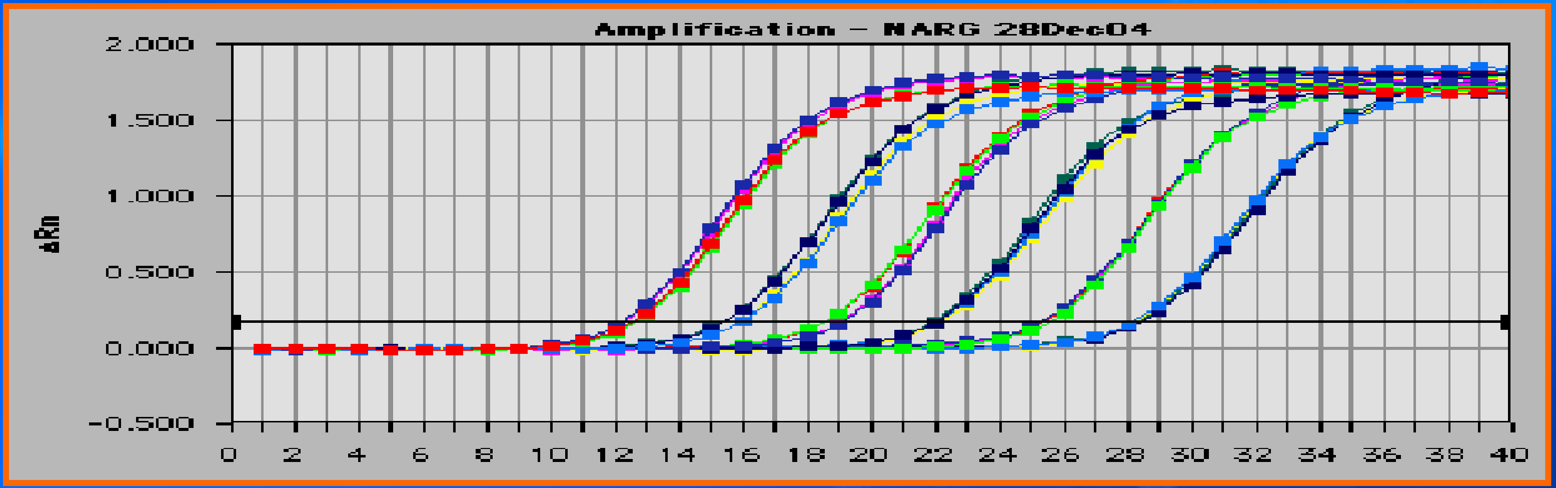
(Baseline set from cycle 3 to cycle 15)

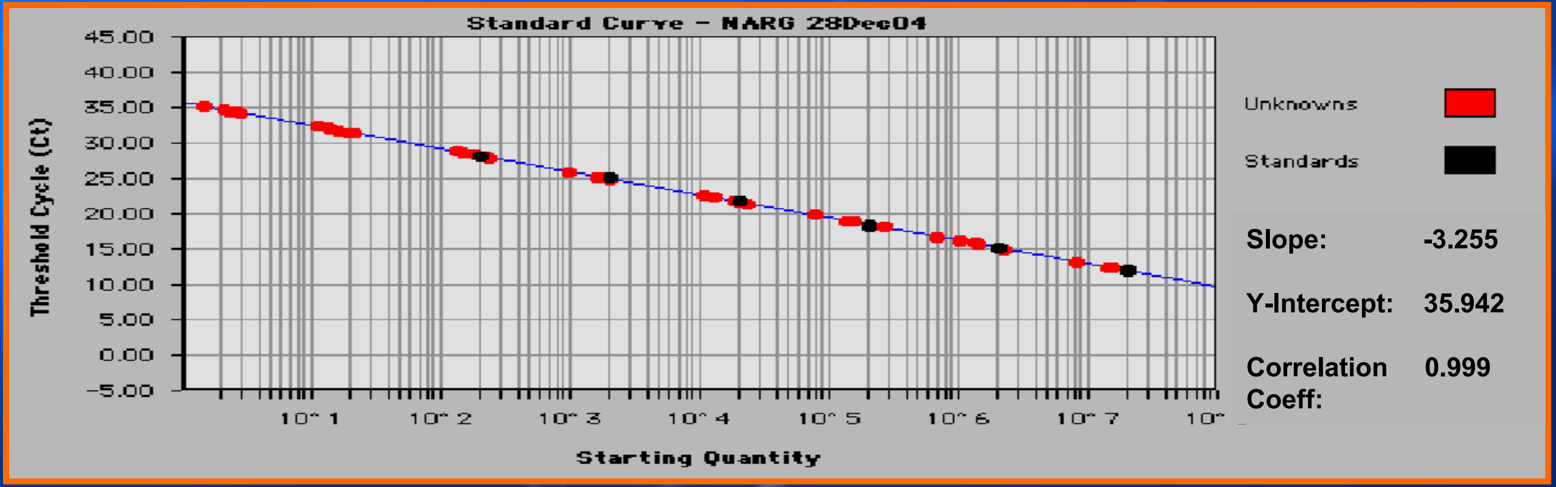




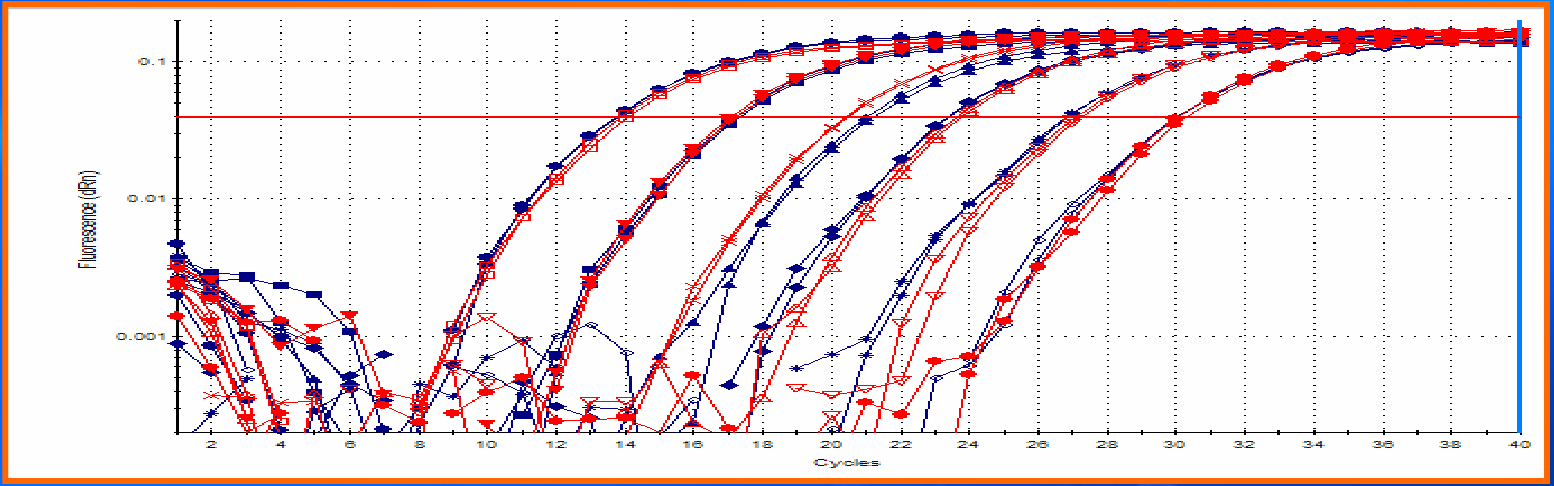
NARG Assay Run On 7700 with Corrected Baseline Setting

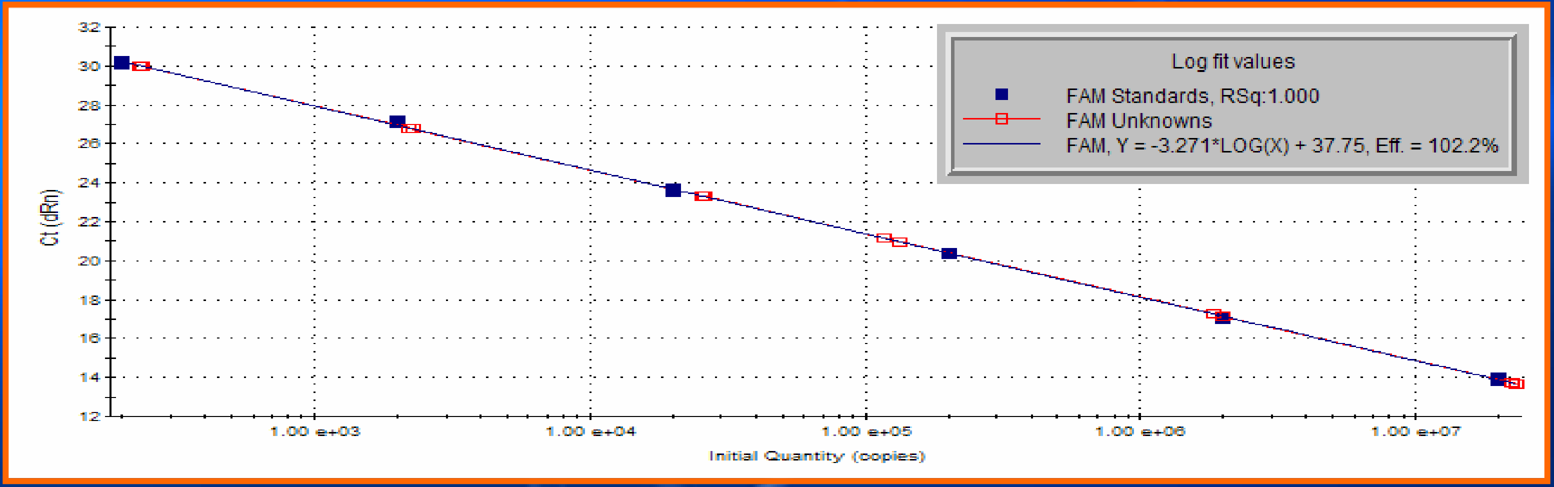
(Baseline set from cycle 3 to cycle 9)



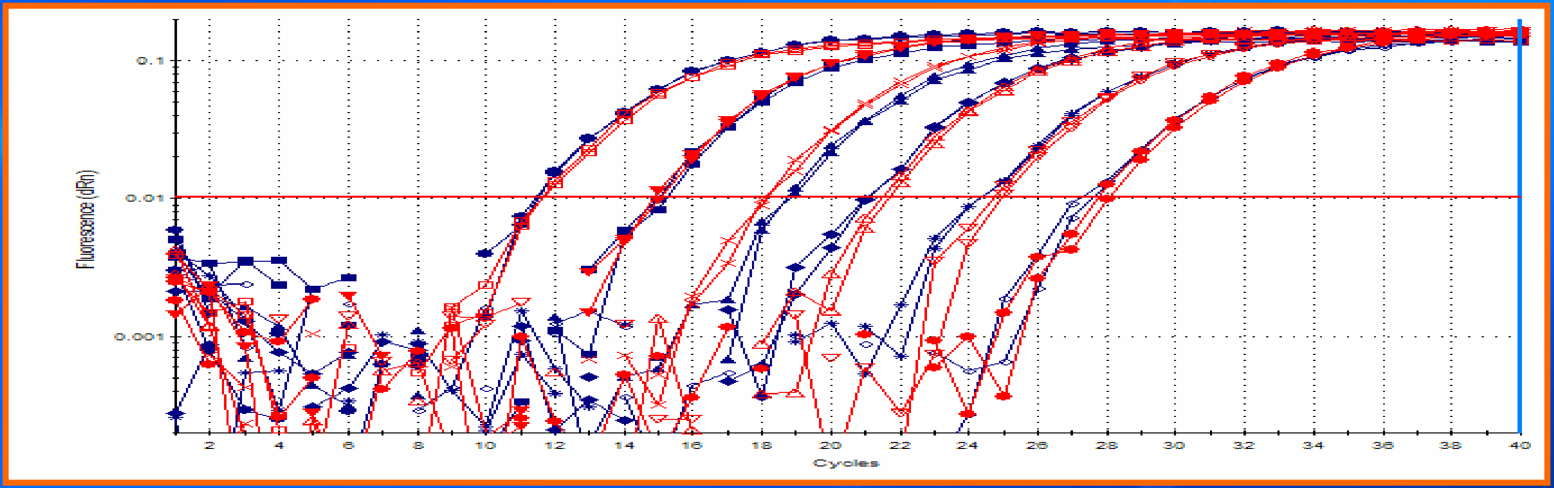


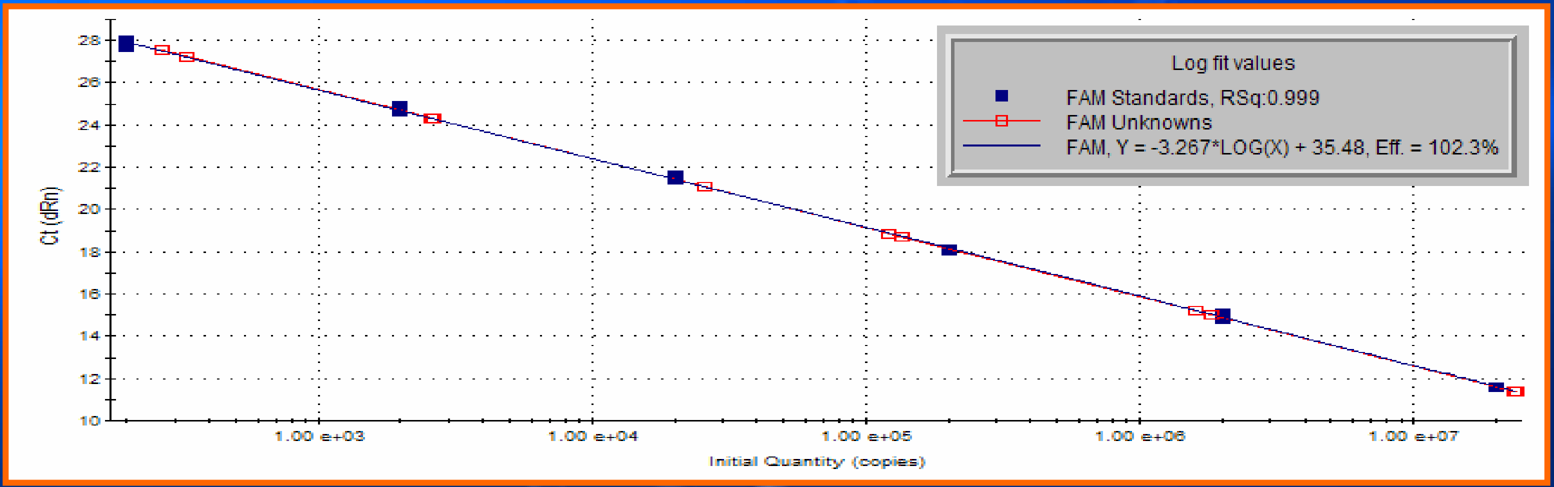
NARG Assay MX3000 Default Settings





NARG Assay MX3000, BBT- Cycle 3-9, Adaptive Baseline

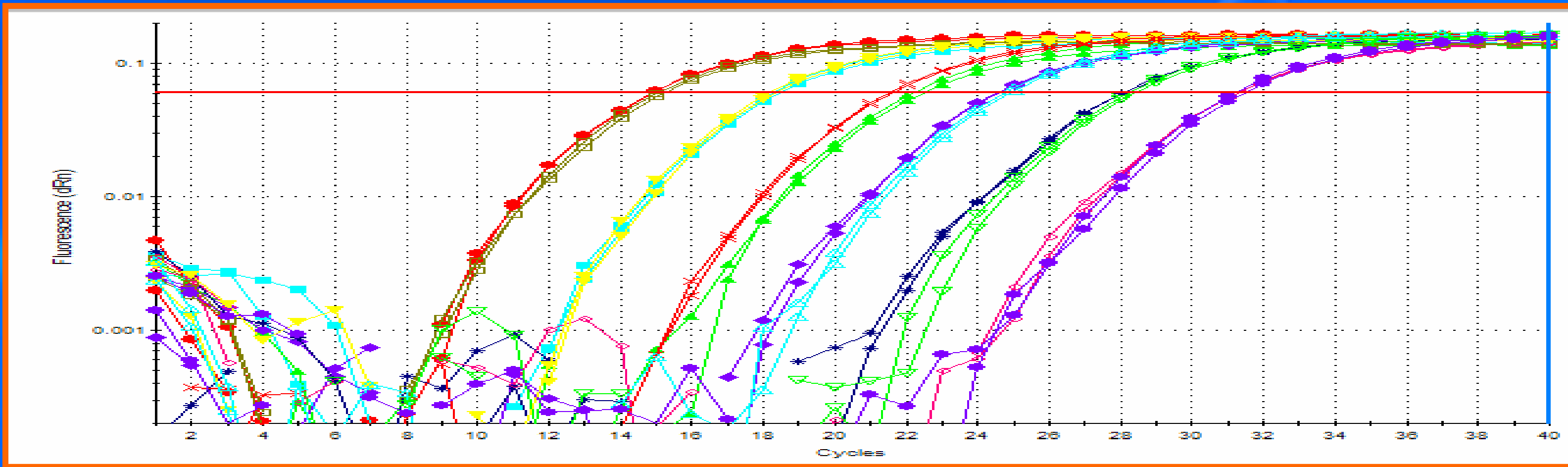




Analysis Settings: Do we really know what they do?

- **Curve smoothing Algorithms**
  - Determine the data points used to generate the log-linear curves, can be set manually or automatically.
- **Baseline setting Algorithms**
  - Determines the background noise from the detector and reagents
  - Fixed number of cycles for all samples or adaptive for each sample
- **Threshold setting Algorithms**
  - A mixed bag of algorithms often not understood by the user which are used to determine the optimal position for a threshold . This determines the reported Ct values including the Ct1 value.

Is there a Single Ideal Position for your Threshold ?



Threshold	Calculated RNA copy #			slope	Ct1	r^2
0.004	2.26E7	2.92E4	3.01E2	3.23	33.86	0.999
0.012	2.51E7	2.46E4	2.72E2	3.25	35.45	0.999
0.020	2.46E7	2.44E4	2.52E2	3.26	36.32	0.999
0.028	2.33E7	2.57E4	2.41E2	3.26	36.98	1.000
0.036	2.30E7	2.58E4	2.40E2	3.27	37.56	1.000
0.044	2.23E7	2.57E4	2.32E2	3.27	38.02	1.000
0.052	2.27E7	2.53E4	2.25E2	3.27	38.45	1.000
0.060	2.30E7	2.51E4	2.17E2	3.26	38.86	0.999
Mean	**2.33**	**2.57**	**2.48**			
std. dev.	**0.10**	**0.15**	**0.27**			

Ct1, Slope, and r^2 values from "Optimal" Analysis Parameters

Instrument	# copies	avg Ct	Slope	r^2	Y-int	Analysis Settings
ABI 7500	3.28E+02	27.48	-3.357(99%)	0.999	**35.93**	manually set threshold, baseline cycles to 3-8
ABI 7700	2.32E+02	28.26	-3.245(103.3%)	0.999	**35.93**	manual baseline cycles to 3-10 10x SD for threshold,manually adj.
BioRad iCycler	2.08E+02	28.12	-3.362(98.4%)	0.999	**35.91**	adaptive baseline, auto threshold best R value, manually adj.
Cepheid Smartcycler I	3.27E+02	28.26	-3.41(96.5%)	0.995	**36.86**	background sub.,primary curve, manual threshold value
Corbitt Rotorgene	3.36E+02	29.03	-3.302(100.8%)	0.998	**37.37**	dynamic tube normalization, manually adjusted threshold
Roche LC 1.2	2.82E+02	29.57	-3.481(93.8%)	0.999	**38.11**	fit points=2, adjusted noise band to 5xSD, baseline set by minimize error
Roche LC 2	2.98E+02	27.53	-3.381(97.6%)	0.999	**35.93**	fit points, minimize error, manual adj of threshold
Stratagene Mx3000p	2.51E+02	28.1	-3.257(102.8%)	1.000	**35.92**	background based threshold 10x SD cycles 3-9. Used adaptive baseline, no moving average, threshold adj
Stratagene Mx4000	2.00E+02	28.30	-3.279(101.8%)	0.999	**35.90**	background based threshold 10x SD cycles 3-9. Used adaptive baseline, no moving average
Mean	2.67E+02	28.3	-3.342(99.2%)	0.998	**35.86**	
Range	2.00E+02-3.36E+02	27.48-29.57	-3.245-3.481 (103.3%-93.8%)	0.995-1.000	**35.90-38.11**	

Conclusions

- All platforms were capable of generating Ct1 values within the theoretically optimal range of 35-38 cycles
- Collectively Ct1, slope and r^2 can be used to determine the quality of an assay
- There is not a single optimal threshold value. Need to be consistent with all subsequent runs
- Curve fitting, baseline and threshold algorithms are not universal across platforms.
- Calculated concentrations will vary across platforms, use of an internal calibrator would be useful when comparing absolute quantitative data between platforms.

Agenda

2004/2005 Real-Time PCR Study Data
Scottie Adams

How to Interpret Your Data:
A Platform Comparison
Brian Holloway

Open Discussion on Quality Control
Greg Shipley
Stephen Bustin