

Nucleic Acids Research Group 2005 Study: A Comparison of Real-Time RT-PCR Technique, Chemistries and Instrumentation in Laboratories Utilizing the Same Assay

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Abstract

The Nucleic Acids Research Group (NARG) study for 2004-05 invited participants to run real-time PCR benchmark tests utilizing an *in vitro* transcribed RNA and a synthetic DNA oligonucleotide template for a human real-time β -Actin assay. The templates, primers, probe and diluent for the templates were provided by the NARG. The experiment entailed making separate 6-log dilution series of the RNA and DNA templates and generating a standard curve for each using the assay reagent's provided. Each laboratory had the choice of running the assays using Taqman[®] or SYBR[®] Green I chemistry and the assay reagents and real-time instrumentation commonly used in their laboratory. The goals of this study were: 1) to provide participants with points of reference to describe their real-time PCR technique from two standard curves generated from a DNA versus a RNA template; 2) to compare how participants analyzed their data, e.g., baseline and threshold settings; 3) to gain information on how the various real-time instruments compare in their ability to detect signals over a 6-log range of template and 4) to compare how the two main real-time PCR chemistries compare using a standardized assay system. For each template, we calculated the slopes and r^2 from a graph of the template numbers (\log_{10}) versus their Ct values to obtain the theoretical number of cycles (Ct) required to amplify one copy of template (the y-intercept at template = 1, defined as Ct1). The results of this study will provide positive feedback for the participants and valuable information on the reagents and instrumentation available to the real-time PCR community.

Research Plan

Participants were sent a kit containing:
 • 1 nmole Forward Primer: h β -Actin-997(+)(CCCTGGCACCCAGCA)
 • 1 nmole Reverse Primer: h β -Actin-1067(-)(GCCGATCCACACGGAGTAC)
 • 0.4 nmole Taqman[®] Probe: h β -Actin-1020(+)(FAM-ATCAAGATCATTCCTCTCTCAGCCGCBHQ)
 • 400 pg synthetic DNA oligo template for the h β -Actin assay
 • 400 pg *in vitro* transcribed RNA template for the h β -Actin assay
 • 200 μ g/ml yeast tRNA in nuclease free water as a diluent
 • Directions for dilutions/suggestions for performing assays
 • Participants were requested to perform RT using the reverse primer on the DNA and the RNA template and run a standard curve using the chemistry and machine(s) in their laboratory.
 Information concerning the chemistry, platform, assay conditions, etc., were submitted using a web based survey form; jpg files of amplification and standard curves were sent with the final exported numerical data via e-mail.

Methods

Primer/Probe Synthesis- The probe and primers were synthesized in the DNA Chemistry Laboratory at the Centers for Disease Control. The probe was synthesized on an Applied Biosystems (Foster City, CA) 3400 DNA synthesizer using standard phosphoramidite chemistry, starting with a BHQ-1 CPG. 0.2 μ mole (Bioscience Technologies, Novato, CA.) and labeled at the 5' end with 6-carboxy-fluorescein (FAM) (Glen Research, Sterling, Virginia). Primers were synthesized on an Applied Biosystems 394 DNA synthesizer using standard phosphoramidite chemistry and 0.2 μ mole columns from Glen Research (Sterling, VA). The probe was purified to greater than 90% purity by reverse-phase HPLC. The quality of both primers and probe were verified by capillary electrophoresis on a Beckman PACE/MDQ system (Beckman Coulter, Fullerton, CA).

Template synthesis- The sDNA template was synthesized by (Invitrogen, Carlsbad, CA). The DNA template is a 71 base oligo, the precise size of the amplified PCR product. The human β -Actin synthetic sRNA template was synthesized by *in vitro* transcription using assay-specific primers in a two-stage PCR to add a T7 RNA polymerase site to the 5' end of the β -Actin assay PCR product in the Quantitative Genomics Core Laboratory at The University of Texas Health Science Center at Houston. *In vitro* RNA synthesis was performed utilizing a Megascript/cript kit (Ambion, Austin, TX).

Diluent preparation- 100 ng/ml rRNA (Invitrogen) in nuclease free water (Ambion) was prepared in the Quantitative Genomics Core Laboratory at the University of Texas Health Science Center-Houston.

Analysis- All standard curves were re-plotted (\log_{10} molecule number vs Ct) using raw data supplied by the participants to allow comparisons between different software/platforms. A linear formula $Y = mX + b$ was fitted by the least square error method, where m is the slope and b is the y-intercept, which represents the extrapolated cycle threshold number for a single molecule, which we have designated as Ct1.

Acknowledgements

We gratefully acknowledge all the participants of this study. We would like to acknowledge the hard work of Ying Wang of UTHSC-Houston, Mark LaMer and Jessica Hoffman of Trudeau Institute, Emmanuelle Nicolas of FOC/CC, Josef Linné and Karen McCaustland of the CDC, and Ashley Price of Penn State University, without whom this study would not have been possible. We would also like to thank our ABR Executive Board *ad hoc*, Dr. Susan Hardin of the University of Houston for her guidance.

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Figure 1 - Participation by Country and Represented Instruments

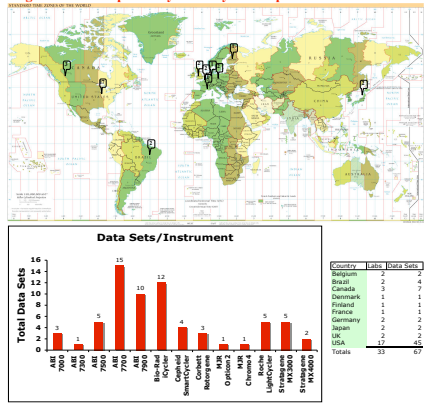
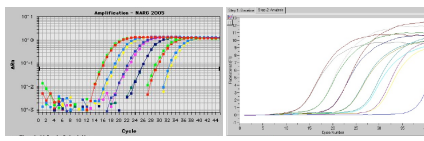
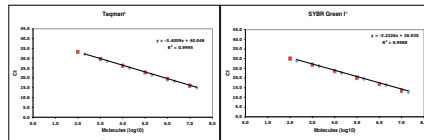


Figure 2- Examples of Optimal Assay Results



Optimal assay results for both the DNA and RNA templates from the same laboratory. The amplification plots for Taqman[®] probe-based and SYBR Green I[®] chemistries came from two different laboratories and utilized different real-time PCR platforms.

Figure 3 - Determining the Number of Molecules from Template Mass for a Real-Time qPCR Standard

Unlike mass alone, molecules are based on mass and length of the template and thus give more information. The NARG suggests everyone plot their standard curves using \log_{10} molecules so that a valid Ct1 value can be obtained for comparison.

To calculate any mass to molecules for a given PCR template:

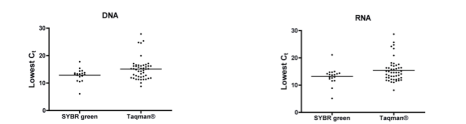
$$\text{Molecules} = \frac{\text{Mass (in grams)} \times \text{Avogadro's Number}}{\text{ave mol wt of a base x template length}}$$

Example: in 1 pg of a single stranded 75 base ssDNA oligonucleotide template, the number of molecules is:

$$1 \times 10^{-12} \text{ gm} \times 6.023 \times 10^{23} \text{ molecules/mole} = 2.43 \times 10^7 \text{ molecules} \\ (330 \text{ gm/mole} \times 75 \text{ bases})$$

Results

Figure 4 - SYBR Green I[®] vs Taqman[®] Comparison



A comparison of SYBR Green I[®] and Taqman[®] assay chemistries based on the lowest Ct values for both the RNA and DNA templates. There was a significant difference between the two assay chemistries when compared by this parameter ($p = 0.03$), due to the signal from multiple fluorescent molecules per template for SYBR Green I[®] versus one per template for a Taqman[®] Probe.

Table 1 - Summary of Real-Time PCR Assay Results

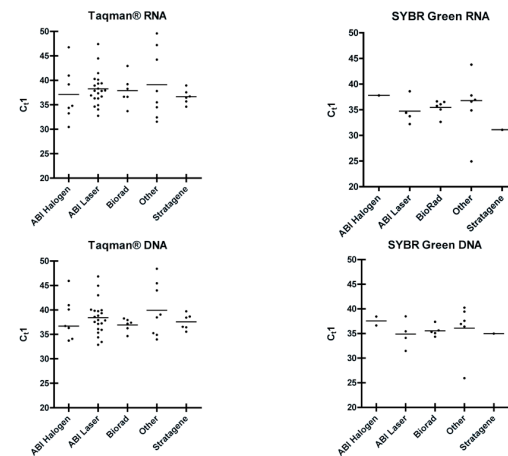
ID	INSTRUMENT	ASSAY TYPE	STEPS	EFF. DNA	EFF. RNA	DNA Y-INT	RNA Y-INT	R ² DNA	R ² RNA	COMPARED
0715	ABI 7700	SYBR green	Two Step	100.0	100.0	37.45	37.62	0.997	0.999	89.08
7228	Roche Light Cycler	SYBR green	Two Step	100.0	100.0	37.37	38.82	0.992	0.991	89.11
3911	Corbett Rotocycler	SYBR green	One Step	95.7	96.4	39.92	34.96	0.998	0.993	88.84
7228A	Roche Light Cycler	SYBR green	Two Step	100.0	100.0	38.39	38.39	0.999	0.999	88.84
3911	Roche Light Cycler	SYBR green	Two Step	98.2	96.1	39.48	37.81	0.996	0.993	88.87
0058A	Bio-Rad iCycler	SYBR green	Two Step	98.1	100.0	39.09	38.46	0.993	0.997	88.88
2005Z	ABI 7700	SYBR green	Two Step	100.0	100.0	38.46	38.76	0.998	0.993	88.8
2005D	ABI 7700	SYBR green	Two Step	100.0	100.0	38.46	38.46	0.998	0.993	88.84
6973	MJ Opticon 2	SYBR green	Two Step	94.9	97.2	38.42	34.88	0.993	0.992	79.7
0052E	ABI 7700	SYBR green	Two Step	100.0	100.0	38.46	38.46	0.998	0.993	79.75
3908B	Bio-Rad iCycler	SYBR green	Two Step	107.8	103.1	31.47	32.22	0.988	0.989	77.58
6148B	Bio-Rad iCycler	SYBR green	Two Step	108.6	101.3	34.35	35.75	0.991	0.993	72.33
0148A	SYBR green	SYBR green	Two Step	100.0	98.0	37.36	36.1	0.999	0.993	71.76
1644	Bio-Rad iCycler	SYBR green	Two Step	116.0	132.9	35.64	35.06	0.993	0.964	68.81
0441	ABI 7700	SYBR green	Two Step	100.0	100.0	38.46	38.46	0.998	0.993	68.15
7273	Stratagene MX200	SYBR green	Two Step	100.0	100.0	34.99	31.11	0.998	0.943	66.18
1115A	Stratagene MX200	SYBR green	Two Step	100.0	100.0	34.99	31.11	0.998	0.943	66.15
1108	Bio-Rad iCycler	SYBR green	Two Step	100.0	100.0	34.99	34.99	0.999	0.971	66.75
0058B	Bio-Rad iCycler	Taqman	Two Step	100.0	100.0	37.34	37.34	0.997	0.997	66.69
0101A	ABI 7700	Taqman	Two Step	100.0	100.0	37.34	37.34	0.997	0.997	66.69
3414	ABI 7700	Taqman	One Step	100.0	100.0	38.23	38.74	0.999	0.999	62.96
7454A	ABI 7700	Taqman	Two Step	98.0	98.2	40.09	40.14	0.999	0.999	62.96
6989	ABI 7700	Taqman	Two Step	98.0	98.0	39.33	39.33	0.992	0.992	61.89
4050	ABI 7700	Taqman	Two Step	98.4	98.9	38.54	38.31	0.997	0.997	61.16
6932T	ABI 7700	Taqman	Two Step	100.0	99.2	39.34	39.36	0.999	0.994	60.33
7468B	Corbett Rotocycler	Taqman	One Step	100.0	99.9	39.04	37.81	0.993	0.993	59.7
0101B	ABI 7700	Taqman	Two Step	98.0	98.4	39.83	37.93	0.999	0.997	59.37
3411	ABI 7700	Taqman	Two Step	98.0	98.9	40.08	40.37	0.999	0.998	58.36
1308H	Stratagene MX200	Taqman	One Step	101.9	103.8	36.46	35.68	0.995	0.999	58.18
1308L	Stratagene MX200	Taqman	One Step	104.6	104.6	36.68	37.56	0.999	0.999	57.79
1308G	Stratagene MX200	Taqman	One Step	101.1	104.7	35.55	34.62	0.995	0.999	58.75
1308B	Stratagene MX200	Taqman	One Step	100.0	100.0	34.99	31.71	0.998	0.999	58.24
2405	ABI 7700	Taqman	Two Step	98.1	98.2	37.55	38.31	0.997	0.993	58.12
2007Y	ABI 7700	Taqman	Two Step	100.0	98.0	37.55	38.08	0.999	0.993	58.03
190A	ABI 7700	Taqman	Two Step	101.6	98.0	38.954	40.29	0.996	0.997	58.34
1308C	Stratagene MX200	Taqman	One Step	102.0	98.0	37.55	34.62	0.999	0.999	58.13
8532E11	ABI 7700	Taqman	One Step	92.1	92.0	38.67	37.87	0.998	0.999	58.34
3205A	ABI 7700	Taqman	Two Step	98.0	92.1	35.46	34.66	0.996	0.997	58.03
8532E12	ABI 7700	Taqman	One Step	99.1	100.2	38.44	38.49	0.999	0.999	52
2008C	Roche Light Cycler	Taqman	Two Step	95.4	95.1	40.42	40.6	0.996	0.993	52.72
6901	ABI 7700	Taqman	Two Step	95.7	92.6	40.1	39.17	0.999	0.999	52.87
8532E14	ABI 7700	Taqman	One Step	95.6	95.7	37.55	38.68	0.999	0.993	53.81
0101	Stratagene MX200	Taqman	One Step	99.1	100.2	38.44	38.49	0.999	0.999	52
1308F	Roche LightCycler II	Taqman	One Step	98.2	100.1	33.99	34.49	0.997	0.996	58.81
8532E15	ABI 7700	Taqman	One Step	98.2	98.2	40.69	41.41	0.999	0.999	58.89
1308E	ABI 7700	Taqman	One Step	91.0	93.7	37.18	36.3	0.997	0.997	58.84
2008A	ABI 7700	Taqman	Two Step	93.2	90.0	42.99	41.48	0.997	0.996	58.13
1644	Bio-Rad iCycler	Taqman	Two Step	113.3	114.6	36.24	36.43	0.994	0.982	70.61
1308D	ABI 7700	Taqman	One Step	97.1	100.8	38.07	34.37	0.997	0.999	77.73
2005D	ABI 7700	Taqman	Two Step	98.2	98.3	40.69	41.41	0.999	0.999	78.94
7271	ABI 7700	Taqman	One Step	116.1	117.7	36.09	34.93	0.993	0.997	75.84
7462D	ABI 7700	Taqman	Two Step	110.0	114.0	36.24	36.44	0.994	0.994	75.87
1308E	Roche LightCycler II	Taqman	One Step	95.3	100.2	35.24	31.95	0.999	0.994	74.49
2200Z	ABI 7700	Taqman	Two Step	110.0	110.0	32.96	32.74	0.997	0.993	73.73
190A	Stratagene MX200	Taqman	One Step	103.7	113.2	35.85	36.7	0.993	0.993	72.84
7462E	ABI 7700	Taqman	Two Step	98.0	98.0	40.69	41.41	0.999	0.999	72.84
6148D	ABI 7700	Taqman	Two Step	100.0	99.1	37.10	39.2	0.997	0.999	72.06
1115B	Stratagene MX200	Taqman	Two Step	91.6	91.7	40.69	41.41	0.992	0.992	71.82
1308C	Corbett Rotocycler	Taqman	One Step	100.4	116.8	34.49	32.44	0.993	0.994	71.26
7462B	ABI 7700	Taqman	Two Step	91.6	98.7	40.69	41.41	0.992	0.992	69.87
1308D	Stratagene	Taqman	One Step	93.9	107.6	38.46	38.51	0.993	0.994	69.82
200Z	ABI 7700	Taqman	Two Step	117.6	117.6	32.72	32.72	0.997	0.997	64.86
1108	Bio-Rad iCycler	Taqman	Two Step	124.1	94.1	37.93	42.93	0.996	0.873	60.26

Summary of the key results obtained in the NARG 2004-05 study. Data are sorted by chemistry and then by comparison score. All standard curves were plotted numerically in a common format so that Ct1 values could be obtained. The composite score was determined using the following formula:
 $0.4^{(60+40 \cdot (1 - (\text{DataSet\#} - \text{Rank\#}) / (\text{DNA-Efficiency} / \text{DataSet\#})) + 0.3^{(60+40 \cdot (1 - (\text{DataSet\#} - \text{Rank\#}) / (\text{Efficiency Differential} / \text{DataSet\#})) + 0.2^{(60+40 \cdot (1 - (\text{DataSet\#} - \text{Rank\#}) / (\text{Ct1-Differential} / \text{DataSet\#})) + 0.1^{(60+40 \cdot (1 - (\text{DataSet\#} - \text{Rank\#}) / (\text{Rsq-DNA} / \text{Rsq-RNA} / 2) / \text{DataSet\#}))$

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Results

Figure 5 - A Comparison of Assay Results from all Instruments in Terms of Ct1



All the instruments showed they could work well in this experiment. Distribution of results for each instrument for both SYBR Green I[®] and Taqman[®] Chemistries with the RNA and/or DNA templates were based on the Resultant Ct1 values. The variability in data is due to variations in laboratory technique.

Summary

Participant laboratories in the NARG 2004-05 study came from many different countries and utilized a broad spectrum of real-time PCR instrumentation (Figure 1).

The study had 4 main goals:

- Goal 1-** To provide feedback to the participants on their real-time PCR technique using different measures of two standard curves, one from a DNA and one from an RNA template using either SYBR Green I, Taqman[®] or both chemistries.
- Results 1-** the 67 data sets received from 33 different laboratories ranged from poor to excellent. Figure 2 shows the expected results. A summary of the results is in Table 1.
- Goal 2-** To compare how participants analyzed their data.
- Results 2-** Due to the different raw fluorescence scales and analysis routines in the software from different manufacturers, it was not possible to compare baseline and threshold settings across all platforms. Further, we could not come up with a universal set of rules for data analysis that would be applicable to all the instruments represented in the study. We did determine that standard curves are not being plotted in the same way by all groups. Many are using \log_{10} mass instead of \log_{10} molecules on the x-axis (see Figure 3). We have defined the y-intercept of the standard curve when plotted this way as the Ct1 value. The Ct1 (number of cycles it would take to amplify 1 molecule) value encompasses all traits of the assay (slope, Ct of first standard point, r^2) and can be used for comparison with other assays.
- Goal 3-** To compare how well the different instruments performed in the study.
- Results 3-** Data for all the instruments in the study can be seen in Table 1. Data for the instrument groups are shown in Figure 5. Reasonable data was derived from all platforms. Outliers were attributed to factors other than instrument performance.
- Goal 4**