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 reagents 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: 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
- 200 µl 100 ng/µl 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 µmole (Biosearch 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 µmole columns from Glenn 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 Megashortscript kit (Ambion, Austin, TX).

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

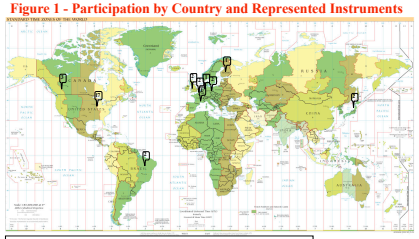
Analysis- All standard curves were re-plotted ($\log_{10}$molecule number vs Ct) using raw data supplied by the participants to allow comparisons between different software/platforms. A linear formula Y= mX + b was fitted by the least square error model, where m is the slope and b is the y- intercept,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

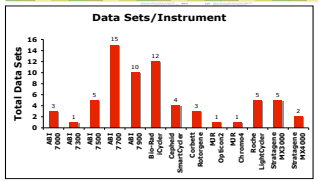
Acknowledgements

We gratefully acknowledge all the participants of this study. We would like to acknowledge the hard work of Ying Wang of UTHSC-Houston, Mark LaMere and Jessica Hoffman of Trudeau Institute, Emmanuelle Nicolas of FCCC, Josef Limor and Karen McCausland 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 ABRF Executive Board *ad hoc*, Dr. Susan Hardin of the University of Houston for her guidance.

Results

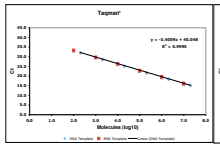
Figure 1 - Participation by Country and Represented Instruments



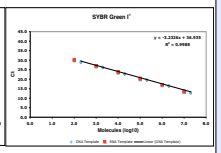


Country	Labs	Data Sets
Belgium	2	2
Brazil	2	4
Canada	3	7
Denmark	1	1
Finland	1	1
France	1	1
Germany	2	2
Japan	2	2
UK	2	2
USA	17	45
Totals	33	67

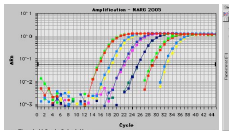
Figure 2- Examples of Optimal Assay Results



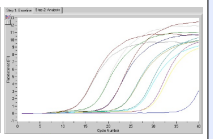
Combined standard curve showing RNA and DNA template results using the Taqman® probe assay.



Combined standard curve showing RNA and DNA template results using the SYBR Green I® assay.



Amplification plot from the DNA template using the Taqman® probe assay.



Amplification plot from the DNA template using the SYBR Green I®assay.

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:

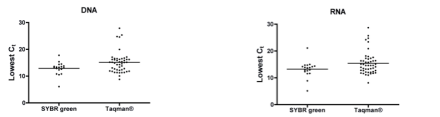
$$\frac{\text{Mass (in grams) x Avogadro's Number}}{\text{(ave mol wt of a base x template length)}}$$

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

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

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® verses one per template for a Taqman® Probe.

Table I - Summary of Real-Time PCR Assay Results

ID	INSTRUMENT	ASSAY_TYPE	CHEMISTRY	Efficiency DNA (%)	Efficiency RNA (%)	DNA Ct1 (Cal)	RNA Ct1 (Cal)	ΔDNA-RNA Ct1	r² DNA (Cal)	r² RNA (Cal)	Composite Score
0715	ABI 7900	SYBR green I	Two Step	102.3	101.1	38.43	37.82	0.62	0.998	1.000	89.9
7222B	Cepheid SmartCycler	SYBR green I	Two Step	100.2	104.0	37.57	36.62	0.95	0.998	0.999	87.1
3511	Corbett RotorGene	SYBR green I	One Step	95.7	96.4	25.92	24.96	0.96	1.000	0.999	86.8
7222A	Roche Light Cycler	SYBR green I	Two Step	103.6	100.4	36.94	36.96	-0.03	0.999	1.000	86.2
3011	Roche Light Cycler	SYBR green I	Two Step	98.2	96.1	39.46	37.81	1.65	0.996	1.000	86.0
0000A	Bio-Rad iCycler	SYBR green I	Two Step	99.1	102.9	35.09	36.66	-1.58	0.993	1.000	84.4
2005Z	ABI 7900	SYBR green I	Two Step	102.4	99.0	35.46	33.76	1.70	0.999	0.999	82.6
2005B	ABI 7900	SYBR green I	Two Step	111.4	110.3	34.11	34.43	-0.32	0.997	0.996	80.5
6973	MJR Opticon 2	SYBR green I	Two Step	94.5	97.2	36.42	34.88	1.54	0.999	0.999	79.7
6932S	ABI 7900	SYBR green I	Two Step	107.4	98.2	38.49	38.62	-0.13	1.000	1.000	78.8
3305B	ABI 7700	SYBR green I	Two Step	107.9	103.1	31.47	32.22	-0.75	0.999	0.999	77.6
3669	Bio-Rad iCycler	SYBR green I	One Step	103.4	116.9	35.35	32.65	2.68	0.976	0.994	73.5
6149B	Bio-Rad iCycler	SYBR green I	One Step	106.6	101.3	34.33	35.75	-1.41	0.998	0.998	72.3
6148A	Bio-Rad iCycler	SYBR green I	Two Step	108.0	96.5	37.36	36.1	1.27	1.000	0.991	71.8
1644	Bio-Rad iCycler	SYBR green I	Two Step	118.0	152.8	35.64	35.06	0.57	0.989	0.986	68.5
0441	ABI 7000	SYBR green I	Two Step	105.5	ND	36.64	ND	ND	0.998	ND	68.2
7275	Stratagene Mx3000	SYBR green I	Two Step	109.4	134.0	34.99	31.11	3.88	0.999	0.994	66.2
1111A	Cepheid SmartCycler	SYBR green I	Two Step	114.5	104.5	40.24	40.82	-0.58	0.985	0.990	65.0
1188	Bio-Rad iCycler	SYBR green I	One Step	ND	123.4	ND	36.58	ND	ND	0.313	60.8
0000B	Bio-Rad iCycler	Taqman®	Two Step	99.3	100.2	38.25	38.27	-0.02	1.000	0.999	95.6
0101A	ABI 7900	Taqman®	One Step	99.0	98.0	37.94	37.83	0.11	1.000	1.000	94.7
3413	ABI 7000	Taqman®	One Step	100.6	101.0	36.32	34.79	1.53	1.000	1.000	93.0
7645A	ABI 7700	Taqman®	One Step	96.8	96.2	40.05	40.14	-0.09	1.000	1.000	93.0
6989	ABI 7700	Taqman®	One Step	95.8	95.8	39.33	39.33	0.00	0.999	0.998	91.9
4058	ABI 7900	Taqman®	One Step	99.4	96.9	35.34	36.31	-0.97	1.000	1.000	91.2
6932T	ABI 7900	Taqman®	One Step	102.0	99.2	39.74	39.38	0.36	1.000	1.000	90.3
7645B	Corbett RotorGene	Taqman®	One Step	100.8	99.5	39.04	37.81	1.23	0.999	0.998	89.7
0101B	ABI 7900	Taqman®	One Step	98.5	99.4	39.83	37.95	1.89	1.000	1.000	89.4
3611	ABI 7500	Taqman®	One Step	95.5	92.9	40.98	40.97	0.00	1.000	1.000	88.4
1358H	Stratagene Mx4000	Taqman®	One Step	99.0	98.0	37.94	37.83	0.11	1.000	1.000	94.7
1358M	Stratagene Mx3000	Taqman®	One Step	104.6	104.6	38.66	37.55	1.12	1.000	1.000	87.8
1358G	Stratagene Mx3000	Taqman®	One Step	103.1	104.7	35.55	34.62	0.93	1.000	0.997	86.8
1358B	Bio-Rad iCycler	Taqman®	One Step	100.0	100.3	34.65	35.71	0.93	0.999	0.998	86.2
2408	ABI 7900	Taqman®	One Step	95.1	95.2	37.55	38.31	-0.75	0.998	0.999	86.1
2005Y	ABI 7900	Taqman®	One Step	98.2	95.5	37.85	36.68	1.18	0.999	0.998	86.0
1904	ABI 7700	Taqman®	One Step	101.6	95.5	39.95	40.29	-0.33	0.988	1.000	85.3
2809A	MJR Chromo4	Taqman®	One Step	102.5	98.2	45.99	44.22	-0.23	0.996	0.999	85.1
8532 SET1	ABI 7700	Taqman®	One Step	92.1	93.5	38.57	37.87	0.70	1.000	1.000	83.9
3305A	ABI 7900	Taqman®	One Step	96.9	92.1	35.46	34.05	1.39	0.999	0.999	83.5
1358	Stratagene Mx3000	Taqman®	One Step	104.4	99.8	39.71	38.95	0.76	1.000	1.000	83.0
2809C	Roche Light Cycler	Taqman®	One Step	95.4	93.1	48.42	49.6	-1.18	1.000	0.998	82.7
5801	ABI 7900	Taqman®	One Step	95.7	92.6	40.1	39.17	0.94	0.999	1.000	82.6
6148C	Bio-Rad iCycler	Taqman®	One Step	106.1	106.7	37.39	36.65	0.74	1.000	0.993	82.0
0358	Stratagene Mx3000	Taqman®	One Step	99.1	105.2	38.44	36.45	1.99	0.999	0.999	82.0
8532a SET2	ABI 7700	Taqman®	One Step	91.5	95.6	37.23	37.33	-0.10	1.000	0.999	80.8
1358F	Roche LightCycler II	Taqman®	One Step	96.2	106.1	33.98	34.49	-0.51	0.999	1.000	80.8
8532a SET1	ABI 7700	Taqman®	One Step	91.1	88.2	38.85	38.93	-0.09	1.000	0.998	80.7
8532 SET2	ABI 7700	Taqman®	One Step	91.5	89.7	37.16	36.3	0.86	1.000	1.000	80.5
1358I	ABI 7700	Taqman®	One Step	103.3	109.1	35.93	34.63	1.30	0.999	0.999	80.3
1358A	ABI 7500	Taqman®	One Step	93.2	90.5	42.99	41.48	1.51	1.000	1.000	79.1
2809D	ABI 7700	Taqman®	One Step	88.2	85.3	46.85	47.41	-0.56	0.997	1.000	77.3
1644	Bio-Rad iCycler	Taqman®	One Step	133.5	134.0	36.24	36.63	-0.39	0.995	0.986	76.5
2005A	ABI 7900	Taqman®	One Step	97.1	106.8	36.7	34.37	2.33	0.999	1.000	77.7
2809G	ABI 7700	Taqman®	One Step	88.2	85.3	46.85	47.41	-0.56	0.997	1.000	77.3
7231	ABI 7700	Taqman®	One Step	116.1	117.7	36.09	34.93	1.16	0.998	0.997	75.9
7952D	ABI 7900	Taqman®	One Step	110.5	114.0	34.08	33.24	0.84	0.998	0.996	75.4
1358E	Roche LightCycler I	Taqman®	One Step	95.3	102.3	35.24	31.55	3.69	1.000	0.998	74.7
23932	ABI 7900	Taqman®	Two Step	173.0	179.0	32.86	32.74	0.12	1.000	0.999	73.7

Summary of the key results obtained in the NARG 2004-05 study. Data are sorted by chemistry and then composite score. All standard curves were plotted from submitted numerical data in a common format so that Ct1 values could be obtained. The composite score was determined using the following formula:

0.4*(60+40*(1-(DataSet#-Rank#[DNA-Efficiency]/DataSet#))+0.3*(60+40*(1-(DataSet#-Rank#[Efficiency differential]/DataSet#))+0.2*(60+40*(1-(DataSet#-Rank#[Ct1-differential]/DataSet#))+0.1*(60+40*(1-(DataSet# -(Rank#[Rsq-DNA]+Rank#[Rsq-RNA])/2)/DataSet#))

Announcements

NARG Research Group Presentation!

Sunday, Feb 6 @ 4:00-5:00 pm

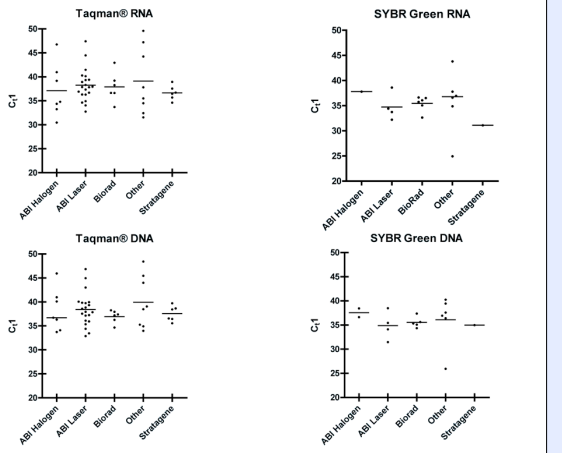
Chatham Ballroom B

Please come and discuss these results with us along with the importance of quality control in your real-time PCR experiments during our Q & A session following a 30 minute presentation.

Please submit poster requests to Gregory.L.Shipley@uth.tmc.edu

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 the 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 I.

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 I. 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- To compare how the different real-time chemistries performed.

Results 4- The study compared the most utilized detection chemistries used in real-time PCR, SYBR Green I and dual-labeled Taqman® oligo probes. As can be seen in Figure 4, there was no significant difference in slope between the two chemistries for either the DNA or RNA templates. However, significant differences were found in Ct1 and lowest Ct with both sDNA and sRNA when comparing the two chemistries (p = 0.03 for both comparisons).

Real-time PCR instruments are extremely sensitive. This characteristic allows for a large dynamic range. It also means that great care in the tools and technique used to set up the assays must be observed to derive the desired results from this methodology.