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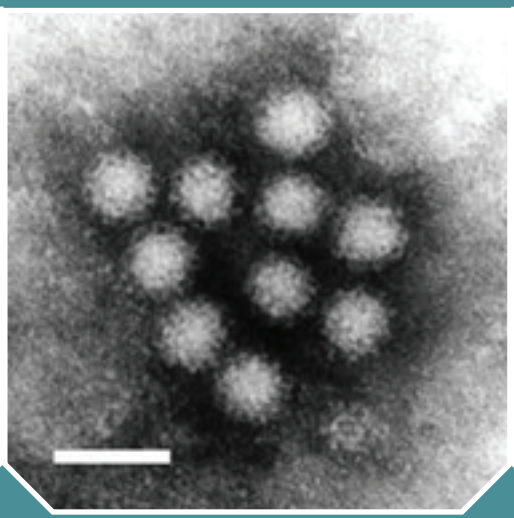
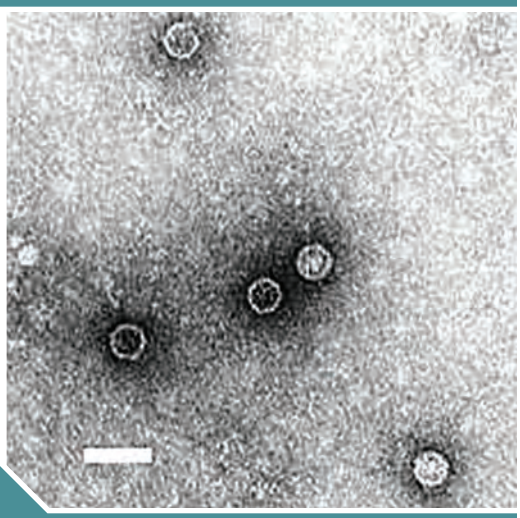
Comparison of EPA Method 1615 RT-qPCR Assays in Standard and Kit Formats





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Comparison of EPA Method 1615 RT-qPCR Assays in Standard and Kit Formats

by

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Cover photos:
Left: Prairie Du Sac, WI Pump house, courtesy of Dr. Mark Borchardt
Middle: norovirus, courtesy of Fred P. Williams; Bar = 50 nanometers
Right: poliovirus, courtesy of Fred P. Williams; Bar = 50 nanometers

Notice/Disclaimer Statement

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Acronyms and Abbreviations

cDNA	complementary deoxyribonucleic acid
Cq	Quantitative cycle
EPA	U.S. Environmental Protection Agency
FCSV	Final concentrated sample volume
GI, GII	Norovirus genogroup I and genogroup II
GC	Genomic copies
GW	Groundwater
kDa	Kilodalton
MWCO	Molecular weight cutoff
NTC	No template control
NoV	Norovirus
PCR	Polymerase chain reaction
PE	Primary effluent from a wastewater treatment plant
qPCR	Quantitative PCR
RG	Reagent grade water
RNA	Ribonucleic acid
RT	Reverse transcription
SE	Secondary effluent from a wastewater treatment plant
UCMR	Unregulated Contaminant Monitoring Rule

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Abstract

EPA Method 1615 contains protocols for measuring enterovirus and norovirus by reverse transcription quantitative polymerase chain reaction. A commercial kit based upon these protocols was designed and compared to the method's standard approach. Reagent grade, secondary effluent, and ground water samples seeded with primary effluent from a local wastewater treatment plant were processed and analyzed for enterovirus and norovirus by both formats. The kit format was easier to use and less labor intensive than the standard assay. The two formats give similar results and it is concluded that either approach may be used for analysis of water samples.

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1. Introduction

Human enteric viruses are found in waterbodies worldwide (Hewitt et al., 2013; Kishida et al., 2012; Lee et al., 2014; Love et al., 2014; Maunula et al., 2012; Miagostovich et al., 2008; Phanuwan et al., 2006; Sassoubre et al., 2012; Xagoraraki et al., 2007). They enter waterbodies through wastewater plant discharges, septage drainage, combined sewer overflows, and other point and non-point sources. Even though diluted upon entering natural waters, a sufficient number of virus particles reach recreational sites and can cause disease. Viruses are thought to be the primary etiological agent of disease at these sites (Soller et al., 2010), but virus monitoring is challenging due to the lack of standard methods and to inherent uncertainty in measurements caused by low virus concentrations. Method 1615, a standardized virus method was developed by the EPA for detection of enteroviruses by both total culturable and molecular assays, and for noroviruses by a molecular assay (Fout et al., 2014). This standardized method was designed for use in the third monitoring round of the Unregulated Contaminant Monitoring Rule (UCMR), with a focus upon groundwater monitoring.

Following the publication of Method 1615, the EPA Office of Science and Technology asked the Office of Research and Development to revise the method for use in monitoring wastewater effluents and recreational waters. In January 2011, EPA Administrator Lisa Jackson, and the Small Business Administration Administrator Karen Mills, announced the formation of Confluence (watercluster.org). Confluence is an organized network of federal/state/local governments, universities, and companies in the Southwest Ohio, Southeast Indiana, and Northern Kentucky region that are partnering together on water-related challenges. The EPA also committed over five million dollars to support water technology innovation in the region. A portion of these resources was set aside for a Water Cluster internal grant process to fund direct interactions between the EPA and industry. A proposal developed in response to the Office of Science and Technology's request to modify Method 1615 was accepted and funded through the Water Cluster internal grant process. The portion of the proposal that dealt with private industry was the development of a kit format for the method's molecular assay. This report summarizes the development and testing of the kit, which has the potential to reduce interlaboratory and intralaboratory variability and labor costs. The objective of the research was to determine whether the kit provides equivalent results to the standard assay approach.

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2. Materials and Methods

2.1 Samples

To provide sufficient and equal virus concentrations for comparing the standard protocols and kits prepared by EMSL Analytical, Inc. in different water matrices, primary effluent (PE) was collected from a local wastewater treatment plant, mixed, divided into 700 mL aliquots, and the aliquots were stored at -70°C. On the afternoon before samples were to be collected, one aliquot of PE per sample was transferred from the -70°C freezer to a 4°C refrigerator. Just prior to use, PE aliquots were thawed in a 37°C waterbath and the entire aliquot used for seeding.

Six samples each of reagent grade water (RG), secondary effluent (SE) from a local wastewater treatment plant using an activated sludge process, and groundwater (GW) from a local drinking water treatment plant were filtered through NanoCeram filters; the sample volumes collected are shown in Table 1. For RG samples, 2.4 g of HEPES was added and the pH was adjusted to between 7.0 and 7.5 prior to filtration. For each SE and GW sample, the majority of the volume was filtered on site and the last nine liters of SE or GW collected and brought back to the laboratory. Each of these portions was seeded with PE and then pumped through the NanoCeram filter that had received the original sample via a peristaltic pump.

Table 1. Samples Analyzed

Sample	Source	Volume (L)
RG #1	Millipore Super Q Water	9
RG #2	Millipore Super Q Water	9
RG #3	Millipore Super Q Water	9
RG #4	Millipore Super Q Water	9
RG #5	Millipore Super Q Water	9
RG #6	Millipore Super Q Water	9
SE #1	Little Miami Wastewater Treatment Plant	87
SE #2	Little Miami Wastewater Treatment Plant	92
SE #3	Little Miami Wastewater Treatment Plant	71
SE #4	Little Miami Wastewater Treatment Plant	82
SE #5	Little Miami Wastewater Treatment Plant	16
SE #6	Little Miami Wastewater Treatment Plant	92
GW #1	Indian Hills Water Works	1662
GW #2	Indian Hills Water Works	1521
GW #3	Indian Hills Water Works	1660
GW #4	Indian Hills Water Works	1654
GW #5	Indian Hills Water Works	1520
GW #6	Indian Hills Water Works	1530

2.2 Sample Processing

Samples were processed according to Method 1615 (Fout et al., 2014) using elution with beef extract and secondary concentration by organic flocculation. Briefly, each NanoCeram filter (Argonide, Sanford, FL) was eluted with 500 mL of 1.5% beef extract, desiccated powder (Becton Dickinson, Franklin Lakes, NJ), 0.375% glycine, pH 9.0, twice using contact times of 1 min for the first elution and 15 min for the second. The combined eluate was adjusted to a pH of 3.5 and stirred for 30 min. The floc was collected by centrifugation at 2,500 ×g for 15 min and dissolved in 30 mL of 0.15 M sodium phosphate, pH 9.0. After removing undissolved material by centrifugation at 4,000 ×g for 10 min, the supernatant was filtered through a 0.2 micron sterilizing filter to remove bacteria and eukaryotes. One third of the filtrate was concentrated further by centrifugal ultrafiltration (Vivaspin 20 with 30 kDa MWCO, Sartorius-Stedim Biotech, Bohemia, NY), resulting in a 0.4 mL final concentrate for each sample. RNA was extracted from 0.2 mL of each final concentrate or from Buffer AE (Negative Extraction Control, Qiagen, Valencia, CA) using the QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions, except that Buffer AVL (Qiagen) was substituted for Buffer AL provided in the kit. The RNA (100 µL final volume) from each sample was stored at -70°C until used for reverse transcription (RT) assays. On the day of the RT assay each sample was thawed and diluted 1:5 and 1:25 in Buffer AE (Qiagen).

2.3 *RT* (Standard Assay)

Master mix RT1 containing 10 ng/µL random primers (Promega, Madison, WI) and 2.5% (v/v) hepatitis G Armored RNA (Asuragen, Austin, TX) and master mix RT2 containing 10 mM Tris, 50 mM KCl, pH 8.3, 3 mM $MgCl_2$, 0.8 mM deoxyribonucleotides (Promega), 10 mM dithiothreitol (Promega), 0.5 units/µL RNase Inhibitor (Promega), and 1.6 units/µL SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY) were prepared, with all concentrations being relative to the final 40 µL RT reaction volume. A volume of 16.5 µL of RT1 was added to wells of 96 well plates (Bio-Rad, Hercules, CA), followed by the addition of 6.7 µL/well of samples or controls. Each sample was diluted 1:5 and 1:25 in Buffer AE and each dilution and the undiluted sample was added to wells in triplicate. No template controls (NTC) consisting of Buffer AE (Qiagen) were distributed throughout the plate. RNA from standard curves, prepared as described in Section 2.8, was added in duplicate. Plates were then covered with Microseal 'A' Film (Bio-Rad) and incubated at 99°C for 4 min followed by a 4°C hold time in a PTC-200 thermal cycler (MJ Research, Waltham, MA). The Microseal film was removed carefully and 16.8 µL of RT2 was added per plate well. Plates were sealed again with Microseal 'F' film (Bio-Rad). Complementary DNA (cDNA) was prepared by incubation at 25 °C for 15 min, 42 °C for 60 min, and 99 °C for 5 min, followed by a 4 °C hold cycle in the PTC-200 thermal cycler (MJ Research). Plates were stored at or below -70°C until analyzed by qPCR.

2.4 *RT* (EMSL Kit Assay)

RT plates containing 16.5 µL or master mix RT1 per well were prepared by EMSL Analytical, Inc., Cinnaminson, NJ, covered with Microseal 'F' film (Bio-Rad, and frozen at -80°C. Master mix RT2 sufficient for the number of wells to be tested was prepared and frozen at -80°C. The RT plates and master mix were shipped to USEPA on dry ice and stored at -80°C until analyzed. Samples were added to the RT plates and incubated as above. Following the denaturation step at 99°C, the master mix was added and cDNA produced as with the standard assay.

2.5 *qPCR* (Standard Assay)

A qPCR master mix consisting of 10 µL per well of 2X LightCycler 480 Probes Master Mix (F. Hoffmann-La Roche, Indianapolis, IN), 0.5 mM ROX reference dye (Life Technologies), and primers and probes (Life Technologies) in the concentrations shown in Table 2 was prepared and 14 µL added per Optical 96-well Fast Plate (Life Technologies) well. 6 µL of cDNA from the RT assays was added and plates were run in a StepOnePlus Real-Time PCR system (Life Technologies) using the Quantitation – Standard Curve setup for TaqMan reagents and the standard instrument run time. The instrument software was programmed to run for 1 cycle at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 sec, and 60 °C for 1 min. The values from Table 3 for the standard curve (see Section 2.8) for the enterovirus and norovirus assays were input into the thermal cycler software.

Table 2. Primer/Probe Concentrations

Primer/Probe [a]	Concentration (mM)	Sequence (5' to 3')
HepF	500	CGGCCAAAAGGTGGTGGATG
HepR	500	CGACGAGCCTGACGTCGGG
HepP	100	6FAM-AGGTCCCTCTGGCGCTTGTGGCGAG-TAMRA
EntF	300	CCTCCGGCCCCTGAATG
EntR	900	ACCGGATGGCCAATCCAA
EntP	100	6FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA
NoVGIBF	500	CGCTGGATGCGNTTCCAT
NoVGIBR	900	CCTTAGACGCCATCATCATTTAC
NovGIBP	250	6FAM-TGGACAGGAGAYCGCRATCT-TAMRA
NoVGIIF	500	ATGTTCAGRTGGATGAGRTTCTCWGA
NoVGIIR	900	TCGACGCCATCTTCATTCACA
NoVGIIP	250	6FAM-AGCACGTGGGAGGGCGATCG-TAMRA

[a] Abbreviations: Hep – hepatitis G assay; Ent – enterovirus assay; NoVGIB – norovirus genogroup I assay; NoVGII – norovirus genogroup II assay; F – forward primer; R – reverse primer; P – probe.

Table 3. Standard Curve Genomic Copies

Standard Curve Concentration	Genomic Copies per RT-qPCR Assay (1)
2.5×10^7	50,250
2.5×10^6	5,025
2.5×10^5	502.5
2.5×10^4	50.25
2.5×10^3	5.025

(1) Place the indicated genomic copy values in the standards section for the real time thermal cycler used

2.6 *qPCR* (EMSL Kit Assay)

A qPCR master mix described in section 2.5 was prepared by EMSL Analytical, Inc., and 14 µL was added per Optical 96-well Fast Plate (Life Technologies) well. Plates were covered with Microseal 'F' film, frozen at -80°C, shipped to EPA on dry ice, and stored at -80°C until used. Plates were thawed just before use. Samples (6 µL of cDNA) were added and run under the same conditions and on the same StepOnePlus thermal cycler as the standard assay. For each assay type, the EMSL kit assay was run in the morning and the standard assay in the afternoon of the same day.

2.7 Inhibition Assay

The hepatitis G PCR assay was run before the enterovirus and norovirus assays. The mean quantitative cycle (Cq) value of each sample dilution was compared to the mean value of NTC and negative extraction controls. The Cq value is the cycle at which the fluorescence of a PCR assay crosses the threshold that defines a positive reaction. Samples that exhibited a mean Cq value greater than 1 Cq unit higher than the mean of the uninhibited NTC/negative extraction controls was considered inhibited and not used, except as indicated in the Results section. The undiluted sample or the first dilution that was uninhibited was used for subsequent enterovirus and norovirus assays.

2.8 Standard Curve

Armored RNA EPA-1615 (Asuragen, Austin, TX; custom order) containing the enterovirus and norovirus regions amplified by the primer sets used (Brinkman et al., 2013) was diluted to 2.5×10^8 genomic copies/mL, divided into 250 µL aliquots, and stored at -20°C. One aliquot was removed from the freezer and thawed. Five serial dilutions were prepared giving concentrations of 2.5×10^7 to 2.5×10^3 genomic copies/mL. RNA from each dilution was extracted and analyzed in the same manner as samples. Table 3 gives the final genomic copy number for each qPCR assay (assuming that the percent loss during extraction is the same for both samples and standards). To be acceptable each standard curve must have an R^2 value of >0.97, a percent efficiency of 80 to 115%, and an overall standard deviation of <1.0.

2.9 Genomic Copy Calculation

The number of genomic copies for each virus was calculated using Equation 1:

$$GC = GC_{PCR} \times 199 \times \frac{FCSV}{S} \quad \text{Equation 1}$$

Where GC_{pcr} is the quantity calculated by the real time thermal cycler software based upon the standard curve, 199 is the factor that correct for the assay volumes used for RNA extraction, RT, and qPCR, *FCSV* is the volume of filtered concentrate after organic flocculation, and *S* is the volume of *FCSV* used for centrifugal ultrafiltration.

2.10 Statistical Analysis

Cq and quantity values (calculated from the standard curve) were analyzed first for normality and equal variance (Sigma Plot version 13.0, Systat Software, San Jose, CA). Many comparisons between the values from the standard and EMSL kit assays were not normal, even when log-transformed. ANOVA on Ranks with Dunn's or Tukey tests (Sigma Plot) was used for comparison of multiple parameters. Finally, the Mann-Whitney Rank U Test (Sigma Plot) was used to determine statistical significance of comparisons of the standard method with the EMSL kit on a per virus basis, considering either each matrix individually or all matrices combined.

2.11 Quality Assurance

EMSL Analytical, Inc. tested each master mix used for kit preparation for activity using EPA-1615 and hepatitis G Armored RNAs as controls. All mixes receiving the Armored RNAs were positive by RT-qPCR for all primer/probe sets and all no template controls were negative. EPA's quality assurance guidelines (Sen et al., 2004) were followed for analysis of samples performed in EPA laboratories. This included the use of a dedicated laboratories and rigorous work flow requirements. Negative extraction and no template controls were included as required by EPA Method 1615. These controls consisted of Buffer AE (Qiagen) in place of sample for RNA extraction or of RNA for RT-qPCR.

The six reagent grade water samples were processed and RNA extracted prior to collection of the other sample types. Two of the reagent grade water samples were analyzed by RT-qPCR to determine if the PE seed contained detectible quantities of each virus type. Cq values for each virus were sufficient to proceed with the remaining sample matrices (data not shown). The two reagent grade samples were analyzed again during analysis of all samples.

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3. Results and Discussion

3.1 Inhibition

No difference in the level of PCR inhibition was observed between the standard and EMSL kit assays for reagent grade and secondary effluent samples (data not shown). In contrast, one additional dilution was required to remove inhibition of groundwater samples when assayed by the standard procedure than when assayed using the EMSL kit format. Secondary effluents were inhibited even at a 1:25 dilution with both formats. The number of available EMSL RT plates for the kit format was limiting. As a result, higher sample dilutions could not be investigated and the 1:25 sample dilutions were used in subsequent virus assays. For groundwater samples the dilutions for subsequent assays were chosen based upon the standard assay to provide direct comparisons of Cq values and genomic copy numbers. The reason for the difference in inhibition between assay types is unclear, but may be related to storage temperature. In support of this assumption the mean Cq value for the hepatitis G PCR assay for all 18 samples tested was 1.8 ± 0.8 Cq units higher for the standard assays than for the EMSL kit assays. Both assay formats used the same reagent lots, but reagents for the standard assay were stored at -20°C while those for the EMSL kit were stored at -80°C. It is likely that lower stability of enzymes or oligonucleotide primers and probes at -20°C led to greater sensitivity to inhibition.

3.2 Assay Performance

The performance of the standard and EMSL kit assays were compared by examining the overall number of detects versus non-detects, difference in mean Cq values between the assay types, and by the derived virus quantities. Tables 4 (enterovirus), 5 (norovirus GI), and 6 (norovirus GII) show the number of detects, the mean Cq values, and quantities for each matrix.

The number of detects and non-detects and Cq values are a function of initial virus concentrations in the PE seed and the dilution required for removal of inhibition. The concentrations were very low for the SE samples, due to the use of 1:25-fold dilutions and the presence of PCR inhibitors, and for the norovirus GII assays. As a result of these low concentrations, Cq values for these assays were in the 39-40 range. Although these values are high, they represent true positive results. Each result checked individually demonstrated typical qPCR profiles well separated from negative samples. Figure 1 shows the profile for the NoVGII standard assays and the degree of separation between positive and negative samples that was seen with all assays.

All samples from each matrix type analyzed for norovirus GI produced positive results with both the standard and EMSL kit assays (Table 5). In contrast, the standard enterovirus and norovirus GII assays showed 7% and 20% more non-detections than the EMSL kit assay, respectively. Each of the enterovirus and norovirus assays resulted in a higher Cq value in the standard assay (overall difference of 0.6±1.0 Cq units) with the greatest difference being the norovirus GII assay (Tables 4 and 6). Both the Cq differences and the numbers of non-detections may be related to storage conditions or shelf life as above. Small decreases in enzyme or primer/probe concentrations would increase the number of cycles required for a sample to become positive

(e.g., higher Cq values) and the number of non-detections of samples with very low starting virus genome concentrations. Differences in Cq values were tested for significance using the Kruskall-Wallis Anova on Ranks to compare data across all matrices for each virus assay and by the Mann-Whitney Rank Sum Test for comparing the standard versus EMSL kit results for each individual matrix. While it appears that the EMSL kit assays were better than the standard assays on the basis of detection rates and Cq values, the differences are not significant by the Kruskal-Wallace test; however, the differences between the standard and EMSL kit results for the reagent grade water matrix and for all matrices combined were significant by the Mann-Whitney Rank Test (Table 6).

The genomic quantity values shown in Tables 4-6 are calculated using Equation 1, which gives the number of genomic copies present in the PE seed added to each sample. These values were based on standard curves that met the acceptance criteria for both the standard and EMSL kits assays (Table 7). Although the Cq values of the samples were higher for the standard assays than for the EMSL kit assays, it was expected that a similar difference would have been observed with the standard curves. This would have normalized the differences, however, the genomic copy quantity values generally were higher for the standard than the EMSL assay. The higher values resulted from a greater Cq (1.3±0.6) difference between the standard curves for the standard assay and the EMSL kit assays versus the Cq difference in samples analyzed by the two formats. As above, the differences are not significant when compared using the Kruskal-Wallace test, but there were significant differences between most enterovirus assays (Table 4) and half of the norovirus GI assays (Table 5) by the Mann-Whitney Test.

Table 4. Comparison of Mean Enterovirus Cq and Genomic Copy Values

Sample	Standard Assay Cq ± S.D. [a]	Standard Assay Log GC ± S.D.	Standard Assay N [b]	EMSL Kit Cq ± S.D.	EMSL Kit Log GC ± S.D.	EMSL Kit n
RG	36.46 ± 1.07	6.75 ± 0.48†	18	35.98 ± 1.07	6.44 ± 0.45†	18
SE	40.01 ± 0.43	6.79 ± 0.14	7	39.62 ± 0.9	6.49 ± 0.31	11
GW	36.02 ± 1.48	7.76 ± 0.31†	18	35.78 ± 1.21	7.36 ± 0.23†	18
Overall	36.85 ± 1.85	7.18 ± 0.62†	43	36.74 ± 1.94	6.80 ± 0.56†	47

[a] S.D. = standard deviation; GC = genomic copies

b Each sample was analyzed in triplicate, resulting in an n of 6 × 3 = 18 when all replicates produced positive values.

† The comparisons indicated by this symbol are statistically significant at P <0.05.

Table 5. Comparison of Mean Norovirus GI Cq and Genomic Copy Values

Sample	Standard Assay Cq ± S.D. [a]	Standard Assay Log GC ± S.D.	Standard Assay n	EMSL Kit Cq ± S.D.	EMSL Kit Log GC ± S.D.	EMSL Kit n
RG	36.35 ± 0.98	4.60 ± 0.51	18	34.28 ± 0.98	4.33 ± 0.37	18
SE	34.17 ± 1.18	5.88 ± 0.34	18	33.44 ± 1.39	5.80 ± 0.39	18
GW	33.31 ± 1.20	5.79 ± 0.25†	18	33.20 ± 1.22	5.22 ± 0.21†	18
Overall	33.81 ± 1.19	5.43 ± 0.70†	54	33.64 ± 1.27	5.22 ± 0.72†	54

† The comparisons indicated by this symbol are statistically significant at P <0.05.

Table 6. Comparison of Mean Norovirus GII Cq and Genomic Copy Values

Sample	Standard Assay Cq ± S.D. [a]	Standard Assay Log GC ± S.D.	Standard Assay n	EMSL Kit Cq ± S.D.	EMSL Kit Log GC ± S.D.	EMSL Kit n
RG	39.24 ± 1.16†	4.22 ± 0.58	13	38.28 ± 1.44†	4.15 ± 0.53	17
SE	39.65 ± 1.17	5.16 ± 0.33	9	38.32 ± 1.46	5.34 ± 0.37	15
GW	38.58 ± 1.42	5.12 ± .030	17	35.78 ± 1.21	5.17 ± 0.23	18
Overall	39.05 ± 1.33†	4.80 ± 0.63	39	38.06 ± 1.42†	4.87 ± 0.66	50

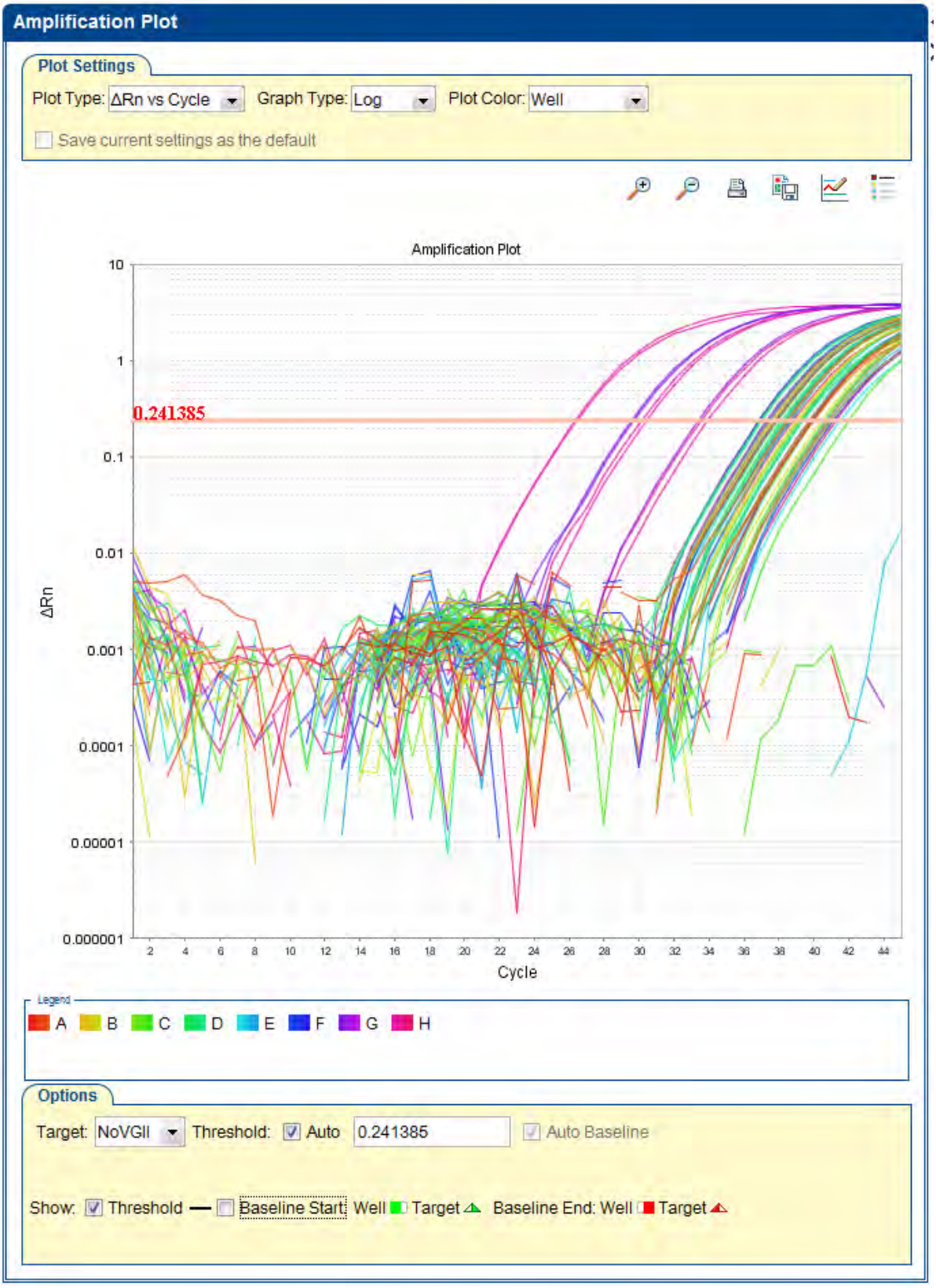
† The comparisons indicated by this symbol are statistically significant at P <0.05.

Table 7. Standard Curve Acceptance Criteria [a]

Criteria	Standard Assay Enterovirus	Standard Assay GIB	Standard Assay GII	EMSL Kit Enterovirus	EMSL Kit GIB	EMSL Kit GII
Slope	-3.85	-3.26	-3.72	-3.11	-3.39	-3.73
% Efficiency	82	103	86	109.5	97	85
R^2	0.99	1.0	.998	1.0	0.99	1.0
Overall S.D.	0.5	0.5	0.2	0.1	0.5	0.4

[a] Acceptable values: % efficiency of 80-110%, R^2 >0.97, and overall standard deviations ≤ 0.5.

Figure 1. Norovirus GII Amplification Plot for Standard Assay



4. Conclusions

It was expected that the standard assay would outperform the EMSL kit. The kit required the RT and PCR enzymes to be frozen under ionic conditions that had not been tested for stability and at -80°C rather than the manufacturers' recommend storage at -20°C. In addition, the use of the EMSL kit requires thawing and refreezing the enzymes which could have led to loss of activity. It is concluded that the conditions used in preparation of the kit do not adversely affect enzymatic activity.

Although there is a statistically significant difference between the standard and EMSL kits for some assays, the differences are very minor and not consistent. The inconsistency stem from the fact that the EMSL kits statistically outperforms the standard assay when Cq values and non-detections are considered while the standard assay is best when genomic quantities are being evaluated. It is concluded that the differences lack biological significance and therefore, either format may be used. Further testing is needed to determine the useable shelf life of the kits.

The EMSL kits were easier to use and less labor intensive than the standard assay. For large studies within a single laboratory or across multiple laboratories the use of the kit should reduce analyst error as well as intralaboratory and interlaboratory variability.

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