Abstract

Evaluation and improvement of real-time PCR methods for detecting norovirus

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Noroviruses are widespread and contagious viruses causing nonbacterial gastroenteritis. Real-time RT-qPCR is currently the gold standard for sensitive and accurate detection for noroviruses and serves as a critical tool for outbreak investigations. However, different surveillance teams may use different assays and the variability of specimen conditions may produce disagreement in results. Furthermore, the norovirus genome is highly variable and continuously evolving. These issues necessitate the re-examination of the real-time RT-qPCR's robustness in the context of

norovirus detection as well as the investigation of practical strategies to enhance assay performance. Four real-time reverse transcription quantitative PCR (RT-qPCR) assays (Assays A-D) were run in parallel to evaluate the performance of different real-time RT-qPCR assays in the detection of norovirus genogroups I and II. Characteristics such as PCR efficiency and limits of quantification and detection were investigated to assess the precision and analytical sensitivities of the assays. RT-PCR assays were also performed to investigate the comparative sensitivity and specificity of the real-time RT-qPCR assays. Overall, Assay D was evaluated to be the most precise and accurate assay in the detection of both norovirus GI and GII. A Zen internal quencher was placed within the probe sequence of Assay D, which further improved the detection range of the assay. This study compared several detection assays for noroviruses and an improvement strategy based on such comparisons provided useful characterizations of a highly optimized real-time RT-qPCR assay for norovirus detection.

Key words: Norovirus, RT-qPCR, sensitivity, limit of quantification, limit of detection, internal quencher

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I. Introduction

Human noroviruses belong to the *Norovirus* genus within the family *Caliciviridae*. They are small (27-40 nm), non-enveloped, icosahedral virions that possess a linear, positive-sense, single-stranded RNA genome[1]. Symptoms caused by human noroviruses are mainly vomiting and diarrhea, but also include nausea, abdominal cramps, and fever, which usually do not last longer than 48 hours[2]. However, there have been reports of infections leading to more severe and chronic illness and even death, particularly for the elderly (>65 years of age) and those with previous medical conditions[3].

Epidemiological studies have consistently provided evidence that human noroviruses are the etiological agents of widespread food and waterborne gastroenteritis via the fecal-oral route. Human noroviruses are characterized by a low infectious dose[4] and are well-known for their persistent survival in the environment including contaminated surfaces[5,6]. It has been demonstrated that virus contamination on surfaces are easily transferred to the hand, and outbreaks commonly occur through contamination of food products by infected food handlers[7-9]. Foodborne norovirus outbreaks have occur through oysters

being contaminated by fecal discharge[10-12], as well as frozen berries contaminated from food handlers[13,14].In a 2015 surveillance report by the U.S. National Outbreak Reporting System, noroviruses were found to be the most frequently reported cause of acute gastroenteritis outbreaks transmitted through person-to-person contact, environmental contamination, and unknown modes of transmission in the USA[15].

Since noroviruses are highly infectious and persistent in the environment, the development of rapid and accurate diagnostic methods has been of key interest in outbreak management[16]. Without a cell culture system, norovirus detection has mainly relied on molecular methods such as detection through immunological methods and nucleic acid amplification methods. Immunological methods such as enzyme immunoassays (EIAs) and the use of recombinant virus-like particles (VLPs) depend upon antibodies to recognize the norovirus viral capsid. However, the antigenic diversity of noroviruses has hindered these methods to be highly sensitive, broadly reactive, nor readily applicable[17-23]. Therefore, detection of noroviruses through nucleic acid amplification and detection through real-time RT-PCR has become the gold standard of accuracy and rapidity[16]. The RNA genome of noroviruses consist of three open reading frames (ORF1, ORF2, and

ORF3), and of these, the short, highly conserved ORF1/ORF2 junction region is used to rapidly detect noroviruses on the real-time RT-qPCR platform[9]. Several studies have successfully developed and established RT-qPCR assays targeting this site[24-27].

Scientific literature on noroviruses has greatly increased in recent years in both high-income and low-income countries precisely due to the increasing use and availability of such molecular diagnostic methods[28]. Real-time RT-qPCR has essentially become the accepted universal method for reporting norovirus detection. Although previously established norovirus real-time RT-qPCR assays have consistently shown similarly high accuracy, the genetic variability of noroviruses inevitably risks poor universality. Studies have already evidenced the frequent genetic recombination events that occur within the norovirus genome, including the supposedly "conserved" ORF1/ORF2 junction region[29,30]. Multicenter evaluations have shown that different laboratories and surveillance systems using different methods can produce different results with the same specimens[31,32]. Another challenge to robust norovirus surveillance is detection in environmental samples, which can come in various conditions and are characterized by very low levels of viral copy numbers[33,34]. Detection

of noroviruses in a wide variety of environmental settings is critical for epidemiological studies, but can be extremely challenging due to several factors such as specimen characteristics and variable viral load. Several European nations have consigned to the ISO method (ISO/TS 15216-1, 15216-2)[35], a recently established protocol for norovirus real-time RT-qPCR. The U.S. CDC also have their own established composite reference method[36], but equally sensitive and universally accepted detection methods may not be widely available to match the global scale of norovirus outbreaks. Real-time PCR is the gold standard for norovirus detection but issues remain to be solved on how to effectively apply the method in a wide variety of settings and to infer accurate descriptions of the epidemiology and genetic evolution of noroviruses[1].

Therefore, evaluation and comparison of different RT-PCR and real-time RT-qPCR assays will provide useful insight to examine consistency between assays. This study investigated real-time RT-qPCR assay characteristics such as PCR efficiency, limits of quantification and detection, as well as sensitivity and specificity in order to make comparisons between several real-time RT-qPCR assays and RT-PCR assays. Based on such assessment, the effect of an internal quencher in

the probe site was investigated on its effect on assay sensitivity.

II. Materials and Methods

1. Sample preparation

Archived, numbered human fecal samples (n=61) from norovirus infected patients were obtained from the Korean Center for Disease Control and stored at -80°C until use. The samples were prepared as 10% suspensions in phosphate-buffered saline (PBS) and were subject to centrifugation at 20,000 × g, for 20 min at 4°C[24]. One hundred microliters of the resulting supernatants were used for RNA extraction using the QIAamp® MiniElute® Virus Spin Kit (Qiagen, Hilden, Germany) and eluted to 100 μL. Extracted RNA were stored at -20°C prior to amplification.

2. Real-time RT-qPCR

Real-time RT-qPCR was performed on archived samples using widely referenced primer sequences and probes[24-27] (hereafter referred to as Assay A, Assay B, Assay C, and Assay D) (Table 1).

Monoplex assays were performed in 25 μL reaction mixtures containing 5 μL of RNA samples, prescribed concentrations of primers and probes (Table 2) for each GI and GII assay, 12.5 μL of 2 × RT-PCR buffer, 1 μL of 25 × RT-PCR enzyme mixture, and 1.67 μL of Detection Enhancer using the AgPath-ID One-Step RT-PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The PCR reaction was performed twice consecutively in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the prescribed conditions for each assay (Table 2).

Viral copy number was quantified using dilutions of Norovirus RNA Positive Control (AccuPower® Norovirus Real-Time RT-PCR Kit; Bioneer, Daejeon, Republic of Korea). All samples were run in duplicates and each assay included a duplicate of no template control. Baseline threshold was maintained at 0.1.

Table 1. Real-time RT-qPCR primer and probe sequences.

Assay	Genogroup	Oligonucleotide (polarity)	Sequence (5' – 3')[a]	Location[b]
A	GI	COG1F (+)	CGYTGGATGCGNTTYCATGA	5291
		COG1R (-)	CTTAGACGCCATCATCATTYAC	5375
		RING1(a)-TP (-)	FAM-AGATYGCGATCYCCTGTCCA-TAMRA	5340
		RING1(b)-TP (-)	FAM-AGATCGCGGTCTCCTGTCCA-TAMRA	5340
	GII	COG2F (+)	CARGARBCNATGTTYAGRTGGATGAG	5003
		COG2R (-)	TCGACGCCATCTTCATTCACA	5100
		Ring2-TP (+)	JOE[c]-TGGGAGGGCGATCGCAATCT-TAMRA[d]	5048
B	GI	JJV1F (+)	GCCATGTTCCGITGGATG	5282
		JJV1R (-)	TCCTTAGACGCCATCATCAT	5377
		JJV1P (+)	FAM-TGTGGACAGGAGATCGCAATCTC-TAMRA	5319
	GII	JJV2F (+)	CAAGAGTCAATGTTTAGGTGGATGAG	5003
		COG2R (-)	TCGACGCCATCTTCATTCACA	5100
		Ring2-TP (+)	JOE[c]-TGGGAGGGCGATCGCAATCT-TAMRA[d]	5048

Table 1. (cont.)

C	GI	NKP1F (+)	GCYATGTTCCGYTGGATG	5282
		NKP1R (-)	GTCCTTAGACGCCATCATCAT	5378
		NKP1P (+)	VIC-TGTGGACAGGAGATCGC-MGB[e]	5319
	GII	NKP2F (+)	ATGTTYAGRTGGATGAGATTCTC	5012
		NKP2R (-)	TCGACGCCATCTTCATTCAC	5100
		Ring2-TP (+)	JOE[c]-TGGGAGGGCGATCGCAATCT-TAMRA[d]	5048
D	GI	COG1F (+)	CGYTGGATGCGNTTYCATGA	5291
		COG1R (-)	CTTAGACGCCATCATCATTYAC	5375
		RING1(a)-TP (-)	FAM-AGATYGCGATCYCCTGTCCA-TAMRA	5340
	GII	BPO-13 (+)	AICCIATGTTYAGITGGATGAG	5007
		BPO-13N (+)	AGTCAATGTTTAGGTGGATGAG	5007
		BPO-14 (-)	TCGACGCCATCTTCATTCACA	5101
		BPO-18 (+)	VIC-CACRTGGGAGGGCGATCGCAATC-TAMRA	5044

[a]Mixed bases in degenerate primers and probes are as follows: Y = C or T; R = A or G; B = not A; N = any; I = inosine; H = A, C, or T

[b]GI primer sequences correspond to position in Norwalk/68 virus (accession no. M87661); GII primer sequences in Assay A correspond to position in Camberwell virus (accession no. AF145896); GII primer sequences in Assay B, Assay C, Assay D, and RT-PCR assays correspond to position in Lordsdale virus (accession no. X86557)

[c]6-Carboxyfluorescein

[d]6-carboxy-tetramethylrhodamine

[e]Minor Groove Binder

Table 2. Primer concentrations and cycling conditions.

		Assay			
		A	B	C	D
GI oligonucleotides (nM)	Forward	400	250	200	400
	Reverse	400	250	200	400
	Probe	600, 200	100	100	200
GII oligonucleotides (nM)	Forward	400	250	200	400, 400
	Reverse	400	250	200	400
	Probe	200	100	100	200
PCR conditions	RT	45°C, 30 m	45°C, 30 m	45°C, 30 m	45°C, 30 m
	Predenaturation	95°C, 10 m	95°C, 10 m	95°C, 10 m	95°C, 10 m
	Denaturation*	95°C, 15 s	95°C, 15 s	95°C, 15 s	95°C, 15 s
	Annealing and extension*	56°C, 60 s	60°C, 60 s	60°C, 60 s	56°C, 60 s
	Number of cycles	45	50	50	45

* = 1 cycle

3. Conventional RT-PCR and Sequencing

RNA samples were subjected to conventional RT-PCR using a semi-nested procedure using COG1F, G1SKF, G1SKR primer sets[37] and GI-F1M, GI-F2, GI-R1M primer sets[38] for GI, and COG2F, G2SKF, G2SKR primer sets[37] and GII-F1M, GII-F3M, and GII-R1M primer sets[38] for GII detection (Table 3). One-step RT was carried out using the QIAGEN® OneStep RT-PCR Kit (Qiagen, Hilden, Germany).

Amplification of the first PCR product was carried out with RT at 45°C for 30 min, initial denaturation at 94°C at 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and final extension at 72°C for 7 min. Semi-nested PCR was performed using the EmeraldAmp® PCR Master Mix (Takara Bio Inc., Kusatsu, Japan). The second product was amplified with initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and final extension at 72°C for 7 min. The products were analyzed on 1.5% agarose gel. The RT-PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced with 3730xl DNA analyzer (Macrogen, Seoul, Republic of Korea). Genotyping was based on nucleotide sequence comparisons and multiple sequence alignments using the BLASTN program (NCBI).

Table 3. RT-PCR primer sequences.

Reference	Genogroup	Oligonucleotide (polarity)	Sequence (5' – 3')[a]	Location[b]
Kojima et al. (2002)	GI	COG1F (+)	CGYTGGATGCGNTTYCATGA	5291
		G1SKF (+)	CTGCCCGAATTYGTAAATGA	5342
		G1SKR (-)	CCAACCCARCCATTRTACA	5671
	GII	COG2F (+)	CARGARBCNATGTTYAGRTGGATGAG	5003
		G2SKF (+)	CNTGGGAGGGCGATCGCAA	5058
		G2SKR (-)	CCRCCNGCATRHCCRTTRTACAT	5401
Kim et al. (2005)	GI	GI-F1M (+)	CTGCCCGAATTYGTAAATGATGAT	5342
		GI-F2 (+)	ATGATGATGGCGTCTAAGGACGC	5358
		GI-R1M (-)	CCAACCCARCCATTRTACATYTG	5649
	GII	GII-F1M (+)	GGGAGGGCGATCGCAATCT	5049
		GII-F3M (+)	TTGTGAATGAAGATGGCGTCGART	5079
		GII-R1M (-)	CCRCCIGCATRICCRTTRTACAT	5367

[a]Mixed bases in degenerate primers and probes are as follows: Y = C or T; R = A or G; B = not A; N = any; I = inosine
[b]GI primer sequences correspond to positions in Norwalk/68 virus (accession no. M87661); GII primer sequences correspond to positions in Lordsdale virus (accession no. X86557)

4. Analysis of data

The performance of real-time RT-qPCR assays were evaluated by their PCR efficiency and limits of quantification and detection (LOQ and LOD, respectively). Cycle threshold (Ct) values of the limits were used as cut-off values. In determining LOD, results showing duplicate consistency and a typical sigmoid curve were redefined as positive. Quantifications of less than 1 genomic copy per reaction were considered as non-specific amplification and thereby defined as negative.

PCR efficiency was determined by the equation:

$$\text{Efficiency (E)} = 10^{-1/slope} - 1 \quad (1)$$

Sensitivity and specificity was determined for each real-time RT-qPCR assay according to sequencing results from RT-PCR reactions.

Sensitivity was determined by the following equation,

$$\text{Sensitivity (Se)} = \frac{Number\ of\ true\ positives}{Number\ of\ true\ positives + Number\ of\ false\ negatives} \quad (2)$$

Specificity was determined by the following equation,

$$\text{Specificity (Sp)} = \frac{Number\ of\ true\ negatives}{Number\ of\ true\ negatives + Number\ of\ false\ positives} \quad (3)$$

Real-time RT-qPCR and RT-PCR were further compared by Venn diagram analysis to determine the most diagnostically and analytically

accurate assay for norovirus detection.

5. Properties of real-time RT-qPCR primers and probes

Targets sites of real-time RT-qPCR primers and probes were compared to 7 GI and 11 GII complete genome sequences obtained from NCBI Genbank through multiple alignment with CLC Genomics Workbench version 6.5.

Primer length and values of melting temperature, and the number and types of degenerate bases used were investigated. Mean values of melting temperature were calculated using the OligoAnalyzer version 3.1 (IDT®, Coralville, Wisconsin, U.S.A.) available at http://sg.idtdna.com/calc/analyzer, based on the qPCR parameter set, targeting RNA, the given oligonucleotide concentrations (Table 2), 50 mM Na^{+}, 3 mM Mg^{++}, and 0.8 mM dNTPs.

6. Assay D modification

A Zen quencher (IDT®, Coralville, IA, USA.), an internal quencher, was added to the probe sequence from assay D (Figure 1). Fresh RNA extractions were used to run Assay D and Assay D-zen each twice consecutively on 30 selected GII samples, using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Assay D-zen followed the same primer concentrations and cycling conditions as Assay D (Table 2). Duplicates of positive GII.4 control, no template control, as well as duplicates of a GI sample and MNV sample were included in the test. Baseline threshold was maintained at 0.1 as in other assays.

Figure 1. Probe D-zen structure. A Zen internal quencher was included in the 9th base from the 5' end.

III. Results

1. Real-time RT-qPCR efficiency

RNA Positive Control was diluted to produce a 6-fold dilution standard curve ranging from 1.0×10^5 to 1.0×10^0 genomic copies per reaction. Standard curves generated for GI (Figure 2) and GII (Figure 3) assays are shown.

The respective PCR efficiencies, correlation coefficient values (R^2), limit of quantification (LOQ), and limit of detection (LOD) are summarized in Table 4. PCR efficiencies were calculated by equation (1). The mean PCR efficiency was 101.9% ± 0.031. The mean R^2 value was 0.993 ± 0.006. With respect to PCR efficiency and R^2 value, the most precise assays were Assay A for GI and Assay C for GII.

LOQ and LOD of each respective assays were defined in genomic copies per reaction and translated to cut-off Ct values. Assay A and D were shown to have the most sensitive limit of quantification as well as limit of detection for both GI and GII.

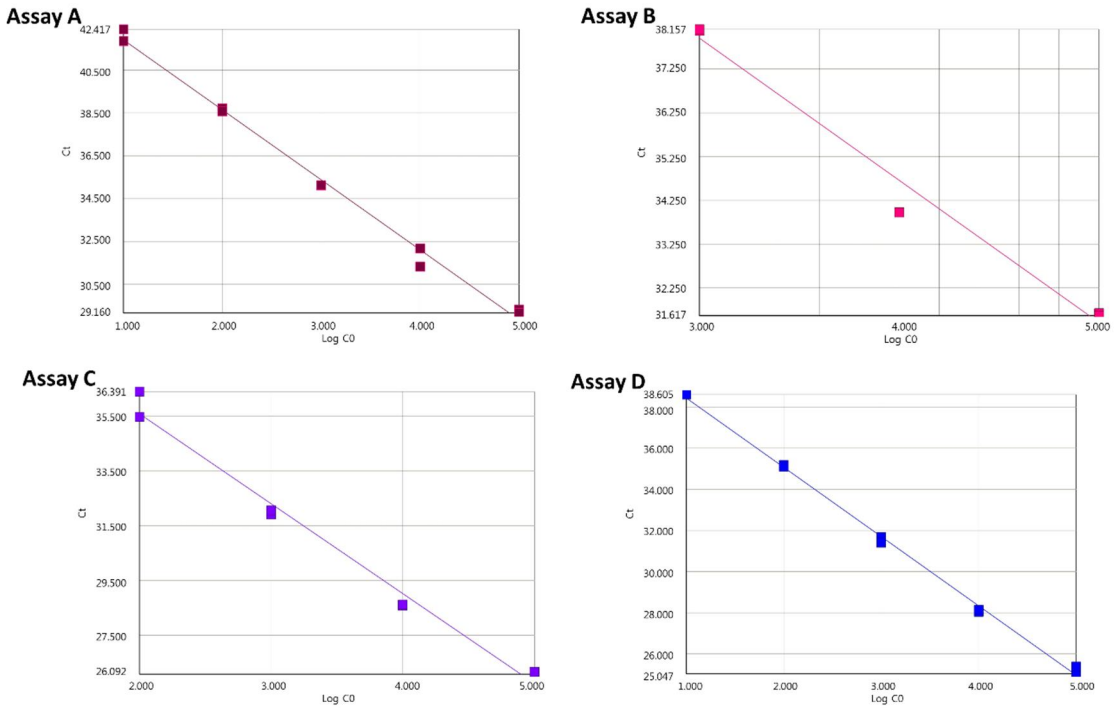

Figure 2. Real-time RT-qPCR standard curves for GI detection. Assay A (R^2: 0.993) and Assay D (R^2: 0.997) are shown to have better efficiency and limits of quantification than Assay B (R^2: 0.984) and Assay C (R^2: 0.986). However, Assay A and B showed abnormally late amplification compared to Assay C and D.

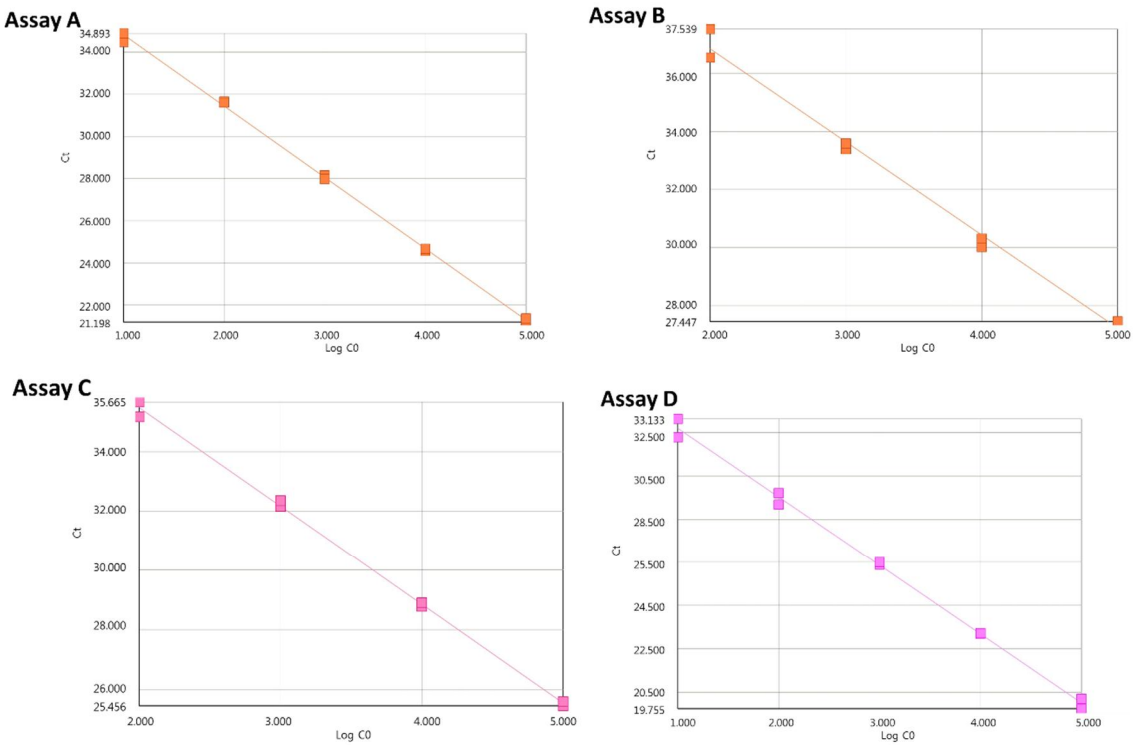

Figure 3. Real-time RT-qPCR standard curves for GII detection. Assay A (R^2: 0.999) and Assay D (R^2: 0.997) are shown to have better limits of quantification than Assay B (R^2: 0.991) and Assay C (R^2: 0.998).

Table 4. Real-time RT-PCR performance calculations for assays A-D.

Genogroup	Performance	Assay			
		A	B	C	D
GI	Efficiency (%)	102.2	103.2	101.9	98.2
	R^2	0.993	0.984	0.986	0.997
	LOQ(copy/rxn)	1	3	2	1
	LOQ(Ct value)	42.1	38.1	35.9	38.1
	LOD(copy/rxn)	1	1	1	1
	LOD(Ct value)	42.1	42.6	41.8	38.1
GII	Efficiency (%)	97.5	104.7	100.6	106.8
	R^2	0.999	0.991	0.998	0.997
	LOQ(copy/rxn)	1	2	2	1
	LOQ(Ct value)	34.7	37.0	35.4	32.7
	LOD(copy/rxn)	0	0	1	0
	LOD(Ct value)	40.1	42.6	43.0	36.6

2. Comparison of real-time RT-qPCR and RT-PCR

Out of a total of 61 samples tested, RT-PCR confirmed 14 positives for GI (22.9%) and 25 positives for GII (40.9%). Sequencing of RT-PCR products confirmed 7 GI genotypes (GI.1, GI.3, GI.4, GI.5, GI.6, GI.8, GI.9) and 5 GII genotypes (GII.2, GII.4, GII.6, GII.13, GII.17).

Positive rates for each respective real-time RT-qPCR assays were calculated and sensitivity and specificity was calculated using equations (2) and (3) using confirmed sequences from RT-PCR products as the gold standard (Table 5). Compared to GII assays, GI assays were less prone to changes in results when the detection range was expanded to LOD. Using the LOD detection range provided 4-7 extra Ct value for signal detection and thereby improved sensitivity by up to 8-fold in the case of Assay C for GII. While this implied improved consistency between real-time RT-qPCR and RT-PCR, it also risked trade-off in specificity, where specificity for Assay D for GII decreased by nearly 2-fold.

Using the LOD range, positive results of real-time RT-qPCR assays and positive results of RT-PCR assays were overlaid on Venn diagrams (Figure 4). By combining positive results of both real-time RT-qPCR and RT-PCR, there were a total of 24 positives for GI and 34 positives

for GII.

Figure 5 shows the distribution of RT-PCR confirmed genotypes in respect to the results of each real-time RT-qPCR assay. Genotypes were more often detected as false negatives rather than as true positives in most of the assays except in Assay A and Assay D for GI.

Table 5. Positive detection rate of 4 real-time RT-PCR assays and their sensitivity and specificity compared to RT-PCR.

Genogroup		Assay A		Assay B		Assay C		Assay D	
		LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD
GI	Positive rate (%)	15/57 (26.3)	15/57 (26.3)	7/57 (12.3)	9/57 (15.8)	4/57 (7.0)	9/57 (15.8)	13/57 (22.8)	13/57 (22.8)
	Se (%)	57.1	57.1	28.6	28.6	14.3	35.7	50.0	50.0
	Sp (%)	83.7	83.7	93.0	88.4	95.3	90.7	86.0	86.0
GII	Positive rate (%)	8/42 (19.0)	15/42 (33.3)	2/42 (4.8)	9/42 (21.4)	1/42 (2.4)	14/42 (33.3)	7/42 (16.6)	18/42 (42.8)
	Se (%)	28.0	44.0	8.0	32.0	4.0	32.0	28.0	78.6
	Sp (%)	94.1	76.5	100.0	94.1	100.0	64.7	100.0	58.8

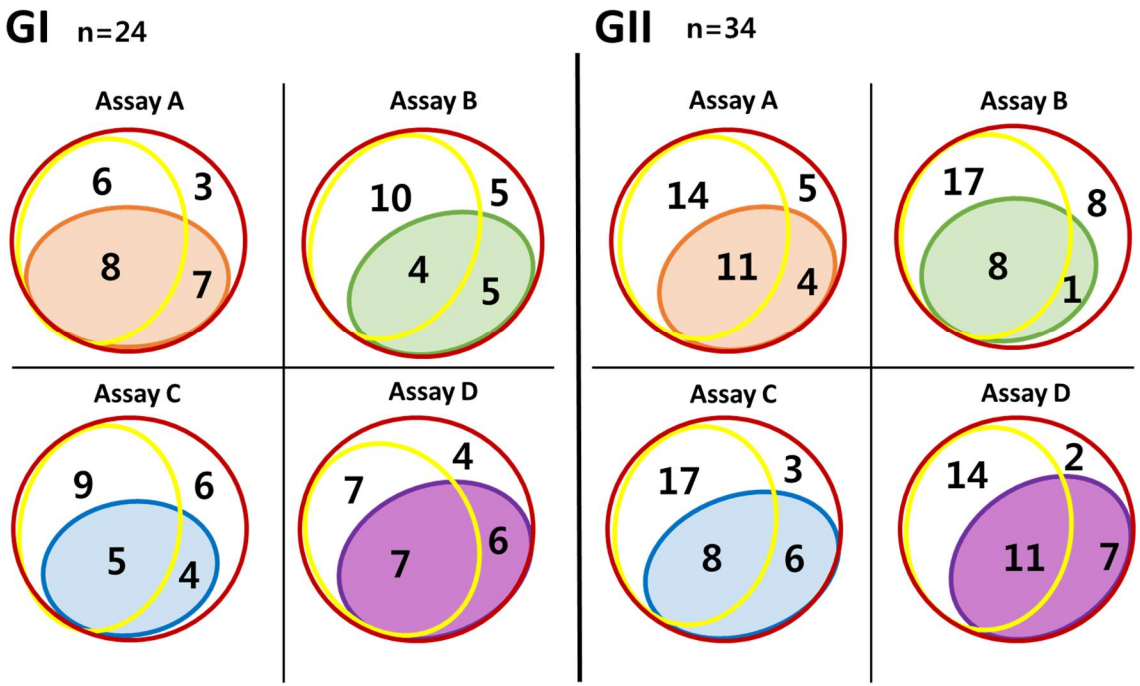

Figure 4. Venn diagram of positive results (left: GI; right: GII). The total number of positive results (24 for GI; 34 for GII) by real-time RT-qPCR and RT-PCR are represented by the red circle. The total number of positive results (14 for GI; 25 for GII) are represented by the yellow circle. The total number of positive results of each respective real-time RT-qPCR assays are represented in shaded circles in their respective colors.

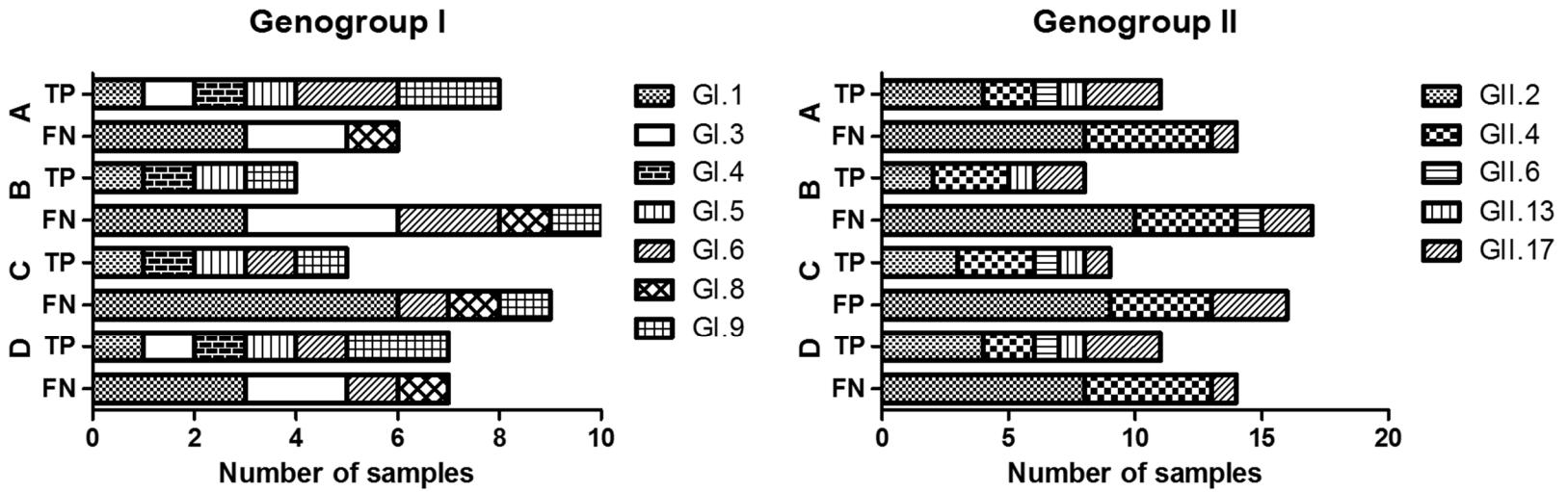

Figure 5. Distribution of norovirus genotypes detected as true positives and false negatives (left: GI; right: GII). Genotypes were more often detected as false negatives rather than as true positives in most assays for both GI and GII detection. In GI detection, Assay A and D performed better than Assay B and C in correctly detecting genotypes. In GII detection, Assay D performed best in correctly detecting genotypes.

3. Primer sequence alignment and primer properties

Seven GI sequences used for alignment with primer and probe sets included the Norwalk GI.1 reference strain, GI.4, GI.6, GI.8, and GI.9, which were detected in this study (Figure 6). Eleven GII sequences used for alignment included the Lordsdale GII.4 reference strain, GII.2, GII.6, GII.13, and GII.17 detected in this study. The sites for the forward primer, TaqMan probe, and reverse primers were identified and the degree of conservation in the target regions were visualized in bar graphs below the alignment. Primer length and values of melting temperature, the number and types of degenerate bases used are summarized in Table 6. Use of degenerate bases were mainly concentrated in the forward primers of all assays to compensate for the higher sequence variability of the region. Forward primers of Assay B and Assay C for GII contained relatively less degenerate bases than Assay A and D. Besides Assay A and D for GI, probe melting temperatures for all assays were calculated to be suboptimal. Detector and quencher fluorophores did not influence primer and probe melting temperatures significantly, except for MGB (Minor Groove Binder) quencher in Assay C for GI.

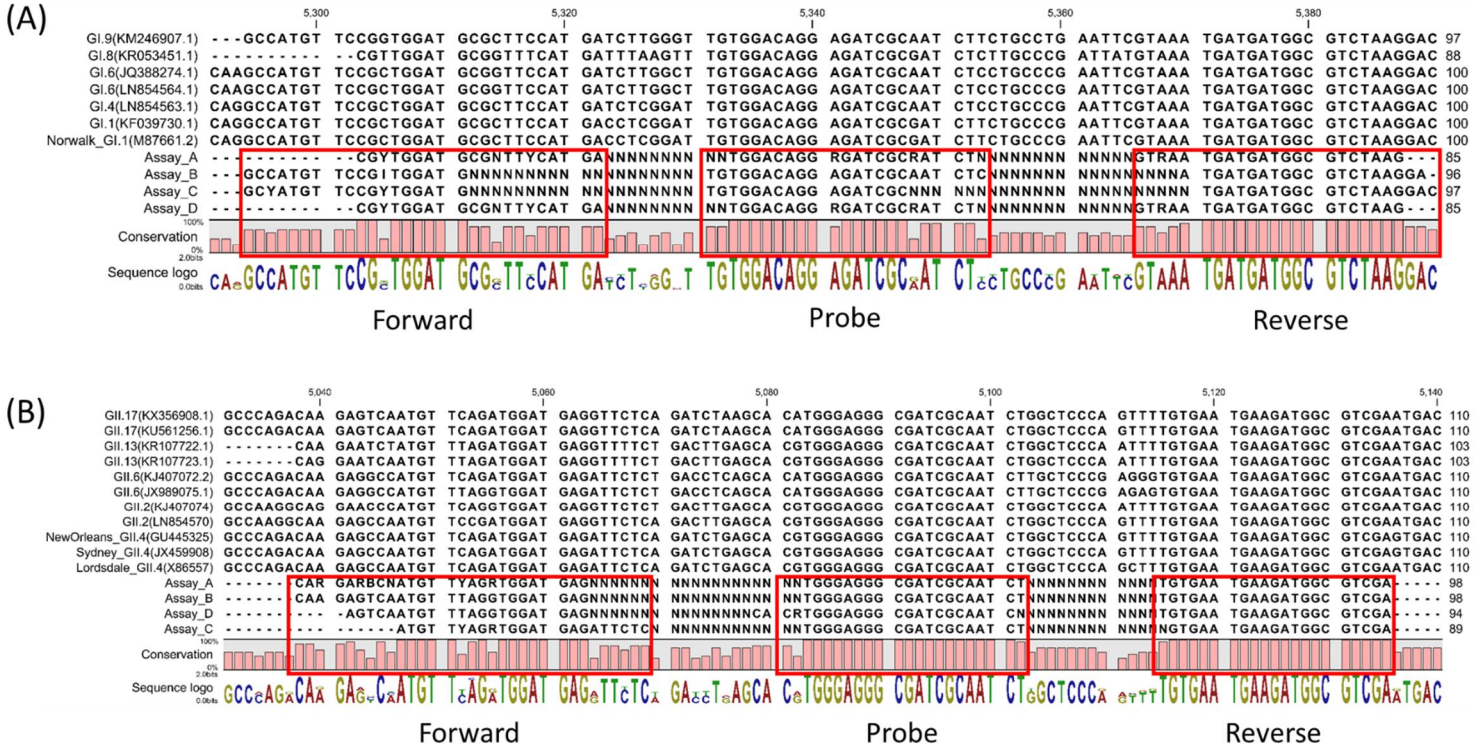

Figure 6. Multiple sequence alignment of real-time RT-qPCR primers and probes with complete genome sequences of representative strains for (A) GI genogroup, (B) GII genogroup. Target regions of all assays as well as the relative sequence conservation in those regions are shown in red boxes.

Table 6. Real-time RT-qPCR primer and probe properties.

		Forward Primer			Reverse Primer			Probe			
		Length	Tm (ºC)	Degeneracy	Length	Tm (ºC)	Degeneracy	Length	Tm (ºC)[a]	Degeneracy	Detector-Quencher
GI	Assay A	20mer	60.8	Y(2), N(1)	22mer	59.0	Y(1)	20mer, 20mer	67.4, 69.0	Y(2)	FAM-TAMRA
	Assay B	18mer	63.7	I(1)	20mer	60.6	-	23mer	60.9	-	FAM-TAMRA
	Assay C	18mer	60.6	Y(2)	21mer	63.4	-	17mer	54.4	-	VIC-MGB[b]
	Assay D	20mer	60.8	Y(2), N(1)	22mer	59.0	Y(1)	20mer	66.0	Y(2)	FAM-TAMRA
GII	Assay A	26mer	60.8	R(3), B(1), Y(1)	21mer	61.6	-	20mer	62.0	-	JOE-TAMRA
	Assay B	26mer	58.1	-	21mer	61.0	-	20mer	61.2	-	JOE-TAMRA
	Assay C	23mer	58.3	R(1), Y(1)	20mer	59.5	-	20mer	61.2	-	JOE-TAMRA
	Assay D	22mer, 22mer	63.5, 57.1	I(3)	21mer	61.6	-	23mer	64.5	R(1)	VIC-TAMRA

[a]Detector-quencher fluorophores were included in probe melting temperature calculations.
[b]MGB (Minor Groove Binder) probes are known to dramatically increase probe melting temperature. Due to licensure, MGB's effect could not be accounted for in the probe Tm calculation.

4. Effect of internal quencher on Probe D

Assay D and Assay D-zen were performed simultaneously on a panel of 30 select GII samples, a positive GII.4 control, no template control, a GI sample and MNV sample in order to test the effect of probe D-zen. Standard curves and amplification plots of the two assay are compared in Figure 7. Assay D-zen is shown to be the more stable reaction with an improved limit of quantification. Sample Ct values and their standard deviations are shown in Table 7.

Positive detection rate using probe D and probe D-zen was 24/30 (80%) and 25/30 (83%) respectively. Among the positives detected by probe D, 6/24 (25%) were within the LOQ range whereas 19/25 (76%) of positives detected by probe D-zen were within the LOQ range.

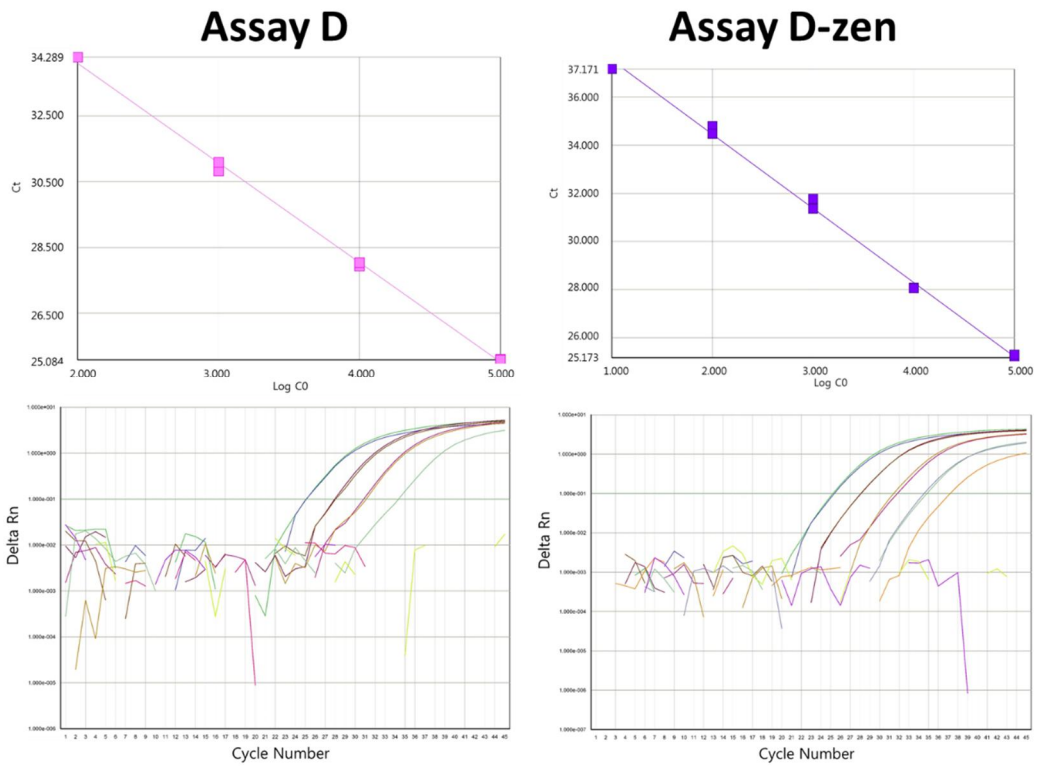

Figure 7. Standard curve and amplification plot comparison of probe D and probe D-zen. The PCR efficiency of Assay D (R^2: 0.998) is 114% and Assay D-zen (R^2: 0.996) is 111%.

Table 7. Effect of internal quencher on sample detection.

Sample no.	Assay D			Assay D-zen		
	Ct	Std dev[a]	Qty[b]	Ct	Std dev[c]	Qty[d]
65	35.3	-	+	37.3	0.144	+
67	32.9	0.873	++	32.5	0.115	++
68	34.9	0.489	+	34.5	0.679	++
69	Undet.	N.A.	N.A.	35.2	-	++
71	36.8	-	+	39.3	-	+
72	Undet.	N.A.	N.A.	38.0	-	+
73	32.4	0.192	++	33.3	0.029	++
74	28.3	0.137	++	29.1	0.192	++
75	35.7	0.948	+	36.9	-	++
76	38.8	2.81	+	35.8	-	++
84	Undet.	N.A.	N.A.	Undet.	N.A.	N.A.
85	36.5	-	+	36.7	0.716	++
86	35.5	-	+	36.6	-	++
87	35.7	0.073	+	36.8	-	++
88	38.1	0.264	+	Undet.	N.A.	N.A.
89	35.7	0.727	+	Undet.	N.A.	N.A.
90	36.1	-	+	36.7	-	++
91	Undet.	N.A.	N.A.	Undet.	N.A.	N.A.
92	33.3	0.314	++	35.6	0.536	++
93	Undet.	N.A.	N.A.	Undet.	N.A.	N.A.
94	29.7	0.651	++	30.4	0.264	++
95	F.P.	N.A.	N.A.	39.3	3.220	+
96	35.1	0.513	+	36.7	0.201	++
97	34.3	0.339	+	36.4	1.100	++
98	Undet.	N.A.	N.A.	Undet.	N.A.	N.A.
99	35.6	N.A.	N.A.	36.4	-	++
100	36.6	1.490	+	39.1	2.910	+
101	35.9	-	+	38.5	4.820	+
102	34.3	0.062	+	34.2	0.205	++
103	F.P.	N.A.	N.A.	Undet.	N.A.	N.A.
P.C.	26.3	0.382	++	27.2	0.056	++
N.T.C.	36.1	0.323	+	Undet.	N.A.	N.A.
GI	33.2	0.153	++	34.2	0.186	++
MNV	Undet.	N.A.	N.A.	Undet.	N.A.	N.A.

[a]Ct standard deviation range for probe D was 0.073-1.490

[b]Samples detected within the LOQ range (cut-off Ct 34.3) are shown as ++, samples detected within the LOD range (cut-off Ct 38.8) are shown as +

[c]Ct standard deviation range for probe D-zen was 0.029-4.820

[b]Samples detected within the LOQ range (cut-off Ct 37.2) are shown as ++, samples detected within the LOD range (cut-off Ct 39.3) are shown as +

IV. Discussion

Norovirus real-time RT-qPCR detection methods have been studied extensively over the past several years. Many well established assays have been and are still widely used in large-scale norovirus investigations[39,34]. These assays remain the mainstay both in outbreak and environmental investigations simply because they have become established routine, but also because the target site for norovirus real-time RT-qPCR cannot be easily changed[40]. The target site is the short, highly conserved ORF1/ORF2 junction region, which was reported to have high nucleotide similarity within a span of less than 300 base pairs for both GI and GII[41]. Outside of this region, noroviruses are renowned for their high genetic variability, several recombination events, and rapid genetic evolution[42-44]. The absolute quantification method is also the most widely used and probably most preferred analysis of norovirus real-time RT-qPCR results. Not only is a reference or a housekeeping gene for relative quantification difficult to define, it would be important to determine the precise quantification of viral load because of the low infectious dose of norovirus[4].

This study used the absolute quantification method to analyze

previously well-established real-time RT-qPCR assays[24-27] in monoplex to cross-compare the methods' accuracy in a same panel of archived samples. Comparisons were made by PCR efficiency, which demonstrates amplification performance, the correlation coefficient (R^2), which demonstrates the reliability of the experimental procedure, as well as the limits of quantification and detection, which demonstrates how sensitively the assay can detect norovirus in low quantities. Such comparisons on four real-time RT-qPCR assays (assays A-D) showed that assays A and D were able to consistently replicate sensitive limits of quantification to at least 1 log genomic copies per reaction, and thereby produced comparatively favorable detection outcomes for both GI and GII genotypes (Table 4).

All samples of this study showed considerably low concentrations of norovirus load. In assays B and C, particularly, the majority of samples were quantified beyond the LOQ range for both GI and GII genogroups (Table 5). The presence of consistent and regular amplification peaks beyond the LOQ range shows that the established real-time RT-qPCR assays are indeed highly optimized and automated platforms to sensitively detect extremely low viral loads independent of standard curve quality. Therefore, broadening the detection range as

much as possible was a crucial strategy for improving detection rate. Precise and accurate quantification of low viral loads by establishing broad and consistent detection range is critical with respect to clinical and environmental surveillance. The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines recommend the elimination of Ct values beyond 40 in order to avoid nonspecific detection[45]. However, this recommendation should be cautiously applied in the case of norovirus. Studies have increasingly emphasized the significance of detecting low norovirus load in clinically asymptomatic individuals[46,47]. Therefore, hasty elimination of large Ct values may cause underestimation of norovirus prevalence and overlook important relationships in norovirus epidemiology. Also, noroviruses are inevitably detected in large Ct values in food and environmental sampling surveys[48] which is most likely associated with poor efficiency in upstream nucleic acid recovery and extraction[49]. The sensitivity of the real-time RT-qPCR platform should be fully optimized in order to compensate for such upstream loss.

Many norovirus studies conduct real-time RT-qPCR on samples previously confirmed with EM (electron microscopy) or RT-PCR. Norovirus genotype identification is also conducted by RT-PCR[50].

Therefore, results of the real-time RT-qPCR assays were compared with semi-nested RT-PCR methods which have also been previously well characterized[37,38]. Sensitivity and specificity values generated and summarized in Table 5 indicate that considerable disagreement may occur between RT-PCR and real-time RT-qPCR results. RT-PCR confirmed that the sample pool consisted of 22.9% of GI and 40.9% of GII, including where the two genogroups were mixed. The diversity of these genotypes were 7 GI genotypes (GI.1, GI.3, GI.4, GI.5, GI.6, GI.8, GI.9) and 5 GII genotypes (GII.2, GII.4, GII.6, GII.13, GII.17). Such detection rates and genotype diversity were not reflective of actual outbreak settings, but nevertheless included clinically and environmentally significant strains GI.1, GI.3, GII.4, and GII.17. Comparison of real-time RT-qPCR and RT-PCR results indicated that all real-time RT-qPCR assays generally showed better performance in specificity rather than sensitivity. Rather than having implications for assay performance, such results likely occurred because controlling sample size of positive and negative specimens in the sample pool is also important[51,36]. Norovirus outbreak investigations using real-time RT-qPCR detection generally report >98% sensitivity and specificity values[32,52,53]. It is suspected that such high sensitivity and specificity

values can be more readily achieved because of the inevitably fresher, larger positive specimen pool while the sequence specificity of real-time RT-qPCR prevents significant trade-off in specificity. Agreement between the two methods were overlaid against each other on Venn diagrams (Figure 4). Comparison of several real-time RT-qPCR assays with RT-PCR assays suggested Assay A and Assay D showed greater diagnostic sensitivity compared to the other assays. Further investigations are required to define the degree of agreement between results from established RT-PCR and real-time RT-qPCR assays. Such investigations may serve to validate current norovirus screening procedures and, if necessary, suggest them for revision.

Studies have shown that noroviruses can display a strain-specific behavior in multiple scenarios such as in disinfection and human immunological susceptibility[54,55]. As shown in Figure 5, confirmed genotype positives were generally found as false negatives more often than true negatives. Assays A and D for GI and Assay D for GII showed less false negative detection than other assays. In GI detection, GI.4 and GI.5 samples were always detected as true positives. In GII detection, GII.6, GII.13, and GII.17 were consistently detected as true positives. These samples appeared as positives in all real-time RT-

qPCR assays with relatively high viral load and almost always quantified within the LOQ range. However, samples consistently showing high viral load in real-time RT-qPCR were negative for RT-PCR as well. None of the GI real-time RT-qPCR assays could detect GI.8. Assay C for GI could not detect any GI.3 samples.

Such differences in assay results may be due to the different sequences and other properties of real-time RT-qPCR primers and probes. Sequence alignment of primers and probe sets with the most representative GI and GII genotypes investigated in this study showed that variability in sequences were compensated by degenerate bases in most primers (Figure 6, Table 6). However, degenerate bases were less incorporated in assays B and C particularly for GII, which may have curtailed the LOQ range and caused delayed detection of samples in the LOD range. Alternatively, greater degeneracy in the forward primers of assays A and D coincided with better reproducibility of linear standard curves and sensitive LOQ. Interestingly, all assays showed suboptimal probe melting temperatures having less than 5°C difference with melting temperatures of primer oligomers[56] (Table 6). Probe sequences, however, were highly conserved, and it has been stated that detection specificity was more dependent upon probe sequences rather than

primer sequences[57].

In interpreting results, it is possible that poor RNA quality resulting from error in RNA extraction as well as the presence of PCR inhibition may have caused hampered nucleic acid amplification[40]. Norovirus detection has become a highly automated procedure with ready-to-use extraction kits and real-time PCR mixtures recommended by public health institutions[50]. The RNA extraction kit used in this study applies an established nucleic acid purification method using size-fractionated silica particles[58] to produce high quality and high yield of nucleic acids. Studies have validated this method to produce high RNA yield in clinical microbiology laboratories[59]. Good laboratory practice has also been adhered to while preparing PCR mixtures for each run. An RNase decontamination product was sprayed on the clean bench surfaces to avoid RNA degradation, and all laboratory equipment including pipettes, tips, and microtubes were sterilized with UV and 70% ethanol before use. Separate rooms were used for each step of mixture preparation. Despite these efforts for quality control, this study's findings may certainly be further validated by cross-confirmation of RNA quality as well as the absence of PCR inhibition.

This study evaluated four different real-time RT-qPCR assays and

demonstrated the importance of producing a broad detection range for reliable quantification, especially for samples with low viral load. Comparisons of real-time RT-qPCR results with parallel screening with RT-PCR further supports this point. Assays A and D, which demonstrated precise PCR reaction with tolerable agreement with RT-PCR results were characterized with more favorable probe Tm and oligo degeneracy to account for norovirus sequence variability. This provided insight into norovirus real-time RT-qPCR optimization approaches. Previously, standard controls were made from RNA transcripts and plasmid vectors to generate consistent, linear standard curves[60-62]. These constructs, however, are based on norovirus sequences which must be intermittently revised and renewed to keep up to date with the continuously evolving norovirus genome[43,63]. Finding and obtaining new strains to construct standard controls require a thorough search of epidemiological data and sequence analysis. Obtaining quality samples of key human norovirus strains may be difficult for research laboratories or environmental scientists without access to clinical samples. Exploring new target sites in the already existing ORF1/ORF2 region can be difficult because the short target site provides less flexibility for primer design[40,64]. An internal quencher

has been told to increase signal sensitivity by decreasing background noise, and has been previously applied to norovirus GII with relative success[65]. However, the study's target site was exceptionally large with complex sample processing including bead-mediated concentration. By applying the Zen internal quencher to probe D, a typical length oligomer for real-time PCR, and comparing it with the original version may better observe the internal quencher's effect on norovirus detection sensitivity.

Simultaneous runs using probe D and probe D-zen showed the zen quencher's potential in improving assay D's LOQ. The standard curve of Assay D determined its LOQ to be 2 log genomic copies per reaction, whereas Assay D-zen's LOQ was 1 log genomic copies per reaction (Figure 7). This demonstrated that the Zen internal quencher on Assay D possibly increased the linear dynamic range of the standard curve from four-fold to five-fold and therefore increased the sensitivity of assay D's LOQ. Ct values and quantification of samples resulting from these simultaneous runs also showed that Assay D-zen detected the majority of the samples (76%) within its LOQ range, whereas Assay D detected 25% of the samples within the LOQ range. Also, Assay D-zen did not produce false positive results which were observed in Assay D

(Table 7). Although the Zen internal quencher did not produce a dynamic change in Assay D, it was able to achieve more reliable detection and quantification. Therefore, the Zen internal quencher is an appealing option for conveniently optimizing existing assays. The simple inclusion of the Zen quencher eliminates time consuming efforts to optimize new primers and probe sets and can aid assay troubleshooting without excessive tampering with primer sequences. Validation of such improvement can be strengthened by testing on various sample types because this study only used archived fecal samples. Confirming the Zen internal quencher's effect on environmental samples would be particularly relevant as they are known for their low viral load and complex matrix which makes it difficult to extract quality RNA[66,67].

In summary, the characterizations of a highly sensitive norovirus real-time RT-qPCR assay were investigated. Increasing the detection range for absolute quantification of norovirus in real-time RT-qPCR suggest improved agreement with RT-PCR results as well as overall improved sensitivity without much trade-off in specificity even in low norovirus concentration samples. The utilization of a Zen internal quencher is a convenient improvement tool that has become available

through recent technological advances. This simple strategy has potential to improve existing norovirus methods precedent to developing entirely new assays to upgrade against the genetic evolution of norovirus strains. The high sensitivity of the real-time RT-qPCR platform, as well as the high genetic diversity of noroviruses, compels researchers to carefully consider potential pitfalls in experimental method and interpretation of results in order to translate laboratory results to public health action. Establishment of a definitive gold standard for norovirus real-time RT-qPCR detection assays in the future may allow for a clearer characterization of the impacts of sample quality and norovirus diversity on real-time RT-qPCR accuracy.

V. References

1. Lopman, B. *et al.* Global burden of norovirus and prospects for vaccine development. *Cdc* 1–46 (2015).

2. Lee, R. M. *et al.* Incubation periods of viral gastroenteritis: a systematic review. *BMC Infect. Dis.* **13,** 446 (2013).

3. Robilotti, E., Deresinski, S. & Pinsky, B. A. Norovirus. *Clin. Microbiol. Rev.* **28,** 134–164 (2015).

4. Teunis, P. F. M. *et al.* Norwalk virus: How infectious is it? *J. Med. Virol.* **80,** 1468–1476 (2008).

5. Cheesbrough, J. S., Green, J., Gallimore, C. I., Wright, P. A. & Brown, D. W. Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis. *Epidemiol. Infect.* **125,** 93–8 (2000).

6. Jones, E. L., Kramer, A., Gaither, M. & Gerba, C. P. Role of fomite contamination during an outbreak of norovirus on houseboats. *Int. J. Environ. Health Res.* **17,** 123–31 (2007).

7. Otter, J. A., Yezli, S. & French, G. L. The role played by contaminated surfaces in the transmission of nosocomial pathogens. *Infect. Control Hosp. Epidemiol.* **32,** 687–99 (2011).

8. Barker, J., Vipond, I. B. & Bloomfield, S. F. Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via

environmental surfaces. *J. Hosp. Infect.* **58,** 42–49 (2004).

9. Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, C. for D. C. and P. Updated norovirus outbreak management and disease prevention guidelines. *Cdc-Mmwr* **60,** 1–18 (2011).

10. Le Guyader, F. S., Atmar, R. L. & Le Pendu, J. Transmission of viruses through shellfish: when specific ligands come into play. *Curr. Opin. Virol.* **2,** 103–110 (2012).

11. European Food Safety Authority. Scientific Opinion on Norovirus (NoV) in oysters : methods , limits and control options. *EFSA J.* **10,** 2500–2539 (2012).

12. Berg, D. E., Kohn, M. A., Farley, T. A. & McFarland, L. M. Multi-state outbreaks of acute gastroenteritis traced to fecal-contaminated oysters harvested in Louisiana. *J. Infect. Dis.* S381-6 (2000). doi:10.1086/315581

13. Andreoletti, O. *et al.* Scientific Opinion on the risk posed by pathogens in food of non-animal origin . Part 2 (Salmonella and Norovirus in leafy greens eaten raw as salad. *EFSA J.* **12,** 1–118 (2014).

14. Falkenhorst, G. *et al.* Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. *Euro Surveill.* **10,** E050922.2 (2005).

15. Wikswo, M. E. *et al.* Outbreaks of Acute Gastroenteritis Transmitted by Person-to-Person Contact, Environmental Contamination, and Unknown Modes of Transmission - United States, 2009-2013. *MMWR. Surveill. Summ.* **64,** 1–16 (2015).

16. Vinjé, J. Advances in Laboratory Methods for Detection and Typing of

Norovirus. *J. Clin. Microbiol.* **53,** 373–381 (2014).

17. Kapikian, A. Z. *et al.* Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J. Virol.* **10,** 1075–81 (1972).

18. Herrmann, J. E., Nowak, N. A. & Blacklow, N. R. Detection of Norwalk virus in stools by enzyme immunoassay. *J. Med. Virol.* **17,** 127–33 (1985).

19. Hayashi, Y. *et al.* Western blot (immunoblot) assay of small, round-structured virus associated with an acute gastroenteritis outbreak in Tokyo. *J. Clin. Microbiol.* **27,** 1728–33 (1989).

20. Jiang, X., Wang, M., Graham, D. Y. & Estes, M. K. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66,** 6527–32 (1992).

21. Belliot, G. L. *et al.* Characterization of Capsid Genes, Expressed in the Baculovirus System, of Three New Genetically Distinct Strains of " Norwalk-Like Viruses " *J. Clin. Microbiol.* **39,** 4288–4295 (2001).

22. Nilsson, J., Rydell, G. E., Le Pendu, J. & Larson, G. Norwalk virus-like particles bind specifically to A, H and difucosylated Lewis but not to B histo-blood group active glycosphingolipids. *Glycoconj. J.* **26,** 1171–1180 (2009).

23. Costantini, V. *et al.* Diagnostic accuracy and analytical sensitivity of IDEIA norovirus assay for routine screening of human norovirus. *J. Clin. Microbiol.* **48,** 2770–2778 (2010).

24. Kageyama, T. *et al.* Broadly reactive and highly sensitive assay for Norwalk-

like viruses based on real-time quantitative reverse transcription-PCR. *J Clin Microbiol* **41,** 1548–1557 (2003).

25. Jothikumar, N. *et al.* Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.* **71,** 1870–1875 (2005).

26. Park, Y., Cho, Y. H. & Ko, G. A duplex real-time RT-PCR assay for the simultaneous genogroup-specific detection of noroviruses in both clinical and environmental specimens. *Virus Genes* **43,** 192–200 (2011).

27. Ministry of Food and Drug Safety. *Guideline for investigation on the cause of food poisoning*. Chungcheonbuk-do, Republic of Korea. (2013).

28. Ahmed, S. M. *et al.* Global prevalence of norovirus in cases of gastroenteritis: A systematic review and meta-analysis. *Lancet Infect. Dis.* **14,** 725–730 (2014).

29. Bull, R. A., Tanaka, M. M. & White, P. A. Norovirus recombination. *J. Gen. Virol.* **88,** 3347–3359 (2007).

30. Victoria, M. *et al.* Bayesian coalescent inference reveals high evolutionary rates and expansion of Norovirus populations. *Infect. Genet. Evol.* **9,** 927–932 (2009).

31. Mattison, K. *et al.* Multicenter comparison of two norovirus ORF2-based genotyping protocols. *J. Clin. Microbiol.* **47,** 3927–3932 (2009).

32. Baert, L. *et al.* Review: Norovirus prevalence in Belgian, Canadian and

French fresh produce: A threat to human health? *Int. J. Food Microbiol.* **151,** 261–269 (2011).

33. Lopman, B. *et al.* Environmental transmission of norovirus gastroenteritis. *Curr. Opin. Virol.* **2,** 96–102 (2012).

34. Masago, Y. *et al.* Comparative Evaluation of Real-Time PCR Methods for Human Noroviruses in Wastewater and Human Stool. *PLoS One* **11,** e0160825 (2016).

35. ISO/TS 15216-2:2013(en), Microbiology of food and animal feed — Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR — Part 2: Method for qualitative detection. Available at: https://www.iso.org/obp/ui/#iso:std:iso:ts:15216:-2:ed-1:v1:en.

36. Gonzalez, M. D. *et al.* Multicenter Evaluation of the Xpert Norovirus Assay for Detection of Norovirus Genogroups I and II in Fecal Specimens. *J. Clin. Microbiol.* **54,** 142–7 (2016).

37. Kojima, S. *et al.* Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* **100,** 107–114 (2002).

38. Kim, S. H. *et al.* Outbreaks of gastroenteritis that occurred during school excursions in Korea were associated with several waterborne strains of norovirus. *J. Clin. Microbiol.* **43,** 4836–4839 (2005).

39. Müller, L. *et al.* Series of Norovirus Outbreaks Caused by Consumption of Green Coral Lettuce, Denmark, April 2016. *PLoS Curr.* **8,** (2016).

40. Stals, A. *et al.* Molecular Detection and Genotyping of Noroviruses. *Food*

Environ. Virol. **4,** 153–167 (2012).

41. Katayama, K. *et al.* Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. *Virology* **299,** 225–239 (2002).

42. Bull, R. A. *et al.* Norovirus recombination in ORF1/ORF2 overlap. *Emerg. Infect. Dis.* **11,** 1079–1085 (2005).

43. Vega, E. & Vinjé, J. Novel GII.12 norovirus strain, United States, 2009-2010. *Emerg. Infect. Dis.* **17,** 1516–8 (2011).

44. Eden, J.-S., Tanaka, M. M., Boni, M. F., Rawlinson, W. D. & White, P. A. Recombination within the pandemic norovirus GII.4 lineage. *J. Virol.* **87,** 6270–82 (2013).

45. Bustin, S. A. *et al.* The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **55,** (2009).

46. Lopman, B., Simmons, K., Gambhir, M., Vinjé, J. & Parashar, U. Epidemiologic implications of asymptomatic reinfection: a mathematical modeling study of norovirus. *Am. J. Epidemiol.* **179,** 507–12 (2014).

47. Kabue, J. P., Meader, E., Hunter, P. R. & Potgieter, N. Norovirus prevalence and estimated viral load in symptomatic and asymptomatic children from rural communities of Vhembe district, South Africa. *J. Clin. Virol.* **84,** 12–18 (2016).

48. Stals, A., Van Coillie, E. & Uyttendaele, M. Viral genes everywhere: Public health implications of PCR-based testing of foods. *Curr. Opin. Virol.* **3,** 69–

73 (2013).

49. Butot, S., Zuber, S. & Baert, L. Sample preparation prior to molecular amplification: Complexities and opportunities. *Curr. Opin. Virol*. **4,** 66–70 (2014).

50. Vega, E. *et al.* Novel surveillance network for norovirus gastroenteritis outbreaks, United States. *Emerg. Infect. Dis.* **17,** 1389–1395 (2011).

51. Phillips, G. *et al.* Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infect. Dis.* **9,** 63 (2009).

52. Mattison, K. *et al.* Analytical performance of norovirus real-time RT-PCR detection protocols in Canadian laboratories. *J. Clin. Virol.* **50,** 109–113 (2011).

53. Miura, T. *et al.* Environmental detection of genogroup I, II, and IV noroviruses by using a generic real-time reverse transcription-PCR assay. *Appl. Environ. Microbiol.* **79,** 6585–6592 (2013).

54. Jin, M. *et al.* Strain-specific interaction of a GII.10 Norovirus with HBGAs. *Virology* **476,** 386–394 (2015).

55. Park, G. W. *et al.* Strain-specific virolysis patterns of human noroviruses in response to alcohols. *PLoS One* **11,** 1–12 (2016).

56. Real-time PCR handbook.*Thermofisher.com* (2014) at <https://www.thermofisher.com/kr/ko/home/life-science/pcr/real-time-pcr/qpcr-education/real-time-pcr-handbook/html>

57. Bustin, S. A. & Nolan, T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.* **15,** 155–66 (2004).

58. Boom, R. *et al.* Rapid and Simple Method for Purification of Nucleic Acids. *J. Clin. Microbiol.* 495–503 (1990).

59. Sefers, S. E. *et al.* QIAamp MinElute Virus kit effectively extracts viral nucleic acids from cerebrospinal fluids and nasopharyngeal swabs. *J. Clin. Virol.* **35,** 141–146 (2006).

60. Pang, X., Lee, B., Chui, L., Preiksaitis, J. K. & Monroe, S. S. Evaluation and Validation of Real-Time Reverse Transcription-PCR Assay Using the LightCycler System for Detection and Quantitation of Norovirus. **42,** 4679–4685 (2004).

61. Lee, S.-G. *et al.* Standardized positive controls for detection of norovirus by reverse transcription PCR. *Virol. J.* **8,** 260 (2011).

62. Martínez-Martínez, M., Diez-Valcarce, M., Hernández, M. & Rodríguez-Lázaro, D. Design and Application of Nucleic Acid Standards for Quantitative Detection of Enteric Viruses by Real-Time PCR. *Food Environ. Virol.* **3,** 92–98 (2011).

63. Zakikhany, K. *et al.* Molecular Evolution of GII-4 Norovirus Strains. *PLoS One* **7,** e41625 (2012).

64. Koehler, R. T. & Peyret, N. Thermodynamic properties of DNA sequences: Characteristic values for the human genome. *Bioinformatics* **21,** 3333–3339 (2005).

65. Xia, H., Gravelsina, S., Öhrmalm, C., Ottoson, J. & Blomberg, J. Development of single-tube nested real-time PCR assays with long internally quenched probes for detection of norovirus genogroup II. *Biotechniques* **60,** 28–34 (2016).

66. Stals, A., Baert, L., Jasson, V., Van Coillie, E. & Uyttendaele, M. Screening of Fruit Products for Norovirus and the Difficulty of Interpreting Positive PCR Results. *J. Food Prot.* **74,** 425–431 (2011).

67. Stals, A. *et al.* Laboratory efforts to eliminate contamination problems in the real-time RT-PCR detection of noroviruses. *J. Microbiol. Methods* **77,** 72–76 (2009).

국문초록

PCR기반 노로바이러스 I, II 유전형 검출법 비교 및 개선 연구

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노로바이러스는 대장염을 일으키는 전염성이 강한 바이러스이다. 현재로 노로바이러스 검출법의 최적표준(gold standard) 방법은 실시간 역전사 중합효소연쇄반응법(real-time reverse transcription PCR; real-time RT-PCR)이며 식중독 대장염 발생에서 대표적인 노로바이러스 검출법으로 매우 중요한 역할을 하고있다. 그러나 세계적으로 노로바이러스 감시체계마다 사용하는 검출 방법이 동일하지 않은 경우가 많으며 노로바이러스를 채취한 시료의 특성이나 상태에 따라 검출 결과가 다르게 나올 수 있다. 또한, 노로바이러스 유전자는 아주 빠르게 진화하는 것으로 알려져 있다. 따라서 노로바이러스 real-time PCR법이 최적표준임에도 불구하고, 방법의 검출 효율을 정기적으로 검증하고 개선 방법을 적용하는 것은 노로바이러스 발생을 효과적으로 감시하고 제어하는 것에 매우 중요한 부분이다. 이 연구는, 현재 많이 쓰이고 있는 4가지의 real-time RT-PCR 방법 (Assay A-Assay D)을 비교하여 사람 노로바이러스 I, II 유전형(GI, GII)에 대한 검출 효율을 평가하였다. PCR 증폭효율, 정량한계, 검출한계 등 검출방법의 특성을 분석하여 가장 효율적인 방법을 선정하였다. Real-time RT-PCR 결과를 역전사 중합효소연쇄반응 (RT-PCR)법과 비교하여 real-time RT-PCR의 민감도 및 특이도를 평가하였다. 결론적으로, Assay D가 가장 효율적이고 정확한 검출법으로 판단되었다. Real-time RT-PCR 방법은 TaqMan probe의 형광물질과 그의 발현을 억제하는 퀀쳐(quencher)물질의 적용 원리를 이용하여 유전자 증폭을 형광신호로 실시간 묘사하는 방법이다. Assay D 서열 내부에 Zen internal quencher라는 퀀쳐 물질을 추가적으로 붙였을 때 정량한계가 향상됨을 볼 수 있었다. 즉, 여러 검출법들

의 효율과 특성을 비교분석하고 개선 방법을 적용하여 노로바이러스 real-time PCR 검출법의 민감도 유지에 필요한 특성을 제시할 수 있었다.

주요 단어: 노로바이러스, real-time RT-PCR, 민감도, 정량한계, 검출한계, zen internal quencher

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