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Development and Characterization of Synthetic Norovirus RNA for Use in Molecular Detection Methods

Eun-Jung Cho, M.D.[1,*], Younggil Cha, Ph.D.[2,*], Su Kyung Lee, M.S.[1], Han-Sung Kim, M.D.[1], Jae-Seok Kim, M.D.[1], Eun Jin Lee, M.D.[3], Nuri Lee, M.D.[1], Ki Ho Hong, M.D.[4], Hee Jin Huh, M.D.[5], Young Joo Cha, M.D.[6], and Hyun Soo Kim, M.D.[1]

[1]Department of Laboratory Medicine, Hallym University College of Medicine, Chuncheon, Korea; [2]Molecular Diagnostic R&D Center, Bioneer, Daejeon, Korea; [3]Department of Laboratory Medicine, Veterans Health Service Medical Center, Seoul, Korea; [4]Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea; [5]Department of Laboratory Medicine, Dongguk University Ilsan Hospital, Ilsan, Korea; [6]Corporate R&D Center for Biological Standards and Control, Resources and Innovation Cooperative, Hanam, Korea

Background: Reference materials are essential for the quality assurance of molecular detection methods. We developed and characterized synthetic norovirus GI and GII RNA reference materials.

Methods: Norovirus GI and GII RNA sequences including the ORF1–ORF2 junction region were designed based on 1,495 reported norovirus sequences and synthesized via plasmid preparation and *in vitro* transcription. The synthetic norovirus GI and GII RNAs were evaluated using six commercial norovirus detection kits used in Korea and subjected to homogeneity and stability analyses. A multicenter study involving five laboratories and using four commercial real-time PCR norovirus detection assays was conducted for synthetic norovirus RNA characterization and uncertainty measurements.

Results: The synthetic norovirus GI and GII RNAs were positively detected using the six commercial norovirus detection kits and were homogeneous and stable for one year when stored at –20°C or –70°C. All data from the five laboratories were within a range of 1.0 log copies/μL difference for each RNA, and the overall mean concentrations for norovirus GI and GII RNAs were 7.90 log copies/μL and 6.96 log copies/μL, respectively.

Conclusions: The synthetic norovirus GI and GII RNAs are adequate for quality control based on commercial molecular detection reagents for noroviruses with high sequence variability. The synthetic RNAs can be used as reference materials in norovirus molecular detection methods.

Key Words: Noroviruses, RNA, Multicenter study, Reference material, Uncertainty, Characterization

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Corresponding author: Hyun Soo Kim, M.D.
Department of Laboratory Medicine, Hallym University Dongtan Sacred Heart Hospital, Hallym University College of Medicine, 7 Keunjaebong-gil, Hwaseong 18450, Korea
Tel: +82-31-8086-2775
Fax: +82-31-8086-2789
E-mail: hskim0901@empas.com

*These authors equally contributed to this study.

INTRODUCTION

Noroviruses, which belong to the family *Caliciviridae*, are non-enveloped, single-stranded, positive-sense RNA viruses that can infect humans and animals and are the leading cause of acute gastroenteritis worldwide [1, 2]. They are composed of linear RNA (~7.5 kb in length) with three open reading frames (ORFs) [3]. ORF1 encodes nonstructural proteins, ORF2 encodes the major capsid protein (VP1), and ORF3 encodes a minor structural protein (VP2) [4]. Based on the VP1 amino acid sequence, noroviruses are classified into at least 10 genogroups (GI–GX) and into more than 40 genotypes [4, 5].

Quality control materials, standards, or reference materials are important for ensuring accurate results in molecular detec-

tion methods [6]. Commonly used types of standards or reference materials for nucleic acid analyses include nucleic acids from clinical samples and synthetic nucleic acids [7, 8]. Several companies and institutions, such as the National Institute for Biological Standards and Control and Paul-Ehrlich Institute, provide international standards and reference materials [9].

Various reference materials are being developed and used in clinical laboratories for quality control of molecular assays. For RNA viruses, virus-positive patient samples, synthetic RNAs, plasmid RNAs, armored RNAs, targeted RNAs from cell lines, and virus-like particles (VLPs) have served as RNA controls and standards [6, 10-13]. VLPs have the advantage of being resistant to nucleases and thus stable, but their manufacturing process is complex. Synthetic RNA can be manufactured in large quantities relatively quickly and easily [13-16].

There is a growing need for universal reference materials irrespective of the high sequence variation among noroviruses. However, only a few manufacturers and institutions produce norovirus reference materials, and detailed information, such as the targeted gene region or exact genotype, is not provided. Moreover, there are no reports on the synthesis and verification of synthetic norovirus RNA reference materials. We developed synthetic norovirus GI and GII RNA reference materials for use as standards or quality control materials and validated and characterized the synthetic RNAs according to ISO Guide 35:2017 [17].

MATERIALS AND METHODS

Preparation of reference materials

In total, 74 and 1,421 norovirus RNA sequences for GI and GII, respectively, were obtained from National Center for Biotechnology Information GenBank. For synthesis, we selected those nucleotide sequences that occurred the most frequently in alignments of the 1,495 sequences. The ORF1–ORF2 junction regions of norovirus GI and GII were selected based on a comparison of the reported sequences. A plasmid for RNA synthesis was prepared based on a previously reported gene synthesis technique, using the MEGAscript T7 Transcription Kit (Ambion, Invitrogen, Carlsbad, CA, USA) [18]. T7 and SP6 RNA polymerases were used to synthesize RNA from the plasmid [14] (Fig. 1). The concentrations of the synthetic norovirus RNAs were 10^7–10^8 copies/μL for GI and 10^6–10^7 copies/μL for GII, as measured using reported methods [19, 20]. Hundred microliters of diluted synthetic RNAs was distributed in microtubes containing RNase Inhibitor (Bioneer, Daejaeon, Korea).

Validation using six commercial norovirus detection kits

We validated synthetic norovirus GI and GII detection using the following six commercial norovirus detection kits: AccuPower Norovirus Real-Time RT-PCR Kit (Bioneer, Daejeon, Korea), PowerChek Norovirus GI/GII Multiplex Real-Time PCR Kit (Kogene Biotech, Seoul, Korea), Allplex Gastrointestinal Panel Assays (Seegene, Seoul, Korea), Luminex xTAG Gastrointestinal Pathogen Panel (Luminex, Austin, TX, USA), BD MAX Enteric Viral Panel (Becton Dickinson, Franklin Lakes, NJ, USA), and FilmArray Gastrointestinal Panel (BioFire Diagnostics, Salt Lake City, UT, USA).

Assessment of the homogeneity between vials

For homogeneity analysis, vials containing the reference materi-

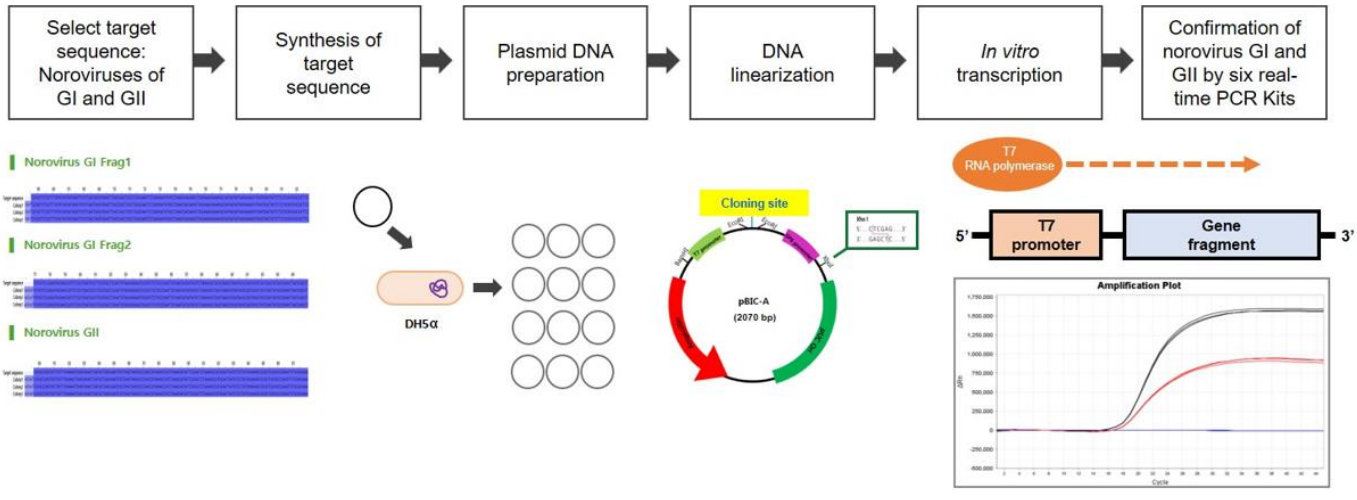

Fig. 1. Overview of the development of the synthetic norovirus GI and GII RNAs.

als were placed at –70°C for 24 hours and thawed at room temperature (20°C–25°C). Three vials per batch were randomly selected from five batches and tested in triplicate using the AccuPower Norovirus Real-Time RT-PCR Kit.

Assessment of stability

Short- and long-term stability of the synthetic norovirus GI/GII RNAs were assessed one year after production, using an isochronous study design. Two vials were stored at –70°C (in a deep freezer), –20°C (in a freezer), 4°C (in a refrigerator), and 25°C (at room temperature) for 3, 7, 14, and 28 days to evaluate short-term stability and at –70°C and –20°C for 3, 6, 9, and 12 months and at 4°C and 25°C for 3, 6, and 9 months to evaluate long-term stability. The number of samples required was 32 vials (two vials×four temperatures×four time points) for short-term stability and 28 vials (two vials×four temperatures×three or four time points) for long-term stability. Each sample was assessed in triplicate using the AccuPower Norovirus Real-Time RT-PCR Kit. Long-term stability was assessed to evaluate expiration within 12 months.

Characterization and determination of uncertainty

To characterize the synthetic norovirus GI/GII RNAs, including determining the uncertainty and assigning a certified value, we conducted a multicenter study involving five laboratories. The contents of synthetic norovirus GI/GII RNAs as candidate reference materials were determined by real-time reverse transcription PCR (RT-PCR). RNA concentrations were determined by absorbance measurements using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) [19, 20]. Five concentrations of norovirus GI and GII plasmids were used to determine the concentrations of the reference materials, which were calculated as logarithmic values. Two vials of candidate reference materials and five concentrations of norovirus GI and GII standard materials were measured in two runs on a single day, with five replicates in each run. For characterization and uncertainty determination, we used the following four real-time PCR assays on two instruments: Allplex GI-Virus Assay (Seegene) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), PowerChek Norovirus GI/GII Multiplex Real-Time PCR Kit (Kogene Biotech) on the CFX96 system, AccuPower Norovirus Real-Time RT-PCR Kit for CFX96 (Bioneer) on the CFX96 system, and AccuPower Norovirus Real-Time RT-PCR Kit on an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer). Standard curves were plotted using five concentrations of standard materials to quantify the RNAs (log copy/μL) based on the cycle threshold (Ct) values. Based on the interlaboratory comparison results, the combined uncertainty (U) was calculated using the following formulas [17]:

$$u_{CRM} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{stab}^2}$$

$$U = k \times u_{CRM}$$

where u_{CRM} is the uncertainty associated with the property value of the certified reference material (CRM), u_{char} is the standard uncertainty associated with a value assigned in a characterization study, u_{bb} is the standard uncertainty associated with homogeneity, u_{stab} is the standard uncertainty associated with a value from a stability study, and k is the coverage factor.

Statistical analysis

MedCalc for Windows version 19.2.6 (MedCalc Software, Ostend, Belgium) and Microsoft Office Excel 2021 (Microsoft, Redmond, WA, USA) were used for statistical analyses. ANOVA was used to examine homogeneity, followed by Bonferroni post-hoc tests. The critical value (F_{crit}) at the 95% confidence interval (CI) was larger than the F-test value (F), i.e., the samples were considered homogeneous. Student's t-test and ANOVA were used to determine instability. Stability analysis results were calculated as the mean, standard deviation (SD), and coefficient of variation and were compared with the initial mean Ct values. The stability test results were subjected to regression analysis to examine linear trends based on the storage temperatures. Samples were considered stable if the Ct values were within +1.5 SD from the initial mean Ct values.

RESULTS

Production of reference materials and validation using six commercial norovirus detection kits

Norovirus GI and GII RNAs were synthesized. According to Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov) results against GenBank, the synthetic norovirus GI and GII RNAs showed the highest sequence similarity to norovirus Hu/GI.1/8K/1979/USA (GenBank accession No. KF429783, 94.4% similarity) and norovirus Hu/GII-4/Toyama3/2007/JP (GenBank accession No. AB541357, 98.7% similarity) genomic RNA, respectively. The synthetic norovirus GI and GII RNAs were positively detected using six commercial norovirus detection kits (Supplemental Data Fig. S1).

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Table 1. Short-term stability of the synthetic norovirus GI and GII RNAs over 28 days

Reference material	Temperature	Ct value (mean ± SD)				
		Day 0	Day 3	Day 7	Day 14	Day 28
Norovirus GI	25°C	20.84 ± 0.40	20.47 ± 0.13	20.51 ± 0.12	22.19 ± 0.22	23.17 ± 0.21
	4°C	20.84 ± 0.40	20.50 ± 0.09	20.37 ± 0.26	20.44 ± 0.13	20.49 ± 0.18
	–20°C	20.84 ± 0.40	20.40 ± 1.13	20.34 ± 1.13	20.46 ± 1.13	20.49 ± 1.13
	–70°C	20.84 ± 0.40	20.30 ± 0.19	20.36 ± 0.24	20.38 ± 0.23	20.33 ± 0.29
Norovirus GII	25°C	18.90 ± 0.30	18.43 ± 0.14	18.55 ± 0.21	20.28 ± 0.17	21.29 ± 0.24
	4°C	18.90 ± 0.30	18.48 ± 0.21	18.51 ± 0.13	18.61 ± 0.15	18.52 ± 0.23
	–20°C	18.90 ± 0.30	18.58 ± 0.17	18.51 ± 1.13	18.54 ± 1.13	18.52 ± 1.13
	–70°C	18.90 ± 0.30	18.55 ± 0.15	18.52 ± 0.25	18.64 ± 0.19	18.62 ± 0.26

Abbreviation: Ct, cycle threshold.

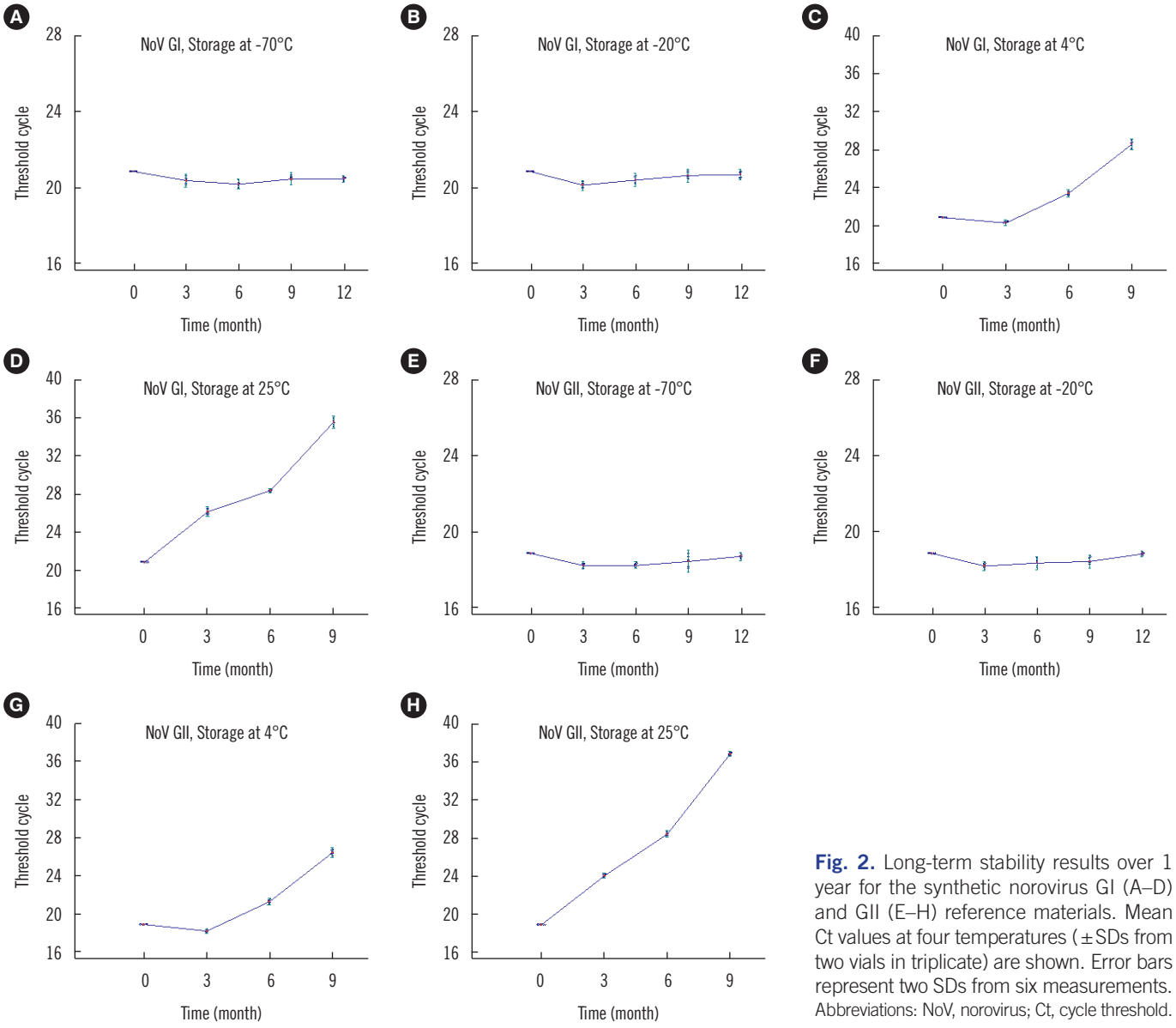

Fig. 2. Long-term stability results over 1 year for the synthetic norovirus GI (A–D) and GII (E–H) reference materials. Mean Ct values at four temperatures (±SDs from two vials in triplicate) are shown. Error bars represent two SDs from six measurements.
Abbreviations: NoV, norovirus; Ct, cycle threshold.

Table 2. Quantification of synthetic norovirus GI and GII RNAs obtained from the five participating laboratories

Lab No.	Assay method		Log copies/μL (Ct values)	
	Reagent	Instrument	Norovirus GI	Norovirus GII
1	Bioneer	CFX96	7.97 ± 0.03 (20.46 ± 0.11)	6.97 ± 0.21 (19.60 ± 0.98)
	Seegene	CFX96	7.94 ± 0.11 (14.14 ± 0.48)	7.09 ± 0.01 (13.18 ± 0.55)
	Kogene Biotech	CFX96	7.91 ± 0.06 (20.67 ± 0.43)	6.87 ± 0.04 (19.34 ± 0.27)
	Bioneer	Exicycler 96	7.82 ± 0.15 (21.01 ± 0.85)	6.96 ± 0.25 (19.56 ± 0.95)
2	Bioneer	CFX96	7.91 ± 0.01 (20.70 ± 0.10)	7.01 ± 0.01 (18.67 ± 0.12)
	Seegene	CFX96	7.76 ± 0.05 (14.90 ± 0.30)	6.96 ± 0.02 (16.26 ± 0.19)
	Kogene Biotech	CFX96	7.98 ± 0.02 (21.34 ± 0.11)	6.88 ± 0.03 (18.53 ± 0.38)
	Bioneer	Exicycler 96	7.92 ± 0.10 (20.46 ± 0.42)	7.04 ± 0.08 (18.83 ± 0.48)
3	Bioneer	CFX96	7.81 ± 0.01 (21.15 ± 0.06)	6.90 ± 0.03 (19.29 ± 0.12)
	Kogene Biotech	CFX96	7.79 ± 0.07 (20.57 ± 0.32)	6.79 ± 0.03 (17.98 ± 0.14)
	Bioneer	Exicycler 96	8.13 ± 0.07 (20.18 ± 0.71)	6.91 ± 0.06 (19.31 ± 0.24)
4	Bioneer	CFX96	7.88 ± 0.02 (20.77 ± 0.10)	6.96 ± 0.03 (19.66 ± 0.17)
	Kogene Biotech	CFX96	7.76 ± 0.02 (20.43 ± 0.07)	6.91 ± 0.03 (17.99 ± 0.11)
5	Bioneer	CFX96	8.02 ± 0.03 (20.66 ± 0.14)	6.99 ± 0.02 (19.74 ± 0.47)
	Kogene Biotech	CFX96	7.94 ± 0.09 (21.03 ± 0.48)	7.15 ± 0.21 (19.31 ± 0.77)

Log copies/μL and Ct values are shown as Mean ± SD.
The following assays and instruments were used: Allplex GI-Virus Assay (Seegene, Seoul, Korea), PowerChek Norovirus GI/GII Multiplex Real-Time PCR Kit (Kogene Biotech, Seoul, Korea), AccuPower Norovirus Real-Time RT-PCR Kit for CFX96 and AccuPower Norovirus Real-Time RT-PCR Kit (Bioneer, Daejeon, Korea); CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer).
Abbreviations: Ct, cycle threshold; Lab, laboratory.

Homogeneity assessment

The between-vial homogeneity analysis showed that the F_{crit} values were 2.04 and 2.04 for the norovirus GI and GII candidate reference materials, respectively (F ratios of 2.02 and 1.18 for norovirus GI and GII, respectively). The F ratios were below the F_{crit} values of the Ct values, i.e., the materials were homogeneous [17].

Stability assessment

We calculated the mean Ct values and SDs for short- and long-term stability at each time point. Table 1 shows the short-term stability results. The differences between the Ct value at the time of measurement and the initial Ct value were all < 1.0 Ct, except for the case of storage at 25°C for more than 14 days, which indicated that the RNAs are stable at –70°C, –20°C, and 4°C for up to 28 days. Fig. 2 shows long-term stability (up to 1 year). The differences between the Ct value at the time of measurement and the initial Ct value were all < 1.5 Ct at –70°C and –20°C for up to one year. However, Ct values after 3 months of storage at 4°C and 25°C were increased compared to the initial Ct value, which indicated that the RNAs disintegrate after 3 months at 4°C or 25°C.

Uncertainty characterization and evaluation

The RNA quantification results based on 13 datasets obtained using four assays in five laboratories are summarized in Table 2. We evaluated the copy numbers based on Ct values using standard curves generated using five concentrations of cloned plasmids. The overall means (95% CI) for the norovirus GI and GII candidate reference materials were 7.90 (7.89–7.92) log copies/μL and 6.96 (6.94–6.98) log copies/μL, respectively. All data from five laboratories were within a range of a 1.0-log copies/μL difference for each RNA, indicating a good concordance among the assays. The assigned values and combined standard uncertainties determined using the AccuPower Norovirus Real-Time RT-PCR Kit were 20.74 ± 1.33 and 19.04 ± 0.89 Ct values for GI and GII at –70°C, respectively, and 20.74 ± 1.35 and 19.04 ± 0.90 Ct values for GI and GII at –20°C, respectively (Table 3).

DISCUSSION

We synthesized norovirus GI and GII RNAs because GI and GII are the most frequently detected genogroups in human infections [21]. The alignment results of 74 nucleotide sequences of norovirus GI strains and 1,421 nucleotide sequences of norovi-

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Table 3. Assigned Ct values and uncertainties of the synthetic norovirus GI and GII RNAs using the AccuPower Norovirus Real-Time RT-PCR Kit

Storage temperature	–70°C		–20°C	
Material	Norovirus GI	Norovirus GII	Norovirus GI	Norovirus GII
Mean ± SD*	20.74 ± 0.48	19.04 ± 0.71	20.74 ± 0.48	19.04 ± 0.71
Total replicates	398	398	398	398
u_{bb}	0.55	0.37	0.55	0.37
u_{stab}	0.18	0.21	0.21	0.22
u_{char}	0.09	0.14	0.09	0.14
u_{CRM}	0.59	0.45	0.60	0.45
Degrees of freedom	9	14	9	15
k	2.26	2.00	2.26	2.00
U	1.33	0.89	1.35	0.90

*Mean is the average value of the characteristic values and was constant regardless of the measured value according to the temperature.
Abbreviations: SD, standard deviation; k, coverage factor; U, combined uncertainty; u_{bb}, standard uncertainty associated with homogeneity; u_{stab}, standard uncertainty associated with a value from a stability study; u_{char}, standard uncertainty associated with a value assigned in a characterization study; u_{CRM}, uncertainty associated with a property value of the certified reference material.

rus GII strains revealed that noroviruses exhibit high sequence variation, and it was difficult to find suitable amplification sites with common sequences using primer and probe combinations. Considerable sequence similarity was found only in the ORF1–ORF2 junction regions of GI and GII noroviruses. Therefore, the ORF1–ORF2 junction region was selected to prepare universal reference materials for norovirus molecular analyses. The synthetic norovirus GI and GII RNAs were positively detected using six commercial norovirus real-time PCR-based detection kits.

The synthetic norovirus GI/GII RNAs were tested for homogeneity and stability. The homogeneity study showed that they were sufficiently homogeneous. Short-term (< 1 month) and long-term (up to 1 year) stability studies of the synthetic RNAs showed no significant disintegration at –20°C and –70°C, and the synthetic RNAs remained stable for 1 year when stored at –20°C or –70°C. The assigned values determined based on 398 replicates were 20.74 and 19.04 Ct values for norovirus GI and GII, respectively. This result indicates that the reference materials show no remarkable deviation, regardless of the reagents used and storage temperature and therefore can be used in commercial molecular norovirus detection assays.

Commercial norovirus detection kits contain norovirus positive control materials, but these can be used only with the reagents in the kit, not with other reagents. Because of the high genome sequence variation in noroviruses, only a few manufacturers and institutions worldwide produce norovirus reference materials that can be used in multiple norovirus detection kits. However, user manuals for these reference materials indicate only that the synthetic RNAs are norovirus GI and GII genes or that the synthetic region of norovirus RNAs is the ORF1–ORF2 junction region. They do not describe the concrete manufacturing process or verification results using commercial norovirus detection reagents, and it is indicated that these RNAs may not be detected with certain norovirus reagents. Because few institutions produce norovirus reference materials that can be used in various norovirus detection kits, there are no published papers on the production and verification of norovirus reference materials. Studies on synthetic DNA/RNAs of other viruses have been published only to the extent that the DNA/RNAs were synthesized and evaluated for stability or homogeneity in a multicenter study [8, 22, 29].

In summary, we prepared synthetic norovirus GI and GII RNAs and conducted homogeneity and stability analyses, a multicenter study, and determination of characteristic values and uncertainties according to ISO Guide 35:2017. As norovirus gene standardization or harmonization has not yet been established, the characteristic values for norovirus concentrations were calculated as copy number per microliter; these were verified using six commercial assays. The synthetic norovirus GI and GII reference materials can be used to ensure reliable results, for quality control of analytical processes, and for validation of commercial detection assays for the highly variable noroviruses.

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AUTHOR CONTRIBUTIONS

Kim HS and Cha YJ designed and supervised the study. Lee SK, Cha Y, Kim HS, and Kim JS performed the measurements. Lee EJ and Lee N performed the statistical analyses. Cho EJ and Cha Y wrote the manuscript. Hong KH, Huh JH, and Cha YJ edited the manuscript. All authors have read and approved the manuscript.

CONFLICTS OF INTEREST

None declared.

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ORCID

Eun-Jung Cho	https://orcid.org/0000-0003-1907-9639
Younggil Cha	https://orcid.org/0000-0003-2671-9059
Su Kyung Lee	https://orcid.org/0000-0002-2536-413X
Han-Sung Kim	https://orcid.org/0000-0002-5481-5390
Jae-Seok Kim	https://orcid.org/0000-0001-6025-0341
Eun Jin Lee	https://orcid.org/0000-0002-4011-2782
Nuri Lee	https://orcid.org/0000-0001-7727-8279
Ki Ho Hong	https://orcid.org/0000-0002-5700-9036
Hee Jin Huh	https://orcid.org/0000-0001-6587-8568
Young Joo Cha	https://orcid.org/0000-0001-7828-8401
Hyun Soo Kim	https://orcid.org/0000-0002-7026-6715

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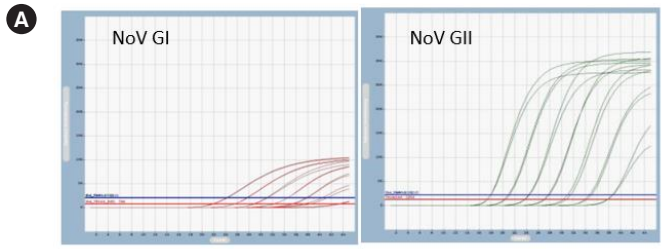

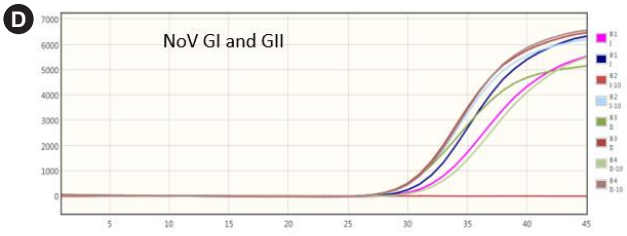

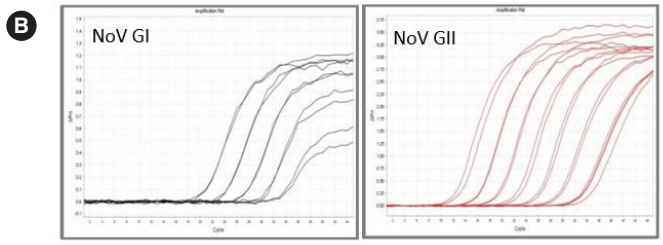

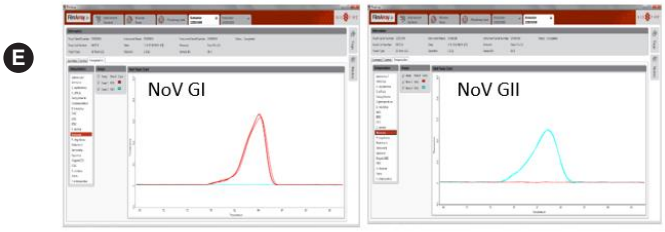

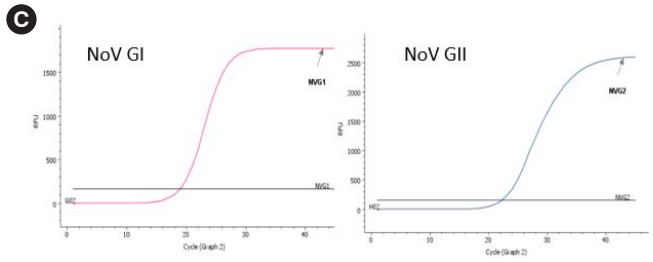

F

Target	Call	Signals (MFI)		S:N Ratio	Call Thresholds
		Raw	Background		
Internal Control	ABS	40.0	40.0	1.0	150
Adenovirus 40/41	NEG	45.0	30.0	1.5	150
Norovirus GI/GII	POS				
GI-Probe		2594.5	25.5	101.7	200
GII-Probe		36.0	20.0	1.8	350
Rotavirus A	NEG	34.0	22.0	1.5	150

Target	Call	Signals (MFI)		S:N Ratio	Call Thresholds
		Raw	Background		
Internal Control	ABS	40.0	40.0	1.0	150
Adenovirus 40/41	NEG	49.0	30.0	1.6	150
Norovirus GI/GII	POS				
GI-Probe		47.0	25.5	1.8	200
GII-Probe		4347.0	20.0	217.3	350
Rotavirus A	NEG	35.0	22.0	1.6	150

Supplemental Data Fig. S1. Verification of the synthetic norovirus GI and GII RNAs using six commercial norovirus detection kits: (A) AccuPower Norovirus Real-Time RT-PCR Kit (Bioneer), (B) PowerChek Norovirus GI/GII Multiplex Real-Time PCR Kit (Kogene Biotech), (C) Allplex Gastrointestinal Panel Assays (Seegene), (D) BD MAX Enteric Viral Panel (Becton Dickinson), (E) FilmArray Gastrointestinal Panel (BioFire Diagnostics), and (F) Luminex xTAG Gastrointestinal Pathogen Panel (Luminex).
Abbreviation: NoV, norovirus.