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# Real-time Nucleic Acid Sequence Based Amplification (Real-time NASBA) for Detection of Norovirus

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Noroviruses (noroV) are the major cause of nonbacterial gastroenteritis in humans worldwide. Since noroV cannot yet be cultured *in vitro* and their diagnosis by electron microscopy requires at least $10^6$ viral particles/g of stool a variety of molecular detection techniques represent an important step towards the detection of noroV. In the present study, we have applied real-time nucleic acid sequence-based amplification (real-time NASBA) for simultaneous detection of NoroV genogroup I (GI) and genogroup II (GII) using standard viral RNA. For real-time NASBA assay which can detected noroV GI and GII, a selective region of the genes encoding the capsid protein was used to design primers and genotype-specific molecular beacon probes. The specificity of the real-time NASBA using newly designed primers and probes were confirmed using standard viral RNA of noroV GI and GII. To determine the sensitivity of this assay, serial 10-fold dilutions of standard viral RNA of noroV GI and GII were used for reverse transcription polymerase chain reaction (RT-PCR) and real-time NASBA. The results showed that while agarose gel electrophoresis could detect RT-PCR products with 10 pg of standard viral RNA, the real-time NASBA assay could detect 100 fg of standard viral RNA. These results suggested that the real-time NASBA assay has much higher sensitivity than conventional RT-PCR assay. This assay was expected that might detect the viral RNA in the specimens which could have been false negative by RT-PCR. There were needed to perform real-time NASBA with clinical specimens for evaluating accurate sensitivity and specificity of this assay.

**Key Words:** Norovirus, Real-time NASBA, RT-PCR

## INTRODUCTION

It is known that norovirus (noroV) is a kind of enteric viruses living in the human intestines as a major cause for gastroenteritis being recently prevalent all over the world in all age groups (Fankhauser RL et al., 2002; Koopmans, 2008). NoroV, about 7.6 kb-sized, is a non-enveloped single-stranded RNA virus belonging to the *Caliciviridae* family, and mostly proliferates in human colonic epithelial cells (Koopmans et al., 2002; Chan et al., 2006). At present, noroV was divided 5 genogroups (GI to GV) and 31 subgenotypes according to its genetic characteristics based on sequence variation of the RNA polymerase and capsid region on the genome. Among 5 genogroups, GI and GII are known to cause diseases to human beings (Rutjes et al., 2006; Zheng et al., 2006).

NoroV causes diseases through secondary infections between human beings or intake of contaminated shellfish, or exposure to excrements or vomits directly contaminated with viruses. Furthermore, noroV also causes infections

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even in an extremely small number of less than 100 virus particles (Fankhauser et al., 1998; Marks et al., 2000). Besides, 30% of those infections are asymptomatic and have a latent period of 24~48 hours, indicating a rapid spread of diseases. For this reason, noroV outbreaks are generally shown in closed environments, e.g. schools, nursing homes, cruises, etc (Mark et al., 2003; Webby et al., 2007). Thus, rapid detection and diagnosis of noroV infection have been required to prevent those diseases from spread.

NoroV have been generally diagnosed by using an electron microscope, since noroV can hardly be cultured *in vitro*. Unfortunately, detection using an electron microscope is available only when $10^5$~$10^6$ particles per stool sample milliliter at least exist (Stiles et al., 2008). Accordingly, it is not capable of detecting samples containing a small number of viruses. Recently, the most commonly used detection method of noroV is reverse transcription-polymerase chain reaction (RT-PCR). This method has higher specificity and sensitivity than an electron microscope. However, RT-PCR is vulnerable to DNA contamination and uses *Taq* polymerase sensitive to several environmental PCR inhibitants. On the contrary, nucleic acid sequence based amplification (NASBA) is isothermal RNA amplification method using T7 RNA polymerase, so this method is not influenced by any contaminated DNA and does not require *Taq* polymerase (Patterson et al., 2006; Lamhoujeb et al., 2009). However, NASBA requires an additional process intended to detect amplified products. As such a process, northern blotting and ECL system is commonly used, but these detection processes have some disadvantages including difficult and complicated operations and the long time required. Thus, a new, easy-to-handle, simple and quick real-time NASBA technique has been developed and used in recent days. This new technique also has the advantageous ability to prevent cross-contamination among amplified products because it is characterized by amplification and detection in a single tube (Loens et al., 2003).

In the present study, we have applied real-time NASBA for simultaneous detection of NoroV GI and GII genogroup using standard viral RNA.

## MATERIALS AND METHODS

### *In vitro* transcription for obtaining standard viral RNA

Plasmids containing capsid gene of noroV GI and GII subtypes were provided from the National Institute of Health (Korea). These plasmids were linearized by cutting with *Xba*I (Fermentas, Boston, Massachusetts, USA) and served as template for making standard viral RNA. *In vitro* transcription was carried out using the MAXIscrpit kit (Ambion, Austin, Texas, USA) according to the protocol provided by manufacturer.

### Real-time NASBA assay

The primers and molecular beacon (MB) probe detecting noroV GI and GII genotypes were designed based on GenBank® published sequences using Primer3 (Table 1) for a real-time NASBA assay. The real-time NASBA processes were performed with a NucliSENS basic kit (version 2; bioMe´rieux, Marcy-l'Etoile, France). Each reaction mixture consisted of 0.2 μM each primer, 0.2 μM MB probe, 2.5 μl of standard viral RNA template, and NASBA enzyme mixture (T7 RNA polymerase, avian myeloblastosis virus reverse transcriptase, RNase H, bovine serum albumin) in a total volume of 10 μl. The mixture of primers, MB probe, and viral RNA was incubated at 65℃ for 5 min and 41℃ for another 5 min for denaturing secondary structure of RNA molecule, and then enzyme mixture was added into reaction tube and real-time NASBA was incubated at 41℃ for 90 min on a NucliSENS EasyQ analyzer (bioMe´rieux, Marcy-l'Etoile, France). The fluorescence signal value was measured at time intervals of 30 sec for each independent reaction at two wavelengths by using the NucliSENS EasyQ Director software (version 2; bioMe´rieux, Marcy-l'Etoile, France).

### RT-PCR

cDNA was synthesized by reverse transcription with standard viral RNA, 0.25 μg of random hexamer (Invitrogen, Carlsbad, California, USA) and 200 U of murine molony leukemia virus reverse transcriptase (MMLV-RT; Invitrogen, Carlsbad, California, USA) for 50 min at 37℃ and 15 min

at 70℃. Subsequent PCR amplification using 1 units of *Taq* polymerase (Cosmo Genetech, Seoul, Korea) was performed in a thermocycler (Applied Biosystems, California, USA) for 30 cycles (94℃ for 30 sec, 50℃ for 30 sec, 72℃ for 30 sec) using GI primer (sense 5'-ctg ccc gaa tty gta aat gat gat-3', antisense 5'-cca acc car cca ttr tac aty tg-3') and GII primer (sense 5'-ggg agg gcg atc gca atc t-3', antisense 5'-ccr cci gca tri ccr ttr tac at-3') (Park et al., 2011). PCR products were electrophoresed on 1.8% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide, and the sizes of the products determined by comparison to 100 bp DNA ladder marker (Bioneer, Daejeon, Korea).

## RESULTS AND DISCUSSION

### Design of primers and MB probes

The primers and MB probes used for real-time NASBA were designed around the capsid gene region which was usually used for diagnosis of noroV by conventional RT-PCR based on the sequences obtained from GenBank®. T7 promoter sequences were added to the 5' terminus of the forward primer for generating RNA molecules in real-time NASBA. Also, in the MB probe, 6 complementary sequences were added to the 5' and 3' terminals forming a

**Table 1.** Primers and MB probes of real-time NASBA used for detecting NoroV GI and GII

| Primer/Probe | Sequence |
|---|---|
| GI-noro(T7)F | 5'-AATTCTAATACGACTCACTATAGGGAGAAGGATCTCTTGCCCGATTATGTA-3' |
| GI-noroR | 5'-GTTCAGCTGTATTTGCCTCT-3' |
| GI- beacon NS | 5'-FAM-CGACTGTCCTTAGACGCCATCATCATCATACAGTCG-DABCYL-3' |
| GII-noro(T7)F | 5'-AATTCTAATACGACTCACTATAGGGAGAAGGACCTGGTGGTGAGTTTACAG-3' |
| GI-noroR | 5'-TGGCCATTGTACATTCTAGC-3' |
| GII- beacon NS | 5'-FAM-CGATCGTTTGGAATTGGGCCCAGAAACGATCG-DABCYL-3' |

*Underlined letters designate T7 promotor sequence. The blue colors are the specific binding sequences to the target RNA.

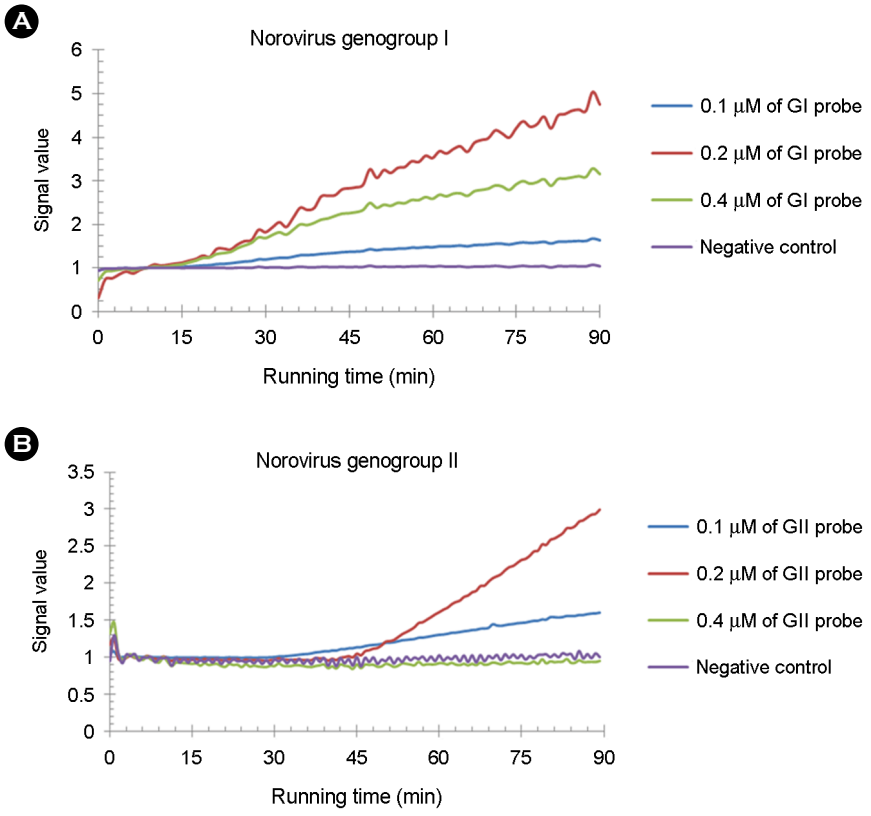


**Fig. 1.** Optimal concentration ratio of primer and MB probe for real-time NASBA. Optimal concentration test of MB probe was performed by controlling concentration ratio between primer and probe. A level of the primer was fixed to 0.2 μM, a level of the MB probe was mixed at ratios of 1:0.5, 1:1, and 1:2, and real-time NASBA was performed. (A) noroV GI, (B) noroV GII; (Blue) Primer: Probe = 2:1, (Red) Primer: Probe = 1:1. (Green) Primer: Probe = 1:2.

stem region of hairpin structure, and at the same time, FAM was labeled at the 5' terminal and Dabsyl at the 3' terminal of MB probe (Table 1).

**Optimization of real-time NASBA**

Real-time NASBA with the designed primers and MB probes was conducted in the NucliSENS EasyQ analyzer (bioMe´rieux, Marcy-l'Etoile, France) using the standard viral RNA (sv-RNA) and the bioMe´rieux's NucliSENS basic kit (version 2). The specific signal of each genogroup was detected, but the signal values were significantly low (data not shown). Therefore, the condition of experiment was needed to improve at this point.

As it was known that a ratio of the primer to the MB probe heavily influences the result in molecular techniques using the fluorescence-labeled probe. For determining the optimal ratio of primers and the MB probe, the primer was mixed at ratios of 1:0.5, 1:1, and 1:2 with the fixed level of the MB probe at 0.2 μM and then real-time NASBA was performed. As the results, irrespective of a level of the primer, no significant differences were shown in signal values (data not shown). Next, the MB probe was mixed at ratios of 1:0.5, 1:1, and 1:2 with the fixed level of the primer at 0.2 μM, and then real-time NASBA was performed. The peak of signal value was shown at 0.2 μM of MB probe, the ratio of primer and MB probe was 1:1 (Fig. 1). Thus, an optimal concentration of primers and MB probe for real-time NASBA was 0.2 μM, respectively.

For deciding the optimal running time of real-time NASBA for detection of noroV, real-time NASBA was performed for up to 150 minutes, the maximum available analysis time, under the determined optimal ratio of the primer and MB probe. The signals began to appear from 30 minutes in both noroV GI and GII, increased by degrees,

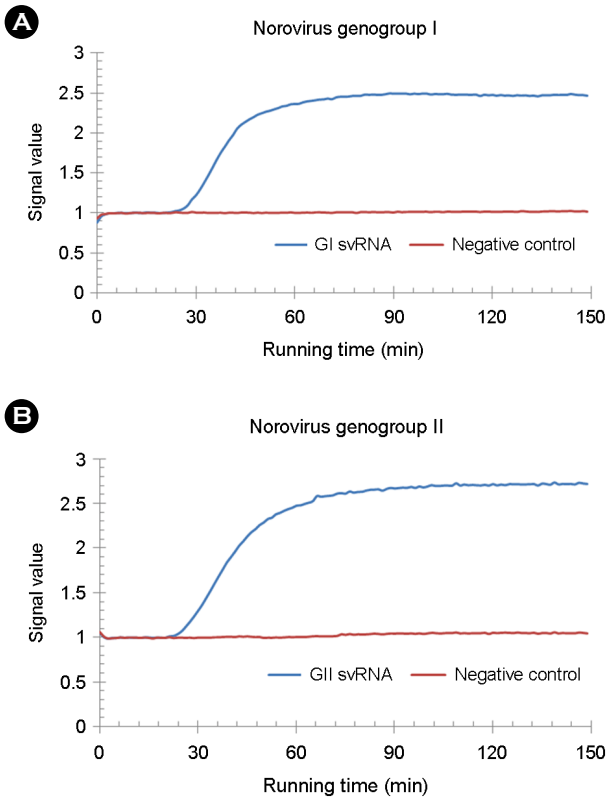


**Fig. 2.** Optimal running time of real-time NASBA. Real-time NASBA was performed for deciding optimal running time to detect noroV RNA. As the result, it was confirmed that optimal running time was 90 minutes for real-time NASBA. (A) noroV GI, (B) noroV GII.

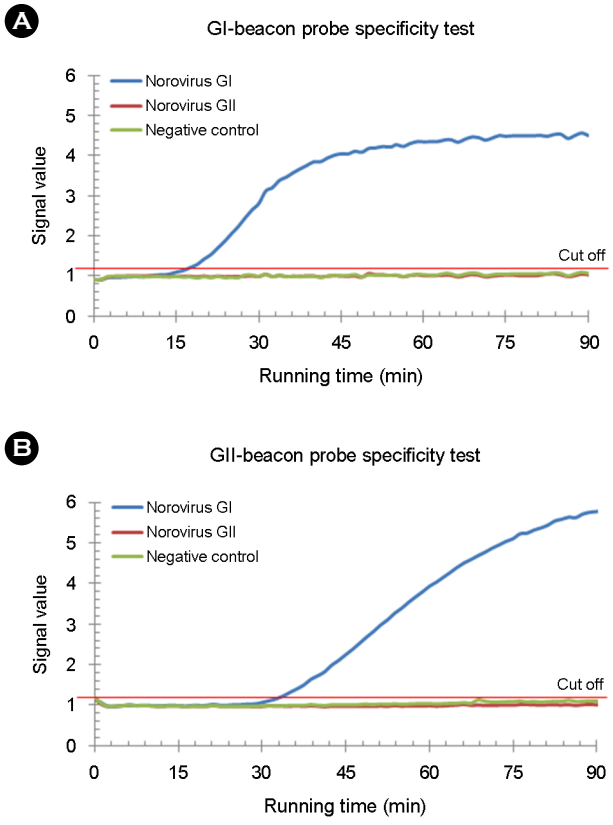


**Fig. 3.** The specificity of noroV GI and GII specific beacon probe used in real-time NASBA. We did experiment with each of genogroup specific primer and probe to confirm specificity. As the result, these data were showed that both genogroup primer and probe have specificity. (A) noroV GI, (B) noroV GII.

and were consistently maintained after 80 minutes. Thus, ninety minutes were decided as the optimal running time of real-time NASBA (Fig. 2).

**The specificity and sensitivity of the noroV GI and GII specific MB probes**

The background signal value corresponded to the mean signal value measured in a well contained specific MB probes and negative control RNA (data not shown). Accordingly, the cut-off value was set at 1.2.

Specificity of the MB probe was tested for sv-RNA, which was designed to detect noroV GI and GII (Fig. 3). Then final signal value of the GI specific MB probe was 4.414 in GI sv-RNA, 1.030 in GII sv-RNA, and 1.045 in negative control, and that of the GII specific MB probe was 6.300 in GI, 1.059 in GII, and 1.065 in negative control. This could be differentiated from the cut-off value. That indicated that each genogroup specific MB probe could correctly detect each genotype in real-time NASBA.

Next, sensitivity of each designed specific MB probe was tested using sv-RNA. Real-time NASBA was performed for sv-RNA, which was 10-fold diluted from 10 ng to 1 fg. Conventional RT-PCR was conducted for the same sv-RNA, and the results of conventional RT-PCR were compared with that of real-time NASBA (Fig. 4). The results showed that while agarose gel electrophoresis of RT-PCR products could detect as small as 10 pg of sv-RNA, the real-time NASBA assay could detect 100 fg of sv-RNA. These results suggested that the real-time NASBA assay has much higher sensitivity than conventional RT-PCR assay and might

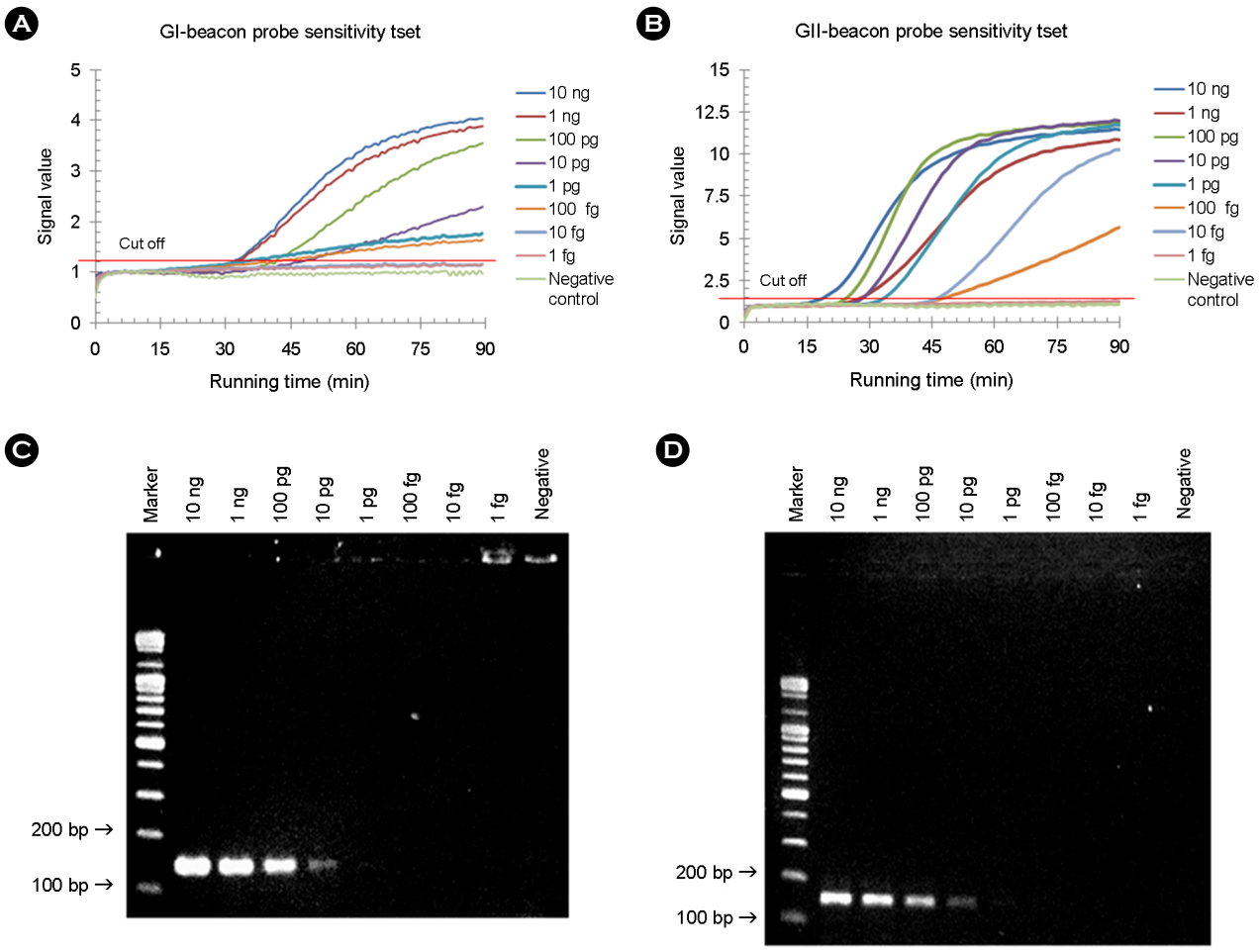


**Fig. 4.** The sensitivity of noroV GI and GII specific beacon probe used in real-time NASBA. To determine the sensitivity of this assay, serial 10-fold dilutions of sv-RNA of NoroV GI and GII were used for RT-PCR and real-time NASBA. The results showed that while RT-PCR could detect 10 pg of sv-RNA, the real-time NASBA could detect 100 fg of sv- RNA. (A), (C) noroV GI; (B), (D) noroV GII.

detect the specimens which could have been false negative by RT-PCR.

In brief, there are five genotypes of noroV as mentioned above, and of them, noroV GI and GII cause primarily diseases in humans. In this context, Patterson et al. (2006) and Lamhoujet et al. (2009) devised real-time NASBA techniques designed to detect only noroV GII. Nevertheless, they could not detect simultaneously all the subgenogroups of noroV GII. For that reason, we attempted to design the primer and MB-probe in more conserved regions so that more subgenogroups can be simultaneously detected. With them, we devised the optimization of real-time NASBA, and subsequently, ascertained that they had remarkably high specificity and sensitivity to sv-RNA. However, the results of real-time NASBA did not indicate any quantitative increases of signal depending upon the concentration of sv-RNA and similar results were also shown in repeated experiments. It suggested that real-time NASBA was not suitable to quantitative detection of target. On the other hand, it was found that the qualitative detection sensitivity was superior to that of conventional RT-PCR. And, according to the results of sequencing, it enabled to detect the patient specimens diagnosed as noroV GI.2 (noroV GI subgenogroup 2) and noroV GII.7 (noroV GII subgenogroup 7). Despite of some uncertainty attributed to the small sample size, we thought that the results of this study suggested its favorable applicability to clinical specimens in clinical practice. For the accurate evaluation of this assay, further clinical studies were needed with more diverse genotypes of noroV and direct specimens.

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