Clinical Chemistry 54:2
406–413 (2008)

Description and Validation of a Novel Real-Time RT-PCR Enterovirus Assay

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BACKGROUND: Enteroviruses are a leading cause of aseptic meningitis in adult and pediatric populations. We describe the development of a real-time RT-PCR assay that amplifies a small target in the 5′ nontranslated region upstream of the classical Rotbart enterovirus amplicon. The assay includes an RNA internal control and incorporates modified nucleotide chemistry.

METHODS: We evaluated the performance characteristics of this design and performed blinded parallel testing on clinical samples, comparing the results with a commercially available RT-PCR assay (Pan-Enterovirus OligoDetect kit) that uses an enzyme immunoassay–like plate end detection.

RESULTS: We tested 778 samples and found 14 discrepant samples between the 2 assays. Of these, the real-time assay detected 6 samples that were negative by the OligoDetect kit, 5 of which were confirmed as positive by sequence analysis using an alternative primer set. Eight discrepant samples were positive by the OligoDetect kit and real-time negative, with 6 confirmed by sequencing. Overall, detection rates of 97% and 96% were obtained for the OligoDetect kit and real-time assays, respectively. Sequence analysis revealed the presence of a number of single nucleotide polymorphisms in the targeted region. The comparative sensitivities of the 2 assays were equivalent, with the limit of detection for the real-time assay determined to be approximately 430 copies per milliliter in cerebrospinal fluid.

CONCLUSIONS: This novel real-time enterovirus assay is a sensitive and suitable assay for routine clinical testing. The presence of single nucleotide polymorphisms can affect real-time PCR assays.

Human enteroviruses belong to the *Picornaviridae* family and include more than 100 serotypes divided among 5 groups (poliovirus, human enterovirus A, human enterovirus B, human enterovirus C, and human enterovirus D) *(1)*. Human enterovirus (HEV)[4] infections are the leading cause of aseptic meningitis in pediatric and adult populations and have been associated with severe disease such as myocarditis, encephalitis, and paralytic poliomyelitis *(2)*. Because the symptoms of viral and bacterial meningitis are very similar but have different treatment regimens, a rapid, sensitive, and specific test is needed to identify the infective agent. Rapid and accurate diagnosis of HEV infection can reduce use of antibiotics, duration of hospitalization, and financial cost *(3, 4)*.

RT-PCR has become the diagnostic methodology of choice due to its sensitivity and rapid turnaround time, allowing significant improvement in patient care and management. A majority of molecular HEV assays target the highly conserved region within the 5′ nontranslated region (NTR) described by Rotbart et al. *(5–10)*. The RT-PCR primer and probe target sequences within this region, although not perfectly homologous, are to a great extent conserved among the human enteroviruses *(11)*. With real-time RT-PCR testing, however, false negative results can occur when single nucleotide polymorphisms (SNPs) located beneath the probe reduce or prevent probe hybridization and fluorescence during detection *(12–15)*.

We describe the development of a novel real-time HEV assay. This assay amplifies and detects a conserved region upstream of the Rotbart amplicon by using primers containing degenerate and modified bases and a minor groove binder (MGB)-conjugated hybridization probe. A noncompetitive RNA internal control (IC) consisting of lyophilized armored RNA was incorporated into the real-time assay and coextracted in each sample to monitor nucleic acid extraction and RT-PCR inhibition. We tested the analytical

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Received July 25, 2007; accepted October 30, 2007.
Previously published online at DOI: 10.1373/clinchem.2007.095414
[4] Nonstandard abbreviations: HEV, human enterovirus; NTR, nontranslated region; SNP, single nucleotide polymorphism; MGB; minor groove binder; IC, internal control; EIA, enzyme immunoassay.

Table 1. Enterovirus and internal control primers and probes.

Primers and probes	Sequence	Nucleotide positions[a]
Rotbart modified design		
EV1	5′-GGCCCCTGAATGCGGCTAAT-3′	454–473[a]
EV2	5′-CAATTGTCACCATAAGCAGCCA-3′	583–604
Probe	5′-MGB-NFQ-CTTTGGGTGTCCGTGT-FAM-3′	549–564
Modified probe	5′-MGB-NFQ-ACTT*T*GGGZ*GZ*CCGT*GT*-FAM-3′	548–564
Upstream design		
EV-F	5′-AATAAATCATAAGAAGAGYCZ*ATTGAGCTA-3′	422–439[a]
EV-R	5′-AATAAATCATAAGGA*TTRGCCGCA*TTC-3′	461–475
Probe	5′-MGB-FAM-TCCGGCCCCTGAATGC-NFQ-3′	451–466
RNA internal control		
IC forward	5′-CCA*TCAAA*GTCGA*GGTGCCTAAAGTG-3′	1513–1538[b]
IC reverse	5′-ACGAACGCCATGCGGCTACAGGAAGCTC-3′	1563–1590
IC probe	5′-MGB-PY-G*TG*TTG*G*TG*G*TG*TAG*AG*C-NFQ-3′	1549–1566

Underlined nucleotides indicate 5′ tails. [a] Cox B5 (acc. no. X67706). [b] ms2 Phage (acc. no. NC 001417). NFQ, nonfluorescent quencher; T*, A*, G*, super T base, super A base, super G base (Nanogen); Z*, neutral base (Nanogen).

performance of this novel design and compared it to the Pan-Enterovirus OligoDetect kit using patient samples submitted to ARUP Laboratories during 2006. In addition, we discuss a hybridization probe–based assay targeting the highly conserved regions described by Rotbart et al. (*5*) and present data illustrating the effect of unforeseen SNPs.

Materials and Methods

VIRAL RNA EXTRACTION

We used 778 patient specimens submitted for molecular HEV testing at ARUP Laboratories from March 2006 through August 2006. Samples of them were analyzed concurrently using the Pan-Enterovirus OligoDetect kit (cat. no. CWO-PCR; Millipore) and real-time RT-PCR assays. All samples used for verification were deidentified and blinded according to institutional protocols. We also tested an additional 27 different known serotypes from clinical isolates by use of the real-time platform. We extracted 70 μL of each sample in duplicate using the Qiagen BioRobot 9604 and eluted the nucleic acids in 86 μL AVE buffer. The RNA IC, consisting of quantified and lyophilized hepatitis C virus (HCV)-armored RNA prepared as described (*16*), was added to the lysis buffer before extraction to yield a final concentration of 300 copies/reaction.

RT-PCR ENZYME IMMUNOASSAY

We performed RT-PCR on duplicate RNA extracts using OneStep RT-PCR kit (Qiagen) and biotinylated PCR primers in the Pan-Enterovirus OligoDetect kit as described (*17*). After RT-PCR, the biotinylated enterovirus amplicon was denatured, hybridized to the enzyme immunoassay (EIA) plate–bound probe, and detected according to the manufacturer's recommendations. Briefly, 10 μL amplicon was transferred to the EIA reaction wells, denature, and allowed to hybridize in the presence of hybridization buffer for 30 min at 37 °C. After a wash step, 100 μL streptavidin/horseradish peroxidase conjugate was added and incubated for 30 min at 37 °C followed by a 2nd wash step. We added 100 μL tetramethylbenzidine substrate and allowed the color change to develop for 10 min at 37 °C before adding 100 μL stop solution. The absorbance was measured at 450 nm on a plate reader.

REAL-TIME RT-PCR ASSAY

The initial real-time design used primers EV1 and EV2, which are similar to those described by Rotbart et al. (*5*) and identical to those in the OligoDetect kit, to generate a 151-bp amplicon. The FAM-labeled Eclipse™ probe (Nanogen), 5′-CTTTGGGTGTCCGTGT-3′, was used for amplicon detection. Primer and probe sequences are listed in Table 1. Each 25-μL reaction contained 10 μL viral RNA, 600 nmol/L EV1 primer, 6 μmol/L EV2 primer, and 200 nmol/L EV probe and was amplified using the OneStep RT-PCR Kit (Qiagen) according to the manufacturer's recommendations. We performed the real-time RT-PCR assay on the Applied Biosystems 7900HT using cycling conditions described (*17*) with the exception of 50 cycles in place of 40 to efficiently detect low positive samples. Melting curve analysis

consisted of 15-s holds at 95 °C, 45 °C, and 95 °C with the ramp rate between 45 °C and 95 °C set to 5%. Fluorescence signal was acquired at the annealing step of each cycle during amplification and throughout the final ramp between 45 °C and 95 °C.

For the novel real-time design, we amplified viral RNA using the QuantiTect RT-PCR Kit (Qiagen) and primers and probes (Nanogen) listed in Table 1. Each 50-μL reaction contained 20 μL RNA, 250 nmol/L EV-F primer, 1.0 μmol/L EV-R primer, and 200 nmol/L EV probe. A subset of samples was analyzed with the EV-F primer at 1.0 μmol/L. The IC primers and probe, at 50 and 200 nmol/L, respectively, amplify and detect a 78-bp region of the ms2 coat protein gene. This gene is packaged, along with a portion of a nonbacteriophage gene (in this instance HCV), inside the ms2 bacteriophage coat proteins as a recombinant RNA. The hybridization probes contain a 5′ MGB and fluorophore with a 3′ quencher. These probes are not hydrolyzed and fluoresce only when bound. The EV probe contained a FAM label, whereas the IC probe was labeled with PY559 dye.

We programmed the Applied Biosystems 7900HT with the following RT-PCR protocol: 10 min at 20 °C for 1 cycle, 30 min at 50 °C for 1 cycle, and 15 min at 95 °C for 1 cycle, followed by 50 cycles of a 3-step PCR (15 s at 95 °C, 30 s at 56 °C, and 30 s at 76 °C). The amplification program was followed by a melting-curve analysis consisting of 15-s holds at 95 °C, 45 °C, and 95 °C. The ramp rate was set at 100% for all steps except the final ramp between 45 °C and 95 °C, which was set to 5%. Fluorescent signal was acquired at the annealing step of each cycle during amplification and throughout the final ramp between 45 °C and 95 °C.

SEQUENCING

Discrepant analysis included sequencing a 284-bp region of the 5′ NTR that spanned the amplicons generated by both assays. We performed the RT-PCR reaction using the Qiagen OneStep RT-PCR kit according to the manufacturer's instructions. We added 10 μL RNA to 15 μL master mix containing 400 μmol/L dNTPs, 0.5 units heat-labile uracil-DNA glycosylase, and 800 nmol/L of the forward (5′-GGCTGCGTTG-GCGGCCTGCC-3′) and reverse (5′-CACCGGATG-GCCAATCCAAT-3′) primers. Cycling conditions were 10 min at 20 °C for 1 cycle, 30 min at 50 °C for 1 cycle, 15 min at 95 °C for 1 cycle, and 50 cycles of a 3-step PCR (30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C), followed by a 10-min cycle at 72 °C.

EVALUATION OF SEQUENCE VARIATION IN PRIMER TARGETS

We generated amplicons representing the genomic target sequence containing known mismatches with the upstream primer of the real-time assay by using modified upstream primers to amplify a plasmid containing a 284-bp region of the coxsackie B5 virus. Each modified primer contained a substituted nucleotide at the desired position to create the mismatch. We then cloned the amplicons into the pCRII vector (Invitrogen) and confirmed the presence of the nucleotide mismatch by sequencing. We quantified the plasmids spectrophotometrically and amplified 2-fold dilutions of each plasmid at the limit of detection in duplicate and compared them to a plasmid containing no mismatches.

LIMIT OF DETECTION AND SPECIFICITY

We used 10-fold dilutions of a plasmid, consisting of a 284-bp region of the coxsackie B5 virus cloned into the pCRII TOPO vector (Invitrogen), to generate a calibration curve to quantify an enterovirus-positive control. The limit of detection was determined by performing 5 or 6 2-fold serial dilutions of the positive control material, from 140 to 2 copies per reaction, in cerebrospinal fluid, serum, and plasma samples before extraction and amplification in duplicate. The lowest dilution detected in both replicates was defined as the limit of detection.

Specificity was determined by assaying nucleic acids from the following RNA viruses: rhinovirus 3, rhinovirus 7, West Nile, influenza A, influenza B, RSV, and norovirus genogroups I and II. Also tested were DNA viruses HSV, VZV, CMV, EBV, HHV6 A and B, BK, and adenovirus, as well as human genomic DNA.

Results

Preliminary attempts at developing a real-time RT-PCR assay for HEV involved using the same primer sequences in the custom Pan-Enterovirus OligoDetect assay along with an Eclipse™ real-time probe containing a 5′ MGB and quencher and a 3′ fluorophore. During side-by-side clinical testing, 6 of 37 positive samples failed to generate amplification curves but produced distinct melting curves approximately 7 °C lower than expected (Fig. 1A). Sequence analysis of these samples identified a T>A SNP beneath the probe at nucleotide position 556 or 558. The probe was redesigned to accommodate these SNPs, using modified neutral base chemistry at these 2 nucleotide positions. These neutral bases have the capacity to complement any nucleotide. Because incorporation of neutral bases decreases the melting temperature (T_m) of an oligonucleotide probe, a number of modified Super T bases were incorporated to compensate for this destabilizing effect. Each Super T base increases the probe T_m approximately 1 °C. Amplification curves and uniform melting curves were subsequently obtained for these polymorphic samples (Fig. 1B). After further optimization and incorporation

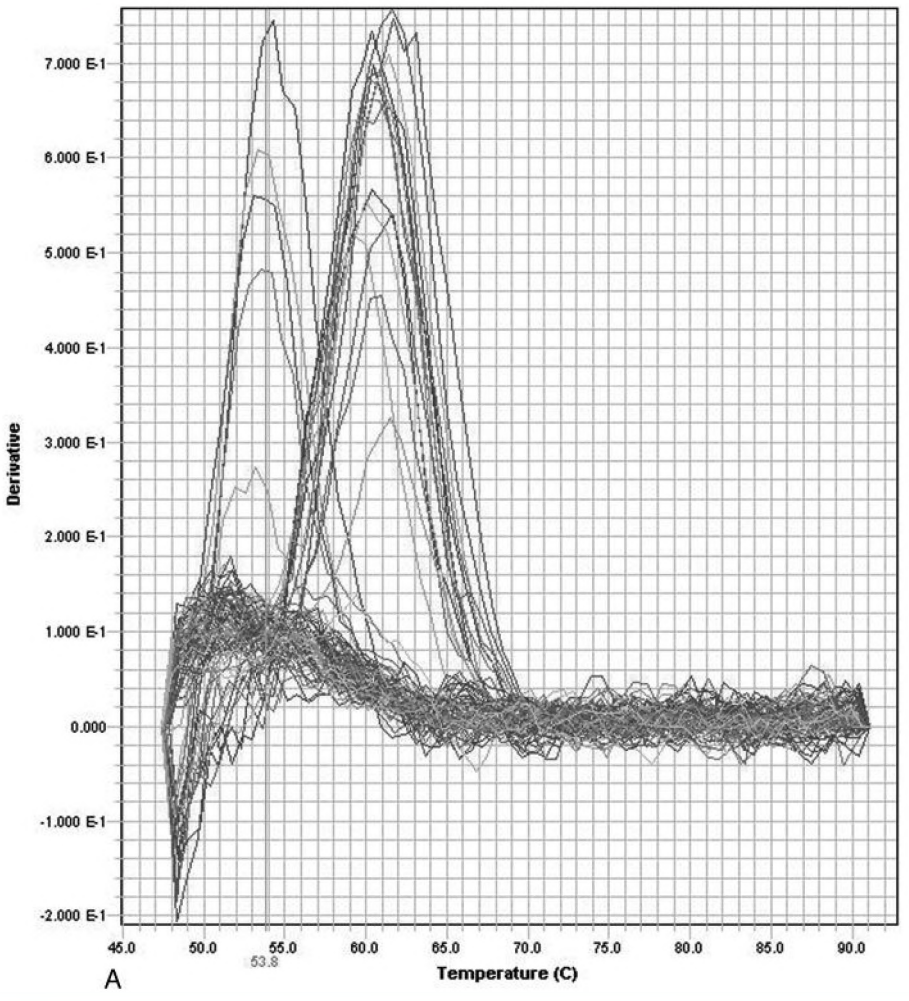

Fig. 1. (A), Melting curves from the initial real-time assay showing the atypical melting peak at 54 °C for a number of clinical HEV-positive samples.

These samples did not produce amplification curves and contained a single nucleotide polymorphism beneath the probe. (B), Melting curves from 6 positive samples that produced atypical melting peaks as shown in (A), as well as the HEV-positive control generated using the modified Eclipse™ probe containing neutral bases.

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of the RNA IC, however, the assay was not as sensitive as the Pan-Enterovirus assay (data not shown).

A novel real-time HEV assay was designed using primers and probes that amplify and detect a 54-bp target upstream of the traditional Rotbart enterovirus amplicon currently used in many real-time and non–real-time HEV assays (Fig. 2). Preliminary experiments with this design also identified a number of discrepant samples that were positive by the Pan-Enterovirus assay but not detected by the real-time assay. Sequence results for the discrepant samples indicated a sequence variant underneath the forward primer. This design was modified to accommodate the polymorphic nucleotide by incorporation of a neutral base. In addition, both HEV primers were modified to include a 12-nucleotide nontemplated AT-rich tail at their 5′ ends. Primer and probe sequences are listed in Table 1.

A breakdown of the sample types tested is listed in Table 2. Of the 778 samples, the real-time assay detected 144 positive samples whereas the Pan-Enterovirus assay detected 147 positives. Results for 14 samples were discrepant after repeat testing by both assays. Six samples were positive only by the real-time assay and 8 samples were positive only by the Pan-Enterovirus assay. As part of the discrepancy analysis, we interrogated the nucleotide sequence for the presence of SNPs that could confound

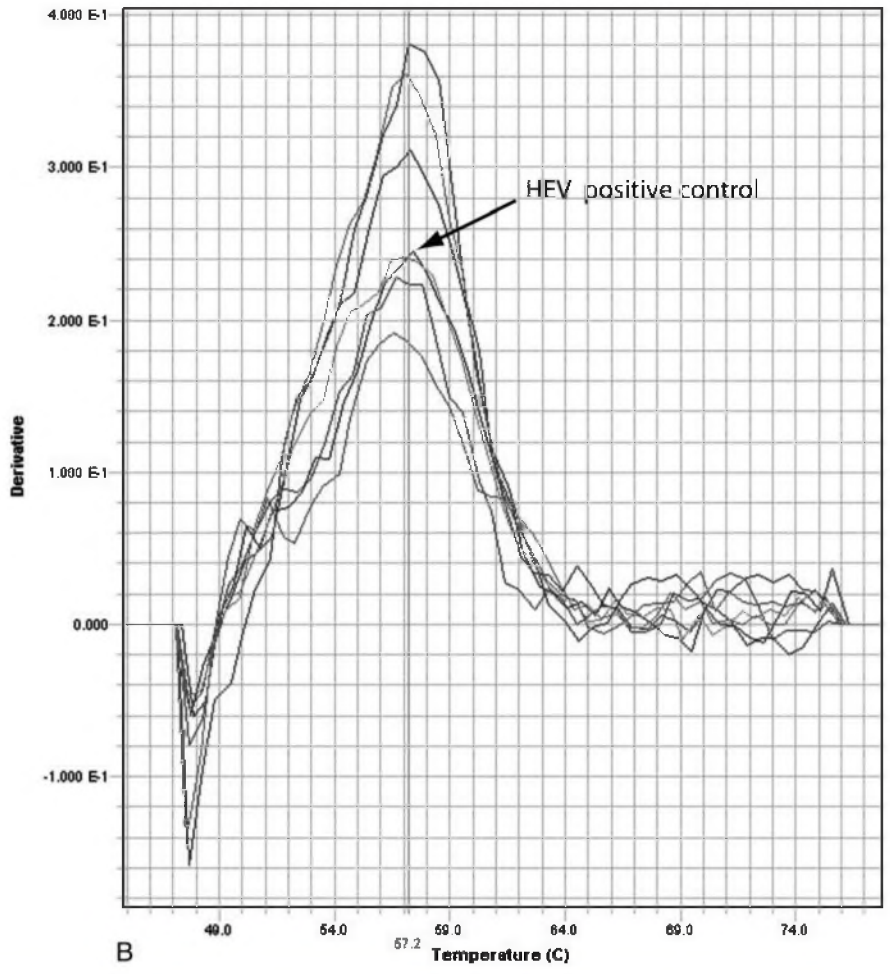

Fig. 1. Continued.

either assay by sequencing a 284-nucleotide region of the 5′ NTR that spans the amplicons generated by both assays. Five of the 6 real-time positives could be sequenced, with 1 sample containing a SNP (T>C) under the 3′ terminal base of the Pan-Enterovirus reverse primer and 1 sample containing a 2-nucleotide mismatch at the 5′ terminal end of the same primer. Six of the 8 Pan-Enterovirus positives could be sequenced, with 4 samples containing SNPs beneath the upstream real-time primer (Fig. 3).

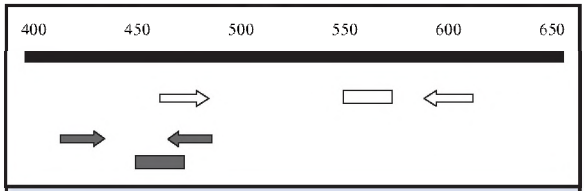

Fig. 2. Illustration showing the relative locations of the primers and probe for the 2 real-time assays described.

Open arrows and open box indicate the respective locations of the primers and probe for the real-time assay modeled after the Rotbart design. Closed arrows and closed box indicate primer and probe location for the newly described upstream design.

To determine the effects of these SNPs in the real-time assay, separate amplicons representing the genomic target sequence containing each polymorphism were generated using modified primers that contained the desired nucleotide substitution. Amplicons were cloned, and 2-fold serial dilutions of each plasmid at the limit of detection were made and amplified in duplicate. The A>G mismatch at the 4th position of the genomic target and the C>T mismatch at the 11th position of the target gave modest decreases in sensitivity of 4- and 6-fold, respectively, compared to a plasmid

Table 2. Breakdown of the total number of true positive and negative samples tested for each sample type.

	Cerebrospinal fluid	Plasma	Serum	Other	Total
Positive	144	6	0	0	150
Negative	484	133	5	6	628
Total	628	139	5	6	778

The number of true positives was determined by using sequence analysis as the tiebreaker when there was a discrepancy between the Pan-Enterovirus OligoDetect and real-time assay. "Other" includes these samples: 1 pericardial fluid, 1 tissue, 1 pleural fluid, and 3 of unknown type.

that contained no mismatches. The G>A mismatch at the terminal position of the upstream primer yielded a more notable 12-fold decrease in sensitivity.

An additional 27 clinical isolates, serotyped by serum neutralization, were successfully analyzed using the real-time assay and generated uniform melting peaks (Fig. 4). These serotypes included coxsackie A virus 9 and 16; coxsackie B viruses 1, 3, 4, 5, and 6; echoviruses 2, 3, 4, 5, 6, 7, 11, 15, 16, 18, 20, 21, 24, 25, 27, 30, and 31; enterovirus 71; and polioviruses 2 and 3.

The detection limits of the 2 HEV assays, measured by extracting and amplifying 2-fold serial dilutions of enterovirus-positive control material, were equivalent as judged by the presence of a crossing threshold and amplification curve in the real-time assay and absorbance above the background in the Pan-Enterovirus assay. For cerebrospinal fluid and serum, the limit of detection in the real-time assay was 7 copies/reaction (430 000 copies/L), and for plasma, 18 copies/reaction (1 110 000 copies/L).

Specificity was determined by amplifying nucleic acids from a number of RNA and DNA viruses; no cross-reactivity was observed.

Discussion

Real-time RT-PCR has become a standard tool for diagnostic testing because of its rapid turnaround time, relatively low risk of contamination, and ease of use. Because fluorescence acquisition for most real-time methodologies is based on the hybridization of a probe to its complimentary target, however, there is a greater possibility of inadvertently missing a positive sample owing to nucleotide mismatches under the probe. The influence of an unknown single nucleotide polymorphism (SNP) on a specific real-time assay is not always obvious, but the number and position of SNPs beneath a real-time probe can determine whether an assay is susceptible to a false-negative result or misquantification *(12, 15, 18–23)*. Another potential disadvantage of real-time PCR is the intrinsic fluorescent background that can affect the ability to detect low positives that generate weak fluorescent signals. This background fluorescence is contingent on the instrumentation, detection chemistry, and dye and quencher selection used *(24–26)*.

The majority of real-time enterovirus assays use the highly conserved regions of the 5′ NTR described by Rotbart et al. *(5)* as primer and probe binding sites. Using these regions, we noted a number of HEV-positive clinical samples that failed to produce amplification curves but were identified by atypical melting peaks. Sequencing of these samples confirmed the presence of 2 different SNPs beneath the probe that reduced its ability to efficiently bind during the annealing stage of the PCR. Melting-curve analysis was performed at lower temperature, however, which allowed probe hybridization and fluorescence detection even in the presence of the SNP. Although these SNPs may confound real-time assays, the Pan-Enterovirus OligoDetect assay could tolerate them because the hybridization step, where the single-stranded amplicon is captured by the plate-bound probe, is performed at low

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GAAGAGTCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAATGCGGCTAATCC Consensus
..........C.......AA.....A.............................. (9)
...A..............A.......................................... (10)
..................GAC....................................... (7)
..................G.......................................... (5)
........................T.................................. (14)
........................T.................................. (4)
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Fig. 3. Sequence alignment indicating the position of the 4 single nucleotide polymorphisms beneath the forward primer (underlined) of the 6 samples that were negative by real-time assay.

Two samples did not contain polymorphisms beneath the forward primer. · indicates nucleotide identical to the consensus sequence.

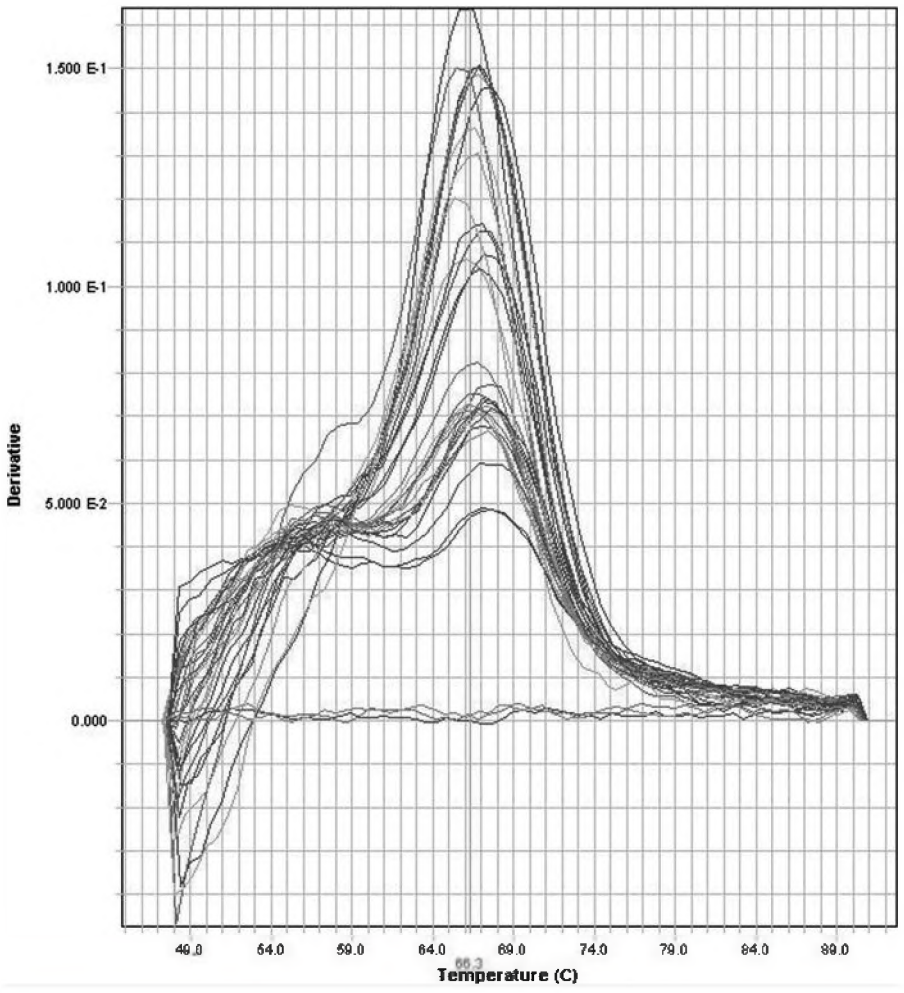

Fig. 4. Melting curves of 27 different human enterovirus isolates tested using the real-time assay, demonstrating uniform melting peaks.

temperature and stringency. The OligoDetect assay is highly sensitive, but its primary disadvantages are the lack of an internal control and labor intensity. Fortunately, modified and neutral base chemistry can be used in real-time assay designs to accommodate SNPs while maintaining thermodynamic stability *(16, 27, 28)*.

This real-time assay amplifies and detects a 54-bp region of the 5′NTR upstream of the traditional Rotbart amplicon. The primers used in this assay include degenerate and modified nucleotides to accommodate known sequence polymorphisms, in addition to 12 nucleotide nontemplated tails on their 5′ ends, which increase PCR efficiency and quantity of product generated. In addition, target detection was achieved using newly described Pleiades hybridization probes that contain a 5′ MGB and fluorophore and a 3′ nonfluorescent quencher. These probes have lower backgrounds and a higher signal-to-noise ratio than other hybridization probes *(26)*. The MGB allows the design and use of shorter probes while maintaining a higher melting temperature, which may be an advantage in designing probes for small conserved regions *(29, 30)*. Moreover, because the hybridization probes are not hydrolyzed during the reaction, they remain available for melting-curve analysis and amplification product confirmation. As demonstrated in this study, the ability to confirm results by melting curves is particularly valuable and may aid in identifying potential false-negative results caused by SNPs beneath real-time probes.

An excellent correlation between the real-time assay and the Pan-Enterovirus OligoDetect assay was observed, with only 14 total discrepant samples of 778 (6 from the Pan-Enterovirus assay and 8 from the real-

time assay). Sequencing of discrepant samples revealed the presence of 3 different SNPs beneath the forward real-time primer and 2 beneath the reverse OligoDetect primer. Studies performed to determine the effect of the 3 SNPs in the real-time assay indicated that the 2 internal nucleotide mismatches had a modest 4- to 6-fold decrease in assay sensitivity, whereas the SNP at the terminal 3′ end of the forward primer had an approximate 12-fold reduction in sensitivity. The remaining discrepant samples between the 2 assays may be due to subtle variations in assay sensitivities, since these samples did not contain any sequence variants.

This study demonstrates how using modified nucleotide chemistries in primers and hybridization probes can reduce the impact of SNPs on real-time assays. It also highlights the inadequacy of current databases routinely used in assay designs and is a reminder of the necessity of an extensive clinical validation.

Grant/funding Support: Nanogen provided free research materials for this study.
Financial Disclosures: W.C.H. has received speaker's honoraria from Nanogen. D.R.H. has received speaker's honoraria and consultation fees and holds equity interest in Nanogen.
Acknowledgments: We wish to thank Maria Erali and Walt Mahoney for critical reading of the manuscript and Derek Vanhille for aid in data collection.

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