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1 RESEARCH

Development of a Quantitative Real-Time Nucleic Acid Sequence-Based Amplification Assay with an Internal Control Using Molecular Beacon Probes for Selective and Sensitive Detection of Human Rhinovirus Serotypes

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Abstract Evidence demonstrating that human rhinovirus (HRV) disease is not exclusively limited to the upper airways and may cause lower respiratory complications, together with the frequency of HRV infections and the increasing number of immunocompromised patients underline the need for rapid and accurate diagnosis of HRV infections. In this study, we developed the first quantitative real-time nucleic acid sequence-based amplification assay with an internal control using molecular beacon probes for selective and sensitive detection of human rhinovirus serotypes. We described a simple method to accurately quantify RNA target by computing the time to positivity (TTP) values for HRV RNA. Quantification capacity was assessed by plotting these TTP values against the starting number of target molecules. By using this simple method, we have significantly increased the diagnostic accuracy, precision, and trueness of real-time NASBA assay. Specificity of the method was verified in both in silico and experimental studies. Moreover, for assessment of clinical reactivity of the assay, NASBA has been validated on bronchoalveolar lavage (BAL) specimens. Our quantitative NASBA assay was found to be very specific, accurate, and precise with high repeatability and reproducibility.

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Introduction

Human rhinoviruses (HRVs) are the most frequent cause of acute upper respiratory tract infections in humans and are usually responsible for 30–50% of cases of common cold [1–3]. However, they may also be associated with more-severe lower respiratory tract infections. Rhinoviruses have been isolated from cases of cystic fibrosis, otitis media, sinusitis, asthma, exacerbations of chronic obstructive pulmonary disease (COPD), and pneumonia, especially in children, in the elderly, and in immunocompromised patients [4–14]. Evidence demonstrating that HRV disease is not exclusively limited to the upper airways and may cause lower respiratory complications, together with the frequency of HRV infections and the increasing number of immunocompromised patients underline the need for rapid and accurate diagnosis of HRV infections. Two nucleic acid amplification techniques (NAATs) are actually available for the detection of HRV: reverse transcription-PCR (RT-PCR) [15, 16], and nucleic acid sequence-based amplification (NASBA) [17, 18]. NASBA has proven to be highly sensitive, specific, and more rapid than RT-PCR technique [19, 20]. Currently, only qualitative NASBA kits for the detection of HRV are commercially available (registered trademarks owned of bioMérieux, Marcy L'Etoile, France), while there are no quantitative NASBA kits. Some quantitative molecular beacon real-time NASBA assays have been described in the literature, mainly for the identification of human immunodeficiency virus (HIV), respiratory syncytial virus A and B, influenza A virus

66 (H1N1), *Trypanosoma brucei*, *Aspergillus fumigatus*,
67 *Plasmodium* species, and *Listeria monocytogenes* [21–29].
68 However, these real-time NASBA assays use mathematical
69 models for the analysis of results that requires employing
70 of specific complex software calibrated to each target [30].
71 Other quantitative NASBA assays, instead, compute the
72 ratio of the time to positivity (TTP) values for both the
73 target RNA and internal control by using standard curves
74 with a correlation coefficient less than 0.99 ($R^2 < 0.99$),
75 index of an insensitive assay [19, 24, 26].
76   Aim of this study was to develop the first quantitative
77 real-time nucleic acid sequence-based amplification assay
78 internally controlled using molecular beacon for selective
79 and sensitive detection of HRV serotypes. Validation and
80 standardization were performed by evaluating diagnostic
81 trueness, precision, and accuracy of real-time NASBA
82 assay.

Materials and Methods

Viral Isolates and RNA Extraction

85 Prototype human rhinovirus serotype 16 (HRV-16) was
86 obtained from the American Type Culture Collection
87 (ATCC, Manassas, Virginia). Rhinovirus serotype 16
88 (ATCC VR-283), originally isolated from a human clinical
89 specimen, was extracted by using an automatic extractor
90 NucliSENS easyMAG platform (bioMérieux, France),
91 according to the manufacturer's recommendations. One-
92 hundred-microliters of HRV-16 were used for the extrac-
93 tion, RNA was eluted in 25 µl of nuclease-free water and
94 stored at −80°C. To evaluate the specificity of the HRV
95 NASBA assay, purified RNA templates from 12 HRV
96 isolates, and 14 selected respiratory viruses other than
97 HRV were used for inclusivity and exclusivity testing
98 (Table 1).

In Vitro RNA Transcription

100 Viral cDNA was generated, first by incubation of random
101 primers (600 ng/µl) and dNTPs (10 mM) (Invitrogen) with
102 10 µl of HRV-16 RNA for 5 min at 70°C. Subsequently, a
103 mix containing buffer 5× [250 mM Tris–HCl (pH 8.3 at
104 25°C), 375 mM KCl, and 50 mM DTT], $MgCl_2$ (25 mM),
105 ImpromII RT (1 U/µl), and Recombinant RNasin® Ribo-
106 nuclease Inhibitor (40 U/µl) (Promega) was added. The
107 total volume (20 µl) of the reaction mixture was incubated
108 for 5 min at 25°C, 60 min at 42°C, and 15 min at 70°C
109 using 9800 Fast Thermal Cycler (Applied Biosystems,
110 Monza, Italy). cRNA production was carried out using
111 T7-RiboMAX Large Scale RNA Production Systems
112 (Promega, USA) at 37°C for 4 h. One-tenth of cRNA

Table 1 Evaluation of real-time NASBA specificity with HRV isolates and respiratory viruses other than HRV

Taxon	Provider	Test specificity for: HRV
Inclusivity testing		
Human rhinovirus 1B	ATCC VR-1366	+
Human rhinovirus 2	ATCC VR-482	+
Human rhinovirus 3	ATCC VR-483	+
Human rhinovirus 7	ATCC VR-1601	+
Human rhinovirus 9	ATCC VR-489	+
Human rhinovirus 16	ATCC VR-283	+
Human rhinovirus 41	ATCC VR-339	+
Human rhinovirus 58	ATCC VR-1168	+
Human rhinovirus 8	QCMD EQA RV.CV10-02	+
Human rhinovirus 42	QCMD EQA RV.CV10-01	+
Human rhinovirus 72	QCMD EQA RV.CV10-03	+
Human rhinovirus 90	QCMD EQA RV.CV10-05	+
Exclusivity testing		
Influenza A virus (H1N1)	QCMD EQA INFRNA10-08	−
Influenza A virus (H3N2)	QCMD EQA INFRNA10-01	−
Influenza B virus	QCMD EQA INFRNA10-06	−
Human parainfluenza virus 1	ATCC VR-94	−
Human parainfluenza virus 2	ATCC VR-92	−
Human parainfluenza virus 3	ATCC VR-93	−
Human parainfluenza virus 4a	ATCC VR-1378	−
Human parainfluenza virus 4b	ATCC VR-1377	−
Human coronavirus OC43	ATCC VR-1558	−
Human coronavirus 229E	ATCC VR-740	−
Human coronavirus NL63	QCMD EQA RV.CV10-10	−
Human coxsackievirus B4	ATCC VR-184	−
Human respiratory syncytial virus A	ATCC VR-1540	−
Human respiratory syncytial virus B	ATCC VR-1400	−

Note: *ATCC* American type culture collection, *QCMD (EQA)* quality control for molecular diagnostics (external quality assessment)

113 product was treated with RQ1 RNase-Free DNase (Pro-
114 mega, USA) at 37°C for 15 min followed by incubation
115 with EDTA for 15 min at 65°C. HRV-16 cRNA was
116 purified using RNAgent kit (Promega, USA) following
117 manufacturer's instructions. HRV-16 cRNA was quantified
118 using Quant-iT DNA BR assay on Qubit™ fluorometer
119 (Invitrogen, Carlsbad, USA), and the number of molecules

120 per microliter calculated from the molecular weight of
121 HRV-16 amplicon (70,950 MW) and Avogadro number
122 (6.023×10^{23}). Ten-fold dilutions of RNA standards were
123 generated in order to amplify from 10^8 to 1 copy per reaction,
124 and frozen at $-80°C$ until use.

Synthesis of Internal Control (IC) RNA

126 For the production of internal control (IC) RNA, we used
127 the human U1A housekeeping gene encoding the "A"
128 protein present in the human U1 small nuclear ribonu-
129 cleoprotein (snRNP) particle. To generate the IC RNA, the
130 U1A molecule was extracted from a clinical specimen,
131 precisely from a human bronchoalveolar lavage sample,
132 and subjected to reverse transcription (RT) reaction by
133 using random primers (600 ng/µl) and ImpromII RT (1 U/
134 µl). IC cDNA product was amplified by using adapted
135 NASBA primers containing the T7 RNA polymerase pro-
136 moter site. Briefly, 2 µl of IC cDNA was added to 28 µl of
137 PCR solution containing Flexi Buffer 5×, 50 mM MgCl₂,
138 1 unit GoTaq® Hot Start Polymerase (Promega), 200 µM
139 of each dNTP, and 25 µM of each U1A primer. After an
140 initial denaturation step of 2 min at 94°C, the first-round
141 PCR amplification was carried out under the following
142 conditions: 95°C for 0 s, 58°C for 15 s, 72°C for 10 s for
143 35 cycles, then one cycle at 72°C for 7 min using the 9800
144 Fast Thermal Cycler (Applied Biosystems). PCR product
145 was transcribed in vitro using T7-RiboMAX, as previously
146 described (see "In vitro transcription" in the "Materials
147 and methods" section). Ten-fold dilutions of IC RNA
148 standards were generated in order to amplify from 10^8 to 1
149 copy per reaction.

Real-Time NASBA Assay and Data Analysis

151 Primers and molecular beacon probes were obtained from
152 literature [31, 32] (Table 2), synthesized by Eurogentec
153 (Seraing, Belgium), and diluted to a final concentration of
154 100 µmol/l. Moreover, to maximize the oligonucleotides
155 stabilization, we added 60% dimethyl sulfoxide (DMSO)
156 to primers and beacons mixture. The HRV beacon was
157 labeled with FAM at its 5′-end and quencher DABCYL at its
158 3′-end, while the IC beacon contained a ROX at its 5′-end
159 and a DABCYL quencher at the 3′-end. The stability and
160 predicted structure of the beacons were analyzed by using
161 the European MFOLD server (http://frontend.bioinfo.
162 rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi). Real-
163 time NASBA reaction was performed on a NucliSens EasyQ
164 analyzer (BioMérieux) using the NucliSENS EasyQ Basic
165 Kit Version 2 (bioMérieux, Lyon, France) for the amplifi-
166 cation according to the manufacturer's manual. To obtain
167 the best amplification efficiency, conditions for the real-time
168 NASBA assay were optimized until the best primers,

Table 2 Primers and molecular beacons for HRV and IC-U1A real-time NASBA assay

Primer or MB[a]	Sequence (5′-3′)
HRVJ P1	**AATTCTAATACGACTCACTATAGGGAG**[b] ACCAMYWTTYTGYSTWGAWAC
HRVJ P2	CTCCGGCCCCTGAATGYGGCT
HRVJMB-FAM	**CCAAGC**[c]GAYGGGACCRACTACTTTGG **GCTTGG**
U1A P1	**AATTCTAATACGACTCACTATAGGG**[b] AGAGGCCCGGCATGTGGTGCATAA
U1A P2	CAGTATGCCAAGACCGACTCAGA
U1AMB-ROX	**CGTACG**[c]GATGAAAGGCACCTTCGTGGA **CGTACG**

[a] MB, molecular beacons. 5′-end of the beacon was labeled with FAM or ROX, and 3′-end was labeled with the nonfluorescent quencher DABCYL. [b] T7 RNA polymerase promoter sequence for NASBA. [c] Stem sequence for the molecular beacons

169 beacons, and KCl concentrations were determined. Titra-
170 tions of IC RNA (between 10 and 10^6 copies) and HRV RNA
171 were performed to determine the optimal amount of internal
172 control to generate the greatest dynamic range for the assay
173 without interference with the detection of HRV RNA (data
174 not shown). As a result, each reaction was run with the
175 addition of 10^5 copies of the IC RNA. Briefly, a total volume
176 of 10 µl of reaction mixture containing 80 mM KCl and
177 0.3 µM of the HRV- and IC-specific primers was incubated
178 with 2.5 µl HRV RNA and 2.5 µl IC RNA in the presence of
179 0.05 µM of HRV- and IC-molecular beacons at 65°C for
180 2 min to denature secondary structure RNA. The reaction
181 was subsequently cooled to 41°C for 2 min to anneal the
182 primers before adding 5 µl of enzyme mixture containing
183 avian myeloblastosis virus retrotranscriptase, RNase H, and
184 T7 RNA polymerase. After a brief centrifugation and gentle
185 mixing by tapping, the tubes were then incubated at 41°C for
186 90 min. To estimate the dynamic range of the real-time
187 NASBA assay (range of concentrations over which the
188 method performs in a linear manner with an acceptable level
189 of trueness and precision), we used HRV standard dilutions
190 from 10^8 copies/µl to 1 copy/µl. Sensitivity of NASBA assay
191 was determined by the lowest standard dilution consistently
192 detectable in replicate reactions at frequency of 100%,
193 whereas the limit of detection by the lowest concentration of
194 target quantified. Nuclease-free water was included as the
195 no-template control (NTC) to serve as a control for back-
196 ground fluorescence. The fluorescence signal was measured
197 with an interval time of 45 s for each independent reaction at
198 two wavelengths using the accompanying NucliSENS
199 EasyQ Director software (Version 2.0). Following amplifi-
200 cation, a specific fluorescence value was chosen as a positive
201 signal (threshold level = 1.1). Using this value for all
202 reactions in one experiment, the time that the target and IC
203 amplification curves reached the threshold level was

recorded as the time to positivity (TTP). HRV TTP values were regressed against the original standard curve to generate a predicted amount of target RNA. In particular, for data analysis, Excel calculation engine was used. NASBA dynamic range was calculated from TTP values regressed against the standard curve by using an Excel spreadsheet that was created "ad hoc" by us. In particular, for each individual HRV standard dilution (from 10^8 copies/reaction to 1 copy/reaction), fluorescent threshold levels of target close to 1.1 and values of the corresponding minutes were extrapolated by the NucliSENS EasyQ Director software. Therefore, minutes were normalized to a value of threshold level = 1.1, and the natural logarithm of normalized minutes was calculated. Quantification capacity was assessed by plotting the TTP values obtained against the logarithm of the number of target molecules in each dilution and calculating a linear regression.

Specificity of Real-Time NASBA Assay

The specificity of the developed real-time NASBA assay was verified by in silico studies (analytical specificity) against publicly available sequence databases (BLAST alignment software (http://blast.ncbi.nlm.nih.gov/Blast.cgi)) to evaluate possible cross-reactions with respiratory viruses other than HRV. Experimental specificity was also verified. In particular, sequences of different respiratory viruses were used for exclusivity testing (Table 1).

Validation and Standardization of Real-Time NASBA Assay

To determine the performance of HRV NASBA assay, we assessed the diagnostic trueness, precision, and accuracy of the technique. Specifically, precision was assessed by evaluating repeatability and intermediate reproducibility of NASBA assay. To determine the repeatability, several replicates containing the various amounts of HRV RNA were tested. The repeatability was determined by 10-fold serial dilutions of the HRV RNA standards. In particular, we used four different dilutions (10^2, 10^3, 10^4, and 10^5 copies/reaction) of quantification standards. Each dilution was analyzed 10 times, with the same method on identical test items in the same laboratory by the same operator using the same equipment. Regarding intermediate reproducibility, each dilution was analyzed with the same method on identical test items in 10 different runs performed by three different operators using different equipment on different days. Moreover, we used the Dixon's test to examine if one measure from 10 replicate measures that we performed (10^2, 10^3, 10^4, and 10^5) could be rejected or not, and the Shapiro–Wilk's test to compare these measures against the normal distribution. Statistical data analyses were performed using the PASW Statistics version 18.0 (SPSS Inc., Chicago, Illinois, USA).

Clinical Specimens

For assessment of clinical reactivity of the assay, respiratory specimens collected from our Virology Unit of the Azienda Ospedaliero-Universitaria San Giovanni Battista, Turin, were tested for HRV by real-time NASBA assay. Clinical specimens included 33 bronchoalveolar lavages (BAL) obtained from 33 transplant patients (M/F, 20/13; mean age, 56.2 years; range 21–88). A number of precautions were undertaken to prevent the occurrence of false-positive results. Each run included control reactions lacking template (no-template controls) to test for the presence of contamination or the generation of nonspecific amplification products under the assay conditions used.

Results

Sensitivity of Real-Time NASBA Assay

Optimal real-time NASBA assay conditions that allowed efficient amplification of the HRV target were established. Sensitivity and limit of detection of NASBA assay were assessed by repeated testing of serial logarithmic dilutions of the HRV RNA standards ranging from 10^8 to 1 copy/reaction. In particular, HRV NASBA dynamic range was calculated from TTP value (time point at which emitted fluorescence exceeds the baseline emission) regressed against the standard curve by using an Excel spreadsheet created "ad hoc" by us (Table 3). Results from linear regression show that HRV real-time NASBA assay was able to quantify from 10^8 to 10 copies/reaction. The standard curve of HRV dilutions plotted versus NASBA amplifications (expressed as TPP) is shown in Fig. 1, whereas plots for the amplification of HRV standard dilutions (from 10^8 to 10 copies/reaction), and the optimal amount of IC RNA (10^5 copies/reaction), are shown in Fig. 2. The consistency of replicates was measured by the correlation coefficient (R^2), which indicates the linearity of TPP values plotted in the standard curve. The R^2 index for HRV was 0.9948. Sensitivity of real-time NASBA assay was determined by the lowest standard dilution consistently detectable in replicate reactions at frequency of 100%. HRV sensitivity was 10 copies/reaction, whereas the limit of detection for reliable quantification was 1 copy/reaction.

Specificity of Real-Time NASBA Assay

Based on the data available at the BLAST alignment software, primers were tested in silico for potential

Table 3 Excel spreadsheet used to calculate HRV NASBA dynamic range

HRV dilutions	Threshold	Minutes	Normalized minutes threshold 1.1	Ln normalized minutes (TTP)	HRV ln dilutions
10^8	1.10476600	15.088	15.02290983	2.709576358	18.4206807
10^7	1.10571300	18.833	18.73569362	2.930430454	16.1180957
10^6	1.10279600	21.82917	21.7738249	3.080708556	13.8155106
10^5	1.11357000	26.3245	26.00370879	3.258239174	11.5129255
10^4	1.10117700	31.57033	31.53658585	3.451148328	9.21034037
10^3	1.10275300	39.81483	39.7154331	3.681739855	6.90775528
10^2	1.10297100	45.81017	45.68677418	3.821808851	4.60517019
10	1.09937600	51.05633	51.0853093	3.933496967	2.30258509

For each individual HRV standard dilution, fluorescent threshold levels close to 1.1 and values of the corresponding minutes were extrapolated by the NucliSENS EasyQ Director software. Minutes were normalized to a value of threshold level = 1.1, and the natural logarithm (ln) of normalized minutes was calculated. Quantification capacity was assessed by plotting the TTP values obtained against the logarithm of HRV dilutions and calculating a linear regression. *TTP* time to positivity
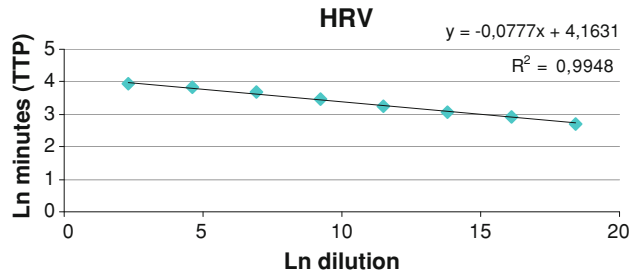

HRV

$y = -0,0777x + 4,1631$

$R^2 = 0,9948$

Fig. 1 Standard curve of HRV real-time NASBA assay. HRV TTP values were regressed against the standard curve to generate a predicted amount of target RNA. HRV ln dilutions (10^8 to 10 copies/reaction) on x-axis are plotted against ln minutes (TTP) on y-axis. The coefficient of correlation (R^2) was 0.9948. *TTP* time to positivity, *Ln* natural logarithm

cross-reactivity with respiratory viruses other than HRV, and demonstrated no significant homologies to any other sequences. Moreover, HRV primer and probe set, tested on respiratory viruses, was able to detect only HRV isolates, thus being the inclusivity of 100% (Table 1). The assay's specificity was further demonstrated by its ability to exclude all respiratory viruses other than HRV listed in Table 1. No positive results were demonstrated for the other respiratory viruses, indicating that this molecular assay is highly specific for HRV isolates, thus being the exclusivity of 100% (Table 1).

Validation and Standardization of Real-Time NASBA Assay

Diagnostic trueness of HRV real-time NASBA method, defined as the degree of agreement between the average value obtained from a large series of test results and an accepted reference value, was evaluated. To establish the level of trueness and concordance with the assigned value, data from 10 replicate measures of each dilution that we

performed (10^2, 10^3, 10^4, and 10^5) were analyzed using a Student's *t* test to compare the mean concentrations from each dilution with an accepted reference value. The mean concentrations from each dilution for the method are shown in Table 4 with the *t* test results, which indicate the significance of the differences between each experimental mean and the assigned value. Analysis of the *t* statistics showed that the method had *t*-calc values lower than the *t*-tab value, demonstrating a significant trueness of HRV assay.

Precision of method was expressed as the coefficient of variation (CV) in the $\log_{10}$ values of the concentration. Repeatability and intermediate reproducibility of HRV assay were evaluated over different concentrations ranging from 10^2 to 10^5 copies/reaction from 10 replicate measures ($n = 10$) of each reference viral quantification standard within a single run or in 10 different run experiments performed by three different operators. The precision associated with each dilution measurement (10^2, 10^3, 10^4, and 10^5) was assessed by calculation of the CV for each. The CVs within a single run (repeatability) ranged from 0.73 to 13.23%; whereas, the CVs in different runs (intermediate reproducibility) ranged from 3.71 to 16.71% (Table 4), indicating that the precision of the assay is satisfactory.

Diagnostic accuracy includes both trueness and precision. The measure of accuracy is usually expressed numerically in terms of bias (lack of agreement). Accuracy shall be within ±25% of the accepted reference value over the whole dynamic range, according to document ISO 16140 [33]. Data for the percentage of inaccuracy HRV method are reported in Table 4.

Clinical Performance of Real-Time NASBA Assay

The developed real-time NASBA assay was able to detect HRV RNA in 7/33 (21.2%) BAL specimens. The TTP values of these NASBA-positive samples ranged between
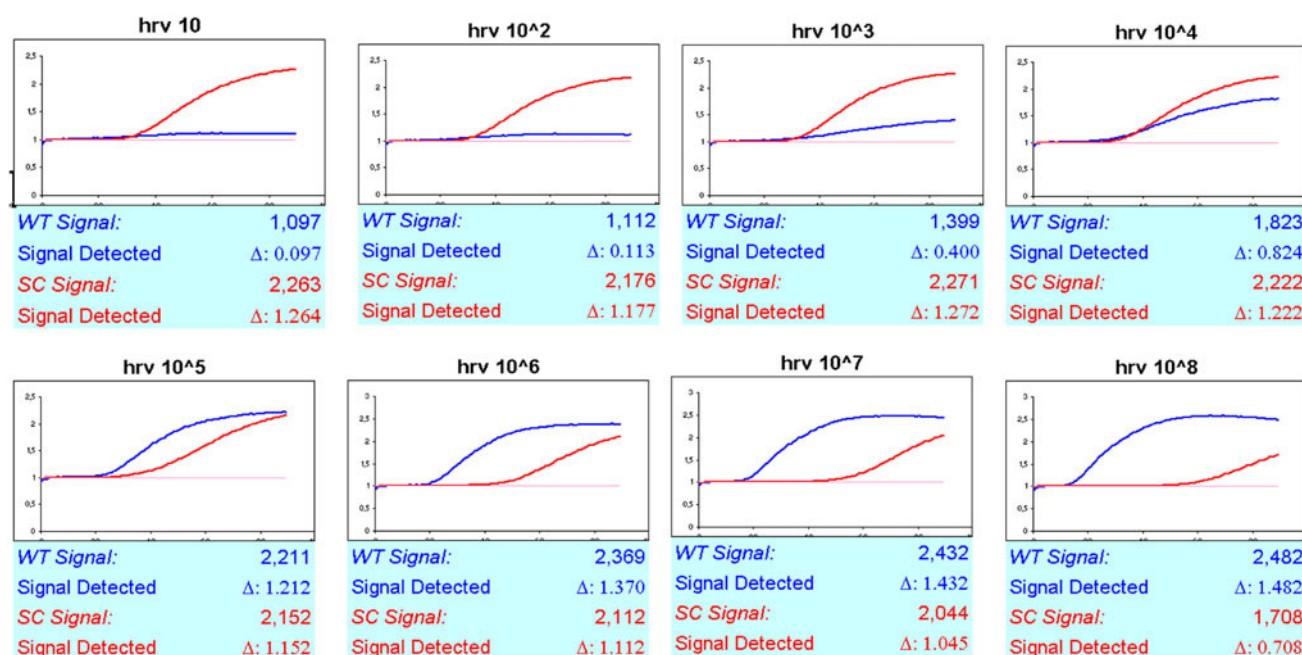
Fig. 2 Real-time NASBA amplification plots of HRV (WT) standard dilutions (from 10^8 to 10 copies/reaction), and the optimal amount of IC (SC) RNA (10^5 copies/reaction). TPP (in minutes) and fluorescence exceeds background are indicated on *x*- and *y*-axis, respectively. *WT* wild type, *IC* internal control, *SC* system control, *TPP* time to positivity

Table 4 Statistical summary of validation and standardisation of HRV real-time NASBA assay

	Ln 10^2	Ln 10^3	Ln 10^4	Ln 10^5
Trueness (*t* test)				
Experimental mean concentration	4.18914	6.66043	9.33646	11.44784
Standard deviation	0.55411	0.27162	0.11906	0.08413
t-calc	1.67897	2.03641	2.36935	1.72916
t-tab ($n = 10$)	2.776	2.776	2.776	2.776
Precision (% coefficient of variation)				
Repeatability	13.22732	4.07810	1.27521	0.73493
Intermediate reproducibility	16.71455	3.70736	4.861	5.53251
Accuracy (% bias inaccuracy)	9.03457	3.58098	1.36973	0.56511

Ln natural logarithm

39 and 51 min when plotted against the standard curve in Fig. 1 (data not shown). All the results were validated by the addition of internal control RNA to rule out inhibition of amplification. In all cases, amplification of the control RNA was observed, thus, confirming that all the negative and positive results are valid. Moreover, all negative control reactions were NASBA negative, demonstrating the absence of amplicon contamination.

Discussion

Viral respiratory tract infections have been recognized as a predominant cause of human disease. To improve clinical management of such patients, it is important to obtain an accurate diagnosis and to identify the causative agent early in infection to ensure appropriate treatment. In this study, we developed the first quantitative NASBA assay for the detection of HRV serotypes. By combining NASBA amplification with molecular beacon probes, this assay becomes a real-time analysis tool that offers faster results than conventional RT-PCR technique. Since NASBA amplification involves three separate enzymes each with their own kinetic parameters, variability in every measurement is inevitable [19]. Weusten et al. [30] were the first to describe a mathematical model for RNA amplification of both target and internal calibrator RNA in a molecular beacon-based NASBA reaction to normalize enzyme efficiency differences between reactions. However, the description of this model did not include all of the essential

parameters needed to operate the model. Consequently, analysis using this model requires software calibrated to each target and is commercially available for only a few specific targets. Here, we describe an alternative method for normalizing NASBA data by using a simple TPP calculation in the presence of an internal control that reduces the variability between replicates and increases the precision, trueness, and accuracy for predicting unknown concentrations of HRV RNA. It has been shown that tube to tube variation within NASBA can be normalized with the addition of an internal control in each reaction [34, 35]. In particular, the optimal concentration for the internal calibrator should be determined as the concentration providing the greatest dynamic range of amplification of both the target and internal control RNA. In our study, a fixed amount of 10^5 copies of the internal calibrator RNA was optimal for the assay reported here (data not shown). Furthermore, the addition of an internal control is fundamental to identify false negative results because of reaction failure, and to monitor the effects of unknown sample factors that might interfere with the amplification kinetics. As concerns the importance of primers and KCl for NASBA optimization, their role has not been emphasized to date. NASBA is an isothermal nucleic acid amplification method able to specifically amplify target RNA by using specific primers in a KCl background. Initially, the concentration of the primers relative to the total concentration of amplifiable RNA is very high, is not rate limiting, and relatively small amounts of primers are consumed in depletion of the initially present pool of RNA copies (linear phase of NASBA process). At some time point, obviously, the primers' concentrations do become rate limiting, and decline to practically zero. At this time point, the DNA intermediate levels have reached their maximum and RNA production proceeds at high speed. From now on, the only reaction that can proceed is T7 RNA polymerase-mediated formation of RNA from the DNA intermediate templates. This time interval represents the second phase of NASBA process characterized by an exponential kinetics. We observed that high concentrations of primers and KCl elongate the linear phase of NASBA process by shorting the exponential amplification; whereas, low concentrations of primers and KCl promote the exponential phase. In particular, in this study we used relatively low concentrations of primers and KCl (0.3 μM and 80 mM, respectively) to elongate the exponential phase of NASBA process, and accordingly, to minimize the reaction-to-reaction variation. By using this simple expedient, we have significantly increased our accuracy, precision, and trueness of prediction over the standard TTP calculations. In summary, we describe a simplified method of calculating unknown concentrations of target RNA using an internal calibrator. This method allowed for greater precision, accuracy, and trueness for predicting HRV RNA over the standard TTP analysis. In conclusion, we described the first real-time NASBA assay capable of accurate quantification of HRV RNA making it a valuable tool in the molecular diagnostics of HRV serotypes.

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