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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.

Keywords Nucleic acid sequence-based amplification · Human rhinovirus · Molecular beacon · Internal control · RNA detection and quantification

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

Francesca Sidoti and Massimiliano Bergallo contributed equally to this work and share first authorship.

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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.

83 Materials and Methods

84 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 μ l 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).

99 In Vitro RNA Transcription

100 Viral cDNA was generated, first by incubation of random
101 primers (600 ng/ μ l) and dNTPs (10 mM) (Invitrogen) with
102 10 μ l of HRV-16 RNA for 5 min at 70°C . Subsequently, a
103 mix containing buffer $5\times$ [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/ μ l), and Recombinant RNasin[®] Ribo-
106 nuclease Inhibitor (40 U/ μ l) (Promega) was added. The
107 total volume (20 μ l) 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 iso-
lates 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)

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

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.

125 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 \times , 50 mM MgCl_2 ,
 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.

150 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 $\mu\text{mol/l}$. 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.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi>). Real-
 162 time NASBA reaction was performed on a NucliSens EasyQ
 163 analyzer (BioMérieux) using the NucliSENS EasyQ Basic
 164 Kit Version 2 (bioMérieux, Lyon, France) for the amplifi-
 165 cation according to the manufacturer's manual. To obtain
 166 the best amplification efficiency, conditions for the real-time
 167 NASBA assay were optimized until the best primers,
 168

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	CTCCGGCCCCCTGAATGYGGCT
HRVJMB-FAM	CCAAGC^cGAYGGGACCR ACTACTTTGG GCTTGG
U1A P1	AATTCTAATACGACTCACTATAGGG^b AGAGGCCCGGCATGTGGTGCATAA
U1A P2	CAGTATGCCAAGACCGACTCAGA
U1AMB-ROX	CGTACG^cGATGAAAGGCACCTTC CGTGG 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. Titrations
 170 of IC RNA (between 10^4 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

204	recorded as the time to positivity (TTP). HRV TTP values	were performed using the PASW Statistics version 18.0	253
205	were regressed against the original standard curve to gener-	(SPSS Inc., Chicago, Illinois, USA).	254
206	ate a predicted amount of target RNA. In particular, for		
207	data analysis, Excel calculation engine was used. NASBA	Clinical Specimens	255
208	dynamic range was calculated from TTP values regressed		
209	against the standard curve by using an Excel spreadsheet that	For assessment of clinical reactivity of the assay, respira-	256
210	was created “ad hoc” by us. In particular, for each individual	tory specimens collected from our Virology Unit of the	257
211	HRV standard dilution (from 10^8 copies/reaction to 1 copy/	Azienda Ospedaliero-Universitaria San Giovanni Battista,	258
212	reaction), fluorescent threshold levels of target close to 1.1	Turin, were tested for HRV by real-time NASBA assay.	259
213	and values of the corresponding minutes were extrapolated	Clinical specimens included 33 bronchoalveolar lavages	260
214	by the NucliSENS EasyQ Director software. Therefore,	(BAL) obtained from 33 transplant patients (M/F, 20/13;	261
215	minutes were normalized to a value of threshold level =	mean age, 56.2 years; range 21–88). A number of pre-	262
216	1.1, and the natural logarithm of normalized minutes was	cautions were undertaken to prevent the occurrence of	263
217	calculated. Quantification capacity was assessed by plotting	false-positive results. Each run included control reactions	264
218	the TTP values obtained against the logarithm of the number	lacking template (no-template controls) to test for the	265
219	of target molecules in each dilution and calculating a linear	presence of contamination or the generation of nonspecific	266
220	regression.	amplification products under the assay conditions used.	267
221	Specificity of Real-Time NASBA Assay		
222	The specificity of the developed real-time NASBA assay was	Results	268
223	verified by in silico studies (analytical specificity) against		
224	publicly available sequence databases (BLAST alignment	Sensitivity of Real-Time NASBA Assay	269
225	software (http://blast.ncbi.nlm.nih.gov/Blast.cgi)) to evalu-		
226	ate possible cross-reactions with respiratory viruses other	Optimal real-time NASBA assay conditions that allowed	270
227	than HRV. Experimental specificity was also verified. In	efficient amplification of the HRV target were established.	271
228	particular, sequences of different respiratory viruses were	Sensitivity and limit of detection of NASBA assay were	272
229	used for exclusivity testing (Table 1).	assessed by repeated testing of serial logarithmic dilutions	273
230	Validation and Standardization of Real-Time NASBA	of the HRV RNA standards ranging from 10^8 to 1 copy/	274
231	Assay	reaction. In particular, HRV NASBA dynamic range was	275
232	To determine the performance of HRV NASBA assay, we	calculated from TTP value (time point at which emitted	276
233	assessed the diagnostic trueness, precision, and accuracy of	fluorescence exceeds the baseline emission) regressed	277
234	the technique. Specifically, precision was assessed by	against the standard curve by using an Excel spreadsheet	278
235	evaluating repeatability and intermediate reproducibility of	created “ad hoc” by us (Table 3). Results from linear	279
236	NASBA assay. To determine the repeatability, several	regression show that HRV real-time NASBA assay was able	280
237	replicates containing the various amounts of HRV RNA	to quantify from 10^8 to 10 copies/reaction. The standard	281
238	were tested. The repeatability was determined by 10-fold	curve of HRV dilutions plotted versus NASBA amplifica-	282
239	serial dilutions of the HRV RNA standards. In particular,	tions (expressed as TPP) is shown in Fig. 1, whereas plots	283
240	we used four different dilutions (10^2 , 10^3 , 10^4 , and 10^5	for the amplification of HRV standard dilutions (from 10^8 to	284
241	copies/reaction) of quantification standards. Each dilution	10 copies/reaction), and the optimal amount of IC RNA	285
242	was analyzed 10 times, with the same method on identical	(10^5 copies/reaction), are shown in Fig. 2. The consistency	286
243	test items in the same laboratory by the same operator	of replicates was measured by the correlation coefficient	287
244	using the same equipment. Regarding intermediate repro-	(R^2), which indicates the linearity of TPP values plotted in	288
245	ducibility, each dilution was analyzed with the same	the standard curve. The R^2 index for HRV was 0.9948.	289
246	method on identical test items in 10 different runs per-	Sensitivity of real-time NASBA assay was determined by	290
247	formed by three different operators using different equip-	the lowest standard dilution consistently detectable in rep-	291
248	ment on different days. Moreover, we used the Dixon’s test	licate reactions at frequency of 100%. HRV sensitivity was	292
249	to examine if one measure from 10 replicate measures that	10 copies/reaction, whereas the limit of detection for reli-	293
250	we performed (10^2 , 10^3 , 10^4 , and 10^5) could be rejected or	able quantification was 1 copy/reaction.	294
251	not, and the Shapiro–Wilk’s test to compare these measures		
252	against the normal distribution. Statistical data analyses	Specificity of Real-Time NASBA Assay	295
		Based on the data available at the BLAST alignment	296
		software, primers were tested in silico for potential	297

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

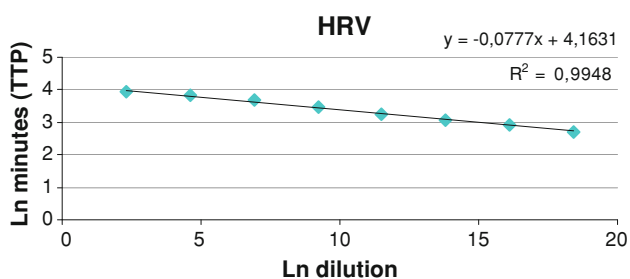


Fig. 1 Standard curve of HRV real-time NASBA assay. HRV TTP values were regressed against the standard curve to generate a predicted amount of target RNA. HRV ln dilutions (10⁸ to 10 copies/reaction) on x-axis are plotted against ln minutes (TTP) on y-axis. The coefficient of correlation (R^2) was 0.9948. *TTP* time to positivity, *Ln* natural logarithm

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

309 Validation and Standardization of Real-Time NASBA 310 Assay

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

performed (10², 10³, 10⁴, and 10⁵) were analyzed using a 317
Student's *t* test to compare the mean concentrations from 318
each dilution with an accepted reference value. The mean 319
concentrations from each dilution for the method are shown 320
in Table 4 with the *t* test results, which indicate the signifi- 321
cance of the differences between each experimental mean 322
and the assigned value. Analysis of the *t* statistics showed 323
that the method had *t*-calc values lower than the *t*-tab value, 324
demonstrating a significant trueness of HRV assay. 325

Precision of method was expressed as the coefficient of 326
variation (CV) in the log₁₀ values of the concentration. 327
Repeatability and intermediate reproducibility of HRV 328
assay were evaluated over different concentrations ranging 329
from 10² to 10⁵ copies/reaction from 10 replicate measures 330
(*n* = 10) of each reference viral quantification standard 331
within a single run or in 10 different run experiments per- 332
formed by three different operators. The precision associ- 333
ated with each dilution measurement (10², 10³, 10⁴, and 334
10⁵) was assessed by calculation of the CV for each. The 335
CVs within a single run (repeatability) ranged from 0.73 to 336
13.23%; whereas, the CVs in different runs (intermediate 337
reproducibility) ranged from 3.71 to 16.71% (Table 4), 338
indicating that the precision of the assay is satisfactory. 339

Diagnostic accuracy includes both trueness and preci- 340
sion. The measure of accuracy is usually expressed 341
numerically in terms of bias (lack of agreement). Accuracy 342
shall be within ±25% of the accepted reference value over 343
the whole dynamic range, according to document ISO 344
16140 [33]. Data for the percentage of inaccuracy HRV 345
method are reported in Table 4. 346

Clinical Performance of Real-Time NASBA Assay 347

The developed real-time NASBA assay was able to detect 348
HRV RNA in 7/33 (21.2%) BAL specimens. The TTP 349
values of these NASBA-positive samples ranged between 350

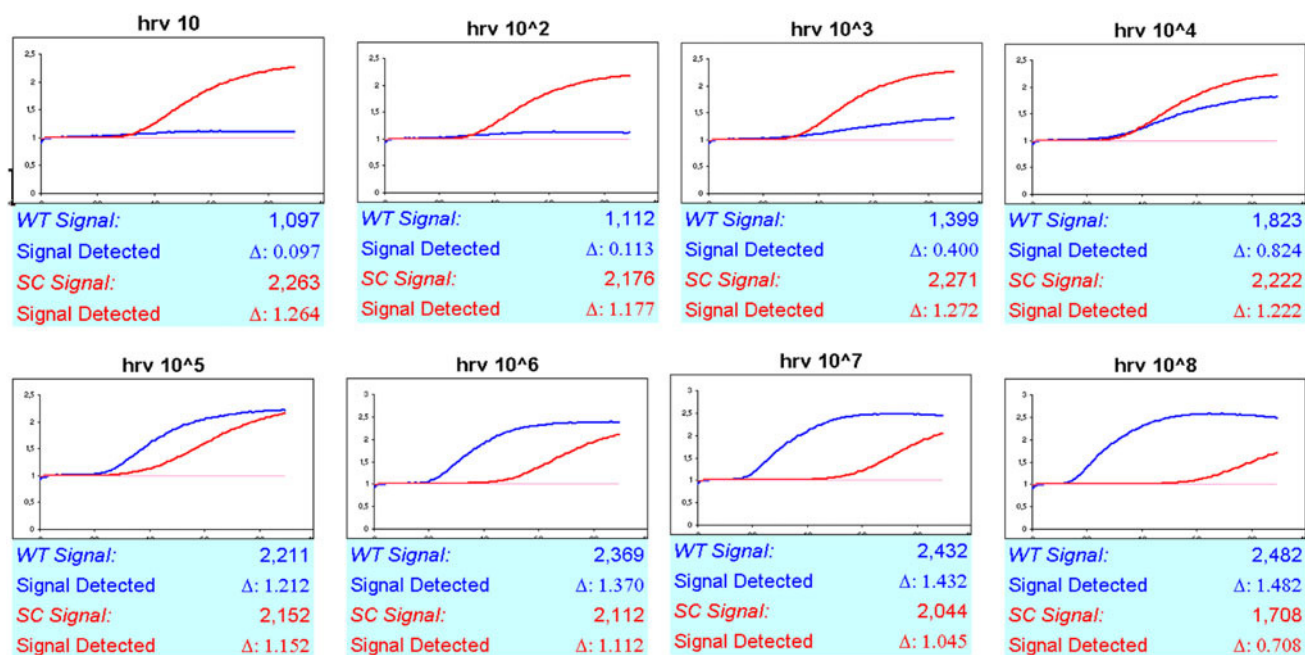


Fig. 2 Real-time NASBA amplification plots of HRV (WT) standard dilutions (from 10^8 to 10^5 copies/reaction), and the optimal amount of IC (SC) RNA (10^5 copies/reaction). TPP (in minutes) and

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 (<i>t</i> test)				
Experimental mean concentration	4.18914	6.66043	9.33646	11.44784
Standard deviation	0.55411	0.27162	0.11906	0.08413
<i>t</i> -calc	1.67897	2.03641	2.36935	1.72916
<i>t</i> -tab (<i>n</i> = 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

351 39 and 51 min when plotted against the standard curve in
 352 Fig. 1 (data not shown). All the results were validated by
 353 the addition of internal control RNA to rule out inhibition
 354 of amplification. In all cases, amplification of the control
 355 RNA was observed, thus, confirming that all the negative
 356 and positive results are valid. Moreover, all negative control
 357 reactions were NASBA negative, demonstrating the
 358 absence of amplicon contamination.

359 Discussion

360 Viral respiratory tract infections have been recognized as a
 361 predominant cause of human disease. To improve clinical
 362 management of such patients, it is important to obtain an

accurate diagnosis and to identify the causative agent early 363
 in infection to ensure appropriate treatment. In this study, 364
 we developed the first quantitative NASBA assay for the 365
 detection of HRV serotypes. By combining NASBA 366
 amplification with molecular beacon probes, this assay 367
 becomes a real-time analysis tool that offers faster results 368
 than conventional RT-PCR technique. Since NASBA 369
 amplification involves three separate enzymes each with 370
 their own kinetic parameters, variability in every measurement 371
 is inevitable [19]. Weusten et al. [30] were the first to 372
 describe a mathematical model for RNA amplification of 373
 both target and internal calibrator RNA in a molecular 374
 beacon-based NASBA reaction to normalize enzyme efficiency 375
 differences between reactions. However, the 376
 description of this model did not include all of the essential 377

378 parameters needed to operate the model. Consequently,
 379 analysis using this model requires software calibrated to
 380 each target and is commercially available for only a few
 381 specific targets. Here, we describe an alternative method for
 382 normalizing NASBA data by using a simple TPP calculation
 383 in the presence of an internal control that reduces the vari-
 384 ability between replicates and increases the precision,
 385 trueness, and accuracy for predicting unknown concentra-
 386 tions of HRV RNA. It has been shown that tube to tube
 387 variation within NASBA can be normalized with the addi-
 388 tion of an internal control in each reaction [34, 35]. In
 389 particular, the optimal concentration for the internal cali-
 390 brator should be determined as the concentration providing
 391 the greatest dynamic range of amplification of both the
 392 target and internal control RNA. In our study, a fixed
 393 amount of 10^5 copies of the internal calibrator RNA was
 394 optimal for the assay reported here (data not shown). Fur-
 395 thermore, the addition of an internal control is fundamental
 396 to identify false negative results because of reaction failure,
 397 and to monitor the effects of unknown sample factors that
 398 might interfere with the amplification kinetics. As concerns
 399 the importance of primers and KCl for NASBA optimiza-
 400 tion, their role has not been emphasized to date. NASBA is
 401 an isothermal nucleic acid amplification method able to
 402 specifically amplify target RNA by using specific primers in
 403 a KCl background. Initially, the concentration of the primers
 404 relative to the total concentration of amplifiable RNA is
 405 very high, is not rate limiting, and relatively small amounts
 406 of primers are consumed in depletion of the initially present
 407 pool of RNA copies (linear phase of NASBA process). At
 408 some time point, obviously, the primers' concentrations do
 409 become rate limiting, and decline to practically zero. At this
 410 time point, the DNA intermediate levels have reached their
 411 maximum and RNA production proceeds at high speed.
 412 From now on, the only reaction that can proceed is T7 RNA
 413 polymerase-mediated formation of RNA from the DNA
 414 intermediate templates. This time interval represents the
 415 second phase of NASBA process characterized by an
 416 exponential kinetics. We observed that high concentrations
 417 of primers and KCl elongate the linear phase of NASBA
 418 process by shorting the exponential amplification; whereas,
 419 low concentrations of primers and KCl promote the expo-
 420 nential phase. In particular, in this study we used relatively
 421 low concentrations of primers and KCl (0.3 μ M and
 422 80 mM, respectively) to elongate the exponential phase of
 423 NASBA process, and accordingly, to minimize the reaction-
 424 to-reaction variation. By using this simple expedient, we
 425 have significantly increased our accuracy, precision, and
 426 trueness of prediction over the standard TTP calculations. In
 427 summary, we describe a simplified method of calculating
 428 unknown concentrations of target RNA using an internal
 429 calibrator. This method allowed for greater precision,
 430 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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