

A strategy to increase the specificity of Syber Green I qRT-PCR in hepatitis A detection

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SUMMARY

Various methods for the recovery and detection of HAV have been suggested, and molecular tests have recently provided an effective replacement for the traditional methods. Real-time RT-PCR technology offers many advantages over conventional RT-PCR in terms of rapidity and specificity. Most procedures are based on the TaqMan chemistry, but some researchers have used the SYBR Green I approach, which is less expensive and simpler to carry out. However the formation of primer-dimers needs to be distinguished from specific products through a melting curve analysis. This study focused on a strategy to increase the specificity of Syber Green I chemistry, thus nullifying the primer-dimers interference. To this end, forward and reverse primers were specially designed for hairpin loop formation, a strategy widely used to improve the specificity and the efficiency of PCR. Two different concentrations of primers were assayed (200 nM and 400 nM) in a one-step, real-time RT-PCR procedure, evaluating the specificity of the amplicons and the optimization of the real-time protocol. We demonstrated that this approach can increase the specificity of the Syber Green I qRT-PCR performance with a good reproducibility of the method. Because of the simplicity of the assay and the lower costs involved, this procedure could be a valid alternative to HAV monitoring from environmental matrices.

KEY WORDS: Syber Green I, qRT-PCR, Specificity, Hairpin primer, Primer-dimers, HAV

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INTRODUCTION

Hepatitis A virus (HAV) is a major cause of acute hepatitis worldwide, contaminated water and food are the primary sources of human infection. Various methods for the recovery and detection of HAV have been suggested by numerous researchers, and recent molecular tests have effectively replaced slow and laborious traditional methods like cell cultures (Nainan *et al.*, 2006). In 1997 Cromeans proposed a reverse transcription polymerase chain reaction (RT-PCR) to amplify viral HAV RNA (Cromeans *et al.*, 1997). This method proved to be more sensitive than immunoassays and it is still the most commonly used method for the detection of HAV in clinical,

food and environmental matrices (Nainan *et al.*, 2006; Pinto *et al.*, 2007). However, the development of a widely applicable technique able to detect and quantify HAV in environmental analysis is still an important goal. Real-time RT-PCR technology (qRT-PCR) offers many advantages over conventional RT-PCR in terms of rapidity and specificity of detection and quantification of viral particles. Several real-time RT-PCR assays have been proposed for HAV detection, and some of these are easy to use and available commercially, such as the Light Cycler instruments (Roche Diagnostics, Mannheim, Germany) (Sánchez *et al.*, 2006; 2007).

The HAV genome consists of positive-strand RNA of approximately 7.5 kb with a 5'-untranslated region (5'-UTR), followed by a single open reading frame (ORF) containing three distinct regions (P1, P2 and P3). The VP1/P2A junction is the segment of the genome used to define genotypes and subgenotypes of HAV strains, because of the presence in this region of the majority of nucleotide variations (>15%) (Robertson *et al.*, 1992;

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Normann *et al.*, 1995; Costa-Mattioli *et al.*, 2002a; Lu *et al.*, 2004). Most real-time procedures are based on the TaqMan chemistry (Jothikumar *et al.*, 2005; Costafreda *et al.*, 2006; Villar *et al.*, 2006), which targets the highly preserved 5'-UTR region, and these are the methods commonly used for detection and quantification (qRT-PCR) of HAV. The sensitivity range covers 60 geq/mL (Villar *et al.*, 2006) and 1 RNA copy per reaction (Costafreda *et al.*, 2006). In contrast, some authors have opted for primers targeting the VP1 capsid region (Brooks *et al.*, 2005), using SYBR green DNA-binding fluorophore. This fluorogenic approach is less expensive and it is simple to apply; however, formation of primer-dimers is common and needs to be distinguished from specific products by a melting-curves analysis after the amplification cycles. When using DNA-binding dye, a good primer design is particularly critical and should comply with the standard PCR guidelines. Specific algorithms are established in primer design to minimize self-complementary primers and interactions between them. Several approaches have been described to circumvent co-synthesis of nonspecific amplification products in PCR, including hot start or touch-down PCR or, in RT-PCR performance, the introduction of a two-step protocol (Kaboev *et al.*, 2000; Vandesompele *et al.*, 2002; Brooks *et al.*, 2005). Moreover, primer designing to promote the formation of looped structures, in order to suppress the primer-dimers or aspecific amplicon production, has been described and applied with success (Ailenberg M. *et al.*, 2000). An intramolecular hairpin structure can be formed when a single strand of DNA contains a complementary sequence region that binds to it (Vallone *et al.*, 2004).

To determine the copy number per reaction in qRT-PCR, it is essential that there be a suitable viral standard at a known concentration, in order to construct an external calibration curve to which to compare the data analysis. In a previous study, we tested different fluorogenic systems and different procedures using standard Armored RNA (AMBION) to draw up a quantitative protocol for enterovirus (Donia *et al.*, 2005) and HAV (Donia *et al.*, 2006) that can be applied in environmental analysis. Specifically, standard Armored RNA Hepatitis A Virus comprises 266 bp of the VP1/VP2 capsid protein interphase re-

gion. The qRT-PCR was performed using the set of primers suggested by the manufacturer (Ambion Diagnostic, USA). Syber Green I technique has shown a good RT-PCR efficiency; the specificity and sensitivity were similar to the TaqMan assay on the Armored RNA, which has served as a valid standard for constructing a calibration curve (Donia *et al.*, 2005; 2006). Since this product is no longer commercially available and, to our knowledge, there is no standard HAV suitable for the construction of a calibration curve on the market, a portion of the virus sequence was cloned and used in qRT-PCR. Besides, in this study, attention was focused on a strategy to increase the specificity of Syber Green I chemistry, nullifying the primer-dimers production, which interferes with data analysis. For this purpose forward and reverse primers, specifically chosen for intramolecular hairpin formation, were selected. In this paper we describe the potential applicability of this approach in the real-time amplification procedure, in order to set out a less expensive method applicable to monitoring the environmental presence of HAV.

MATERIAL AND METHODS

A qRT-PCR procedure, using Syber Green I strategy, has been assessed, with attention focused on the primer design, evaluating the specificity of the amplicons and optimizing the real-time protocol. For this purpose, two different concentrations of primers were assayed: 200 nM and 400 nM.

Virus and viral RNA extraction

The HM-175 strain of HAV was used in this study; it was supplied by A. Bosch (University of Barcelona, Dept. of Microbiology) and was replicated on FRhK4 cells (Venuti A. *et al.*, 1985).

The viral RNA was extracted using the QIAmp viral RNA kit (Qiagen, Milan, Italy), following the manufacturer's instructions and dissolved in RNase-free water containing 0,5 U/ μ L of RNase inhibitor (Promega, Milan, Italy). The concentration of viral RNA was determined by broad spectrum fluorescence analysis, at an optical density of 260 nm (NanoDrop - Euroclone - Milan, Italy; Micro-Volume Full-Spectrum Fluorometer), and converted in genome copy

TABLE 1 - Primer sequences used in this study.

	Stem length	Loop size	ΔG Kcal/mol	Primer sequence
Forward	2	3	-1,30	5'-TGAACAGGTATACAAAGTCAGC-3'
Reverse	7	3	-1,93	5'-ATACCAACTTGGGGATCTGGAAC-3'

equivalent (gce) (our initial stock was at $3, 7 \times 10^7$ gce/ μ L).

Primer design

Primers (forward position 2017-2038 and reverse position 2281-2259) directed to the VP1/VP2 capsid protein interphase region of a complete sequence HM-175 HAV strain (available at GenBank, Acc. n° M14707) were designed by OligoExplorer 1.2 software, and the choice was steered towards the presence of a secondary structure (hairpin) in oligonucleotides for the existence in the 5'-end of a complementary tract to the 3'-end. Algorithms of selected hairpin primers were established under certain basic criteria: the melting temperature of the primers and the free energy (ΔG) of the secondary structures were calculated by the nearest neighbor method (Breaslaue *et al.*, 1986); no more than 4-5-bases for the loop structure were required, with a minimum of 2 base pairs in the stem (3'-end) (Vallone *et al.*, 2004). The characteristics of the primer set used in this study are described in Table 1. The same primer set was used in the standard cloning and in the real-time performances.

Standard cloning

A 265 bp fragment of the VP1/VP2 capsid protein interphase region of a complete sequence HM-175 HAV strain was generated by conventional RT-PCR (Macaluso *et al.*, 2006), using the primer set previously described. The PCR product was purified and sequenced to identify HAV homology and then cloned in pCR 4 TOPO vector (Invitrogen- Milan, Italy), in accordance with the manufacturer's recommendations. The amplicon (265 bp) ligated with the T7 phage RNA polymerase promoter was transcribed in vitro (T7 RNA polymerase - Promega, Milan, Italy) to generate a large quantity of synthetic HAV RNA. After transcription, a digestion step was performed, with Dnase and Rnase-free RQ1 (Promega, Milan, Italy), to eliminate remaining plasmid DNA; after

being extracted as described above, the purified RNA yield was determined spectrophotometrically (NanoDrop- Euroclone - Milan, Italy) and converted into a genome copy equivalent (gce) ($1,6 \times 10^9$ gce/ μ L, stock solution).

Standard curves

Two external calibration curves were constructed: serial ten-fold dilutions, in ultrapure Rnase-free water, of transcribed (plasmid) and extracted viral RNAs ranging from 10^5 to 10^{-1} gce/ μ L were prepared to determine detection limits; the curves generated were then compared.

Syber green I real-time RT- PCR (qRT-PCR)

The quantification of HAV was performed by a one-step, real-time RT-PCR, using Syber Green I chemistry. Assays were run with the BioRad iCycler instrument, using QuantiTect Syber Green I RT-PCR kit (Qiagen- Milan, Italy).

Amplifications were carried out in 25 μ L of reaction volume, mixing 5 μ L of RNA target and 20 μ L of the master mix containing forward and reverse primers. Two different concentrations of primers were tested: 200nM and 400 nM. Each dilution of the standard was assayed in five replicates per test, and each test was repeated at least three times. Positive controls, an HM-175 HAV strain at a concentration of 10^3 to 10^{-1} gce/well, plus a negative control not containing nucleic acid, were added to each run. Reactions were performed with the following thermal profile: 50°C for 30 min, for the reverse transcription step, a hot-start denaturing step of 95°C for 13 mins., followed by 45 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. The fluorogenic signal emitted was read during the annealing-extension step and analyzed by iCycler IQ software. Immediately after amplification, a melting curve protocol was produced by increasing each cycle by 0.5°C, starting from the set-point temperature (55.0°C), for 80 cycles, each one of 10s. Threshold cycle (Ct) values were used to calculate a linear regression line by plotting, on

a graph, the starting copy numbers logarithm, expressed by increasing the fluorescence values of amplification dilutions. The slope of the calibration curves determined the PCR efficiency (EFF), in accordance with the equation: $EFF = 10^{[-1/\text{slope}] - 1}$; the slope values were $-3.3 \approx 100\%$ EFF. The real-time RT-PCR reproducibility was represented by correlation coefficients (CC) with values ranging between 0 and 1 (Donia *et al.*, 2005; 2006).

RESULTS

Sensitivity of Syber green I qRT-PCR

The sensitivity of the qRT-PCR assay was investigated by serial ten-fold dilutions using, in ultrapure Rnase-free water, the *in-vitro* transcribed RNA (plasmid) and the viral RNA extracted as described previously.

The parameters of the primer design were based on the presence of a hairpin structure, due to the presence, in the 5'-end, of oligonucleotides complementary to those in the 3'-end (ΔG ranging from -1.6 to -5.8 kcal/mol) (Nazarenko *et al.*, 2002a; 2002b). The effect of the hairpin structure on PCR efficiency was established by comparing the values of the threshold cycle (Ct) and the slope of the amplification curves of the qRT-PCRs, utilising the previously investigated ARMORED HAV RNA primers (Armored primers, suggested by Ambion) (Donia *et al.*, 2006) and the loop primers (Table 2). No significant differences were observed in RT-PCR efficiency and sensitivity

among the external calibration curves constructed. Using the Armored standard and primers, we obtained the best calibration curve at a primer concentration of 400 nM (slope -3.3, CC=0.992, EFF=100%, Ct=22° cycle for 10^4 copies of target) (Donia *et al.*, 2006). Similar values were obtained using a 400 nM concentration of hairpin primers in both calibration curves, constructed, respectively, on plasmid and viral RNA dilutions: slope -3.3, CC=0.995, EFF 100%, Ct=23° cycle for 10^4 copies of target (plasmid dilutions); slope -3.3, CC= 0.993, EFF=97%, Ct=23° cycle for 10^4 copies of target (virus dilutions). Using 200 nM of hairpin primer concentration, the values of the calibration curves proved more uniform in terms of efficiency: slope -3.3/-3.2, CC=0.995/0.990, EFF=100%/99%, Ct = 25° cycle for 10^4 copies of target respectively (plasmid/virus). The detection limit using hairpin structured primers was 1 gce of plasmid RNA in 100% of the tests and 1 gce of HAV viral RNA in 66% of the tests, in contrast to 10 gce in 100% of the tests of standard ARMORED RNA HAV investigated previously using suggested primers targeting the same VP1/VP2 capsid protein interphase region of the genome (Donia *et al.*, 2006). The positive control concentrations, included in each run as an unknown sample, were confirmed by qRT-PCR in all cases; the negative controls did not produce any amplification, demonstrating an absence of interaction between forward and reverse hairpin primers (primer-dimers and/or primer-primer interactions during amplification reaction).

TABLE 2 - Results of Real-time RT-PCR.

Primer concentration	Slope		CC		EFF		Detection limit		Melting peak °C	
	Plasmid	HAV	Plasmid	HAV	Plasmid	HAV	Plasmid	HAV	Plasmid	HAV
200 nM	-3.2	-3.7	0.990	0.997	105%	85%	1 gce	1 gce	79.5	79.0
	-3.3	-3.2	0.995	0.991	100%	103%	1 gce	1 gce	79.5	79.5
	-3.2	-3.1	0.991	0.986	105%	109%	1 gce	10 gce	79.5	79.0
means	-3.2	-3.3	0.992	0.991	103%	99%				
400 nM	-3.1	-3.3	0.995	0.995	105%	97%	1 gce	10 gce	79.0	79.5
	-3.1	-3.6	0.962	0.985	108%	98%	1 gce	1 gce	79.0	79.5
	-3.8	-3.3	0.997	0.993	82%	86%	1 gce	1 gce	79.0	79.5
means	-3.3	-3.3	0.984	0.991	98%	91%				

CC = correlation coefficient; EFF = efficiency; gce = genome copy equivalent.

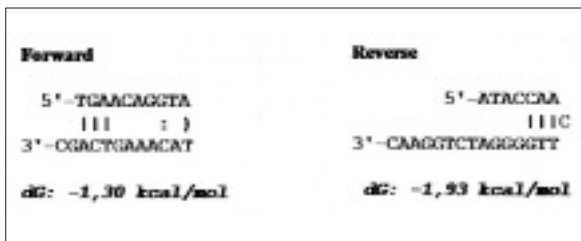


FIGURE 1 - Hairpin primer structures.

Specificity of Syber green I qRT-PCR

Special primers with hairpin loop structures have been purposefully chosen to enhance the specificity of the reaction. These primers contain three nucleotides in the self-annealing sequence - at the 5' end and 3' end - that, at the preparation temperature of the mix, are self-looped and unable to initiate either the transcription or the amplification (Figure 1). The heating produces the melting primers that can initiate the RT-PCR reaction (Kaboev *et al.*, 2000). The use of hairpin primers has been shown to improve the specificity of qRT-PCR by nullifying primer-dimer formation, especially in the absence or a lower amount of target (Nazarenko *et al.*, 2002a). The best EFF (amplification efficiency), determined on the basis of the slope values of the calibration

curves, was between 100% and 99%, with the primers concentration at 200 nM, in relation to the slope values obtained: -3.3 to -3.2, respectively, depending on the target used (plasmid or virus). The reproducibility of the reaction was determined by the correlation coefficient (CC), which was approximately 0.993 total mean values, both for the curve constructed with the plasmid and for that constructed with the virus. The amplicon specificity was checked both through melting-curve analysis and gel analysis, in order to evaluate the potential formation of primer-dimers. The primers set selected produced a single melting peak at $79.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, in each assay (Figure 2). The gel analysis and melting peaks corresponded, in all cases, with a single band of expected size (265 bp) produced after electrophoretic migration.

DISCUSSION

Several real-time reverse transcription-polymerase chain reaction (RT-PCR) assays have revolutionized HAV detection, leading to rapid, sensitive and reproducible analyses (Costa-Mattioli *et al.*, 2002b; Sánchez *et al.*, 2007). A DNA-binding fluorogenic molecule, like Sybr Green I, has been

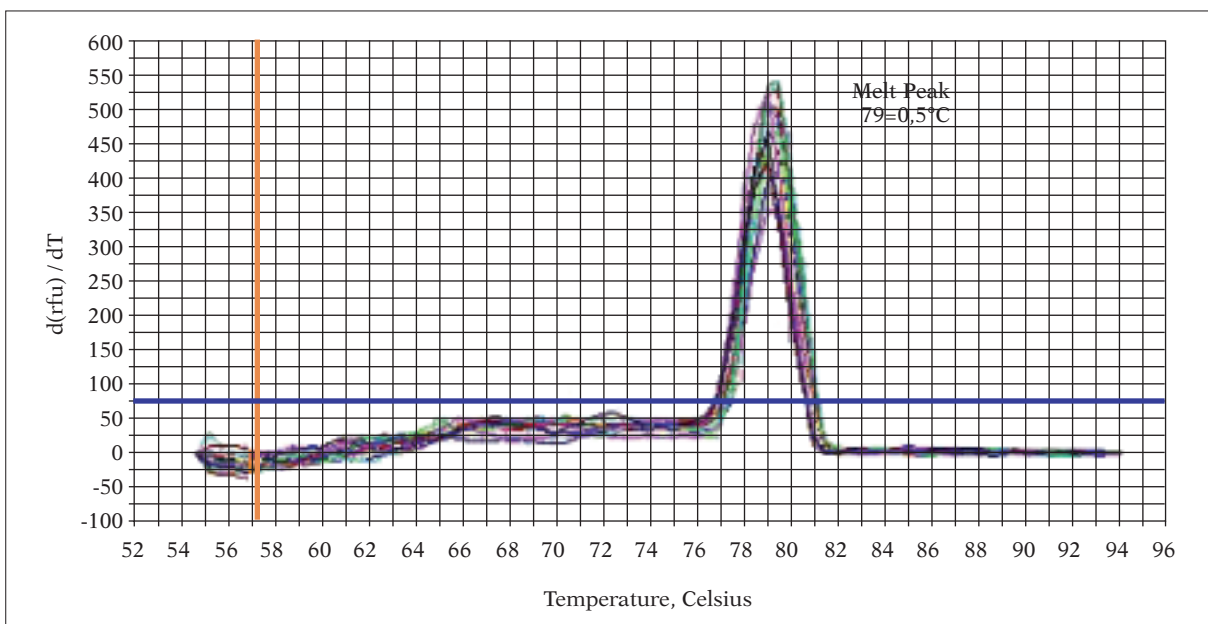


FIGURE 2 - Example for single melt peak of amplicons.

widely used because of its simplicity. This approach requires less specialised knowledge than the design of fluorogenic oligoprobes, and is suitable for large-scale measurement of nucleic acid in environmental matrices (Vandesompele *et al.*, 2002; Mackay *et al.*, 2002). However, the association of a DNA-binding fluorophore with the formation of primer-dimers or other aspecific amplification products (double DNA strands) can confuse the interpretation of the results (Mackay *et al.*, 2002).

In selecting the forward and reverse primers for real-time RT-PCR, mainly using the Syber Green I strategy, the PCR-primer design guidelines recommend that primers sets which present secondary structures be avoided. The formation of a single stranded loop with a large negative free energy value is an index of the stability of the structure, while activation of the loop primer is typically unfavorable. Nevertheless a 3'-end hairpin with a ΔG of -2 Kcal/mol and an internal hairpin with a ΔG of -3 Kcal/mol is generally tolerated. Short partially self-complementary sequences forming hairpin structures can be used, in certain cases, as probe or primers in more practical applications (DNA hybridization and amplification reactions) (Nazarenko *et al.*, 2002a; 2002b). Primers with hairpin loop ΔG approaching -3Kcal/mol (at 25°C) are troublesome when their 3'-end is "tied up", because this can cause internal primer extension and eliminate a given primer from the reaction (Rychlik W. *et al.*, 1993). Singh (2000) reported that, in terms of PCR efficiency, the stem length of the hairpin primer is the most important characteristic, but ΔG value is not significant.

The primers chosen in this study present a ΔG value of -1.30 Kcal/mol and -1.93 Kcal/mol for forward loop primers and reverse loop primers, respectively; the 3'-ends are free in the hairpin structure to allow primer extension by Taq DNA polymerase, demonstrating that only expected products are amplified. This was confirmed by the melting-curve analysis for the increased specificity of the qRT-PCR procedure and with potential elimination of post-PCR manipulations. Indeed, the nonspecific PCR fragments would have been visualized, in our case, as distinct melting peaks. Other authors describe the use of the hairpin-primers constructed by adding five or six nucleotides to the 5'-end, complementary to the

3'-end, in order to obtain the thermodynamic parameters required for design of the beacon probes. A GC-rich sequence in the stem of the loop increases the T_m of the loop-structured primers (Kaboev *et al.*, 2000). In our case, the hairpin-loop primers present a high T_m without external nucleotides added but determined by the nucleotides present in their sequence from thermodynamic calculation (OligoExplorer 1.2 software). The possibility of employing specific primers with a higher melting temperature (T_m) permits the use of higher temperatures for reverse transcription (RT) and for primer annealing (T_a) in the thermal protocol for amplification, increasing the specificity and reducing the background of reaction.

The one-step qRT-PCR is a closed-tube format assay that reduces chances of contamination and false positive results, but it is also a complex reaction susceptible to artifacts. Therefore, further optimization may be required.

Using this approach, it is easier to optimize the reaction by varying the concentration of the primers and the thermal protocols of amplification. In this regard, we have seen that, by using primers with secondary structures, it was possible to reduce the effective concentration to 200 nM, obtaining the best reaction efficiency in both calibration curves (EFF=100% for plasmid, EFF=99% for viral RNA). Hairpin-shaped primers make possible hot-start PCR, because they anneal to the target only at high temperatures, upon denaturing of the stem (Broude, 2005). The post-amplification melting-curve analysis is a straightforward way to check real-time PCR reactions for primer-dimer artifacts and potential contaminations, and to ensure reaction accuracy (Ririe *et al.*, 1997). Besides, thanks to this characterization of analysis products, further confirmation by time-consuming gel electrophoresis can be avoided.

When constructing the external calibration curve, the crucial point in the qRT-PCR is to have valid standard RNA available, at a known absolute concentration, in order to quantify a RNA target in the analytical samples. There are two possibilities for molecules that can be amplified, and whose concentrations can be estimated in advance: an ssRNA molecule obtained after *in vitro* transcription of a cloned cDNA corresponding to the amplicon and, the second possibility, the actual

virus genome (HM-175 strain) (Costafreda *et al.*, 2006). The molecule concentrations can be determined by optical density at 260 nm and then converted into a genome copy equivalent/ μL . In this study we used both RNA molecules and the cloned amplicon, with the latter proving more stable than the extracted viral RNA. *In vitro* transcription is the method that has been used most frequently to produce RNA controls and standards (Costafreda *et al.*, 2006), even though viral RNA dilutions have also been widely used (Costa-Mattioli *et al.*, 2002b; Casas *et al.*, 2007). The major disadvantage of both options is their susceptibility to enzymatic and chemical degradation. The use of Rnase inhibitor in the medium of RNA resuspension and the use of fresh RNA extracted at each run can reduce this degradation, as Costafreda *et al.*, (2006) have demonstrated and suggested. Nevertheless, the use of cloned amplicons as the standard presents the advantage of cold storage at $+4^{\circ}\text{C}$ for some weeks or at -20°C for lengthy periods. The cDNA (plasmid) is known to be more resistant to chemical degradation, and the RNA transcribed *in vitro* is retested each time, before diluting it to construct a calibration curve.

With respect to the labeled probe detection system, the Syber Green I chemistry offers the possibility of amplifying highly variable regions of the virus genome for which probe design is often difficult (Nainan *et al.*, 2006). The strategy of using hairpin primers to enhance PCR specificity and sensitivity is one possible application. All design software employs standard algorithms and thermodynamic parameters for predicting secondary structures and intermolecular interaction events (self and cross-dimers) in the rapid screening of short probe and primer oligonucleotides. Nevertheless, these important characteristics, present in a hairpin structure, are not considered in evaluating their potential use as primers. This study demonstrated that the approach in question is able to increase the specificity of the Syber Green I qRT-PCR performance with a good reproducibility of the method. Since only the specific amplification product is detected, this method results in a highly accurate assay. Therefore, given the simplicity of the assay and the cost saving, we believe that the procedure could provide a valid alternative for HAV monitoring from environmental matrices.

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