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The Development of a Qualitative Real-Time RT-PCR Assay for the Detection of Hepatitis C Virus

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ARTICLE

The development of a qualitative real-time RT-PCR assay for the detection of hepatitis C virus

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Abstract Real-time polymerase chain reaction (PCR) represents a favourable option for the detection of hepatitis C virus (HCV). A real-time reverse transcriptase PCR (RT-PCR) assay was developed as a qualitative diagnostic screening method for the detection of HCV using the ABI PRISM® 7500 Sequence Detection System. The primers and probe were designed to target the 5′-untranslated region of the hepatitis C viral genome. A second heterologous probe assay was developed for the detection of the haemagglutinin gene of phocine distemper virus (PDV) and was used as an internal control. A semi-automated HCV extraction method was also implemented using the ABI PRISM™ 6100 Nucleic Acid PrepStation. The HCV assay was optimised as a qualitative singleplex RT-PCR assay with parallel testing of the target and internal control. The assay results (n=200) were compared to the COBAS AMPLICOR™ HCV Test v2.0 assay. The assay demonstrated a high rate of sensitivity (99%), specificity (100%) and an acceptable limit of detection (LOD) of 100 IU/ml. The development of a qualitative multiplex assay for the simultaneous detection of HCV and internal control indicates the same high rates of sensitivity and specificity. This sensitive real-time assay may prove to be a valuable method for the detection of HCV.

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Introduction

Hepatitis C virus (HCV) is responsible for most cases of blood-borne hepatitis and is the leading cause of chronic liver disease worldwide, with a global prevalence of hepatitis C infection of approximately 2% [1]. Traditional laboratory assays for the diagnosis and management of HCV infection include serological tests to detect and classify antibody response and to determine the HCV RNA genotype. Many limitations are, however, associated with the serological diagnosis of hepatitis C. These limitations include a requirement for confirmation by other assays (e.g. immunoblots), reduced specificity in low-risk populations, reduced sensitivity in cases of early infection and immunosuppression, and, most importantly, the inability to distinguish between resolved and chronic infection [2, 3]. Reverse transcriptase polymerase chain reaction (RT-PCR) is considered to be the "gold standard" for the detection of viral genomic RNA in serum or plasma. The detection of RNA exclusively demonstrates active viral infection [3]. Therefore, unlike serology, HCV RNA testing can be used for the diagnosis of acute hepatitis before seroconversion, in seronegative patients with immune deficiency and for investigating congenital HCV infection. HCV RNA detection is also useful for confirming indeterminate serological results and for monitoring response to treatment [3, 4].

In the last ten years, many commercial nucleic acid amplification assays for qualitative and quantitative HCV detection have become available. However, these methods can be laborious and time-consuming. Automated technologies such as real-time PCR represent a more favourable option for accurate qualitative and quantitative hepatitis C detection. Unlike conventional, end-point PCR, where amplicon amplification and detection involve separate steps and run the risk of cross-contamination between samples and/or PCR product and where sensitivity is often limited by the resolution of the detection method (e.g. agarose gel electrophoresis), real-time PCR systems offer rapid combined amplification and real-time, probe-based detection of amplicons in a closed automated system. However, whilst the commercial real-time PCR assays are appealing, the cost may prohibit their introduction into routine diagnostic laboratories. Nevertheless, many open-channel real-time PCR platforms are now available. These flexible systems function as analytical platforms that allow full customisation of the diagnostic assay design.

Despite the advantages offered by real-time detection platforms, sample preparation remains the major rate-limiting step. Conventional manual nucleic acid extraction methods are time-consuming, labour-intensive and susceptible to contamination. Fully automated sample preparation systems are increasingly available to accompany real-time platforms. These systems offer the rapid isolation of nucleic acids on a high-throughput scale. Such systems are, however, costly. Semi-automated nucleic acid preparation systems, such as the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA), could provide a more cost-effective alternative. This system is a small, bench-top instrument comprised of a programmable vacuum manifold and a 96-well filtration plate, which uses solid-phase extraction chemistry for the purification of DNA or RNA from a range of sample types. Development of a combined ABI 6100 HCV extraction method with a real-time ABI 7500 RT-HCV PCR assay may, therefore, facilitate the rapid and accurate detection of HCV.

Materials and methods

A total of 650 serum samples were used for the development and optimisation of the RT-PCR assay. A further 200 samples were used in a clinical trial. All samples were collected from randomly selected patients attending the hepatology clinic at St. James's Hospital, Dublin, Ireland. Samples were separated and frozen at −20°C within 4 h of collection. A 200-μl aliquot of serum was removed and tested for HCV-RNA by the COBAS AMPLICOR™ HCV Test v2.0 assay (Roche Diagnostics GmbH, Mannheim, Germany). A 500-μl aliquot of serum was removed and tested using the ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA).

A dilution series of the WHO Second International Standard 2003 for HCV RNA (National Institute for Biological Standards and Control (NIBSC), code 96/798, Hertfordshire, UK) was used to determine the lower limit of detection (LOD) of the RT-PCR assay. The HCV RNA Genotype Panel for Nucleic Acid Amplification Techniques (NIBSC, code 02/202, Hertfordshire, UK) and the Quality Control for Molecular Diagnostics (QCMD) 2002 and 2004 HCV Proficiency Panels (QCMD, Glasgow, Scotland) were also used to assess assay performance.

Template RNA was extracted from 500 μl of serum samples using the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems). A 70-μl aliquot of master mix consisting of 100 μg of proteinase K (Applied Biosystems), 25 μg of polyadenylic acid (Sigma-Aldrich IRL Ltd., Dublin, Ireland) and 10 μl of phocine distemper virus (PDV) internal control (IC) was added to the samples. The PDV IC was kindly supplied as a 1,000× stock cell culture by Dr. Hubert Niesters, Department of Virology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands [5]. Samples were mixed and then incubated at room temperature for 1 h. The lysates were applied to the ABI PRISM™ 6100 Nucleic Acid PrepStation and vacuum-based wash and elution steps were performed according to the manufacturer's instructions.

Prior to use, the ABI 6100 extraction protocol was evaluated by comparison with the QIAamp® UltraSens™ virus extraction method (QIAGEN GmbH, Hilden, Germany). The manual extraction was performed according to the manufacturer's instructions using a 500-μl start volume. Forty serum samples were extracted by both methods.

All RNA eluates were converted to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). The cDNA conversion was performed according to the manufacturer's instructions with the following modifications. In the preparation step, the total reaction volume was optimised to 50 μl and RT reaction conditions were optimised to 25°C for 10 min, followed by 37°C for 1 h.

HCV-specific primers and probes were selected using Primer Express Software™ Version 2.0 (Applied Biosystems) (Table 1). The HCV-specific primer set, HCV-F (5′-AGCGTCTAGCCATGGCGTT-3′) and HCV-R (5′-GCAAGCACCCTATCAGGCAGT-3′) was designed to generate a 238-bp amplicon. The probe (5′-TCTGCGGA ACCGGTGAGT-MGB-3′) was designed as a 5′-FAM-labelled minor groove binding (MGB) hybridisation probe. The PDV haemagglutinin gene (GenBank accession #AF479274) was selected as the target for the PDV IC primer–probe set, details of which were supplied by Dr. Niesters (personal communication). The original primer set, PDV-F (5′-GGTGGGTGCCTTTTACAAGAAC-3′) and PDV-R (5′-ATCTTCTTTCCTCAACCTCGTCC-3′), was modified to generate an 83-bp amplicon. The probe (5′-ATGCAAGGGCCAATT-MGB-3′) was re-designed as a 5′-VIC-labelled MGB hybridisation probe (Table 2).

Table 1 Multiple sequence alignment for hepatitis C virus (HCV)-specific primer–probe set for the 5′UTR of HCV genotypes

HCV forward primer (nucleotide position 74–92[a])	HCV-MGB probe (nucleotide position 147–164[a])	HCV reverse primer (nucleotide position 288–308[a])
HCV1a AGCGTCTAGCCATGGCGTT	HCV1a TCTGCGGAACCGGTGAGT	HCV1a ACTGCCTGATAGGGTGCTTGC
HCV1b AGCGTCTAGCCATGGCGTT	HCV1b TCTGCGGAACCGGTGAGT	HCV1b ACTGCCTGATAGGGTGCTTGC
HCV1c AGCGTCTAGCCATGGCGTT	HCV1c TCTGCGGAACCGGTGAGT	HCV1c ACTGCCTGATAGGGTGCTTGC
HCV2a AGCGTCTAGCCATGGCGTT	HCV2a TCTGCGGAACCGGTGAGT	HCV2a ACTGCCTGATAGGGTGCTTGC
HCV2b AGCGTCTAGCCATGGCGTT	HCV2b TCTGCGGAACCGGTGAGT	HCV2b ACTGCCTGATAGGGTGCTTGC
HCV2c AGCGTCTAGCCATGGCGTT	HCV2c TCTGCGGAACCGGTGAGT	HCV2c ACTGCCTGATAGGGTGCTTGC
HCV3a AGCGCCTAGCCATGGCGTT	HCV3a TCTGCGGAACCGGTGAGT	HCV3a ACTGCCTGATAGGGTGCTTGC
HCV3b AGCGTCTAGCCATGGCGTT	HCV3b TCTGCGGAACCGGTGAGT	HCV3b ACTGCCTGATAGGGTGCTTGC
HCV4a AGCGTCTAGCCATGGCGTT	HCV4a TCTGCGGAACCGGTGAGT	HCV4a ACTGCCTGATAGGGTGCTTGC
HCV4b AGCGTCTAGCCATGGCGTT	HCV4b TCTGCGGAACCGGTGAGT	HCV4b ACTGCCTGATAGGGTGCTTGC
HCV4c AGCGTCTAGCCATGGCGTT	HCV4c TCTGCGGAACCGGTGAGT	HCV4c ACTGCCTGATAGGGTGCTTGC
HCV4d AGCGTCTAGCCATGGCGTT	HCV4d TCTGCGGAACCGGTGAGT	HCV4d ACTGCCTGATAGGGTGCTTGC
HCV4e AGCGTCTAGCCATGGCGTT	HCV4e TCTGCGGAACCGGTGAGT	HCV4e ACTGCCTGATAGGGTGCTTGC
HCV4f AGCGTCTAGCCATGGCGTT	HCV4f TCTGCGGAACCGGTGAGT	HCV4f ACTGCCTGATAGGGTGCTTGC
HCV5a AGCGTCTAGCCATGGCGTT	HCV5a TCTGCGGAACCGGTGAGT	HCV5a ACTGCCTGATAGGGTGCTTGC
HCV6a AGCGTCTAGCCATGGCGTT	HCV6a TCTGCGGAACCGGTGAGT	HCV6a ACTGCCTGATAGGGTGCTTGC
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Primer AGCGTCTAGCCATGGCGTT	Probe TCTGCGGAACCGGTGAGT	Primer GCAAGCACCCTATCAGGCAGT

[a] Numbering according to Wang et al. (1993), where the ATG start codon for the polyprotein precursor gene is located at nucleotide position 342 [6]
Primer–probe sequences are presented in the 5′ to 3′ orientation

The assay was designed as a qualitative singleplex RT-PCR assay with parallel detection of HCV and PDV in separate wells. Both the HCV and PDV reactions were optimised to a final reaction volume of 50 μl, containing 10 μl of cDNA and 40 μl of PCR mixture. This PCR mixture contained 25 μl of TaqMan® 2× Universal PCR Master Mix (Applied Biosystems), 0.3 μM of HCV-specific primers and 0.25 μM of HCV-specific MGB probe, or 0.3 μM of PDV-specific primers and 0.25 μM of PDV-specific MGB probe. Primer and probe concentrations were optimised for both reactions using “matrix guidelines” from Applied Biosystems. The amplification protocol for both gene targets included an initial denaturation step at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

Following the optimisation of a singleplex assay, efforts were made to develop the assay into a qualitative multiplex format. The PDV amplification reaction was optimised as a primer-limited assay using the “limiting primer matrix” (Applied Biosystems 2001). For the multiplex assay, 10 μl of cDNA was added to 40 μl of reaction mixture containing 25 μl of TaqMan® Universal PCR Master Mix, 0.3 μM of HCV-specific primers, 0.1 μM of PDV-specific primers and 0.25 μM of both HCV-specific and PDV-specific MGB probes.

The LOD for the HCV assay was determined using a dilution series of the WHO Standard, covering a range from 10 IU/ml to 10,000 IU/ml. The sensitivity of the assay was also monitored by testing the external QCMD 2002 and 2004 HCV Proficiency Panels. Genotype specificity of the

Table 2 Multiple sequence alignment for phocine distemper virus (PDV)-specific primer–probe set for the haemagglutinin gene of PDV

PDV forward primer (nucleotide position 41–62[a])		PDV probe (nucleotide position 64–78[a])		PDV reverse primer (nucleotide position 101–123[a])	
DK881a(H)	GGTGGGTGCCTTTTACAAGAAC	DK881a(H)	ATGCAAGGGCCAATT	DK881a(H)	GGACGAGGTTGAGGAAAGAAGAT
DK884a(H)	GGTGGGTGCCTTTTACAAGAAC	DK884a(H)	ATGCAAGGGCCAATT	DK884a(H)	GGACGAGGTTGAGGAAAGAAGAT
4a(H)	GGTGGGTGCCTTTTACAAGAAC	4a(H)	ATGCAAGGGCCAATT	4a(H)	GGACGAGGTTGAGGAAAGAAGAT
(H)	GGTGGGTGCCTTTTACAAGAAC	(H)	ATGCAAGGGCCAATT	(H)	GGACGAGGTTGAGGAAAGAAGAT
	**********************		***************		***********************
Primer	GGTGGGTGCCTTTTACAAGAAC	Probe	ATGCAAGGGCCAATT	Primer	ATCTTCTTTCCTCAACCTCGTCC

[a] Numbering according to Nielsen (2002), where the ATG start codon for the haemagglutinin gene is located at nucleotide position 21 [7]
Primer–probe sequences are presented in the 5′ to 3′ orientation

assay was monitored by testing the HCV RNA Genotype Panel for Nucleic Acid Amplification Techniques (NIBSC code 02/202). To determine reproducibility, clinical samples (n=32), the WHO dilution series and the QCMD panels were tested in three separate assay runs using the same batches of reagents.

The singleplex assay was evaluated by comparing the results obtained for 200 serum samples (99 HCV-positive, nine HCV-low-positive and 92 HCV-negative) with results obtained from the COBAS AMPLICOR assay. An HCV-low-positive sample was defined as a sample which yielded an equivocal HCV result (absorbance at 660 nm≥0.15<1.0) on initial testing and subsequently demonstrated absorbance values≥0.15 on repeat testing in duplicate. The results of the nine HCV-low-positive samples were also compared with the results obtained by the VERSANT® HCV RNA assay v3.0 (Bayer Diagnostics, Berkeley, CA). The multiplex assay was evaluated by testing representative samples from the clinical trial group (n=28). A sample was considered as a true positive/negative if an HCV-positive/-negative result, respectively, was obtained by the COBAS AMPLICOR assay.

Results

HCV cycle threshold (Ct) values for the ABI 6100 RNA extracts (mean=36.0) were within one Ct value of those obtained from UltraSens™ extracts (mean=35.2), with a 95% confidence interval (CI) of 0.6 to 0.9 (P<0.001). In the singleplex assay, Ct values for HCV- and PDV-positive signals ranged from 22.5–42.5 and 35.7–39.3, respectively. Using a dilution series of the WHO Standard for HCV, the LOD was determined as 100 IU/ml (Table 3). In accordance with the qualitative QCMD rating, the assay achieved a maximum performance score for QCMD HCV Proficiency Panels 2002 and 2004 (Table 4). All HCV genotypes in the HCV RNA Genotype Panel for Nucleic Acid Amplification Techniques were detected (Table 3). The singleplex assay also displayed good reproducibility with the results for clinical samples (n=32), the WHO dilution series and the QCMD panels, falling within 1.5 Ct values of each other.

Table 3 Observed cycle threshold (Ct) values for dilution series of HCV RNA WHO Standard (Panel A) and HCV genotype panel (NIBSC) (Panel B)

Panel A		Panel B	
Standard (IU/ml)	Observed Ct	Genotype 1,000 IU/mL	Observed Ct
10,000	31.2	1	37.0
5,000	32.4	2	35.2
1,000	34.6	3	36.8
500	35.2	4	36.4
250	36.2	5	36.5
100	37.2	6	38.4
50	Undetected		

In the singleplex assay, 107 of the 108 HCV-positive samples tested yielded a positive result, thereby, demonstrating a sensitivity of 99.0%. The resulting negative predictive value was 98.9%. The positive sample that remained undetected in the clinical trial had previously failed to be detected by the VERSANT® assay (LOD<3,200 copies/ml), but yielded a positive result by the COBAS assay (LOD=50 IU/ml), albeit within the low-positive category, as defined above. All 92 HCV-negative samples in the clinical trial yielded a negative result. The specificity and positive predictive value for the singleplex assay were, therefore, 100%.

In the multiplex assay, Ct values for HCV-positive signals demonstrated a wider range than the singleplex assay (22.0–48.5). HCV amplification plots demonstrated a mean increase of 1.5 in Ct values (95% CI of 0.4–2.5, P=0.01) compared to the singleplex assay. For PDV-positive signals, the range of Ct values also widened (35.9–48.0), with a mean increase of 3.6 in individual Ct values (95% CI of 2.5–4.8, P<0.001) (Table 5). Nevertheless, using the multiplex format, the PDV internal control was detected in all samples. The LOD of the multiplex assay was the same as that observed for the singleplex assay (100 IU/ml). Of the 28 HCV samples tested in multiplex, 27 yielded the correct HCV result, with only one false-negative occurring. This sample also yielded a false-negative result in the singleplex assay.

Discussion

The direct detection of HCV RNA has become an essential tool for the diagnosis of hepatitis C infection. The qualitative RT-PCR assay developed demonstrated high sensitivity (99.0%) and an acceptable LOD of 100 IU/ml, which is comparable to other in-house HCV PCR detection assays [8, 9]. This LOD falls within the recommended detection limits for HCV RNA assays (100 IU/ml) [10], although many commercial assays have a claimed an LOD of 50 IU/ml. Only one of 108 HCV-positive samples tested in the clinical trial was missed. This sample was undetected in the VERSANT assay, which has an LOD value of <3,200 copies/ml. However, since the sample demonstrated a low-positive result by the COBAS AMPLICOR system, which has an LOD of 50 IU/ml, it is likely to represent a true positive with a low viral load.

The assay also displayed excellent specificity, with the detection of all genotypes in the NIBSC HCV RNA Genotype Panel and the QCMD HCV Proficiency Panels

Table 4 Observed cycle threshold (Ct) values for QCMD HCV proficiency panels

QCMD 2002	Target concentration range (IU/mL)	Observed Ct	QCMD 2004	Target concentration (IU/mL)	Observed Ct
HCV 1	2–20×10^4 (+)[a]	30.5 (+)	HCV 17	8.3×10^3 (+)	32.2 (+)
HCV 2	1–4×10^5 (+)	27.2 (+)	HCV 18	1.7×10^3 (+)	33.3 (+)
HCV 3	BLD[b]-1,200 (+)	39.5 (+)	HCV 19	1.7×10^2 (+)	39.0 (+)
HCV 4	1–5×10^4 (+)	30.9 (+)	HCV 20	8.3×10^4 (+)	28.5 (+)
HCV 5	1–8×10^3 (+)	37.2 (+)	HCV 21	8.3×10^3 (+)	31.5 (+)
HCV 6	1–8×0^3 (+)	36.4 (+)	HCV 22	3.4×10^3 (+)	33.2 (+)
HCV 7	BLD-1,200 (+)	Undetected (–)	HCV 23	8.3×10^4 (+)	26.8 (+)
HCV 8	Neg (−)[c]	Undetected (–)	HCV 24	Negative (−)	Undetected (−)

[a] (+)=positive signal
[b] BLD=below limit of detection
[c] (−),=negative signal

Table 5 Comparison of cycle threshold (Ct) values observed for HCV and PDV target in the singleplex and multiplex assays

Sample no.	Singleplex HCV Ct	Multiplex HCV Ct	Singleplex PDV Ct	Multiplex PDV Ct
1	34.0	34.1	37.7	42.2
2	34.0	33.4	37.1	47.4
3	37.3	39.6	37.4	47.0
4	37.9	42.3	37.3	45.9
5	40.1	45.9	37.2	43.3
6	40.9	48.5	38.2	47.4
7	40.7	45.5	37.9	38.6
8	25.3	24.7	36.8	38.4
9	22.5	22.1	36.0	37.4
10	22.8	22.0	35.9	36.5
11	24.7	23.8	38.7	39.9
12	25.5	24.7	36.9	37.5
13	22.5	23.5	37.1	37.8
14	22.8	23.5	37.6	37.7
15	23.1	23.8	36.1	35.9
16	24.4	25.2	36.3	36.8
17	22.9	23.9	37.4	44.1
18	42.5	38.	39.3	43.3
19	38.8	39.1	35.9	48.4
20	36.7	38.3	37.1	48.0
21	40.8	47.1	37.2	43.6
22	36.1	37.2	36.8	38.9
23	36.6	39.5	35.7	37.9
24	35.1	35.5	36.1	40.1
25	37.3	40.0	36.4	38.1
26	Undetected	Undetected	35.8	37.7
27	Undetected	Undetected	37.6	39.9
28	Undetected	Undetected	37.3	39.4
Mean	31.7	33.1	37.0	40.7
Standard deviation	7.8	9.5	0.9	3.8
95% lower–95% upper	0.4–2.5		2.5–4.8	

2002 and 2004. Using the singleplex assay, full marks were obtained for both panels. In addition to high sensitivity and specificity, the hybridisation probe assay yielded highly reproducible results, with results not varying by more than 1.5 Ct values on repeat testing.

Although the HCV results of samples tested in multiplex remained unchanged from the singleplex assay, the Ct values of HCV and PDV target increased in the multiplex format. The reason for this Ct increase is currently unknown, but may be due to primer limitation or primer–dimer formation. Nevertheless, the multiplex assay yielded sensitivity (95.8%), specificity (100%) and LOD (100 IU/ml) rates similar to the singleplex assay. Preliminary results of the multiplex clinical trial are promising and indicate that this assay may be used for the qualitative detection of HCV.

It is increasingly recognised that the use of a universal internal control helps to enhance the performance of molecular virology techniques [11, 12]. This study, therefore, incorporated the design of a second RT-PCR assay for the detection of a viral RNA internal control. PDV was added directly to the samples in low concentration as a viral substrate. As PDV behaves in a more similar manner to the HCV target, it can simulate the extraction, amplification and detection of HCV. This methodology confirmed that there was no significant loss of sample during the extraction procedure and that there were no inhibitors to reverse transcription or amplification in the samples. International collaboration among diagnostic laboratories to implement PDV as a universal RNA viral internal control could represent a step towards the standardisation of molecular virology assays [11].

Aside from the design of an accurate HCV detection assay, this study also addressed other important design facets of molecular diagnostic assays. Many nucleic acid extraction systems are designed for dedicated commercial assays, but offer little flexibility for in-house assay design. This study incorporated the development of a high-yield, high-purity HCV RNA extraction method using the semi-automated ABI PRISM™ 6100 Nucleic Acid PrepStation

(Applied Biosystems, Foster City, CA). This system enabled rapid high-throughput purification, thus, helping to overcome the cost issues associated with automation and the time factors associated with manual extraction procedures. The lack of PCR inhibitors observed in this study reflects the high purity of viral RNA isolated using the optimised semi-automated ABI 6100 extraction protocol. Furthermore, the results for the ABI 6100 extracts compared well to the results for extracts from the manual UltraSens™ Kit.

The HCV assay developed in this study represents a robust and reliable RT-PCR method for the detection of hepatitis C in serum samples. The assay design incorporated the use of semi-automated extraction, a viral substrate internal control, a two-step RT-PCR procedure and a flexible singleplex or multiplex format and represents an attractive alternative to commercial HCV PCR systems.

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