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Validation of One-Step Real-Time RT-PCR Assay in Combination with Automated RNA Extraction for Rapid Detection and Quantitation of Hepatitis C Virus RNA for Routine Testing in Clinical Specimens

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Abstract A one-step real-time quantitative RT-PCR assay in combination with automated RNA extraction was evaluated for routine testing of HCV RNA in the laboratory. Specific primers and probes were developed to detect 302 bp on 5'-UTR of HCV RNA. The assay was able to quantitate a dynamic linear range of 10^7–10^1 HCV RNA copies/reaction (R^2=0.997). The synthetic HCV RNA standard of 1.84±0.1 (mean±SD) copies developed in this study corresponded to 1 international unit (IU) of WHO International Standard for HCV RNA (96/790 I). The detection limit of the assay was 3 RNA copies/reaction (81 IU/ml) in plasma samples. The assay was comparable to the Amplicor HCV Monitor (Monitor) assay with correlation coefficient r=0.985, but was more sensitive than the Monitor assay. The assay could be completed within 3 h from RNA extraction to detection and data analysis for up to 32 samples. It allowed rapid RNA extraction, detection, and quantitation of HCV RNA in plasma samples. The method provided sufficient sensitivity and reproducibility and proved to be fast and labor-saving, so that it was suitable for high throughput HCV RNA test.

Key words: Automated RNA extraction, HCV RNA, quantitation, real-time RT-PCR

The hepatitis C virus (HCV) is a positive single-stranded RNA virus that has been identified as a major agent responsible for post-transfusion and community-transmitted non-A, non-B hepatitis infections [4, 7, 16, 32]. HCV has been the most serious complication of transfusions of blood products in connection with a series of worldwide outbreaks of hepatitis C, which was associated with intravenous immunoglobulin administration [28, 31, 38, 39]. Thus, effective detection of HCV in blood and blood-derived products became extremely important [3]. From 1 July 1999 the European Committee for Proprietary Medicinal Products (CPMP) recommended nucleic acid amplification techniques (NATs) to test only batches derived from plasma pools, using validated test methods of suitable sensitivity and specificity [5].

Many various NATs have been developed for quantitation of HCV RNA. Most NATs are based on conventional reverse transcription-polymerase chain reaction (RT-PCR) [30, 37, 42]. Alternatives are based on branched-DNA (bDNA) assay [1, 20, 34] or in-house competitive PCR (cPCR) [14, 19, 21, 26, 41]. Real-time RT-PCR based assays have been developed and described as a faster and more sensitive approach to detect HCV RNA using a molecular beacon probe [40] or TaqMan probe and the ABI Prism 7700 sequence Detection System [18, 22, 33]. Real-time PCR allows convenient quantitation and eliminates the need for post-PCR manipulation, since amplification, detection, and quantitation of PCR products occur simultaneously in the same reaction vessel [10, 40]. Further advanced real-time PCR technology, using LightCycler (LC) instrument (Roche Molecular Biochemicals), has been introduced [15, 25, 35]. It allows rapid cycling with fluorescence measurement in glass capillaries. Recent studies have used SYBR Green Dye [9, 15], 'TaqMan' probes [35], or fluorescently labeled probes [25] for real-time detection of PCR products by LC instrument. A recent assay by the LC system using hybridization probes, which adopts two-round RT-PCR in single closed capillaries, shows high sensitivity [24].

Most HCV-NATs use conventional RNA extraction methods using Tri-Reagent-BD (Molecular Research Center)

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[40] or Trizol® reagent (Life Technology) [22]. Some other advanced methods use highly pure viral nucleic acid kit (Roche Molecular Biochemicals) and QIAamp UltraSens virus kit (Qiagen, Inc.) [24]. These extraction methods are labor-intensive, time-consuming, and require many steps with attendant risk of carryover contamination [29, 36]. Therefore, they are not suitable for routine testing for HCV RNA in a diagnostic setting or in the quality control laboratory where large numbers of clinical samples or final products are tested. To apply HCV-NAT for routine clinical testing, a simple, rapid, and reproducible nucleic acid extraction as well as a sensitive, precise, and rapid quantitative detection are required. Recently, fully automated instruments for nucleic acid extraction such as BioRobot M48 [13], BioRobot 9604 instrument (Qiagen, Inc.) [6], or MagNA Pure LC (Roche Molecular Biochemicals) [13, 23, 27] have commonly been used for routine diagnostic PCR, since they minimize time and labor and provide reliable and reproducible test results.

In the present study, we have validated one-step real-time RT-PCR using LightCycler with specific hybridization probes, in combination with automated RNA extraction on MagNA Pure LC. We evaluated the assay for applicability to routine testing for HCV RNA in clinical specimens in quality control laboratories.

MATERIALS AND METHODS

Primers and Hybridization Probes

The primers CFB73 (sense) 5'-ATGGCGTTAGTATGAGTGTCGT-3' and CRG374 (antisense) 5'-GGTGTTACGTTTGGTTTTTCT-3' were designed to target the highly conserved 302 bp sequences between the position 73 and 374, coding the 5'-untranslate region (5'-UTR) of HCV RNA. The sequence-specific oligonucleotide probes CFL 5'-GCCGAGTAGTGTTGGGTCGCGAAA-3' labeled with fluoresceine at the 3' end and CLC 5'-CCTTGTGGTACTGCCTGATAGGGTGC-3' labeled with LC-Red 705 at the 5' end were designed to anneal to an internal sequence of the amplicon, rendering a 2 bp gap between probes. For construction of synthetic HCV RNA standard, a primer CFLApaI (sense) 5'-GCCGAGTAGTGTTGGGGCCCGAAA-3' containing an *Apa*I-restriction site with the altered nucleotide sequence of original T to G at position 257 and G to C at position 259 was used with primer pair CFB73/CRG374, so that it could be readily distinguished from the wild-type sequence using melting curve analysis.

Preparation of Synthetic HCV RNA Standard

HCV RNA standard for the one-step real-time RT-PCR assay was prepared from a fragment of 302 bp (73-374) on 5'-UTR of HCV genomic RNA by site-directed mutagenesis [2], cloning, and *in vitro* transcription. A 2 bp mismatch was incorporated into the probe-binding region (CFL-binding region) of the 302 bp HCV target fragment for melting curve analysis. In brief, total RNA extracted from a HCV-positive plasma was reverse transcribed into cDNA using SuperScript II reverse transcriptase (GibcoBRL) and random primer (GibcoBRL). The cDNA was amplified by PCR with primer pair CFLApaI/CRG374. The PCR amplified product was separated on a 1% low-melting agarose gel and purified with QIAquick Gel Extraction kit (Qiagen) and then used as a megaprimer for the second-round PCR. The second-round PCR was carried out with primer CFB73 and the megaprimer. The PCR-amplified product was confirmed by restriction enzyme *Apa*I digestion, gel-purified, and cloned into the pCR 4 Blunt Vector using Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). The presence and orientation of the inserts were determined using restriction enzyme *Eco*RI digestion and DNA sequencing. The positive strand RNA was transcribed from the *Spe*I-linearized recombinant plasmid with T7 RNA polymerase (Roche Diagnostics). The DNA templates were removed by digestion with DNase I (Roche Diagnostics) following the transcription reaction. The RNA transcripts were purified by Tri-Reagent (Molecular Research Center, U.S.A.) according to the manufacturer's instruction. Purified RNA was resuspended in 60 µl of diethylpyrocarbonate (DEPC)-treated PCR-grade water (Invitrogen) and tested for contamination of the recombinant plasmid DNA by LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Quantitation of the purified RNA was carried out by spectrophotometry at A_{260}. RNA copy number was calculated, based on the total HCV RNA transcripts and its molecular weight [17]. We used this synthetic RNA as a standard to quantitate HCV RNA in plasma specimens.

Clinical Specimens

A total of 105 plasma samples collected in Korea were used for the study. The samples were examined by in-house RT-nested PCR and Abbott AxSYM HCV Version 3.0 MEIA (AxSYM) (Abbott Diagnostics, Chicago, IL, U.S.A.), and 51 samples were found to be HCV RNA-positive and 54 samples were found to be negative from the two assays. For the RT-nested PCR, reverse transcription of viral RNA was carried out with antisense primer (SKC390 5'-ATGGTGCACGGTCTACGAGA-3') for 60 min at 42°C and resulting cDNA was amplified by PCR with primer pair SKC178 5'-CCACCATGAATCACTCCCCT-3' and SKC390 by Taq DNA polymerase (TaKaRa), with an initial incubation at 94°C for 2 min. This was followed by 35 cycles, with each cycle consisting of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. Cycles were followed by incubation for 10 min at 72°C. Nested PCR amplification was performed with the RT-PCR amplification product with primer SKC184 5'-CTCCCCTGTGAGGAACTATTG-

3' and SKC328 5'-TATCAGGCAGTACCACAAGGC-3', for 35 cycles as described above. The assay with the AxSYM was carried out in accordance with the manufacturer's instructions. Genotypes of HCV RNA-positive samples were identified using the VERSANT™ HCV Genotype Assay (LiPA) (Bayer Corporation, Tarrytown, NY, U.S.A.).

Automated RNA Extraction Using MagNA Pure LC

Total nucleic acid was extracted from 100 µl of each plasma sample in accordance with the manufacturer's instructions by using the MagNA Pure LC total Nucleic Acid Isolation Kit (Roche Diagnostics) on MagNA Pure LC by the extraction protocol 'Total NA Serum_Plasma_Blood'. The RNA was eluted in a final volume of 100 µl in a sample cartridge. The MagNA Pure LC software, Ver. 3.0, automatically calculated reagent volumes and numbers of reaction tips needed for the run. After completion of RNA extraction, the purified RNA and RT-PCR master mixture [6 mM magnesium chloride, 0.2 µM forward primer (CFB73), 0.1 µM reverse primer (CRG374), 0.5 µM FL probe (CFL), 0.25 µM LC probe (CLC), 4 µl of RT-PCR reaction mix, and 0.4 µl RT-PCR enzyme mix] were pipetted and transferred into pre-cooled LC capillaries positioned in the cooling block. The MagNA Pure LC automatically performed all steps of the procedure by using the robotic arm according to the pre-programmed protocols. The remnant of RNA in the sample cartridge was taken from the MagNA Pure LC and stored at −80°C. The World Health Organization (WHO) International Standard for HCV RNA (96/790 I) was serially diluted in normal human plasma and extracted as described above.

Real-Time RT-PCR with the LightCycler

The one-step real-time RT-PCR analysis, from cDNA synthesis to PCR amplification, was optimized with LightCycler instrument using LightCycler RNA Amplification Kit Hybridization Probes (Roche Diagnostics) [9]. The reaction mixture for the one-step real-time RT-PCR contained 5 µl of HCV RNA template and 15 µl of the master mixture in a final reaction volume of 20 µl. The optimum concentration of the primers and probes was determined experimentally. RNA template and the master mixture were automatically pipetted into each glass capillary positioned in an LC carousel by the MagNA Pure LC. Capillaries were sealed, centrifuged in the LC carousel-centrifuge, and placed in the LC rotor. The one-step real-time RT-PCR consists of five consecutive programs. Program 1 was set for reverse transcription of the template HCV RNA by incubation at 55°C for 10 min. Program 2 was set for denaturation of the resulting cDNA by incubation at 95°C for 30 s. Program 3 was set for 40 cycles of PCR amplification of the resulting cDNA with quantitation analysis mode of denaturation at 95°C for 5 s; annealing at 50°C for 15 s with single acquisition mode; amplification at 72°C for 12 s with a 2°C/s temperature transition rate. Program 4 was set for melting curve analysis to confirm PCR product identity by 1 cycle with analysis mode of melting curve at 95°C for 0 s, annealing at 45°C for 40 s, and continuous acquisition at 95°C for 0 s with a 0.1°C/s temperature transition rate. The last program was set for cooling the rotor and thermal chamber at 40°C for 30 s. In every RT-PCR run, a negative template control consisting of PCR-grade water and a positive control, calibrated working reagent (HCV RNA working reagent for NATs, NIBSC Code 01/408), were processed to achieve routine quality control of the assay. For RNA quantitation by commercial method, Cobas Amplicor HCV Monitor Test (Monitor) (2.0) was carried out according to the manufacturer's instructions.

Calibration of Synthetic HCV RNA Standard to WHO International Standard for HCV RNA

The calibration of the synthetic HCV RNA standard was performed by quantitating the serial dilutions of the WHO standard (50,000 IU/0.5 ml) by the one-step real-time RT-PCR. WHO standards were reconstituted in deionized water to a concentration of 100,000 IU/ml and serially diluted (factor 2) in normal human plasma. Total RNA was extracted by MagNA Pure LC and then subjected to the one-step real-time RT-PCR in parallel with the dilution series of the synthetic HCV RNA standard. The relation between HCV RNA copies and WHO HCV IU was analyzed.

Statistical Analysis

The coefficient of variation (CV) of crossing point (C_p) values of each dilution of samples between runs (intra-assay) and within runs (inter-assay) was calculated using: (Standard Deviation of each C_p/mean values of a set of C_p) ×100. For the comparison of values between the one-step real-time RT-PCR and the Monitor, paired t-test was performed using SAS version 8.1.

RESULTS

Linearity, Range of Quantitation, and Melting Curve Analysis

In order to prepare a standard curve for quantitation of HCV RNA, seven 10-fold serial dilutions of synthetic HCV RNA standard (10^7–10^1 copies/reaction) in dilution buffer were amplified by the one-step real-time RT-PCR. After reaction was completed, an external standard curve was obtained by the Second Derivative Maximum method of LC software version 3.5 by plotting the cycle number against the starting concentration of each dilution. The fluorescence detected at the first amplification that reached the threshold cycle (C_T) number was expressed as C_p value by the LC software, and the C_p data were used to generate a

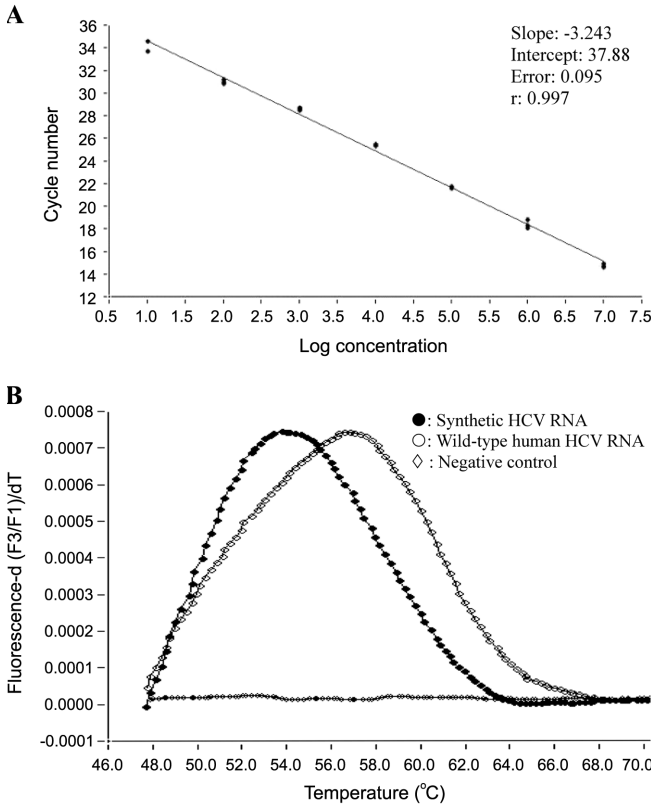

Fig. 1. Real-time RT-PCR.
(A) External standard curve generated from 10-fold serial dilutions (10^7–10^1 copies/reaction) of the synthetic HCV RNA standard. A linear range was demonstrated. Cycle number was plotted versus the logarithm of initial concentration of each dilution point. All experiments were analyzed in triplicate and showed regression coefficient (r) of above 0.99. (B) The melting curves of the synthetic HCV RNA standard (T_m 54°C) and wild-type human HCV RNA (T_m 57°C). The negative derivative of fluorescence (F) with respect to temperature (T), -d(F3/F1)/dT, was plotted.

standard curve [9]. Amplification profiles of the synthetic HCV RNA standard at different concentrations showed the linear range of 10^7 to 10^1 copies per reaction with a mean correlation coefficient r=0.997 in triplicate analysis (Fig. 1A). The cut-off for the quantitation of HCV RNA by the one-step real-time RT-PCR was at 10 copies per reaction (271 IU/ml) with linearity.

The melting curve was plotted as fluorescence signal intensity (the negative derivatives) versus temperature (Fig. 1B) by the LC data analysis software program's melting curve analysis following amplification. Melting curve analysis could confirm PCR product identity, since synthetic HCV RNA standards designed to have a 2 bp mismatch with the probe resulted in a 3°C difference in melting temperature (T_m) from the wild-type HCV target sequence. The T_m values of the HCV target sequence and the synthetic HCV RNA standards were 57°C and 54°C, respectively, so that the synthetic HCV RNA standard was distinguishable from the wild-type HCV target sequence in this experiment.

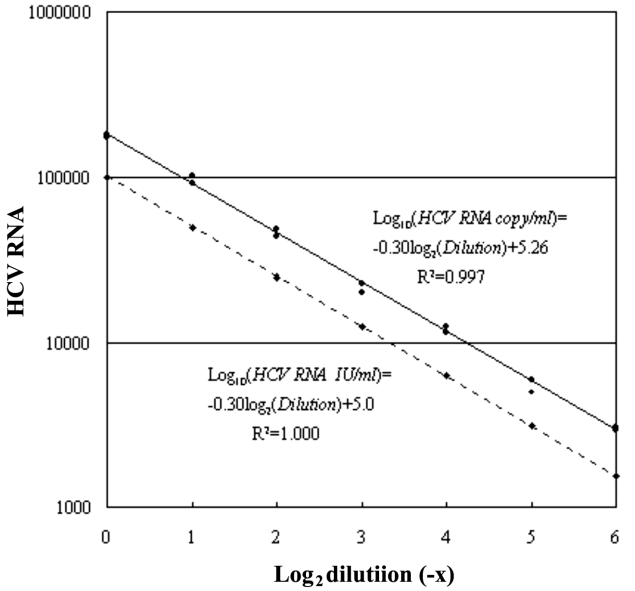

Fig. 2. Relationship between WHO HCV IU and the synthetic HCV RNA copies.
From the regression curve, 1.84±0.10 (Mean±SD) RNA copies were equivalent to 1 IU WHO HCV. ◆: HCV RNA IU/ml; ●: HCV RNA copy/ml.

Synthetic HCV RNA Copies Equivalent to WHO HCV IU

HCV RNA copies were converted to WHO HCV IU based on the C_p values obtained from the one-step real-time RT-PCR. The ratio of HCV RNA copies per HCV IU was about 1.84±0.10 (Mean±SD) (Fig. 2).

Detection Limit

In order to determine the detection limit of the assay, RNAs were extracted from four serial half-logarithm dilutions from 10 copies to 0.3 copies (from 271 IU/ml to 8 IU/ml) of a plasma sample by MagNA Pure LC and subjected to the one-step real-time RT-PCR. The assay was performed in triplicate for each dilution point on different days. Detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated as an exact value [8]. The one-step real-time RT-PCR of clinical sample in combination with automated RNA extraction showed a positive cut-off point (the concentration giving at least 95% positive results) between 1 and 3 copies per reaction (27 and 81 IU/ml) (Table 1). The assay had an estimated sensitivity of 1 copy per reaction in plasma samples (approximately 50 copies/ml or 92 WHO IU/ml).

Precision of the Assay

The precision was assessed within each run (intra-assay) and between runs (inter-assay). The C_p values obtained from the assay using HCV RNA, extracted from half-logarithm serial dilutions from 6×10^3 to 6×10^1 copies (from 3.3×10^3 to 3.3×10^1 IU) of a HCV-positive plasma sample on MagNA Pure LC, were used for the assessment.

Table 1. Detection limit of the one-step real-time RT-PCR assay following automated RNA extraction by MagNA Pure LC.

HCV RNA[a] concentration (copies (IU)/reaction)	Run[b]			
	1	2	3	Positivity (%)
10 (5.4)	3[c]	3	3	100.0
3 (1.6)	3	3	3	100.0
1 (0.5)	2	1	2	55.5
0.3 (0.2)	0	0	0	0

[a]Each RNA dilution was obtained from automated RNA extraction after half-logarithm serial dilution of plasma samples with known RNA concentration.
[b]Each run was performed in triplicate on different days.
[c]Data represent the number of positives out of the triplicate for each concentration.

The assay was carried out in triplicate at each dilution point in a total of three independent experiments on different days. The intra-assay provided high repeatability, showing CVs of the range of 0.10 to 1.43%. The mean of the inter-assay CVs between runs on different days was 1.09%, indicating high intermediate precision (Table 2).

Diagnostic Sensitivity and Specificity

In order to evaluate the practical application of the one-step real-time RT-PCR in combination with automated RNA extraction, we tested 51 HCV RNA-positive plasma samples and 54 negative plasma samples. The RNA concentration of HCV RNA-positive samples was in the range of 10^7–10^4 copies per ml (5.4×10^6–5.4×10^3 IU/ml) by the assay. The positive samples that had been tested positive for RT-nested PCR and the AxSYM were also found to be positive by automated RNA extraction and one-step real-time RT-PCR. The negative samples were also found to be negative (data not shown). Upon repeated tests, all samples showed constant results. This indicated that both the diagnostic sensitivity and specificity of the assay were 100% in human plasma samples.

Comparison of One-Step Real-Time RT-PCR with the Cobas Amplicor HCV Monitor Test

Thirty-nine of 51 HCV RNA-positive plasma samples were quantitated by the Cobas Amplicor HCV Monitor Test, version 2.0 (Monitor) (Roche Molecular Systems, Branchburg, NJ, U.S.A.) and by the one-step real-time RT-PCR. The positive samples consisted of 4 genotypes (type 1: 1 sample; type 1b: 31 samples; type 2a/2c: 6 samples; type 4a: 1 sample), which had been identified using the LiPA. These 4 HCV-genotypes of our samples are prevalent in Korea [12]. RNA was extracted from the samples by MagNA Pure LC and then subjected to the two assays. The HCV RNA concentrations obtained from each assay were compared. Linear regression analysis was carried

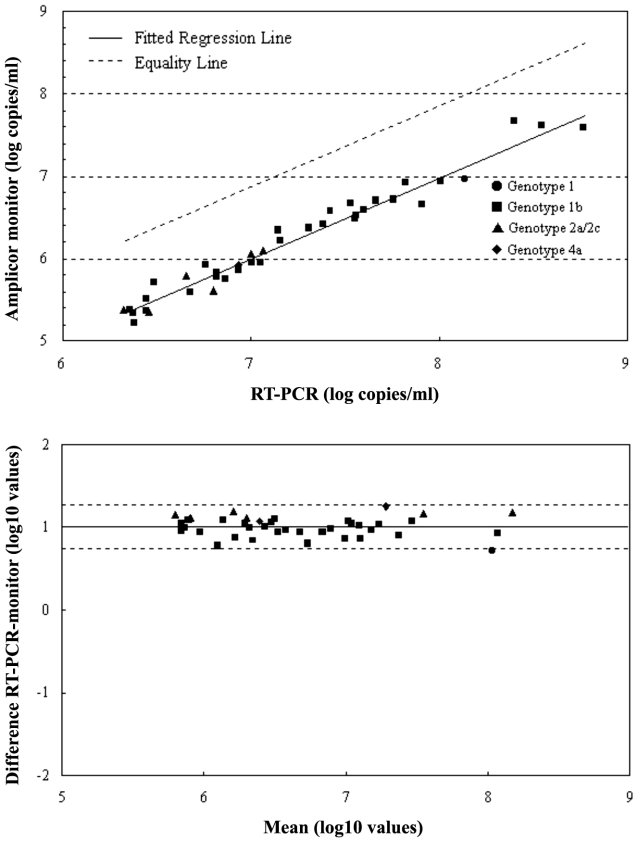

Fig. 3. Correlation plot of $\log_{10}$ HCV RNA values obtained with the one-step real-time RT-PCR assay and the Monitor assay on 39 HCV RNA-positive plasma samples.
The differences between the two values are shown against the mean.

Table 2. Repeatability (intra-assay CV) and intermediate precision (inter-assay CV) of the one-step real-time RT-PCR assay following automated RNA extraction by MagNA Pure LC.

Copies (IU)/reaction	Average C_p values for three tests (intra-assay CV (%))			Inter-assay CV (%)
	1	2	3	
$6\ (3.3)\times10^3$	26.91 (0.23)	26.18 (1.20)	26.33 (0.61)	1.43
$6\ (3.3)\times10^{2.5}$	27.62 (1.43)	27.53 (0.39)	27.47 (0.64)	0.85
$6\ (3.3)\times10^2$	28.31 (0.10)	28.07 (0.41)	28.54 (1.36)	1.02
$6\ (3.3)\times10^{1.5}$	29.10 (1.02)	29.07 (0.25)	29.94 (0.50)	1.56
$6\ (3.3)\times10^1$	31.10 (0.37)	31.14 (0.82)	31.31 (0.37)	0.58
CV mean				1.09

out to investigate the relationship between the quantitative data of the one-step real-time RT-PCR assay and those of the Monitor assay (Fig. 3). The correlation coefficient between Monitor and RT-PCR $\log_{10}$ values for specimens (n=39) was 0.98 ($P<0.001$). The fitted regression line was given by the equation: $\log_{10}$ (Monitor IU/ml)=−0.98+1× $\log_{10}$ (RT-PCR IU/ml). The 95% CI for the estimated intercept was −1.43, −0.53. The 95% CI for the estimated slope was 0.93, 1.06. The differences were plotted against the mean in the results of each unknown sample using the Monitor and the RT-PCR. The mean (±SD) difference for Monitor and RT-PCR in the $\log_{10}$ values was 1.0 (±0.12). The titers obtained from our assay were about 10-times higher than the Monitor assay for plasma samples (paired t-test, $P<0.001$).

DISCUSSION

We evaluated the one-step real-time RT-PCR based on the LC instrument combined with automated RNA extraction on the MagNA Pure LC, and found that this method was capable of rapid sample preparation, RNA amplification, and real-time data analysis without post-PCR manipulation. The entire assay process of 32 samples required about 3 h (15 min for set-up, 70 min for RNA extraction, 20 min for post-elution process, and 75 min for thermocycling amplification and final data analysis). Therefore, it would be very useful for a wide range of routine diagnostic testing of HCV RNA in clinical samples in quality control laboratories.

We employed the LC hybridization probe format for sequence-specific detection of HCV RNA in plasma samples. Probes prevent false-positive results due to nonspecific amplification products and guarantee specificity of results [23−25]. The optimized one-step real-time RT-PCR assay using synthetic HCV RNA standard provided a linear external standard curve for quantitation of HCV RNA in clinical samples. The assay showed an accurate measurement of RNA concentration at a broad range of virus RNA amount from 10^7 to 10^1 copies using the external standard curve. The quantitation limit (the concentration of 100% frequency of quantitation) of our assay was 10 HCV RNA copies per reaction (271 IU/ml).

Physical separation with one-way flow of samples is possible by using automated RNA extraction by MagNA Pure LC programmed to place automatically the purified RNA into the glass capillary and one-step real-time RT-PCR on LightCycler, which simultaneously carries out reverse-transcription, PCR amplification, and detection in a closed capillary. It could avoid the need for post-PCR manipulation, thereby minimizing a risk of contamination (false-positive) [10, 23]. In addition, melting curve analysis could be a useful tool to detect probable carryover contamination by high-copy synthetic RNA standards during manipulation of samples in this study. Melting curve analysis proved that synthetic HCV RNA standard was practically differentiated from the wild-type HCV target sequence. In this study, each run was carefully monitored for overall contamination by using a negative control and by carrying out melting curve analysis.

In calibration analysis of our synthetic HCV RNA standard against the WHO HCV standards, the 1.84±0.10 (Mean±SD) copies of the synthetic RNA standard were shown to be equivalent to 1 IU WHO Standard. The parallelism and the linear correlation of the synthetic HCV RNA standard with WHO standard revealed that the synthetic HCV RNA had an equal activity of the viral RNA in quantitation. Our synthetic RNA standard showed very similar amplification profiles with HCV viral RNA templates. It means that the specific primers, CFB73 and CRG374, and probes, CFL and CLC, were a good combination for the assay.

In biological sample assay, it is important to consider the loss of target material during the extraction procedure of the plasma or serum [22]. Therefore, we evaluated the overall sensitivity and precision of the one-step real-time RT-PCR following automated RNA extraction on MagNA Pure LC, and obtained an estimated sensitivity to detect 1 copy (92 IU/ml) of HCV RNA in plasma. There was no significant variation of CV in intra-assay between triple runs at each dilution point and in inter-assay between runs on different days, therefore, the assay showed excellent repeatability and intermediate precision as well. The automated extraction system produced nucleic acids of high purity, removing the possible PCR inhibitors in samples without much loss of nucleic acids, and prepared the assay for consistent detection results.

When it was applied to clinical samples, the assay showed 100% diagnostic sensitivity and specificity to determine whether the samples were HCV-positive or -negative, which had been cross-examined by two other assay methods (data not shown). This demonstrates that the method evaluated in this study is so robust that it maintains the validity during the procedures.

The relationship between the one-step real-time RT-PCR assay on LC instrument and the Monitor assay was significant. There was a good correlation between the two assay results, and the one-step real-time RT-PCR assay provided more sensitive data.

The selection and validation of a suitable standard material preparation play a key role in assay validation [11]. Our synthetic HCV RNA standard proved to be a good external standard material for quantitation of HCV RNA in clinical samples. This synthetic standard should be constant in quality and could be the alternative to the traditional HCV RNA standard (a dilution of HCV-positive serum) and precludes the hazardous material from the experimental procedure.

In conclusion, the one-step real-time RT-PCR assay in combination with automated RNA extraction was found to be fast and labor-saving and easy to carry out. The assay using our specific probes and the synthetic HCV RNA standard provided practical, rapid, reliable, sensitive, and precise detection and quantitation of HCV RNA. Therefore, it is suitable for routine screening of clinical samples or blood-derived products or for viral clearance validation [9] in quality control laboratories of manufacturers or national regulatory authority (NRA).

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