Evaluation of the Abbott Investigational Use Only RealTime Hepatitis C Virus (HCV) Assay and Comparison to the Roche TaqMan HCV Analyte-Specific Reagent Assay[▽]

Michael T. Pyne,^{1*} Eric Q. Konnick,^{1†} Amit Phansalkar,^{1‡} and David R. Hillyard^{1,2}

ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, Utah 84108,¹ and
University of Utah Department of Pathology, 15 North Medical Drive East, Salt Lake City, Utah 84112²

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The accurate and sensitive measurement of hepatitis C virus (HCV) RNA is essential for the clinical management and treatment of infected patients and as a research tool for studying the biology of HCV infection. We evaluated the linearity, reproducibility, precision, limit of detection, and concordance of viral genotype quantitation of the Abbott investigational use only RealTime HCV (RealTime) assay using the Abbott *m2000* platform and compared the results to those of the Roche TaqMan Analyte-Specific Reagent (TaqMan) and Bayer Versant HCV bDNA 3.0 assay. Comparison of 216 samples analyzed by RealTime and TaqMan assays produced the following Deming regression equation: $\text{RealTime} = 0.940 (\text{TaqMan}) + 0.175 \log_{10} \text{HCV RNA IU/ml}$. The average difference between the assays was 0.143 $\log_{10}$ RNA IU/ml and was consistent across RealTime's dynamic range of nearly 7 $\log_{10}$ HCV RNA IU/ml. There was no significant difference between genotypes among these samples. The limit of detection using eight replicates of the World Health Organization HCV standard was determined to be 7.74 HCV RNA IU/ml by probit analysis. Replicate measurements of commercial genotype panels were significantly higher than TaqMan measurements for most samples and showed that the RealTime assay is able to detect all genotypes with no bias. Additionally, we showed that the amplicon generated by the widely used Roche COBAS Amplicor Hepatitis C Virus Test, version 2.0, can act as a template in the RealTime assay, but potential cross-contamination could be mitigated by treatment with uracil-*N*-glycosylase. In conclusion, the RealTime assay accurately measured HCV viral loads over a broad dynamic range, with no significant genotype bias.

Methods for accurate quantitation of serum and plasma hepatitis C virus (HCV) RNA levels have become key tools both for understanding the biology of HCV infection and for the clinical management of patients under treatment. The ability to predict likelihood of response to combination interferon/ribavirin therapy by assessing rates of HCV viral load decline has provided a more individualized treatment algorithm that can identify nonresponsive patients early in treatment, sparing them significant morbidity and cost. New algorithms that examine the kinetics of HCV viral decline provide an even more refined tool for management and complement the information provided by HCV genotype determination. Finally, HCV viral kinetics data are essential for the understanding of new therapeutics such as the class of protease inhibitors. For all applications, accurate quantitation of all HCV types over a broad dynamic range is critical. The advent of real-time PCR methods provides a powerful tool for a broad dynamic range of quantitation of viruses; however, targets such as HCV require careful assay design to avoid errors due to sequence variations intrinsic to RNA viruses.

Abbott Molecular, Inc. (Des Plaines, IL), recently released the *m2000* system and tests for human immunodeficiency virus type 1, HCV, and chlamydia/gonorrhea (*Chlamydia trachomatis*/*Neisseria gonorrhea*) in the European Union with Conformité Européenne-marked certification, and the system was recently approved for human immunodeficiency virus type 1 by the U.S. Food and Drug Administration. The *m2000* system consists of an eight-channel liquid handling platform (the *m2000sp*) for performing automated nucleic acid extraction and PCR preparation and of a real-time PCR platform (the *m2000rt*) for detection and quantification. We used the *m2000* system to evaluate the sensitivity, reproducibility, linearity, and concordance of viral genotype quantitation of the Abbott Molecular investigational use only (IUO) RealTime HCV (RealTime) assay and compared aspects of its performance to the Roche TaqMan Analyte-Specific Reagent (ASR) (TaqMan) (Roche Molecular Systems, Inc., Branchburg, NJ) and Bayer Versant HCV bDNA 3.0 (bDNA) assay. In addition, we examined the potential for contamination by the Roche COBAS Amplicor Hepatitis C Virus Test, version 2.0 (Amplicor) (Roche Molecular Systems, Inc., Branchburg, NJ) and TaqMan amplicons and the effect of uracil-*N*-glycosylase (UNG) treatment in mitigating contamination from these widely used tests.

MATERIALS AND METHODS

Sample selection, preparation, and storage. Plasma and/or serum samples previously submitted to ARUP for HCV viral load testing by TaqMan were collected for use in this study. Remaining patient samples were retrieved from

* Corresponding author. Mailing address: ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108. Phone: (801) 583-2787, ext. 3431. Fax: (801) 584-5048. E-mail: michael.pyne@aruplab.com.

† Present address: University of Utah School of Medicine, 30 N 1900 E, Salt Lake City, UT 84132.

‡ Present address: Cytel Inc., 675 Massachusetts Ave., Cambridge, MA 02139.

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frozen storage, deidentified, thawed at room temperature, transferred to bar-coded 4-ml Simport tubes (Beloil, Quebec, Canada), and stored at -70°C before testing.

WHO reference material. World Health Organization (WHO) reference material (second international standard for HCV RNA for genomic amplification technology assays, National Institute for Biological Standards and Controls code 97/798; Potters Bar, United Kingdom) (9) was used to calibrate the TaqMan assay and to prepare the dilutions for the determination of the limit of detection in the RealTime assay. To verify assay calibration, aliquots of a 1:3 dilution of the reference material were tested in the RealTime, TaqMan, and bDNA assays.

Roche TaqMan assay. The procedures involved in nucleic acid extraction, PCR preparation, and assay calibration for TaqMan at ARUP have been described in detail previously (7). To summarize, 220 μl of serum or plasma from 96 patient samples and controls (representing high, medium, low, and negative virus titers) was processed using a Qiagen BioRobot 9604 employing a QIAamp Virus BioRobot 9604 Kit (Qiagen, Valencia, CA) modified to incorporate and coextract the Roche internal quantitation standard RNA template. HCV and quantitation standard RNAs were extracted by lysis of the sample, and the released RNA was bound to a silica matrix by vacuum filtration; the membrane was washed to remove impurities, and the extracted RNA was eluted into a collection plate. TaqMan PCR master mix was prepared, aliquoted into reaction vessels, and inoculated with the extracted RNA on the BioRobot 9604 platform.

The inoculated PCRs were sealed, cycled, and analyzed by the Roche COBAS TaqMan instrument using calibration coefficients derived from replicate testing of a dilution series of HCV Armored RNA genotype 1b material (Ambion Diagnostics, Austin, TX) normalized to the WHO HCV reference material (7, 9).

ARUP's implementation of the TaqMan ASR on the Qiagen BioRobot 9604 processes, amplifies, and analyzes 96 samples at a time in approximately 8 h, including 1.5 h of hands-on time.

Abbott RealTime assay. Nucleic acid extraction and PCR preparation for the RealTime assay were performed on the *m2000sp* instrument using manufacturer-provided protocols, reagents, and software. The provided protocol extracts nucleic acid from 0.5 ml of serum or plasma using magnetic particles and has been previously described (6). Test samples and controls were removed from the freezer and allowed to thaw at room temperature while the *m2000sp* instrument and reagents were prepared. The system processes, amplifies, and analyzes 48 samples in approximately 7 h, 1 h of which is hands-on time.

Microtiter plates containing the PCRs prepared by the *m2000sp* system were sealed and thermocycled on the *m2000rt* instrument using protocols supplied by the manufacturer. The primers and probe sequences target conserved regions in the 5' untranslated region of the HCV genome. The assay uses a linear probe with a fluorophore at the 5' end and a quencher at the 3' end. In the absence of target, the probe self-quenches by random coiling. In the presence of target, the probe anneals, separating the fluorophore from the quencher. Fluorescence is generated primarily by probe hybridization since the read temperature for detection is quite low (35°C) although some signal is generated by 5'→3' exonuclease cleavage. Quantification was performed automatically by the *m2000rt* software and was based on a stored calibration curve derived from replicate testing of high and low calibrators, provided by Abbott, which are standardized to the WHO reference material. Calibration is required with each new lot of extraction and/or PCR reagents although during this study, a single lot of reagents was used.

PCR contamination control. To minimize contamination potential, the sample preparation, nucleic acid extraction, and PCR setup were performed in separate rooms from the amplification and detection. Additionally, both systems are closed processes that do not require opening postamplification reactions for detection, thus minimizing potential contamination events. The TaqMan system incorporates dUTP/UNG contamination control in the master mix. The RealTime assay does not utilize dUTP or UNG, but an optional protocol provided by Abbott for the addition of 1 unit of UNG (Invitrogen Corp., Carlsbad, CA) per reaction to the master mix was performed for all study samples since the amplicon produced by Amplicor can be amplified with the Abbott RealTime chemistry (see Results section). Following reaction setup and plate sealing, the plate was incubated at room temperature for 10 min to allow UNG digestion, as directed by the Abbott protocol.

Cross-contamination studies. To evaluate the potential for cross-contamination between the TaqMan, Amplicor, and RealTime assays and to evaluate the effectiveness of the UNG protocol, we conducted cross-contamination experiments. PCR products generated from clinical samples in the TaqMan and Amplicor assays were quantitated by agarose gel electrophoresis and diluted in Basematrix Diluent (BBI Diagnostics, West Bridgewater, MA) to levels that produced signals of approximately 1 to 5 $\log_{10}$ HCV RNA IU/ml after extraction

and amplification with the RealTime assay. Replicate measurements of these samples were made with and without the addition of 1 unit of UNG per reaction mixture as previously described and incubation at room temperature (as recommended by the Abbott protocol) or 44°C (since the bacteria-derived enzyme likely has higher activity at elevated temperatures) for 10 min prior to thermocycling. PCR product from a low-titer control in the RealTime assay was diluted by a factor of 10^6 in Basematrix and extracted and amplified in the TaqMan and Amplicor assays in duplicate.

Versant HCV bDNA 3.0 assay. The bDNA assay was performed using the manufacturer's protocol with the Bayer System 340 bDNA Analyzer (Bayer HealthCare, Diagnostics Division, Tarrytown, NY).

Correlation samples. A total of 218 samples with viral loads spanning the ranges of both assays were selected to evaluate assay correlation. When available, HCV genotype information (determined by parallel testing at ARUP) of the samples was maintained so that genotype quantitation could be examined.

Negative samples. The limit of detection of the TaqMan assay performed at ARUP (~ 75 HCV RNA IU/ml) has been previously described (7). Fifty samples with viral loads below the limit of detection of the TaqMan assay were selected for retesting in the RealTime assay.

Limit of detection. To determine the limit of detection in the RealTime assay, 11 serial dilutions of the WHO reference material were prepared in Basematrix. Eight replicates at each concentration (25, 20, 15, 12.5, 10.5, 8.5, 7.5, 5, 3.5, 2, and 1 HCV RNA IU/ml) were tested on two runs (four replicates per run). Probit analysis was performed to determine the limit of detection and 95% confidence interval.

Reproducibility and linearity. Dilutions of three patient samples (unknown genotypes) with high viral titers ($>7 \log_{10}$ HCV RNA IU/ml) and a high-titer ($>10 \log_{10}$ HCV RNA IU/ml) HCV Armored RNA genotype 1b sample (Ambion Diagnostics, Austin, TX) were used to evaluate intra- and interassay reproducibility and linearity. These four samples were serially diluted in Basematrix diluent through 11 levels to cover the expected range of the RealTime assay. Sufficient volume for six aliquots of each dilution was prepared and stored at -70°C until use. For each sample, four of six replicates were tested on the same run to evaluate intra-assay reproducibility and linearity. Interassay reproducibility was evaluated by performing two additional runs containing one aliquot of each sample dilution on each run. The average values for the intra-assay replicates were combined with the values for the two additional runs to determine interassay reproducibility and linearity. Reproducibility was evaluated by determining the mean and standard error of the mean for each dilution. Linearity was evaluated by linear regression analysis, excluding the highest-concentration aliquots, which were set at identity to establish the expected values.

Genotype panels. To assess the assays' abilities to measure viral loads of samples containing diverse genotypes, two genotype panels were tested. HemaCare Bioscience HCV genotype panels HCVGTP-004c and HCVGTP-005a (Ft. Lauderdale, FL) containing a total of 15 samples representing genotypes 1a, 1b, 2, 3a, 4, 5a, 6a, 6b, 7c, 8c, and 9b were tested (genotype designations were provided by the manufacturer; they were not confirmed by independent analysis). Each panel member was diluted 10-fold in Basematrix diluent, aliquoted, stored at -70°C , and tested in triplicate by TaqMan, RealTime, and bDNA assays using standard procedures. The significance of the results was evaluated using analysis of variance and the Tukey honestly significant differences test for multiple comparison.

RESULTS

A total of 218 clinical samples previously tested in the TaqMan assay were tested in the RealTime assay. Results were obtained for 216 samples; 1 sample previously measured in the TaqMan assay at $2.57 \log_{10}$ HCV RNA IU/ml was considered "not detected" according to the RealTime assay, and another sample measured at $6.41 \log_{10}$ HCV RNA IU/ml in the TaqMan assay failed to produce an internal control amplification, and so no result was calculated. The estimates of Deming regression analysis are as follows: $\text{RealTime} = 0.940 (\text{TaqMan}) + 0.175$; $R^2 = 0.861$, $S_{y/x|D} = 0.439$; $n = 216$ samples. Bland-Altman (1) analysis was performed on these 216 samples (Fig. 1) to compare the average result with the difference between the methods for each sample. On average, the TaqMan assay result was $0.143 \log_{10}$ HCV RNA IU/ml

FIG. 1. Bland-Altman analysis. The correlation data were analyzed by the method of Bland and Altman (1) by comparing the average $\log_{10}$ HCV RNA IU/ml for the two methods versus the difference in $\log_{10}$ HCV RNA IU/ml of the two methods. The genotypes are indicated where known. The average difference ($0.143 \log_{10}$ HCV RNA IU/ml), average plus 2 standard deviations ($[SD] 1.059 \log_{10}$ HCV RNA IU/ml), and average minus 2 standard deviations ($-0.773 \log_{10}$ HCV RNA IU/ml) are indicated by the heavy solid lines.

higher than the RealTime assay result. The range for the mean ± 2 standard deviations was between -0.773 and $1.059 \log_{10}$ HCV RNA IU/ml. Sixty-six percent of the samples differed by less than $0.5 \log_{10}$ HCV RNA IU/ml, and 97% of the samples differed by less than $1.0 \log_{10}$ HCV RNA IU/ml between the two assays. Table 1 shows the mean difference between the assays and the standard deviations for the 216 samples grouped by genotype, indicating no significant difference between these genotype sets.

Fifty samples previously tested in the TaqMan assay that produced results below the level of detection (<75 HCV RNA IU/ml) were tested in the RealTime assay. Forty-eight of these samples were "not detected" in the RealTime assay. One sample was measured at 12 HCV RNA IU/ml but had insufficient remaining sample for retesting; the other sample was detected but not quantified. Upon retesting, this sample measured 20 HCV RNA IU/ml.

The WHO HCV reference material (1:3 dilution) was tested in all three assays. The results from all three assays (TaqMan,

4.45 $\log_{10}$ HCV RNA IU/ml; RealTime, 4.48 $\log_{10}$ HCV RNA IU/ml; and bDNA, 4.43 $\log_{10}$ IU/ml) correlated well with the expected value (4.52 $\log_{10}$ HCV RNA IU/ml).

Eight replicates each of a dilution series prepared from the WHO reference standard (1 to 25 HCV RNA IU/ml) were tested and analyzed by probit analysis. The limit of detection (95% detection rate) of the RealTime assay was determined to be 7.74 HCV RNA IU/ml (95% confidence interval, 4.84 to 12.35 HCV RNA IU/ml).

To assess the linearity, reproducibility, and precision of the RealTime assay, serial dilutions of three high-titer patient samples and one Armored RNA construct were created in replicate. The expected range of the high-titer samples was approximately $1 \log_{10}$ HCV RNA IU/ml to $7 \log_{10}$ HCV RNA IU/ml; the expected range of the Armored RNA sample was approximately $1 \log_{10}$ HCV RNA IU/ml to $8.7 \log_{10}$ HCV RNA IU/ml. Four replicates were tested in the same run to evaluate the intrarun performance, and two additional aliquots were run in additional runs to evaluate the interrune performance. The highest concentration of Armored RNA could not be quantified in the assay; since the manufacturer claims an upper quantification limit of $8 \log_{10}$ HCV RNA IU/ml, this result was expected. A total of nine replicates from all four sample sources at the lowest concentration were detected but not quantified. At the next-to-lowest concentration, one replicate for sample CH05 did not produce a result due to the failure of the internal control. The mean $\pm$ standard error of the mean and linear regressions for each sample are shown in Tables 2 and 3.

Two genotype panels containing a total of 15 samples representing genotypes 1a, 1b, 2, 3a, 4, 5a, 6a, 6b, 7c, 8c, and 9b were tested in triplicate in the TaqMan, RealTime, and bDNA assays (Fig. 2). Results were obtained for all samples except

TABLE 1. Assay genotype bias among correlation samples

Genotype (no. of samples)	Correlation sample data ($\log_{10}$ HCV RNA IU/ml) ^a	
	Mean difference in assay results (TaqMan – Real Time values)	SD
1 (103)	0.30	0.33
2 (21)	0.22	0.45
3 (22)	-0.07	0.34
4 (3)	-0.36	0.56
Unknown (67)	-0.04	0.56

^a There is no significant difference between genotypes in this data set.

TABLE 2. Intra- and interassay precision

Dilution no. and comparison type	Mean $\pm$ SEM log ₁₀ HCV RNA IU/ml in the indicated sample (<i>n</i>) ^a			
	CH01	CH03	CH05	CRNA
Intra-assay				
1	6.758 $\pm$ 0.023	7.060 $\pm$ 0.036	6.620 $\pm$ 0.041	NC
2	6.207 $\pm$ 0.027	6.495 $\pm$ 0.029	6.095 $\pm$ 0.018	7.965 $\pm$ 0.015
3	5.657 $\pm$ 0.008	5.938 $\pm$ 0.012	5.518 $\pm$ 0.018	7.262 $\pm$ 0.017
4	5.037 $\pm$ 0.020	5.360 $\pm$ 0.029	4.955 $\pm$ 0.024	6.395 $\pm$ 0.013
5	4.468 $\pm$ 0.019	4.765 $\pm$ 0.016	4.327 $\pm$ 0.014	5.680 $\pm$ 0.018
6	3.837 $\pm$ 0.016	4.138 $\pm$ 0.018	3.755 $\pm$ 0.006	4.888 $\pm$ 0.009
7	3.238 $\pm$ 0.019	3.592 $\pm$ 0.024	3.225 $\pm$ 0.022	4.117 $\pm$ 0.013
8	2.638 $\pm$ 0.005	3.060 $\pm$ 0.014	2.740 $\pm$ 0.015	3.285 $\pm$ 0.021
9	2.070 $\pm$ 0.029	2.550 $\pm$ 0.004	2.240 $\pm$ 0.024	2.537 $\pm$ 0.011
10	1.478 $\pm$ 0.044	2.022 $\pm$ 0.048	1.873 $\pm$ 0.003 (3)	1.887 $\pm$ 0.054
11	1.110 (1)	1.680 $\pm$ 0.041	1.220 $\pm$ 0.039 (3)	1.170 (1)
Inter-assay				
1	6.799 $\pm$ 0.040	7.097 $\pm$ 0.027	6.650 $\pm$ 0.017	NC
2	6.192 $\pm$ 0.027	6.478 $\pm$ 0.014	6.082 $\pm$ 0.021	7.972 $\pm$ 0.009
3	5.606 $\pm$ 0.026	5.916 $\pm$ 0.023	5.499 $\pm$ 0.011	7.247 $\pm$ 0.019
4	5.039 $\pm$ 0.017	5.373 $\pm$ 0.007	4.898 $\pm$ 0.040	6.415 $\pm$ 0.028
5	4.446 $\pm$ 0.018	4.748 $\pm$ 0.036	4.326 $\pm$ 0.003	5.700 $\pm$ 0.015
6	3.806 $\pm$ 0.019	4.183 $\pm$ 0.024	3.728 $\pm$ 0.016	4.886 $\pm$ 0.014
7	3.226 $\pm$ 0.013	3.631 $\pm$ 0.019	3.242 $\pm$ 0.010	4.122 $\pm$ 0.009
8	2.659 $\pm$ 0.012	3.067 $\pm$ 0.018	2.697 $\pm$ 0.022	3.328 $\pm$ 0.022
9	2.070 $\pm$ 0.029	2.563 $\pm$ 0.024	2.253 $\pm$ 0.013	2.526 $\pm$ 0.006
10	1.533 $\pm$ 0.085	2.057 $\pm$ 0.020	1.828 $\pm$ 0.076	1.799 $\pm$ 0.058
11	1.140 $\pm$ 0.024 (2)	1.730 $\pm$ 0.060	1.270 $\pm$ 0.029	1.210 $\pm$ 0.033 (2)

^a The intra- and interassay precision was evaluated by fourfold serial dilutions of four samples (the three high-titer patient samples, CH01, CH03, and CH05, and an Armored RNA construct, CRNA). Six replicates were prepared and tested in the RealTime assay. Four replicates were tested on a single run to evaluate intra-assay reproducibility, and the remaining two replicates were tested on a second and third run (and compared to the mean of the intra-assay data) to evaluate interassay reproducibility. The expected values were calculated by setting the highest concentration at identity and using the dilution factor to calculate the lower concentrations. Since the means for each sample at the highest concentrations were set at identity, they were not included in the regression analysis. NC, not calculated (the expected concentration was 8.7 log₁₀ HCV RNA IU/ml, which is above the limit of the assay; see text). *n* = 4 unless noted otherwise in parentheses (the number of replicates that produced quantitative results was less than the number of samples tested).

one replicate of genotype 1b in the TaqMan assay, which produced an “invalid” result. For each sample, the Tukey honestly significant differences test was applied. All samples showed significant differences ($P \leq 0.05$) between the methods, except for the following: one genotype 7c, which showed no significant difference between any of the three methods; genotype 5a, which had no significant difference between the RealTime and bDNA measurements; one genotype 2, which had no significant difference between the TaqMan and bDNA measurements; and one genotype 6c, which had no significant difference between the TaqMan and bDNA measurements. The average difference for the pairs where the *P* value was >0.05 was 0.09 log₁₀ HCV RNA IU/ml while the average difference for the pairs when *P* was ≤ 0.05 was 0.48 log₁₀ HCV RNA IU/ml.

Each of the 16 runs on the RealTime assay had three control samples (high, low, and negative virus titers). The expected value of the high-titer control was 5.87 log₁₀ HCV RNA IU/ml,

and the median was 5.91 log₁₀ HCV RNA IU/ml. The expected value of the low-titer control was 2.50 log₁₀ HCV RNA IU/ml, and the median was 2.65 log₁₀ HCV RNA IU/ml. The percent coefficients of variation were 1.28% and 3.13% for the high- and low-titer controls, respectively. All 16 negative controls produced “not detected” results.

The results of the cross-contamination studies indicate that only the Amplicor amplicon can be amplified in the RealTime assay; the TaqMan amplicon did not amplify in our tests using the RealTime assay. The addition of the UNG protocol to the RealTime assay decreased the signal produced by the contaminating Amplicor amplicon by approximately 1.5 log₁₀ HCV RNA IU/ml when the samples were incubated at room temperature and approximately 2 log₁₀ HCV RNA IU/ml when the samples were incubated at 44°C for 10 min (Fig. 3). When challenged with significant amounts of RealTime amplicon, neither the TaqMan nor Amplicor assay produced a positive result.

TABLE 3. Intra- and interassay linear regression analysis^a

Sample	Intra-assay regression analysis				Interassay regression analysis			
	Slope	Slope SE	Intercept	Intercept SE	Slope	Slope SE	Intercept	Intercept SE
CH01	0.981	0.006	0.176	0.024	0.965	0.008	0.194	0.034
CH03	0.911	0.009	0.547	0.037	0.988	0.005	0.061	0.022
CH05	0.892	0.009	0.640	0.033	0.992	0.006	0.015	0.025
CRNA	0.988	0.006	0.099	0.030	0.992	0.007	0.085	0.033

^a See the note to Table 2 for an explanation of the method and calculations.

FIG. 2. Genotype panels. Tenfold dilutions of HemaCare Bioscience's HCV genotype panels HCVGTP-004c and HCVGTP-005a containing a total of 15 samples representing genotypes 1a, 1b, 2, 3a, 4, 5a, 6a, 6b, 7c, 8c, and 9b were tested ($n = 3$, except for TaqMan 1b, where $n = 2$). Samples with genotypes of 2, 3a, 6a, and 7c are represented twice in these panels. The mean $\pm$ standard deviation is shown. The pairs indicated by asterisks are not significantly different ($P > 0.05$). The remaining samples showed significantly different values between the methods.

DISCUSSION

Evolving clinical algorithms for the diagnosis and monitoring of HCV infection continue to rely on highly sensitive molecular assays which also provide accurate quantification over a broad dynamic range. The Abbott IUO RealTime HCV assay provides an important new tool for clinical testing with significant innovations in test chemistry and informatics and utilizes

instrumentation capable of generic molecular testing. This study focuses on several critical issues of assay performance. Workflow and cost comparisons to other platforms have been described in several other publications (2, 4, 12, 14).

The limit of detection in the RealTime assay was determined to be 7.74 HCV RNA IU/ml using the WHO standard and probit analysis. The calculated copies per reaction mixture

FIG. 3. Cross-contamination and effect of UNG. PCR product produced by the Amplicor assay was preliminarily quantitated by agarose gel electrophoresis, diluted in Basematrix, and then extracted and amplified in the RealTime assay without UNG or with UNG and incubated at room temperature (RT) or 44°C for 10 min prior to thermocycling. The mean $\pm$ standard deviation is shown ($n = 3$, except for the samples without UNG at an input of 5 log₁₀ HCV RNA IU/ml, where $n = 2$). The fractions in the chart indicate the number of samples that were detected but not quantitated out of the total number of replicates tested at the given input level.

using WHO standard material is approximately 6, suggesting that the assay is highly efficient and is essentially limited in sensitivity by the volume of the patient sample input. Current and future HCV testing algorithms may benefit from increasingly sensitive assays (11). However, the risk of false-positive results may also increase with more sensitive assays, and the reliability of quantitative or qualitative results near the statistical limits of detection should be considered carefully. In addition, care should be taken when transitioning any new molecular test into the laboratory, with special attention to the potential risk of amplicon carryover from earlier open-tube platforms or when the assays will be used concurrently. This study demonstrates the effectiveness of UNG to mitigate potential carryover from built-up amplicon which could be used in addition to spatial isolation of the new testing platform. No evidence for false-positive detection was noted in this study although rigorous assessment of this question would demand repeat testing of low-positive samples in the setting of long-term clinical testing. A potential recommendation for HCV clinical testing using any platform is to confirm low-positive results used for critical clinical decision making, such as when assessment of treatment success or extension of time of therapy is being considered.

The RealTime assay demonstrated a broad, linear dynamic range of approximately $7 \log_{10}$ HCV RNA IU/ml (1.08 to $8.0 \log_{10}$ HCV RNA IU/ml) as determined by serial dilutions of high-titer patient samples and Armored RNA. The upper limit of quantification is limited by the built-in data analysis procedure. Samples of Armored RNA at an expected concentration of approximately $8.7 \log_{10}$ HCV RNA IU/ml produced typical amplification curves but failed one of the data analysis parameters. However, since the first 10 cycles are not read, the cycle numbers for these samples were very low (~ 2.8 threshold cycles), which is inadequate for baseline signal processing (data not shown). Based on the distribution of HCV RNA-positive samples previously described (7), it is expected that only approximately 0.25% of positive samples would require dilution and repeat testing using the RealTime assay. The assay precision was good; the percent coefficient of variation was less than 2% for viral loads above $2 \log_{10}$ HCV RNA IU/ml and less than 10% below $2 \log_{10}$ HCV RNA IU/ml.

The genotype bias of the correlation samples shown in Table 1 was not significant. A previous study comparing clinical samples of genotypes 1 to 4 (6) in the TaqMan and RealTime assays indicated higher average results for TaqMan in genotype 1 samples, lower average results in genotypes 2 and 4, and similar results for genotype 3, but the significance of these results was not reported, and the numbers of patients were lower than in the current study for all genotypes except genotype 4. It is unclear if the differences between that study and this study are due to natural variances in the assays' reproducibility, differences in the assay versions themselves (e.g., ASR versus the Conformité Européenne-marked assay), differences in calibration methods (e.g., WHO standard versus secondary standards), or a combination of these factors. It should be noted that the TaqMan assay used in this study (ASR) is being replaced by an FDA-cleared in vitro diagnostics assay (Roche COBAS Ampliprep/COBAS TaqMan HCV Test). Although these assays should have similar performance characteristics,

differences between the in vitro diagnostics and ASR assays cannot be excluded.

Using commercially available genotype panels, we observed significant differences ($P \leq 0.05$) between assays for nearly every tested genotype. In all but one instance, the RealTime assay had higher values than the TaqMan or bDNA assays. The low-read temperature (35°C) of the RealTime assay increases the probe's mismatch tolerance and likely contributes to the assay's ability to measure different genotypes equally.

Previous studies have highlighted the challenge of unbiased detection of different HCV genotypes (3, 6, 8, 10, 13). However, these and other studies (including this study) have limited ability to measure genotype bias for several reasons. First, due to the limited numbers of clinical samples available with known genotypes, most studies have insufficient statistical power and sample variability to definitively measure genotype bias. Second, genotype panels typically contain single examples of each genotype. Individual samples likely contain small variations, even within a given genotype, which may affect an assay's ability to accurately quantitate a given sample. Third, while the introduction of an international standard has greatly improved the ability to compare results from different laboratories, there are still two problems. Studies have shown that even when assays are calibrated to the same international standard, results from different assays cannot be reliably compared (3, 5, 7). Finally, the international standard represents a single instance of a genotype 1 specimen. Therefore, standardization does not take into account variability either between genotypes or within genotypes. Although some algorithms for HCV testing rely on relative viral load changes for which genotype-specific variation in quantification may have less impact, other applications may be more seriously impacted. Measuring the consistency of assay viral load determination for the broad diversity of circulating HCV viruses in various populations remains a challenging task.

A significant step forward with the RealTime system includes increased quality measures to aid in the assessment of the accuracy and reliability of viral load determinations both at the time of verification and as a long-term tool to assess clinical testing. The RealTime assay uses novel data reduction algorithms that allow analysis of results without user interaction or inspection. The data reduction encompasses several validity checks and transformations, including raw fluorescence thresholds, baseline correction, slope correction, normalization, internal control evaluation, reaction efficiency evaluation, and an innovative method of determining the crossing threshold (E. B. Shain, J. M. Clemens, T.-W. Jeng, and G. J. Schneider, U.S. patent application 20,050,130,211).

The RealTime assay appears to have excellent accuracy, precision, and dynamic range combined with low detection limits and little to no genotype bias that should prove useful in the diagnosis and treatment of patients infected with HCV.

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