High-Speed Detection of Blood-borne Hepatitis C Virus RNA by Single-Tube Real-Time Fluorescence Reverse Transcription-PCR with the LightCycler, Dieter Ratge, Birgit Scheiblhuber, Maren Nitsche, and Cornelius Knabbe (Department of Clinical Chemistry and Laboratory Medicine, Robert Bosch Hospital, Auerbachstrasse 110, D-70376 Stuttgart, Germany; *author for correspondence: fax 49-711-8101-3618, e-mail Dieter.Ratge@RBK.de)

Hepatitis C virus (HCV) has been identified as the agent responsible for the vast majority of cases of posttransfusion non-A, non-B hepatitis. Although generally asymptomatic, ~85% of the infections become chronic with a wide spectrum of outcomes (1).

Current assays developed to detect antibodies against HCV proteins are successful in detecting most cases of chronic HCV infection. Antibody tests may be negative, however, in cases of acute HCV infection during the window that precedes seroconversion. No immunoassay for direct detection of HCV antigen is available at the present time. With nucleic acid amplification tests, it is possible to detect HCV viremia an average of 59 days before immunological seroconversion (2, 3).

Nucleic acid amplification tests for detection of HCV sequences in blood products became compulsory in Germany on April 1, 1999 (4, 5). Because HCV, with its extremely heterogeneous genome, circulating in the blood in concentrations that range from undetectable (<50 copies/mL) up to 10^6 copies/mL (6), a detection limit of 5000 IU/mL (according to WHO, 1 IU corresponds to 2–5 genome copies, depending on the HCV-RNA method) in a single blood specimen is acceptable by the criteria of the Paul-Ehrlich-Institut (PEI, Langen, Germany).

In blood bank settings, the Cobas Amplicor Hepatitis C Virus Test, Ver. 2.0 (Roche Molecular Biochemicals) (7), is the most frequently used assay. Testing of only a few samples with this assay is quite expensive. Furthermore, turnaround time of PCR and detection exceeds 4 h. For selected single donations of thrombocytes collected by selected single donations of thrombocytes collected by thrombocytapheresis, e.g., for severely ill patients with HLA antibodies, the time period between preparation and release of the result often is too long.

The recent development of real-time quantitative PCR based on the LightCycler (Roche Molecular Biochemicals) (8) offers the opportunity for detection of HCV RNA in up to 32 samples within 70 min after RNA isolation. Here we report the development of a practical, rapid, and sensitive single-step reverse transcription (RT)-PCR method.

We analyzed 187 serum samples, including 100 samples from thrombocyte donors and 87 patients with confirmed or suspected HCV disease. All blood samples were drawn into tubes without additives (Sarstedt) and centrifuged within 2 h of collection. PEI reference preparation HCV RNA 75/98 with 25 000 IU/mL, calibrated against the WHO Standard HCV RNA (96/790) and diluted to 5000 IU/mL was used for run control. All samples were aliquoted and stored at −30 °C until further testing. RNA from thrombocyte donors was partly isolated before freezing without any effect compared with stored aliquots. Viral RNA was extracted from 200 μL of each sample, using the High Pure Viral Nucleic Acid reagent set (Roche Molecular Biochemicals). Nucleic acids were extracted in 50 μL of nuclease-free water. At least one positive and one negative control were processed in parallel with each batch of samples.

We used the primers KY78s and KY80s, which identify a 244-bp sequence of the highly conserved 5' untranslated region of the HCV genome (9). The donor probe KY FL (5'-GCCGCCCTCAGGGCCCTCCTAC-3') is labeled with 5,6-carboxyfluorescein attached to 3' -O-ribose; the adjacent acceptor probe KY LC (5'-CCCGGAGAGCCATAGTTGTCGTG3') was labeled with LightCycler Red 640 attached to the 5' terminus (both from TIB MOLBIOL). RT-PCR reactions were performed in a final volume of 20 μL. The reaction mixture consisted of 2.1 μL of water, 2 μL of 10× PCR Buffer (100 mmol/L Tris-HCl, pH 8.3, 500 mmol/L KCl, 15 mmol/L MgCl₂, 0.1 g/L gelatin), 2.4 μL of 25 mmol/L MgCl₂, 2 μL of 20 g/L nonacetylated bovine serum albumin (Sigma-Aldrich), 1 mmol/L each dNTP, 700 nmol/L each primer, 0.5 μL of enhanced AMV Reverse Transcriptase (20 U/μL), and 0.5 μL of AccuTaq LA DNA Polymerase (5 U/μL). The buffer, MgCl₂, dNTP mixture, and the enzymes were part of the Enhanced Avian RT-PCR reagent set (Sigma-Aldrich). The probes were added to the RT-PCR mixture to a final concentration of 300 nmol/L. A 14-μL aliquot of this reaction mixture was transferred to LightCycler glass capillaries, and 6 μL of RNA solution was added to each tube. HCV RNA was reverse-transcribed into cDNA (25 min at 48 °C) and subsequently amplified by PCR in the same single tube. The additional temperature profile consisted of denaturation at 95 °C for 3 min, followed by 50 cycles of denaturation for 1 s, annealing with fluorescence monitoring at 62 °C for 15 s, and extension at 72 °C for 13 s, with a temperature transition rate of 20 °C/s.

The fluorescence profiles generated from diluted PEI reference preparation with HCV RNA concentrations between 5000 and 156 IU/mL and a negative control are shown in Fig. 1A. Fluorescence intensity increased with increasing HCV RNA content in the samples. In contrast, the threshold cycle (CT), which is the first cycle in which the fluorescence is increased above background in a log-linear fashion, decreased. Analysis of the fluorescence curves using CT as the predictive value of the concentration of target RNA present in the samples was unreliable in samples with no or low HCV RNA concentrations. Because the PEI guideline does not demand a quantitative evaluation, we decided to define a fluorescence cutoff value. In 149 HCV RNA-negative serum samples (100 donors of thrombocytes, 49 patient samples), the highest final fluorescence intensity was 0.12 (131 samples <0.08; 12 samples 0.09–0.1; 6 samples 0.11–0.12). All of these samples were confirmed to be HCV RNA negative by two other methods [Cobas Amplicor Hepatitis C Virus Test, Ver. 2.0, and GEN-ETI-K DEIA (Sorin Biomedica)] (10), using the QIAamp viral RNA purification protocol (Qiagen).
When the noise band of 0.04 in Fig. 1A was set to a cutoff value of 0.12 (Fig. 1B), only plots with a higher final fluorescence from samples with suspect or positive HCV RNA content were shown on the screen. This approach was used for routine screening of samples of thrombocytapheresis. The detection limit was <1000 IU of HCV RNA/mL of serum, which is far below the PEI requirement for HCV RNA testing. A concentration of 1000 IU/mL HCV RNA corresponded to 24 IU of HCV RNA in a test tube, which led to a final fluorescence of 0.12–0.19 in different assays.

Eightfold amplifications of a sample containing 5000 IU/mL HCV RNA on five different working days with two lots of reagents gave highly reproducible final fluorescence intensities, with intraassay CVs of 3.7–9.1% and an interassay CV of 15%. The intraassay CV calculated by C\textsubscript{T} analysis was even lower (<1%).

The accuracy of the method was demonstrated by comparison of all LightCycler results (149 negative samples with HCV RNA <1000 IU/mL and 38 samples with HCV RNA >1000 IU/mL), with both our standard HCV nucleic acid amplification tests giving a concordance rate of 100%. Moreover, the real-time RT-PCR showed similar sensitivities for the genotypes 1a, 1b, 2a, 2b, 2c, 2i, 3a, 4, and 5a in a genotype panel (data not shown).

The real-time RT-PCR is well suited for quantification, using C\textsubscript{T} analysis on the basis of an RNA calibration curve. From amplification plots using a series of 10-fold dilutions, the final fluorescence of each sample was estimated. The results are shown in Fig. 1B, which displays the logarithmic plot of all fluorescence data vs cycle number.

![Fig. 1. Fluorescence vs cycle number in serially diluted PEI reference preparations and in a negative control (A), and logarithmic plot of all fluorescence data vs cycle number (B).](image_url)

(A), serially diluted PEI reference preparations contained 5000 (run control) to 156 IU/mL HCV RNA. The noise band was set to 0.04, which corresponds to the final fluorescence intensity of the negative sample. Background subtraction was used, and the fluorescence was set to channel 2[channel 1]. (B), the cutoff value was adjusted to 0.12, which was the highest final fluorescence intensity in 149 HCV RNA-negative samples. Only samples with sufficient HCV RNA content to generate fluorescence >0.12 (~600 IU/mL in this serial dilution experiment) are seen on the screen. △, 5000 IU/mL; □, 2500 IU/mL; ○, 1250 IU/mL; ♦, 625 IU/mL; ●, 312 IU/mL; •, 156 IU/mL; dotted line, negative control.
dilutions of a genotype 2a sample, we obtained a linear correlation between the C_{\text{t}} and the template at concentrations of 10^{-5}–10^{-6} IU/mL HCV RNA (r = 0.96). In clinical samples, the calibration curve could be extrapolated up to 6.3 × 10^{-3} IU/mL HCV RNA (data not shown). The results from 20 HCV RNA-positive patients with HCV RNA titers between 2.1 × 10^{3} and 6.3 × 10^{2} IU/mL HCV RNA were then compared with those of the Cobas Amplicor HCV Monitor assay. The correlation was statistically significant (r = 0.92). The mean values of the results obtained with our RT-PCR protocol and the Amplicor assay were comparable.

Single-tube real-time quantification of HCV RNA using TaqMan technology (Roche) and the ABI PRISM 7700 system (Perkin-Elmer) has been described recently (11, 12). Compared with our test, these real-time tests showed comparable reproducibility with lower (11) and higher sensitivity (12), but sequence variation among HCV targets presented problems (12). The Light Cycler test has the advantage of a running time of only 70 min compared with 150 min with the PRISM 7700, but the PRISM 7700 has a higher throughput and is better suited for automation.

Our method has been approved by PEI for HCV virus testing in blood samples and is ideally suited for rapid analysis of smaller sample numbers. This convenient approach is very economic without the risk of carryover contamination.

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References


Analytical and Clinical Performance of the Immulite Cardiac Troponin I Assay, Alain Lavoinne, Bruno Cauliez, Hélène Eltchaninoff, René Koning, and Alain Criteria (1 Laboratoire de Biochimie Médicale et 2 Service de Cardiologie, Hôpital Charles Nicolle, CHUR de Rouen, 76031 Rouen cedex, France; * author for correspondence: fax 33-2-32-88-87-80, e-mail alain.lavoinne@chu-rouen.fr)

Cardiac troponin I (cTnI) is now regarded as one of the most specific markers for cardiac injury, and an increasing number of commercial methods are available. We report here a study of the Immulite cTnI assay manufactured by DPC.

The Immulite cTnI assay is a sandwich immunoassay that uses a monoclonal antibody immobilized on beads and a goat polyclonal antibody labeled with alkaline phosphatase as a tracer (1); both antibodies recognize epitopes localized in the N-terminal part (residues 33–110) of the protein. The chemiluminescent substrate used for the enzymatic reaction is an ester of adamantyl dioxetane phosphate. We used as calibrators purified human cTnI in horse serum. The within- and between-run imprecisions (CV) were <3.9% and 3.6%, respectively, at a mean cTnI of 1.5 µg/L (n = 20). The minimal cTnI value quantified was 0.2 µg/L, and no detectable cTnI was observed in 20 runs of assay diluent. The lowest cTnI concentration giving rise to a within-assay CV <20% was 0.33 µg/L. Linearity was good up to 150 µg/L (r = 0.996).

As observed with the Stratus cTnI assay routinely used in our laboratory, the results obtained for plasma (y) were ~10% lower than those obtained for serum (x): y = 0.88x – 0.7; r = 0.999; n = 24. To simulate moderate and severe icterus, we added up to 375 µmol/L unconjugated bilirubin to three serum samples (cTnI concentrations of 1.3, 3.7, and 21.6 µg/L). A decrease in the cTnI concentration was observed at 100 µmol/L bilirubin (~10% for each sample). No detectable interference was observed for hemoglobin (up to 3.4 g/L) or triglycerides (22 g/L).

In 90 healthy individuals (34 males and 56 females; ages 19–57 years), only two results were above the detection limit (0.34 and 0.41 µg/L). Specificity was determined in 10 patients with rhabdomyolysis (total creatine kinase activity >2000 U/L) and 33 patients with chronic renal failure (creatinine >110 µmol/L) without any cardiac injury. Immulite cTnI was undetectable in 4 of the 10 patients with rhabdomyolysis (with a maximum cTnI value in the 6 remaining patients of 0.51 µg/L) and in 29 of the 33 patients with chronic renal failure (with a maximum cTnI value in the 4 remaining patients of 0.48 µg/L).

The relationship between the Immulite and Stratus systems was compared in 365 heparinized samples after exclusion of specimens with cTnI values outside the linear reportable range of the test methods. Of the 365 samples, 37 (10%) had one of the two results below the detection