Rapid and accurate quantification of different HCV genotypes by LightCycler Real Time PCR and direct sequencing of HCV amplicons

Paola Menegazzi[1], Elisa Reho[1], Massimo Ulivi[3], Oliviero E. Varnier[4], Flavia B. Lillo[2], Luigi Tagliaferro[1]

[1]Department of Virology and Molecular Biology, "Dr. Pignatelli" Laboratory, Lecce, Italy;
[2]Pathology and Microbiology Laboratory, Fondazione Istituto San Raffaele G. Giglio, Cefalù (Palermo) Italy;
[3]LightCycler Application Laboratory, TIB Molbiol s.r.l., Genova, Italy;
[4]Institute of Microbiology, School of Medicine, University of Genova, Italy

───── **SUMMARY** ─────

Follow-up of chronically infected HCV patients is the primary clinical goal in therapy administration. In the absence of an HCV vaccine, the timely monitoring of HCV viral load combined with the information of the viral genotype could contribute to patient disease management. A LightCycler Real Time RT-PCR assay was developed and optimized allowing rapid and accurate quantification of HCV RNA over an extended dynamic range using a single human reference standard. A total of 5,096 plasma samples, collected over almost 5 years, were tested and HCV RNA was quantified in 2,435 samples with levels ranging from 5.7×10^1 to 2.52×10^9 IU/ml. The precision and reproducibility of the test are documented by various inter-assay parameters of the reference standard obtained in 409 RT-PCR runs. This Real Time RT-PCR protocol uses the LightCycler cDNA amplicons for direct sequence analysis and reduces the sequencing time to approximately 3 hours. Nearly all HCV genotypes were identified. Viral sequences showed a similarity level close to 100%, independently from the viral load, while the LightCycler melting temperature analysis did not correlate with HCV genotypes. All this makes the LightCycler Real Time RT-PCR protocol a suitable tool for the diagnosis and monitoring of HCV infections.

KEY WORDS: HCV, LightCycler, Real time PCR, RNA quantification, DNA sequencing, HCV genotypes

INTRODUCTION

The hepatitis C virus (HCV) was identified in 1989 and it rapidly became evident that it was the causative agent of a major worldwide health problem (Alter *et al.,* 1999; Sheehy *et al.,* 2007; Strader *et al.,* 2004) Based on phylogenetic analyses, HCV has been sub-classified into 6 major genotypes and several sub-types, which show a

─────
Corresponding author
Luigi Tagliaferro
Laboratory "Dr. Pignatelli"
Dept. Virology & Molecular Biology
Via Martiri d'Otranto, 2
73100 Lecce, Italy
E-mail: virologia@labpignatelli.it.

level of homology ranging from 50% to 70% in their nucleotide sequences (NIH, 2002). In the European Union, genotypes 1, 2, and 3 are commonly detected, while genotype 4 has only been reported in the last 10 years (Tagliaferro *et al.,* 1997). Approximately 15% to 30% of HCV infected patients recover spontaneously, whereas the remaining 70% to 85% develop chronic infection (Hoonfagle *et al.,* 1997). Several diagnostic tests are available for detection of anti-HCV antibodies, core antigen, genomic RNA and viral typing (Castelain *et al.,* 2004; Cook *et al.,* 2004; Farma *et al.,* 1996; Germer *et al.,* 2005; Murphy *et al.,* 2007; Nolte *et al.,* 2003); however, for the clinical follow-up of chronically HCV infected patients and the confirmation of active viral replication in naive HCV antibody positive patients,

an accurate, reproducible, rapid and sensitive laboratory assay is needed for the determination of HCV viral load (Terrault *et al.*, 2005). The clinical application of HCV quantitation and genotyping is also relevant for the different outcome expected after therapy administration. We developed and optimized a protocol to quantify HCV RNA in plasma samples accurately and rapidly using LightCycler Real Time PCR, which also permits direct sequence analysis of the cDNA amplicons for identification of the HCV genotype.

MATERIALS AND METHODS

Clinical samples
Whole blood (6-8 ml) was collected from 5,096 HCV infected patients from October 2002 to February 2007, in vacutainers with sodium citrate or ethylenediaminetetraacetic acid. Plasma was separated by centrifugation within 2-3 hours of collection, and aliquots (0.3-0.5 ml) were stored at -70°C until RNA extraction.

RNA extraction
RNA was extracted from 200 µl of plasma using the High Pure Viral RNA kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions, and eluted in 50µl of nuclease-free PCR-grade water. The RNA was stored at -70°C until analysed.

HCV quantitative Real Time RT-PCR
HCV RNA quantification was performed using a Real-Time RT-PCR assay developed in-house. Eight microliters of purified RNA were retrotranscribed to cDNA and amplified in a single-tube reaction containing 7.4 µl of LightCycler-RNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany),

3.25 µM of $Mn(OAc)_2$, 0.5 µM of each primer and 0.2 µM of each probe (Table 1) (TIB MolBiol s.r.l., Genova, Italy) (Ratge *et al.,* 2000).
The primers amplified a 234 base pair fragment from the HCV 5'-NC region, which is conserved among the 6 main HCV genotypes. Reverse transcription was performed at 61°C for 30 min, the mixture was then heated at 95°C for 2 min and then PCR was performed for 45 cycles of 95°C for 0 s (denaturation), 60°C for 10 s (annealing) and 72°C for 10 s (extension). The fluorescence was measured at the end of each annealing phase. On completion of the amplification, there was a brief denaturation step at 95°C for 20 s, followed by a melting curve profile which started at 40°C for 20 s and increased to 80°C at 0.2°C/s with continuous monitoring of the fluorescence signal.

Reference standards
Hepatitis C virus RNA was also extracted from an International World Health Organization human reference standard (Accurun HCV RNA Positive Control series 400 or series 500, BBI Diagnostics, Boston, MA, USA) using the procedure described above. Following elution, the Human Reference Standard (HRS) HCV RNA stock solution was divided into 10 µl aliquots corresponding to $1.7x10^5$ IU/ml or $9.1x10^5$ IU/ml of HCV RNA, and stored at -70°C. Initially, ten-fold serial dilutions were made from an aliquot of HRS stock solution to prepare standard curve dilutions from $1.7x10^5$ to $1.7x10^2$ IU/ml or from $9.1x10^5$ to $9.1x10^2$ IU/ml. Eight microliters of each standard curve dilution were amplified and HCV RNA quantification was performed by on line fluorescence monitoring to identify the PCR cycle number (crossing point) at which the logarithmic linear phase could be distinguished from the background. The results obtained were stored for use as external standard curve data. The clinical

TABLE 1 - *Primers and probes used for real-time HCV RNA single step RT-PCR.*

Primer or probe	Nucleotide sequence (5' to 3')	Position	T_m (°C)
Primer KY80for	AgCgTCTAgCCATggCgT	74-91	59.1°C
Primer KY78rev	CAAgCACCCTATCAggCAgT	308-288	57.2°C
Probe HCV 3FL	gCAgCCTCCAggACCCCCC FL	107-125	67.5°C
Probe HCV 5LC	LC640-CCCgggAgAgCCATAgTggTCTg ph	128-150	66.9°C

RNA samples were amplified together with 8 µl of undiluted HRS stock solution, corresponding to $1.7x10^5$ IU/ml or $9.1x10^5$ IU/ml, which was included as a single reference standard. The concentration of HCV RNA (expressed in IU/ml) in the clinical samples was calculated by comparing the crossing points of the logarithmic linear phase of the samples with the crossing points of the external standard curve, which was re-calibrated using the data obtained for the single reference standard used in the actual PCR run. The use of this LightCycler application meant it was possible to use the same external standard curve data to quantify HCV RNA in different PCR runs. Eight microliters of sterile water were used as a no RNA template control.

Sequencing of HCV amplicons
The 234 bp HCV amplicons, produced in the LightCycler RT-PCR, were directly sequenced without the need for additional reverse transcription and amplification steps. The PCR products were purified using the PCR Purification kit (QIAquick, Qiagen GmnH) with minor modifications of the manufacturer's protocol. Briefly, the amplicons were heated at 94°C for 5 minutes to dissociate the hybridization probes, as their presence could potentially impair the sequencing process. Sequencing was performed using the TruGene HCV Genotyping Assay (Bayer HealthCare, Tarrytown, NY, USA) with slight modifications to the protocol to integrate the two systems. Ten microliters of HCV PCR product were added to a 15 µl final volume of CLIP master mix, consisting of 2.5 µl 5'-NC buffer, 2.5 µl DMSO, 2.0 µl 5'NC CLIP Primer Mix, 1.25 µl AmpliTaq FS, and 6.75 µl sterile H_2O. Five microliters of the master mix were aliquoted in each of the 4 CLIP™ tubes containing 3 µl of the appropriate CLIP™ terminator mix. At the end of the reaction, 6 µl of the Stop Loading Dye were added to each tube. An increased volume (up to

15 µl of amplicon) was used for samples containing less than 10^3 HCV RNA IU/ml.
The amplified sequence was matched to all HCV isolates sequences stored in two different versions of *Open Gene* libraries and a report was produced. Precisely, the older HCV sequences database *(Gene objects 3.2 HCV_5NC_311.gnl)* comprised 119 isolates and the score was calculated basing on 78 hyper-variable nucleotides. The new database version (*Gene objects 3.2 HCV_5NC_312.gnl*) comprises, instead, 143 HCV isolates and analyses the amplified sequences basing on 66 hyper-variable nucleotides (59 of which common to the first version).

RESULTS

HCV quantification
A single step quantitative LightCycler Real Time RT-PCR was developed and optimized. The quantitative calibration standard curves showed optimal correlation between theoretical and calculated HRS IU/ml concentration and a linear range from $1.7x10^5$ to $1.7x10^2$ HCV RNA IU/ml with a correlation coefficient (r) of -1.00. The analytical sensitivity of our method was 1.1 copies per reaction mixture corresponding to 57 IU/ml (data not shown).
A total of 5,096 plasma samples, collected in almost 5 years, were tested and HCV RNA was quantified in 2,435 samples with levels ranging from $5.7x10^1$ to $2.52x10^9$ IU/ml. HCV viral loads showed a typical Gaussian distribution, with a middle range of $1x10^5$-$1x10^6$ IU/ml.
To evaluate the reproducibility of HCV quantification, we monitored the crossing point of the HSR in each amplification run. Two hundred and three amplification runs were performed using the Accurun 400 HRS (corresponding to $1.7x10^5$ IU/ml). As shown in Table 2, the crossing points showed an inter-assay average of 30.66 (min

TABLE 2 - *Inter-Assay Reproducibility of the Human Reference Standard.*

HCV RNA IU/ml	N. of PCR assays	Crossing point number			
		Min - Max	*Mean*	*SD*	*CV (%)*
$1.7x10^5$	203	28.02 - 33.71	30.66	0.92	3.01
$9.1x10^5$	206	25.27 - 32.38	27.87	1.14	4.08

28.02, max 33.71), with a median of 30.78, a standard deviation (SD) of 0.92 and a coefficient of variation (CV) of 3.01%. A further 206 HCV amplifications were performed using Accurun 500 HRS (corresponding to 9.1×10^5 IU/ml) and the crossing point inter-assay average was 27.87 (min 25.27, max 32.38), with a median of 27.71, an SD of 1.14 and a CV of 4.08%.

HCV genotyping by direct sequencing of Real Time PCR amplicons

We sequenced a total of 467 HCV *LightCycler* amplicons, observing 5 of the 6 principal HCV genotypes known worldwide, identifying 14 types and subtypes, with a homology average of 99.9%. Along with genotypes 1-4, HCV 5 has been found in Italy as well (Tagliaferro *et al.,* 1997).

We compared the sequencer old data with a newer software version, containing a recent HCV sequences database able to distinguish 143 HCV isolates (instead of 119).

In our case record the older HCV sequences database *(Gene objects 3.2 HCV_5NC_311.gnl)* distinguished 56 different HCV isolates, with 192 of 467 samples (41%) typed but not subtyped, due to

the presence of some indistinguishable sequences common to several strains. The most frequently identified genotypes in native patients were 1b (38%) and 2 (30%), with a clear prevalence of the *K0014*, *HCJ5* and *S83* isolates (Figure 1a).

Since the last sequence database version comprises a different pattern of HCV isolates, we compared the previous sequences with the new database version (*Gene objects 3.2 HCV_5NC_312.gnl*), which identified 63 HCV isolates, so that the subtyped samples increased to 432 (93%), the unsubtyped therefore decreasing from 192 (41%) to 35 (7%) (Figure 1b).

The last database does not comprise HCV 1c *HCG9*, HCV 2c *S83*, HCV 3c *NE48*, HCV 3e *NE145*, HCV 4c *GB116* and 4d *DK13* isolates. Consequently, in Figure 1b HCV 1c *HCG9* isolate disappeared; unsubtyped genotypes 2 and 4 are drastically reduced due respectively to the elimination of 2c *S83* and 4d *DK13* isolates; unsubtyped genotypes 3 disappeared, due to the elimination of 3a *T1787* isolate.

Observing the HCV phylogenetic tree (Figure 2), comprising all HCV isolates typed by both software versions and elaborated basing on the am-
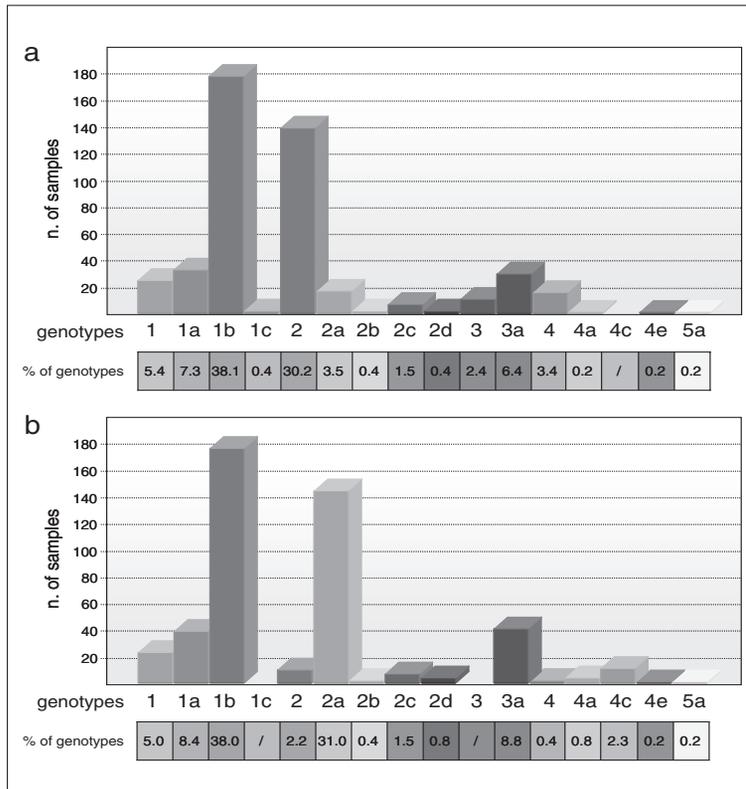

genotypes	1	1a	1b	1c	2	2a	2b	2c	2d	3	3a	4	4a	4c	4e	5a
a % of genotypes	5.4	7.3	38.1	0.4	30.2	3.5	0.4	1.5	0.4	2.4	6.4	3.4	0.2	/	0.2	0.2
b % of genotypes	5.0	8.4	38.0	/	2.2	31.0	0.4	1.5	0.8	/	8.8	0.4	0.8	2.3	0.2	0.2

FIGURE 1 - *Distribution of HCV genotypes, identified in 467 LightCycler RT-PCR positive HCV cDNA samples, basing on (a) the older HCV sequences database (Gene objects 3.2 HCV_5NC_311.gnl) and (b) the latest one (Gene objects 3.2 HCV_5NC_312.gnl): genotypes 1, 2 and 4 are fractions of amplicon sequences that cannot be sub-genotyped.*
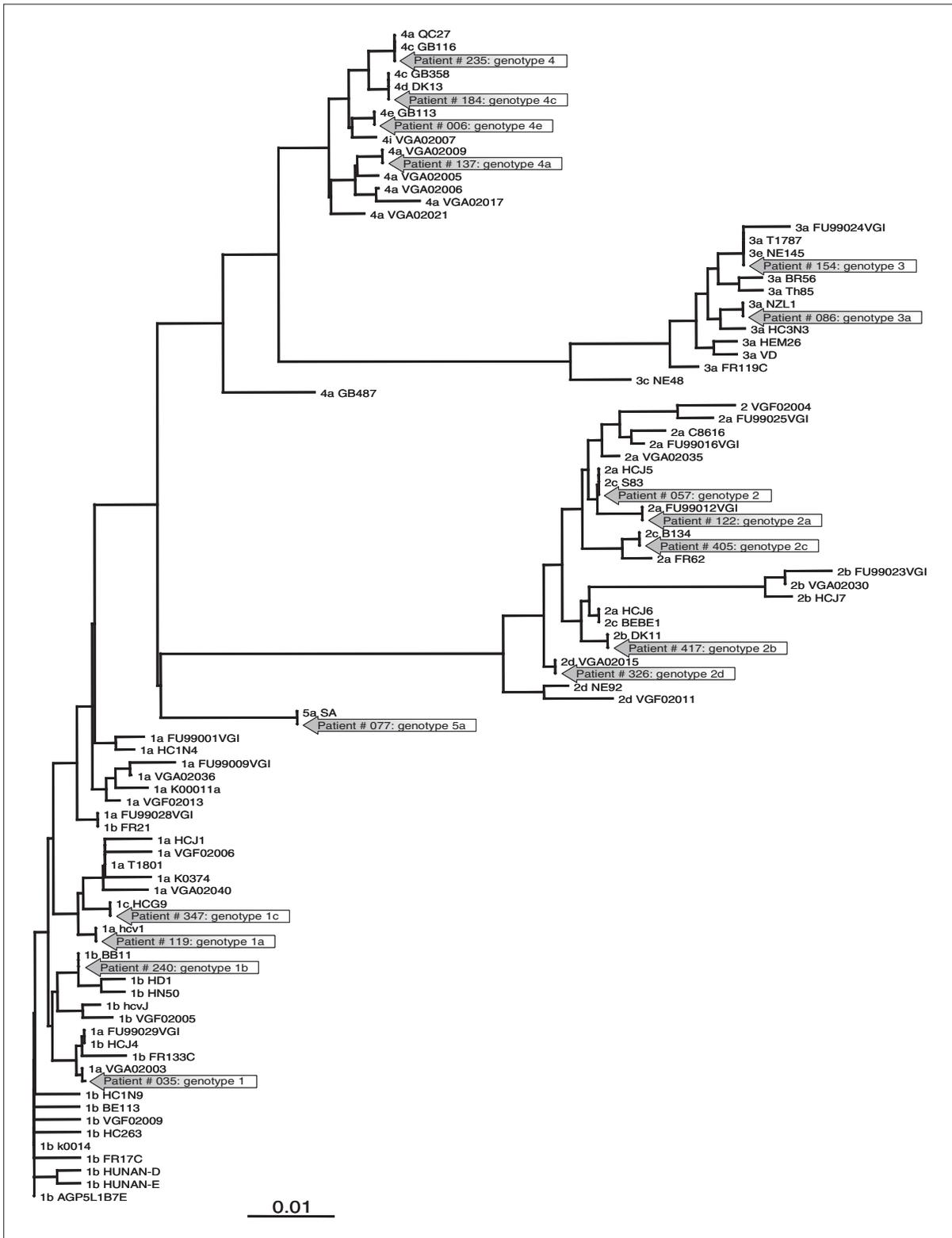
FIGURE 2 - *HCV phylogenetic tree: several patients' sequences, representative of each identified HCV types and sub-types, are included.*

plified HCV region (5′-NC), 2a *HCJ5* and 2c *S83*, 3a *T1787* and 3e NE145, 4c *GB358* and 4d *DK13* isolates are closely positioned; this might partially support the fact that often several typing tests (e.g.: sequencer, Inno-LiPA, etc.) may not distinguish among subtypes, like *2a/2c*, so that the strain is expressed as type 2. Furthermore, several patients' sequences, representative of each identified HCV types and subtypes, were included showing the good effectiveness of the test.

Lastly, revising our case-record with the new HCV sequencer database, it was observed that in the Apulia region (southern Italy) the most frequently identified genotypes in native patients were 1b *K0014* (24%) and 2a *HCJ5* (22%) (Figure 1b).

The melting temperatures of HCV amplicons genotyped by direct sequence analysis were compared, but overlapping melting temperatures were observed amongst different HCV genotypes and subtypes, hampering the possibility to use this method for typing.

DISCUSSION

The LightCycler Real Time RT-PCR assay, developed and optimized in this study, allowed the rapid (approximately 1 hour) and accurate quantification of HCV RNA over a large dynamic range (10^1 to 10^9 IU/ml) using a single human reference standard. This is the first report describing the routine testing of a large number of clinical samples (more than 5,000) over a long period of time (almost five years). The assay was precise and reproducible as shown by the inter-assay SD (1.03) and CV (3.55%) mean values of the Accurun HRS crossing points obtained in 409 RT-PCR runs. The analytical sensitivity of this assay, i.e. the smallest amount of HCV RNA tested and reliably quantified, was 57 IU/ml, since the linearity of the standard curve permitted interpolation of values located below the lowest HRS. Furthermore, participation in the *European Quality Control for Molecular Diagnostics (QCMD) 2007 Hepatitis C Virus Proficiency Programme* assessed the effectiveness of this protocol with the maximum score (data available).

The innovative aspect of this Real Time RT-PCR protocol was the use of the LightCycler cDNA amplicons for direct automated sequence analysis, which bypassed the need for additional reverse transcription and amplification steps, reducing costs and sequencing time to approximately 3 hours. Nearly all HCV genotypes (1 to 5) were identified. The comparative analysis of the viral sequences obtained from the LightCycler amplicons revealed an interesting cost-effectiveness, saving much time and money, and was able to subtype the 93% of observed HCV genotypes with a very high level of sequence homology (99.9%), irrespective of the viral load.

The absence of correlation between the LightCycler melting temperature and HCV genotypes, probably due to polymorphisms in the region covered by the hybridization probes, suggested that HCV genotyping cannot be done by temperature melting analysis, confirming assertions by Schroter *et al.* (2002) and requires DNA sequence analysis.

Furthermore, a phylogenetic analysis of all HCV sequences obtained by both software versions unambiguously showed that the adopted method allows a reliable typing of HCV RNA sequences.

Follow-up of chronically infected HCV patients is the primary clinical goal in therapy administration. In the absence of an HCV vaccine, the timely monitoring of HCV viral load combined with the information of the viral genotype could contribute to patient disease management (Strader *et al.*, 2004; Terrault *et al.*, 2005). Thus, modern diagnostic laboratories need to have an accurate, reproducible, rapid and cost-effective HCV-RNA assay to provide clinicians with reliable data (Gourlain *et al.*, 2005). In conclusion, the ease of use, high speed of performance and the ability to detect most HCV genotypes makes the described LightCycler Real Time RT-PCR protocol a suitable tool for the diagnosis and monitoring of HCV infections.

Although available methods for HCV typing have reached a good level of reliability for clinical purposes (Murphy *et al.*, 2007) an External Quality Assessment (EQA) program is desirable.

REFERENCES

ALTER M.J., KRUSZON-MORAN D., NAINAN O., ET AL. (1999). The prevalence of hepatitis C virus in the United States. *N Engl J Med.* **341**, 556-562.

CASTELAIN S., DESCAMPS V., THIBAULT V., ET AL. (2004). TaqMan amplification system with an internal positive control for HCV RNA quantification. *J Clin Virology.* **31**, 227-234.

COOK L., NG K.W., BAGABAG A., COREY L., JEROME K.R. (2004). Use of the MagNA pure LC automated nucleic acid extraction system followed by Real-Time reverse transcription-PCR for ultrasensitive quantitation of Hepatitis C virus RNA. *J Clin Microbiol.* **42**, 4130-4136.

FARMA E., BOERI E., BETTINI P., ET AL. (1996). Single step PCR: a tool for molecular diagnosis of hepatitis C virus infection. *J Clin Microbiol.* **34**, 3171-3174.

GERMER J.J., HARMSEN W.S., MANDREKAR J.N., MITCHELL P.S., YAO J.D.C. (2005). Evaluation of the COBAS TaqMan HCV test with automated sample processing using the MagNA Pure LC instrument. *J Clin Microbiol.* **43**, 293-298.

GOURLAIN K., SOULIER A., PELLEGRIN B., ET AL. (2005). Dynamic range of hepatitis C virus RNA quantification with the cobas ampliprep-cobas amplicor HCV monitor v2.0 assay. *J Clin Microbiol.* **43**, 1669-1673.

HOOFNAGLE J.H. (1997). Hepatitis C: the clinical spectrum of disease. *Hepatology.* **26**, 15S-20S.

MURPHY D.G., WILLEMS B., DESCHENES M., ET AL. (2007). Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C Virus with reference to C/E1 and 5' untranslated region sequences. *J Clin Microbiol.* **45**, 1102-1112.

NATIONAL INSTITUTES OF HEALTH. (2002). NIH consensus development conference statement: management of hepatitis C 2002: a preliminary draft statement, June 10-12, 2002. Bethesda, Maryland: *NIH Consensus Development Program.*

NOLTE F.S., GREEN A.M., FIEBELKORN K.R., ET AL. (2003). Clinical evaluation of two methods for genotyping hepatitis C virus based on analysis of the 5' non-coding region. *J Clin Microbiol.* **41**, 1558-1564.

RATGE D., SCHEIBLHUBER B., NITCHE M., KNABBE C. (2000). High-speed detection of blood-borne hepatitis C virus RNA by single-tube real-time fluorescence reverse-transcription PCR with the LightCycler. *Clinical Chemistry.* **46**, 1987-1989.

SCHROTER M., ZOLLNER B., SCHAFER P., ET AL. (2002). Genotyping of Hepatitis C virus 1, 2, 3 and 4 by one-step LightCycler method using three different pairs of hybridization probes. *J Clin Microbiol.* **40**, 2046-2050.

SHEEHY P., MULLAN B., KENNY-WALSH E., ET AL. (2007). In vitro replication models for the hepatitis C virus. *J of Viral Hepatitis.* **14**, 2-10.

STRADER D.B., WRIGHT T., THOMAS D.L., SEEFF L.B. (2004). Diagnosis, management, and treatment of hepatitis C. *Hepatology.* **4**, 1147-1171.

TAGLIAFERRO L., CORBELLI M., CROVATTO M., SANTINI G.F. (1997). Detection of Hepatitis C Virus (HCV) genotype 4 in Italy. *New Microbiol.* **20**, 87-88.

TERRAULT N.A., PAWLOTSKY J-M., MCHUTCHINSON, ET AL. (2005). Clinical utility of viral load measurements in individuals with chronic hepatitis C infection on antiviral therapy. *J Viral Hepatitis.* **12**, 465-472.