

Monitoring HCV RNA viral load by locked nucleic acid molecular beacons real time PCR

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Abstract

Locked nucleic acids (LNA) based real time PCR was used in particular situations where there are difficulties in primer design due to sequence complexity. In this study a new real time RT-PCR assay was developed using LNA modified primers and LNA molecular beacon probes to monitor hepatitis C virus (HCV) viral load in plasma and serum samples. The technique did not suffer from an heterogeneity of the HCV genome and, in addition, an internal RNA control was amplified in the same reaction tube with different short primers and beacon probe. Due to the short consensus LNA primers length, the PCR efficiency was close to 100% with no formation of hairpin loop structures. In summary a new LNA molecular beacon based real time RT-PCR assay was used successfully to measure quantitatively the total level of HCV RNA in both experimental and clinical specimens. The high sensitivity (50 IU/ml), the wide range of genotype detection, increased specificity and robustness obtained with this test are particularly useful for screening large number of specimens and measuring viral loads to monitor the progress of the disease.

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1. Introduction

Hepatitis C (HCV) virus, a single-stranded RNA virus belonging to the *Flaviviridae* family, is a major pathogen of post-transfusion and community-transmitted non-A non-B hepatitis. HCV is now the most common blood-borne infection in the USA and Europe. HCV is a major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma worldwide. Accurate diagnosis is essential to identify the presence of HCV RNA in apparently symptom-free carriers, or to monitor the viral load of treated patients. HCV circulates in blood at a low copy number and its genome is heterogeneous. Many investigators have used different methods to quantify the viral load in patients infected with HCV. These methods involve amplifica-

tion procedures based either on branched-DNA (bDNA) assay, or on conventional RT-PCR methods (Simmonds et al., 1990), or real time RT-PCR (Yang et al., 2002; Castelain et al., 2004).

The application of fluorescence techniques to PCR and RT-PCR, together with suitable instrumentation capable of combining amplification, detection and quantitation, has led to the development of kinetic PCR and RT-PCR methods that permit accurate nucleic acids quantitation. Molecular beacons are fluorescent DNA probes that have been used in real time PCR to detect newly synthesized amplicons (Tyagi and Kramer, 1996) forming a stem-and-loop structure; the loop portion of the molecule is complementary to the target nucleic acid and the stem is formed by the annealing of complementary arm sequences at the ends of the probe sequence (Tyagi and Kramer, 1996). The specificity of this system is such that it can detect readily single nucleotide differences (Giesendorf et al., 1998; Marras et al., 1999), which can be difficult to achieve with the 5' nuclease assay system.

More recently locked nucleic acids (LNA) based real time PCR was developed in particular situations where there are dif-

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Table 1
Primers and molecular beacon table: LNA bases are preceded by a +

Name	5' labeling	Sequence	3' labeling
HCVLNAF		gCCATg + gCgTTAg + TATgAgT	
HCVLNAR		ACTCg + CAAgCA + CCCTATC	
GBV-CLNAF		CCCAgA + AACCGAC + gCCTATT	
GBV-CLNAR		CCgggA + TTTACg + ACCTACCA	
HCVBeacon	FAM	CCGGTGAAGAGCCA + T + AG + TG + G + TCTGCGGAATCACCGG	BHQ1
GBV-CBeacon	HEX	CCGGTCTGTGGT + G + AT + GG + G + TGATGCCGACCGG	BHQ1

LNA primers and LNA beacon probes were synthesized by Prologo (www.prologo.com).

difficulties in primer design due to sequence complexity (Latorra et al., 2003a,b). LNA is a novel type of nucleic acid analogue that contains a 2'-O, 4'-C methylene bridge. This bridge-locked in 3' endo conformation restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and exceptional biological stability. Introducing LNA chemistry into a real-time quantitative PCR, the probe increases thermal duplex stability and improves specificity of probe hybridization to its target sequence. As such, background fluorescence from spurious binding is reduced and the signal-to-noise ratio is increased. The LNA monomer chemical structure enhances the stability of the hybridization of the probe to its target. As a result, the duplex melting temperature (T_m) may increase by up to 8 °C per LNA monomer substitution in medium salt conditions compared to a DNA fluorescent probe for the same target sequence depending on the target nucleic acid. This increase in hybridization creates a significant broadening of the scope of assay conditions and allows for more successful single-tube multiplexing. Due to enhanced hybridization characteristics of LNA and significant T_m contribution, shorter LNA-containing quantitative PCR probes can be synthesized, allowing flexibility in design. These shorter probes can be used to address traditionally problematic target sequences, such as AT- or GC-rich regions, or as in this case not very conserved region among different HCV genotypes. Short LNA primers are indicated for multiplex PCR because they are less prone to primer–primer interactions, and they permit the inclusion in the same reaction LNA-primers and LNA-probe recognizing an internal RNA control in order to monitor the whole assay as indicated in the European guidelines for in vitro diagnostic tools (Directive 98/79/EC, 1998; Liikanen, 2002).

In this study an high-throughput LNA molecular beacon based assay was developed. This diagnostic tool is able to monitor the viral load of hepatitis C virus, including the six recognized genotypes and subtypes with the same efficiency using serum or plasma samples (Table 1).

2. Materials and methods

2.1. Reference and clinical samples

The standards used in this study consisted an NAP HCV RNA provided by Acrometrix, CA (www.acrometrix.com) (Jorgensen and Neuwald, 2001). These samples were prepared by diluting quantitatively HCV RNA positive human serum or plasma

in defibrinated, delipidized normal human plasma. The value assignments for each standard are based upon the World Health Organization (WHO) First International Standard for HCV RNA and were expressed in International Unit/ml. With respect to other standards such as DNA plasmids or long oligoes, they were treated as unknown samples, reducing any possible variable due to multistep processing during RNA extraction and reverse transcription. Clinical samples and HCV RNA negative samples were retrieved from plasma samples tested by the AMPLICOR HCV MONITOR TestTM, v2.0 or the COBAS AMPLICOR HCV MONITOR TestTM, v2.0 from the Unit of Immunohaematology, Istituto Nazionale Tumori (Milan).

2.2. Internal control

In order to avoid false negative results due to user errors or PCR inhibitors, an internal RNA control was amplified by different primers in the same reaction tube. For this purpose, Armored RNA[®] GBV-C (Ambion, Milan) was used because it is ribonuclease resistant, sequence-defined RNA molecule, non-infectious and easy to handle. Armored RNA was added directly to a plasma or serum sample, thus serving as an internal control (Schlueter et al., 1996; Pasloske et al., 1998; WalkerPeach et al., 1999).

2.3. Positive and negative controls

Armored RNA HCV1b (Ambion, Milan) was used as a positive control. With respect to other naked positive controls it permitted to start from the RNA extraction step without affecting recovery. With respect to DNA positive controls, it allowed to monitor the whole assay from the beginning, without losing any variable of the RNA extraction and reverse transcription steps (WalkerPeach et al., 1999). As negative controls, HCV RNA free serum samples tested by the AMPLICOR HCV MONITOR TestTM (Roche, Milan) were used, and as a negative HCV RNA template the phage MS2 RNA (Roche-Applied-Science, Milan) was adopted.

2.4. RNA extraction

In order to obtain the best sensitivity, 2 ml of serum or plasma samples were centrifuged at 4 °C at 21,000 × *g* for one hour. 1.8 ml of supernatants were discarded and the pellets were resuspended in the remaining 200 µl of serum as described previously

(Forman and Valsamakis, 2004). The protocol from the QIAamp MinElute RNA extraction kit (Qiagen, Milan) was followed with a final elution with 20 μ l of AE buffer. Acrometrix standard, the positive and negative controls were extracted in parallel during the assay with clinical samples. Samples with less than 2 ml of plasma were tested centrifuging the total amount of plasma available with the same experimental conditions. The derived final IU/ml was calculated multiplying for the index ratio: 2/test sample quantity.

2.5. Reverse transcription

Eleven microlitres of total RNA was subjected to reverse transcription using MMLV-RT (Promega, Milan) with 150 ng of random primers (Invitrogen, Milan), 5 U of RNase inhibitor (GE Healthcare, Milan), 0.5 mM dNTPs (SIGMA, Milan) incubating the reaction directly at 37 °C for 50 min, followed by a denaturing step at 65 °C for 15 min to inactivate the reverse transcriptase.

2.6. Primer design

The primers and molecular beacons probes used in this study were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The target sequences from each of the six known HCV genotypes and subtypes were retrieved from Los Alamos HCV database (www.hcv.lanl.gov). Multiple sequence alignment was done by ClustalW software (www.ebi.ac.uk, see Fig. 3). Unconserved nucleotides were converted to N in order to avoid primer and probe design in those nucleotides. Flanked molecular beacon arms were designed using the OLIGO 6.0 software reaching a temperature of 55 °C in the stem loop conformation. LNA nucleotide were added to primers and beacon probes as described previously (Latorra et al., 2003b,c) using a T_m prediction tool available at www.exiqon.com. Amplicons were tested by MFOLD (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>) for hairpin in primers and probe positions (Fig. 1).

2.7. Real time PCR

Four microlitres of cDNA were added to 21 μ l of master mix composed of: 0.8 μ M of HCVLNA primers, 1.6 μ M of HCVBeacon, 0.4 μ M of GBV-CLNA primers, 1.6 μ M of GBV-CBeacon, 1 \times ROX Dye (Invitrogen Cat. No.), 10 mM Tris (pH 8.3), 50 mM KCl, 4.0 mM MgCl₂, 200 mM deoxynucleoside triphosphates, 1 U of ABSuperTaq polymerase (AB Analytica, Padova). Following dye normalization at 50 °C for 2 min and template denaturation for 4 min at 95 °C, amplification conditions were as follows: 38 cycles (each) of 30 s at 95 °C, 40 s at 60 °C (plate reading), 40 s at 72 °C. Amplification was carried out on ABI SDS 7000. Every reaction was done in duplicate. For every assay the negative template control (cDNA from phage MS2 RNA), the positive control (cDNA from Armored 1b), and four dilutions of the cDNA obtained from the Acrometrix standard (5 \times 10⁶ IU/ml) were loaded in duplicate to calibrate the system.

2.8. HCV genotyping

Acrometrix standards and a set of clinical samples were genotyped by reverse dot/blot method as described previously (Di Tommaso et al., 2003) and confirmed by automated sequence analysis using primers KY78 and KY80 (Young et al., 1993). Briefly PCR product were separated by electrophoresis on 2% agarose gel and purified using the MiniElute Gel extraction kit (Qiagen GmbH, Hilden Germany). Sequencing analysis was then undertaken on a purified product using the DTCS-Quick start Kit (Beckman Coulter, Inc., Fullerton, CA) and the CEQ2000 XL automatic DNA sequencer. The strands were screened using the forward and reverse primers. Sequences were compared to HCV database at www.hcv.lanl.gov to identify the correct genotype and subtype using HCV-BLAST.

3. Results

3.1. Performance evaluation

Performance evaluation of this test was done following the European directives described previously in the Official Journal of European Community (Directive 98/79/EC, 1998; Liikanen, 2002).

3.1.1. Quantitation and sensitivity

The analytical detection limit (sensitivity limit) of this assay was determined using the ABI7000TM Instrument with the candidate international HCV standard OptiQuantTM HCV RNA [WHO International Standards for Hepatitis C Virus (HCV) RNA nucleic acid amplification (NAT) Assays, Acrometrix cod 94–2011]. These known calibrated samples were subjected to RNA extraction using the QIAamp[®] MinElute Virus Spin Kit with several modifications from the instruction of the supplier. In order to obtain the optimum sensitivity, an ultracentrifugation step was introduced before RNA purification at 21,000 \times g for 60 min at 4 °C as described previously (Forman and Valsamakis, 2004) with the following modifications: the starting plasma samples was 2 instead of 1 ml and the centrifuging time was 1 h instead of 2. By this protocol a sensitivity of 50 IU/ml was confirmed by the 24 replicates with a 95% cut-off value (Liikanen, 2002) (see Table 2).

Table 2
Quantitation testing was performed using ABI7000TM Instrument and the candidate international HCV standard OptiQuantTM HCV RNA

Acrometrix OptiQuant TM HCV RNA (IU/ml)	Mean values detected
5,000,000	4,800,000
500,000	430,397
50,000	49,765
5000	5432
500	812
50	126 ^a
0	0

^a This data was obtained by ultracentrifuging 2 ml of 50 IU/ml plasma samples.

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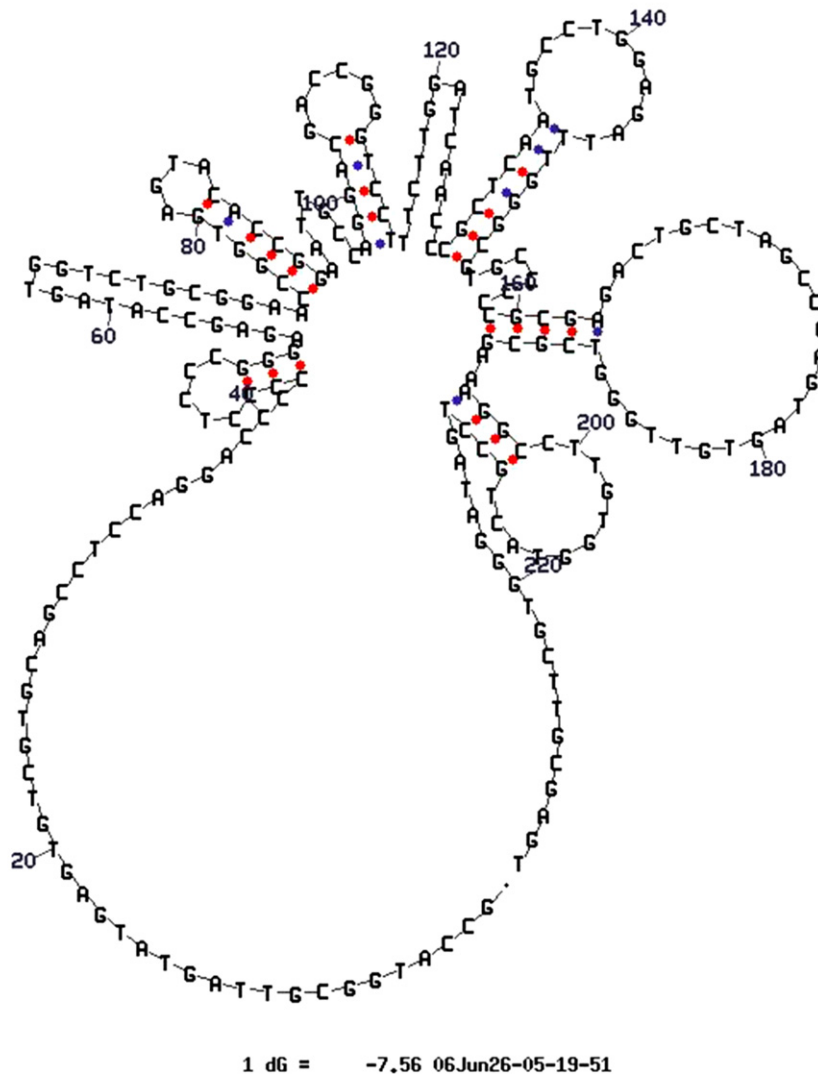


Fig. 1. 5' UTR HCV amplicon was analyzed for nucleic acid folding and hybridization prediction. Primer forward (HCVLNAF), position from 1 to 20, and reverse (HCVLNAR), position from 213 to 231 were free of secondary structures.

3.1.2. Intra and inter-assay variability

The standard deviation of C_t values in intra-runs for a 2,500,000 IU/ml standard was estimated as 0.115, while the standard deviation of C_t values in inter-runs was estimated as 0.254 for standard 1, 0.193 for standard 2, 0.270 for standard 3 and 0.270 for standard 4. The dynamic range of the assay has been tested for 5×10^1 IU/ml and 10^8 IU/ml.

3.1.3. Robustness

The robustness of the internal control (Armored GBV-C RNA) was assessed by purification and analysis of 100 HCV negative plasma samples. The total failure rate of HEX signal (specific for GBV-C) was 0%. Inhibitors were not observed due to the high efficiency of RNA purification.

3.1.3.1. Cross-contamination. During the evaluation tests, 22 runs were tested alternating high positive and negative samples without false positives.

3.1.3.2. Whole system failure rate. During the evaluation tests, 24 samples with $3 \times$ the positive cut-off concentration (150 IU/ml), and 8 samples with $2 \times$ positive cut/off (100 IU/ml) were evaluated for failure rate. There was no failure with any of the HCV samples. Thus, the robustness was detected as $\geq 99.9\%$.

3.1.4. Specificity of the assay

The primers and probe designed for this assay are specific strictly for HCV and they do not contain any perfect match sequences for any other known viruses, bacteria or genomic

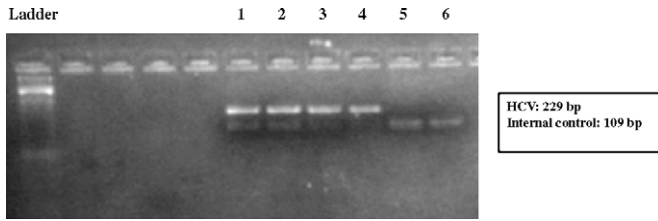


Fig. 2. MW: 100 bp ladder. 1: standard 1; 2: standard 2; 3: standard 3; 4: standard 4; 5: blood donor 1; 6: blood donor 2.

DNA, including other related viruses such as GB virus C (GBV-C) or yellow fever virus (YFV). As indicated in European directives (Liikanen, 2002), 20 tests with GBV-C 5' UTR RNA were evaluated with no positive signals; in addition 100 blood donors were tested with no positive signals (specificity >99.9%). The gel in Fig. 2 shows absence of aspecific bands and visualize only the HCV amplicon (the upper one: 229 bp) and the internal control amplicon (the lower one: 109 bp).

3.1.5. Genotype/subtype detection and quantitation efficiency

The genotype/subtype detection efficiency was evaluated by analyzing the four different sample tests from Acrometrix genotyping panel: hepatitis C genotype panel types 1, 2, 3, and 4 Cod. 94–2000. This panel does not provide information about the real quantity of each sample even if Acrometrix declares that every sample is >5000 IU/ml. Due to the fact that primers and probes are designed to match perfectly every genotype and known subtype (see HCV sequence database on <http://hcv.lanl.gov>), this diagnostic tool revealed no difficulty in amplifying the four major genotypes with good IU/ml estimations for the dilutions series. In addition, genotypes 5 and 6a obtained from clinical samples gave similar results.

4. Discussion

The amount of HCV RNA in plasma or serum samples is believed to represent the steady state of viral replication and clearance. Therefore, it is important to have a highly specific and sensitive assay to quantify precisely HCV RNA in the blood of patients with HCV. This is particularly helpful for monitoring the effect of anti-HCV therapy. It is well established that in patients with chronic HCV infection the response to alpha interferon therapy is correlated with serum HCV RNA. Although the viral load has been suggested to correlate with HCV activity and the degree of liver damage, the studies by different investigators have generated controversial data. These results may be caused by the lack of a reliable method for HCV RNA quantitation (Yang et al., 2002).

A newly Real Time LNA molecular beacon based assay was developed amplifying with the same efficiency different HCV genotypes starting from serum or plasma samples.

Every assay requires a standard curve made by four dilution of a standard RNA with known International Units concentration. This standard is an NAP HCV RNA provided by Acrometrix (www.acrometrix.com, CA) (Jorgensen and Neuwald, 2001),

prepared by quantitatively diluting HCV RNA positive human serum or plasma into defibrinated, delipidized normal human plasma. The value assignments for each standard are based upon the World Health Organization (WHO) 1st International Standard for HCV RNA. To monitor every RT-PCR reaction, an internal control based on Armored RNA technologies (WalkerPeach et al., 1999) was added in the first step before RNA extraction, allowing the monitoring of the assay for performances and affordability. Armored GBV-C RNA was amplified in the same tube, using same experimental conditions with respect to HCV RNA, and it is recognized by another beacon probe labeled by a different Dye (HEX).

To detect HCV, it was necessary to select a primer set and molecular beacons to generate a relatively short amplicons, with the ability to detect with the same sensitivity any of the six well known HCV genotypes and subtypes. Consensus regions are difficult to retrieve from the most conserved HCV genome, the so called 5' UTR (untranslational region) or 5' NCR (non-coding region). Additionally repetitive sequences must be avoided as well as sequence primers that form hairpin loop which decrease the PCR efficiency. Consensus primers described previously (Yang et al., 2002; Young et al., 1993; Grassi et al., 1995) were tested by MFOLD (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>). These primers showed secondary structures that make these oligonucleotides not ideal. A new set of primers was designed with no hairpin structures (see Fig. 1), simply decreasing primer length remaining in the same consensus regions and increasing the T_m by the use of LNA oligoes. LNA primers have the potential for use in multiplex PCR protocols without being affected by multiple primer interactions as in this case.

The probe sequence was chosen so that it would hybridize to a sequence within its target amplicon that is conserved among most clinical subtypes (see Fig. 3). The arm sequences of the probe was chosen so that it would hybridize to each other at the PCR annealing temperature but not to the probe sequence, and to ensure that it would form a hairpin structure containing an unstructured probe sequence.

The primers and probe designed for this assay are specific for HCV and they do not contain any perfect match sequences for any other virus, bacteria or genomic DNA. Evaluation tests for HCV virus specificity was done attempty to amplify GBV-C 5' UTR, a member of the *Flaviviridae* family without having any positive signal.

The real time PCR test is made in double with a standard deviation calculation related to the threshold cycle (C_t) value and to the IU/ml of each sample to evaluate errors due to sample pipetting. Every assay needs a standard curve made by four dilution of a standard RNA with known International Units concentration. This standard is a NAP HCV RNA provided by Acrometrix, CA (Jorgensen and Neuwald, 2001) based upon the World Health Organization (WHO) First International Standard directive for HCV RNA. The dynamic range of the assay is wider than other available tests available on the market, and it spans between 5×10^1 and 10^8 IU/ml.

The variability of the intra-runs and inter-runs was not significant statistically. Applying this diagnostic tool to clinical

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