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Abstract

Based on recent LightCycler techniques developed for the quantitation of serum HCV RNA, we have developed a quantitative method for the intracellular hepatitis C virus (HCV) RNA using LightCycler PCR. A simple real-time PCR assay, based on the SYBR Green I dye and LightCycler fluorimeter and with no probe requirement, is described. In the presence of 0.5 microg of cellular RNA, it was demonstrated that as few as 25 copies of HCV RNA could be specifically detected with a set of primers that amplify a 144-base pair sequence unique to the 5'-noncoding region of HCV RNA. We demonstrated that this method was useful for the evaluation of antiviral reagents using HCV-infected human cultured cells.

KEYWORDS: hepatitis C virus, real-time PCR, LightCycler

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Short Communication

Quantitative Method of Intracellular Hepatitis C Virus RNA using LightCycler PCR

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Based on recent LightCycler techniques developed for the quantitation of serum HCV RNA, we have developed a quantitative method for the intracellular hepatitis C virus (HCV) RNA using LightCycler PCR. A simple real-time PCR assay, based on the SYBR Green I dye and LightCycler fluorimeter and with no probe requirement, is described. In the presence of 0.5 μg of cellular RNA, it was demonstrated that as few as 25 copies of HCV RNA could be specifically detected with a set of primers that amplify a 144-base pair sequence unique to the 5'-noncoding region of HCV RNA. We demonstrated that this method was useful for the evaluation of antiviral reagents using HCV-infected human cultured cells.

Key words: hepatitis C virus, real-time PCR, LightCycler

Hepatitis C virus (HCV) is the main causative agent of chronic viral hepatitis. Most patients progress to liver cirrhosis and then to hepatocellular carcinoma. HCV is an enveloped virus belonging to the *Flaviviridae,* whose genome contains a 9.6 kilobase positive-stranded RNA encoding at least 10 viral proteins. While a number of studies have investigated the genetics of HCV, the mechanism of HCV replication in infected cells is poorly understood [for review, see 1]. We previously reported that the human MT-2C T-cell line [2] and human hepatocyte-derived PH5CH8 cell line [3] could support HCV replication. These cell culture systems were useful for evaluation of antiviral reagents, due to the fact that bovine lactoferrin (LF), a milk glycoprotein belonging to the iron transporter family, markedly inhibited HCV infection in the cells [4].

To monitor replication of HCV in these HCV-infected cell culture systems, we measured HCV RNA semi-quantitatively by a conventional reverse transcription-nested polymerase chain reaction (RT-nested PCR) method, checking the amplification level of the 5'-noncoding (NC) region in every fifth cycle of the second PCR [2]. Although we could roughly estimate the level of intracellular HCV RNA (10^2-10^3 copies/μg RNA), this method was labor intensive and time consuming because it required sampling at every fifth cycle and agarose gel electrophoresis for the detection of PCR products.

Recently, a new PCR method that can finish within 30 min, using real-time PCR technology and a LightCycler, was adapted to the quantitative detection of HCV RNA in clinical serum samples [5, 6]. This method does not require a probe for the detection, and the PCR product is monitored continuously by SYBR Green I dye binding to double stranded DNA during one-step PCR. We confirmed that 10 copies of HCV RNA from human serum could be detected, according to the protocol using the primer sets reported [5, 6] (data not shown). Using

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this method, we tried to detect the HCV RNA from *in vitro* HCV-infected PH5CH8 cells. However, we failed to detect the intracellular HCV RNA (10^2–10^3 copies/μg RNA), because cellular RNA (0.5 μg) lowered the specificity and sensitivity of this one-step PCR method. This result led us to modify the protocol of one-step PCR using the LightCycler, and we subsequently applied LightCycler PCR instead of a second PCR in a previous report on our semiquantitative method [2]. Amplification was performed in 20 μl of LightCycler FastStart DNA Master SYBR Green I mix containing 2.5 mM $MgCl_2$ by using 1 μl of the first PCR product obtained by the method described previously [2] and primers 104 and 197R [2], resulting in amplification of the 144-base pair of the 5'-NC region. LightCycler PCR was performed in 35 cycles of 15 sec at 95 °C (denaturation), 5 sec at 57 °C (annealing), and 8 sec at 72 °C (extension) with fluorescence detection at 88 °C after each cycle. After the final cycle, melting-point analysis of the samples was performed within the range of 65 to 95 °C. In this condition, we confirmed the successful detection of about 100 copies of HCV RNA in the RNA specimen (0.5 μg) from *in vitro* HCV-infected PH5CH8 cells (approximately 5×10^4 cells).

Using HCV RNA synthesized *in vitro* as described previously [2], we next examined the sensitivity and specificity of LightCycler PCR under this condition. RNA (0.5 μg) derived from PH5CH8 cells was added to each reaction tube so as to be equivalent to the amount of RNA in the actual experimental specimens. Standard

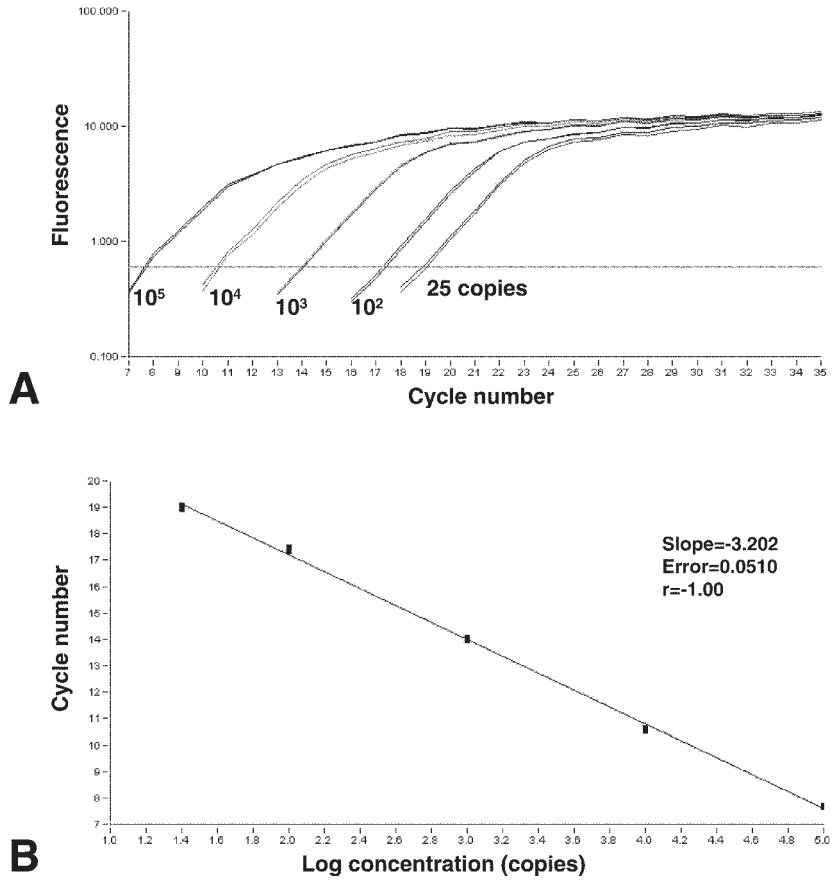

Fig. 1 Standard curve obtained by LightCycler PCR using *in vitro* synthesized HCV RNA. (A) Logarithmic curve of fluorescence versus cycle numbers for each sample. Cellular RNA (0.5 μg) from PH5CH8 cells was added to each reaction tube before RT so as to be equivalent to the amount of RNA in the actual experimental specimens. The horizontal line corresponds to the baseline as described in the text. The results of duplicate samples are indicated. (B) Crossing points (cycle numbers) plotted against the logarithmic concentration of the initial copies of HCV RNA.

curves were constructed from serial 10-fold dilutions (10^5 to 10^1 copies) and 25 copies of synthetic HCV RNA. After real-time PCR was completed, logarithmic values of fluorescence for each dilution were plotted against the cycle number. A baseline was set above the fluorescence background and a crossing point was determined using the amplification curves obtained during the exponential phase of amplification. As shown in Fig. 1A, as few as 25 copies of HCV RNA could be detected, although 10 copies of HCV RNA were not detected. There was a good relationship between the cycle number corresponding to the crossing point and the log concentration of initial copies of HCV RNA used, as shown in Fig. 1B. Good linearity was obtained in the range of 10^5 to 25 copies of HCV RNA. As shown in Fig. 1, high reproducibility of our quantitative method was obtained. The specificity of the amplified product was determined by melting curve analysis. Melting curve acquisitions were performed immediately after PCR was performed within the range of 65 to 95 °C. The melting temperature (Tm) of the PCR product appeared to be 89 °C, although the primer dimer had a Tm of 81 °C (Fig. 2). The other merit of our method is that a reproducible standard curve is obtained in each LightCycler PCR, using the stable first PCR products, which were amplified from *in vitro* synthesized HCV RNA and stored at −80 °C as the templates. Using this PCR system, we actually performed the quantitation of HCV RNA in RNA specimens derived from *in vitro* HCV-infected PH5CH8 cells. The results showed that the amount of HCV RNA in HCV-infected cells (7–14 days postinoculation) was 10^2–10^3 copies per μg RNA. These values were comparable to those obtained in a previous semi-quantitative analysis [2]. These results indicate that our PCR method using the LightCycler is useful for quantitation of intracellular HCV RNA. In contrast, the intracellular HCV RNA (10^2–10^3 copies/μg RNA) was not detected by the usual PCR methods using LightCycler PCR [5, 6].

To demonstrate the usefulness of LightCycler PCR, we examined whether this method could be applied as the evaluating system of anti-HCV activities in bovine and human LFs, which prevent HCV infection in PH5CH8 cells [4]. Using the same assay conditions [4], excepting the shifts to small scale (5×10^4 cells in 96-wells plate) and LightCycler PCR technology, we attempted to determine the IC_{50} dose of LF against HCV infection. As shown in Fig. 3, the quantification of intracellular HCV RNA by LightCycler PCR demonstrated that the prevention of HCV infection with bovine LF occurred in a dose-dependent manner. From this result, we determined that the IC_{50} dose of bovine LF was 0.12 mg/ml. Using the same assay method, the IC_{50} dose of human LF was also determined to be 0.4 mg/ml (data not shown). These results suggest that the HCV-inhibiting activity of bovine LF was relatively stronger than that of human LF. These results indicate that our quantitative

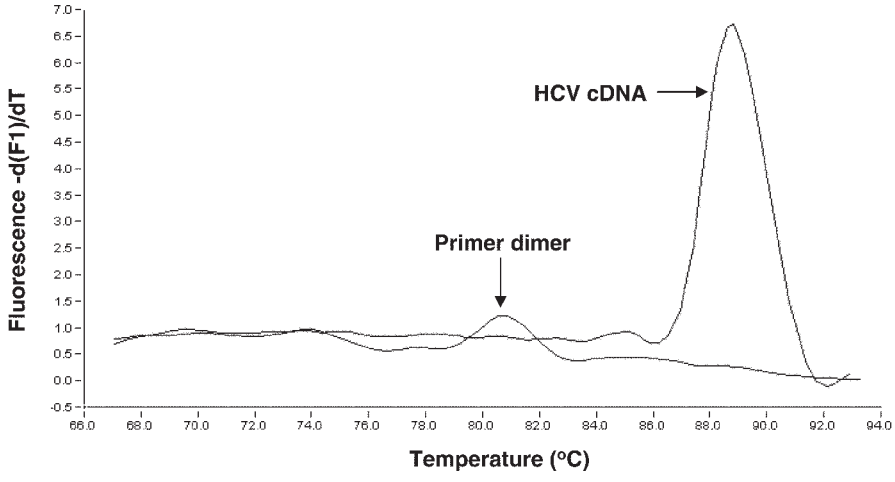

Fig. 2 Schematic diagram of a Tm analysis as accomplished at the end of PCR. HCV PCR product has a Tm of 89 °C and is easily distinguishable from primer dimer with a Tm of 81 °C. The graph displays the negative first derivative of the melting curve data (-dF/dT) versus temperature.

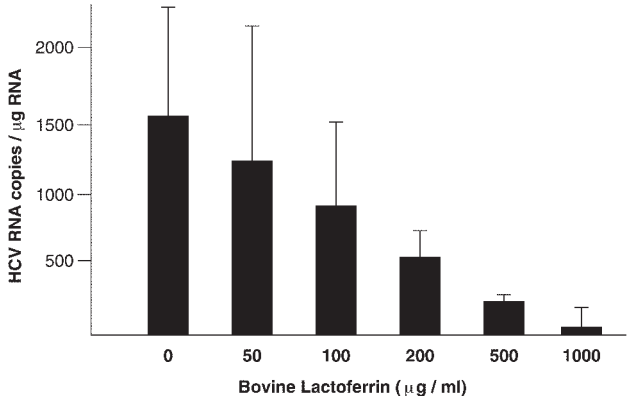

Fig. 3 HCV-inhibiting activity of bovine LF in PH5CH8 cells. PH5CH8 cells and inoculum 1B-2 were used for the HCV-inhibiting assay of LF as described previously [4]. Quantitative RT-nested PCR using LightCycler PCR was performed using RNA samples in greater than triplicate. The number in the axis of the ordinate indicates the copies of HCV RNA per μg RNA.

method using LightCycler PCR is highly useful for the evaluation of anti-HCV reagents. Furthermore, this method may be useful for the quantitative analysis not only of HCV genomes but also of other RNA viral genomes derived from infected cells.

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