Table 2 Correlation of interferon-λ_3 (*IFN-λ_3*) with clinical or immunological parameters in patients with chronic hepatitis C

Factors	CC with IFN-λ_3	*p*
Age (years)	−0.10	–
WBC (/mm^3)	−0.05	–
Hb (g/dL)	0.07	–
Plt ($\times 10^4$/mm^3)	−0.09	–
TP (g/dL)	0.07	–
Alb (g/dL)	−0.01	–
AST (U/L)	0.34	<0.0001
ALT (U/L)	0.34	<0.0001
T-bil (mg/dL)	0.03	–
T-chol (mg/dL)	−0.22	0.02
AFP (ng/mL)	0.30	0.001
HCV RNA (log IU/mL)	−0.05	–
Fibrosis score	0.07	–
Histological activity score	0.25	0.01
FIB-4 score	0.10	–
APRI	0.29	0.001
IP-10 (pg/mL)	0.53	<0.0001
MIP-1α (pg/mL)	−0.08	–
MIP-1β (pg/mL)	−0.18	–
RANTES (pg/mL)	0.26	–
PDGF-BB (pg/mL)	0.40	<0.0001

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *APRI* aspartate aminotransferase platelet ratio index, *AST* aspartate aminotransferase, *CC* correlation coefficient by Spearman's analysis, *FIB-4* fibrosis-4, *Hb* hemoglobin, *HCV* hepatitis C virus, *IP-10* interferon-γ-inducible protein 10, *MIP-1α* macrophage inflammatory protein 1α, *MIP-1β* macrophage inflammatory protein 1β, *PDGF-BB* platelet-derived growth factor BB, *Plt* platelets, *RANTES* regulated on activation, normally T cell expressed, and secreted, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *WBC* white blood cells

intestine are capable of producing IFN-λ in response to rotavirus to protect the host from infection [26]. Although both hepatitis E virus and hepatitis A virus are RNA viruses that are transmissible by the enterofecal route, the difference in serum IFN-λ_3 levels suggests that there are distinct mechanisms of recognition of hepatitis E virus and hepatitis A virus by the hosts. Further investigation is needed to disclose which pattern recognition receptors are utilized in hepatocytes or immune cells for the recognition of such viruses to produce IFN-λ.

The regulatory mechanisms of transcription and translation of IFN-λ_3 have not been well documented. The *IFNL3* SNPs (rs8099917) are located 8.9 kb upstream of the promoter region of the *IFNL3* gene [8, 9, 11]. Because of such localization, it is less likely that the genetic variation has some impact on the transcriptional level of *IFNL3*. With regard to the relationship between the *IFNL3* genotype and its transcripts, controversial results have been reported thus far. Some groups reported that IFN-λ_3 messenger RNA levels in peripheral blood mononuclear cells were higher in patients with the *IFNL3* major genotype than in those with the minor genotype [9]. In contrast, others showed that in hepatocytes such levels were comparable regardless of *IFNL3* SNPs. In the search for some genetic factors influencing *IFNL3* transcription, Sugiyama et al. [27] reported the existence of variable-length TA repeats in the promoter of the *IFNL3* gene. Other investigators showed that a certain structure of the 3′ untranslated region in the *IFNL3* gene is involved in the durability/stability of the gene [28]. Nevertheless, the contribution of such factors is not enough to fill in the gap, suggesting that certain other regulatory factors for *IFNL3* are still to be revealed.

Reports concerning serum IFN-λ in C-CH patients are limited. Langhans et al. [29] showed that serum levels of IFN-λ, which includes IFN-λ_2 and IFN-λ_3, were higher in patients with the *IFNL3* major genotype than in those with the minor genotype. One of the limitations of their study seems to be the lack of specificity for the measurement of IFN-λ_3. Since the homology of *IFNL2* (which encodes IFN-λ_2) and *IFNL3* is quite high, it is difficult to quantify specifically IFN-λ_3 by excluding contamination by IFN-λ_2. To exclude such a possibility, we used the newly developed chemiluminescence enzyme immunoassay for IFN-λ_3, which enables one to quantify IFN-λ_3 without any influence from IFN-λ_2 in the range from 0 to 1,000 pg/mL. By means of this system, we found that serum levels of IFN-λ_3 are not statistically different between patients with the *IFNL3* major genotype and those with the minor genotype.

On primary HCV exposure, the significance of IFN-λ family members as an antiviral protein is evident. However, such impact of IFN-λ_3 in chronically HCV-infected patients is still elusive. Langhans et al. [29] reported that serum IFN-λ levels in patients who had spontaneously cleared HCV were higher than in patients with chronic HCV infection, implying that a higher level of IFN-λ somewhat contributed to HCV eradication. In this study, we aimed to clarify the significance of IFN-λ_3 in patients with chronic HCV infection with different approaches. Firstly, we searched for the factors influencing serum IFN-λ_3 quantity by correlation analysis with clinical markers and multiple cytokines/chemokines. We found that AST, ALT, and α-fetoprotein levels and histological activity were positively correlated with serum IFN-λ_3 levels. In addition, one of the noninvasive fibrosis markers, APRI, was weakly correlated with serum IFN-λ_3 levels. Among the chemokines examined in this study, serum IFN-λ_3 was positively correlated with IP-10 and PDGF-BB. IP-10 (CXCL10) is induced in HCV-infected hepatocytes as one of the IFN-stimulated genes, and attracts CXCR3-positive T cells and natural killer cells and subsequently activates inflammation. IP-10 is also reported to be involved in the early stage of liver fibrosis [30, 31]. A similar fibrotic

Table 3 Factors associated with sustained virological response in patients with chronic hepatitis C who underwent 48 weeks of pegylated inferferon-α plus ribavirin therapy

	UVA			MVA	
	SVR	Non-SVR	*p*	OR (95 % CI)	*p*
Number	74 (38 male, 36 female)	45 (31 male, 14 female)	0.06		
Age (years)	55.4 ± 10.1	58.2 ± 10.0	0.122		
WBC (/mm^3)	5,043 ± 1,695	5,248 ± 1,363	0.247		
Hb (g/dL)	14.3 ± 1.5	14.4 ± 1.6	0.504		
Plt (×10^4/mm^3)	18.2 ± 4.6	16.9 ± 6.0	0.186		
TP (g/dL)	7.5 ± 0.6	7.6 ± 0.5	0.292		
Alb (g/dL)	4.2 ± 0.4	4.1 ± 0.4	0.575		
AST (U/L)	47.5 ± 27.9	66.5 ± 50.0	0.049	1.012 (0.997–1.027)	0.108
ALT (U/L)	66.4 ± 47.9	80.0 ± 62.9	0.286		
T-bil (mg/dL)	0.7 ± 0.3	0.9 ± 0.4	0.101		
T-chol (mg/dL)	178.1 ± 36.8	174.3 ± 37.7	0.717		
AFP (ng/mL)	7.1 ± 7.8	14.1 ± 18.8	0.062		
HCV RNA (log IU/mL)	6.3 ± 0.7	6.3 ± 0.5	0.753		
IFNL3 rs8099917 (TT/non-TT)	70:4	30:15	<0.0001	17.25 (3.34–89.13)	0.001
Histological activity score (A0-A1/A2-A3)	45:20	24:15	0.454		
Fibrosis score (F1–F2/F3-F4)	57:8	27:12	0.023	0.239 (0.072–0.798)	0.02
IFN-λ$_3$ (pg/mL)	17.3 ± 31.7	11.8 ± 14.9	0.262		
IP-10 (pg/mL)	458.0 ± 404.9	504.7 ± 364.0	0.208		
MIP-1α (pg/mL)	13.1 ± 36.1	4.2 ± 5.6	0.026	0.66 (0.457–0.956)	0.028
MIP-1β (pg/mL)	195.7 ± 204.3	154.9 ± 81.5	0.865		
RANTES (pg/mL)	18,125 ± 8,076	16,597 ± 7,946	0.187		
PDGF-BB (pg/mL)	3,931 ± 1,846	3,312 ± 1,803	0.079		

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *CI* confidence interval, *Hb* hemoglobin, *HCV* hepatitis C virus, *IFN-λ$_3$* interferon-λ$_3$, *IP-10* interferon-γ-inducible protein 10, *MIP-1α* macrophage inflammatory protein 1α, *MIP-1β* macrophage inflammatory protein 1β, *MVA* multivariate analysis, *OR* odds ratio, *PDGF-BB* platelet-derived growth factor BB, *Plt* platelets, *RANTES* regulated on activation, normally T cell expressed, and secreted, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *UVA* univariate analysis, *WBC* white blood cells

function was reported for PDGF-BB, the level of which is reported to be increased in patients with advanced/fibrosis stages of HBV infection [32, 33]. These reports support the notion that IFN-λ$_3$ is related to liver inflammation and fibrosis. As well as in B-CH patients, a positive correlation was observed between serum IFN-λ$_3$ levels and inflammation (AST levels) and fibrosis markers (FIB-4 score and APRI). Secondly, we examined whether serum IFN-λ$_3$ and chemokines are involved or not involved in the SVR to PEG-IFN-α plus RBV therapy for C-CH patients. We confirmed that *IFNL3* genotypes, fibrosis score, and MIP-1α are associated with SVR in this cohort, but failed to do so with IP-10 and serum IFN-λ$_3$. Several studies showed that pretreatment IP-10 levels could be a predictor of SVR in PEG-IFN-α plus RBV therapy for C-CH [34], the significance of which became stronger in combination with *IFNL3* genotypes [35, 36]. One of the reasons why the IP-10 levels failed to be significant in this study may be a bias for the enrollment of patients from multiple hospitals and medical centers.

In summary, serum IFN-λ$_3$ levels are increased in patients with chronic HCV infection regardless of the *IFNL3* genotype, the level of which is associated with liver inflammation and fibrosis. The biological role and clinical impact of IFN-λ$_3$ in patients with chronic HCV infection need to be investigated further.

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Conflict of interest The authors declare that they have no conflict of interest.

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High-throughput and sensitive next-generation droplet digital PCR assay for the quantitation of the hepatitis C virus mutation at core amino acid 70

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ABSTRACT

The next-generation droplet digital polymerase chain reaction (ddPCR) assay employs an emulsion-based endpoint to quantitate the amount of target DNA and is more robust than real-time PCR when analyzing sequence variations. However, no studies have applied this technique to quantitate mutations in polymorphic viral genomes. To develop this approach, a ddPCR-based assay was designed to quantitate with high-throughput and sensitivity mutations and their frequencies in codon 70 of the hepatitis C virus (HCV) gene that encodes the Core protein. The assay was linear from 2.5 to 10^5 copies per assay, and the limit of detection of mutants in the presence of a 20,000-fold excess of wild type was 0.005%. The results correlated well with those obtained using the COBAS® TaqMan® HCV Test, which is a real-time PCR assay for the quantitative detection of HCV RNA in human serum ($n = 87$; range, 2.3–7.7 $\log_{10}$ IU/mL; Pearson's $R^2 = 0.9120$; $p < 0.0001$). The median frequencies of mutations by ddPCR were 0.262% ($n = 55$; range, 0–37.951%) and 99.687% ($n = 32$; range, 52.191–100%) for the wild-type and mutant sequences, respectively, by direct sequencing. The ddPCR assay should be useful for quantitating mutations in other polymorphic viral genomes.

1. Introduction

The development of quantitative polymerase chain reaction (qPCR) technology has a rich and diverse history (Syvänen et al., 1988; Becker-andr and Hahlbrock, 1989; Gilliland et al., 1990; Porcher et al., 1992). Over the past few decades, the overwhelming majority of qPCR technology has relied on some version of real-time PCR using TaqMan® probe (Higuchi et al., 1993). However, the accuracy of real-time PCR is limited by relative quantitation using a standard curve, and this technique cannot detect less than 1% mutant DNA in a wild-type DNA background (Hindson et al., 2011). In 1999, digital PCR (dPCR) (Vogelstein and Kinzler, 1999) was developed for the absolute quantitation of target DNA. This technique does not require generation of a standard curve by the real-time PCR. Recently, new next-generation digital PCR-droplet PCR (ddPCR) has been developed, which is more precise than the real-time PCR (Morisset et al., 2013; Strain et al., 2013) as a high-throughput assay using conventional TaqMan assays with a 96-well plate format (Hindson et al., 2011).

On the other hand, the mutation rate of RNA viruses is high, ranging from 10^{-1} to 10^{-4} base substitutions per genome per year (Holland et al., 1982), because of the absence of proofreading enzymes that assure the fidelity of DNA replication. This high mutation frequency is coupled with high replication rates. The rates of viral genomic RNA mutation exceed those of chromosomal mutations of the host by a factor of one million. The ddPCR proved useful for the quantitative detection of single nucleotide polymorphisms in the human genome (BRAF-V600E, EGFR, and KRAS) (Hindson et al., 2011; Pekin et al., 2011; Hubers et al., 2013), but its application to quantification of highly polymorphic viral genome mutations has not been reported.

The hepatitis C virus (HCV) is an RNA virus of the family Flaviviridae, genus *Hepacivirus*, and it infects approximately 185 million people worldwide, with 3–4 million new infections annually (Thomas, 2013). Persistent HCV infection is usually clinically mild, but 20% of infections progress to severe chronic hepatitis and cirrhosis that occasionally culminate in hepatocellular carcinoma (HCC) (Niederau et al., 1998; Liang et al., 2000). More than

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350,000 people die each year from HCV-related liver failure and HCC (Kiyosawa et al., 1990; Yang and Roberts, 2010). The HCV genome consists of a 9.6-kb RNA with a large open reading frame that encodes a polyprotein of 3010 amino acids (Grakoui et al., 1993). Putative structural proteins, located in the N-terminal one-fourth of the polyprotein, include the capsid protein (Core protein), followed by two possible virion envelope proteins (E1 and E2) and nonstructural proteins (NS), including NS2, NS3, NS4A, NS4B, NS5A, and NS5B, which are required for RNA replication. A mutation in the sequence of amino acid 70, encoding the Core protein (Core a.a.70), is associated with the progression of liver disease and is an independent risk factor for the development of HCC (Miura et al., 2011, 2013; Akuta et al., 2012). Although the mutation can generally be detected by direct sequencing (Akuta et al., 2005), it is difficult to determine the presence of substitutions when the sequencing data are superimposed. This has hindered attempts to elucidate the relationship between the progression of liver disease and mutations in the HCV genome. Ultra-deep pyrosequencing (UDPS) results show that 89.9% hepatitis C viruses harbor mutations in the genomes (Miura et al., 2013). However, performing UDPS analysis requires skill, considerable effort, and does not generate sufficiently reproducible and accurate data (Noguchi et al., 2006; Hoff, 2009; Glenn, 2011).

As mentioned earlier, ddPCR can be used for the quantitative detection of single nucleotide polymorphisms in the human genome (Hindson et al., 2011; Pekin et al., 2011; Hubers et al., 2013). However, its application for the quantitation of mutations in highly polymorphic viral genomes (such as those of HIV and HCV) has not been reported. Therefore, the goal of the present study was to design a novel ddPCR assay to quantitate mutations in HCV Core a.a.70.

2. Materials and methods

2.1. Sample collection

From May 2011 through May 2013, serum samples were obtained from 87 to 69 patients infected with HCV-1b. The patients were from a hospital in Japan, which was located within the same premises as our participating institutions. The serum samples were stored at −80 °C for further use. All patients were positive for antibodies against HCV antigen (anti-HCV) and for HCV RNA. To confirm false positives in the ddPCR assay, twenty clones in 32 clinical samples were picked up and analyzed the frequency of aa70 mutations by direct sequencing (Supplementary data 1), and seven samples, which were HCV RNA negative using the COBAS TaqMan® HCV Test, were used (Supplementary data 2). Two different sample groups were used in the assay to alleviate bias. The first group of 87 samples (38% were obtained from men; mean age, 67.0 ± 12.28 years) was used for design purposes, and the other group of 69 samples (Supplementary data 3; 34% were obtained from men; mean age, 54.5 ± 10.13 years) was used for validation purposes. The ethics committees of the participating institutions approved the study protocol, which conforms to the 2008 Declaration of Helsinki. Written informed consent was obtained from each patient.

2.2. RNA extraction and cDNA synthesis

Total nucleic acids from clinical samples were isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit-High Performance (Roche Diagnostics, Tokyo, Japan) on a MagNA Pure LC 2.0 instrument (Roche Diagnostics), according to the external lysis protocol provided by the manufacturer (Edelmann et al., 2013). HCV RNA was extracted from 0.5 mL of serum and eluted in 0.05 mL of elution buffer included in the kit. cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random primers, following the manufacturer's protocol.

2.3. Detection of mutations in Core a.a.70 using direct nucleotide sequence analysis

The Core gene was amplified using nested PCR and sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit and Genetic Analyzer 3130xl (Life Technologies, Tokyo, Japan). The Core a.a.70 substitutions were identified as described by Akuta et al. (2005).

2.4. Production of a plasmid standard

The amplicons containing the region encompassing wild-type and mutant Core a.a.70 sequences (nucleotide position: 330–684 bp) were cloned into the pCR4-TOPO vector (Life Technologies, Japan), and the identities of eight clones were verified by nucleotide sequencing. The concentration of plasmid DNA was measured using a Nanoview Spectrophotometer (GE Healthcare, Baie d'Urfe, Quebec, Canada), and optical density (OD) at 260 nm and the ratio of ODs at 260/280 nm, respectively, were used to determine the quantity and purity of plasmid DNA. The initial copy number of standard plasmids per microliter was calculated using a DNA copy number calculator (Thermo Scientific, 2013). Serial dilutions of plasmid DNA were prepared in Easy Dilution Solution (Takara Bio, Otsu, Japan).

2.5. Analysis of Core a.a.70 and assay design

Direct nucleotide sequence analysis of the 87 patients with HCV infection was performed to determine mutation patterns in and around the target Core a.a.70 site. Throughout this article, the nucleotide positions of the amino acids were numbered according to the full-length genome sequence of HCV genotype 1b strain NC1 (GenBank accession number AB691953.1). Different complex patterns of mutations were revealed; however, polymorphisms within 13 nucleotides (544–556 bp) up- or downstream of the target codon were limited to seven patterns. Bases presented in italics indicate mutations and those underlined indicate the codon of Core a.a.70 (Table 1). The Mutant type changed from arginine to glutamine (pattern 3 and 4) or histidine (pattern 7) at position 70 of amino acids encoding the HCV Core protein. The sequence of Core a.a.70 obtained from 84 of the 87 patients (96.55%) included the top 1–4 nucleic acid sequence patterns. These common sequences were confirmed in 69 patients with HCV infection (Supplementary data 3). Real-time PCR primers were designed according to the conserved sequences using DNA database (Hepatitis Virus DataBase Server, http://s2as02.genes.nig.ac.jp/) and PrimerExpress 3.0 software (Applied Biosystems, Foster City, CA, USA) as follows: sense, 5′-CGT GGA AGG CGR CAA CCT AT-3′ (516–535 bp); antisense, 5′-CRC GGG GKG ACA GGA GCC A-3′ (645–627 bp). Fluorescence resonance energy transfer probe sequences were designed according to the sequence patterns (Table 1 and Supplemental data 3) as follows: 5′-VIC-CTCGCCGRCCCGA-MGB-3′ (wild-type probe: 544–556 bp) for detecting the wild-type sequences and 5′-FAM-CTCGCCARCCCGA-MGB-3′ (mutant probe: 544–556 bp) for detecting the mutant sequences.

2.6. ddPCR

The QX100™ Droplet Digital™ PCR System (BioRad, Hercules, CA, USA) was used according to the manufacturer's instructions. The reaction mixtures for ddPCR contained the following components: 10 μL of ddPCR Master Mix (Bio-Rad, Pleasanton, CA, USA), 3 μL of primers (final concentration, 900 nM each), 2 μL of probes

Table 1
Frequency of sequence patterns of 13 bp nucleotides up–downstream of the target HCV Core a.a.70.

Pattern	Sequences (544–556 bp)*	Core a.a.70	Core a.a.70-type	Frequency
1	5′-ctcgc cgg cccga-3′	Arginine	Wild-type	45/87; 51.71%
2	5′-ctcgc cg*a* cccga-3′	Arginine	Wild-type	8/87; 9.20%
3	5′-ctcgc c*ag* cccga-3′	Glutamine	Mutant-type	25/87; 28.74%
4	5′-ctcgc c*aa* cccga-3′	Glutamine	Mutant-type	6/87; 6.90%
5	5′-Ytcgc cgg cccga-3′	Arginine	Wild-type	1/87; 1.15%
6	5′-ctcgc c*gt* cccga-3′	Arginine	Wild-type	1/87; 1.15%
7	5′-ctcgc c*at* cccga-3′	Histidine	Mutant-type	1/87; 1.15%

Direct nucleotide sequence analysis of the 87 patients with HCV was performed to determine mutation patterns. The letters in bold italics indicate the mutation in the target sequence to the probes, which were substitution to pattern 1 (51.71%; the most major sequence pattern in this study), and the underlines indicate three bases of the core a.a. 70 codon.

* The nucleotide position (bp) was numbered according to the full-length genome sequence of HCV genotype 1b strain NC1, GenBank accession number AB691953.1. *Y*: *c*+*t*.

(final concentration, 500 nM each), and 5 μL of cDNA. Droplets were generated using the droplet generator (DG) with 70 μL of DG oil per well with a DG8 cartridge and cartridge holder, 20 μL of PCR reaction mix, and a DG8 gasket. Droplets were dispensed into each well of a 96-well PCR plate by aspirating 40 μL from the DG8 cartridge. The PCR plate was subsequently heat-sealed with foil and placed in a PCR thermal cycler Mastercycler® Nexus gradient (Eppendorf AG, Hamburg, Germany). The cycling conditions were as follows: an initial denaturation cycle of 10 min at 95 °C, followed by 45 cycles of denaturation for 30 s at 94 °C, annealing for 2 min at 49 °C, extension for 1 min at 72 °C, and a final incubation for 10 min at 98 °C. After cycling, the droplets were immediately analyzed or stored at 4 °C, and the PCR plates were transferred to the QX100 Droplet Reader (Bio-Rad). The ddPCR workflow and data analysis were performed as described previously (Pinheiro et al., 2012). Positive and negative amplicon-containing droplets (accepted droplets) were discriminated using the fluorescence amplitude threshold, as determined with QuantaSoft software (Bio-Rad). The frequency (%) of mutations in Core a.a.70 was determined by calculating the ratio of mutant versus wild-type + mutant copies.

2.7. HCV RNA quantitation

HCV RNA values were quantitated using the COBAS TaqMan® HCV Test (Roche Diagnostics).

2.8. Quality control

To avoid generating false positives, general guidelines published online (Roche-PCR-Chapters, 2009) were applied. The procedure included the physical separation of areas for extraction, PCR setup and amplification/detection, and use of filtered tips. To monitor contamination, one sample of PBS for every 32 samples was processed and extracted. In addition, a template control (water) was added to the PCR plate. Over the course of the study, no false positives were detected.

2.9. Statistical analysis

The linear range of the ddPCR assay was determined by plotting the data and comparing them with a line of the same slope. Pearson's correlation coefficients were calculated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), and linear regression analyses were performed and displayed as scatter plots using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Mutations in Core a.a.70 determined by direct nucleotide sequencing

Wild-type and mutant sequences were detected in 55 and 32 samples, respectively (Table 1). The elution profiles for five samples indicated the presence of more than one sequence (Fig. 1). Even after seven analyses, the samples represented two mutant strains and two wild-type strains; however, it was difficult to determine whether sample 1 was a mutant. This sample was eventually determined as a mutant by direct sequences. UDPS analysis of the viral genomic region that encodes the HCV Core protein in clinical samples recently showed a mixture of mutant and wild-type sequences in Core a.a.70 in 71 of 79 (89.9%) cases, and the ratio of mixtures increased as liver disease advanced to liver cirrhosis and HCC (Miura et al., 2013). Therefore, it was considered necessary to develop a more sensitive assay for the quantitation of mutations in Core a.a.70 to predict the progression of liver disease.

3.2. Optimization of the ddPCR assay

It was essential to determine the optimum annealing temperature for the ddPCR assay to detect mutations in Core a.a.70. Therefore, plasmid-DNA (100,000 copies/well) was annealed at the following temperatures: 43.0, 43.5, 44.6, 46.5, 48.9, 51.3, 53.7, 56.1, 58.5, 60.4, 61.5, and 62.0 °C. The amplitudes of FAM (mutant, Fig. 2A) and VIC (wild type, Fig. 2B) signals are displayed as rain plots. In this example, the chosen annealing temperature was 49 °C.

3.3. Specificity

The specificity of the assay was determined using samples containing wild-type (160,000 copies/well) or mutant plasmids (160,000 copies/well). Subsequent experiments were conducted at 49 °C for 45 cycles (Fig. 2C and D). No positive droplet was detected when using FAM in the presence of wild type plasmid, and when using VIC in the presence of mutant plasmid For wild-type and mutant plasmid (n = 4; FAM: Fig. 2C and VIC: Fig. 2D, rain plot). The rate of false positives was 0% for both mutant and wild-type plasmids.

3.4. Sensitivity and linearity of detection

The ddPCR assay was used to determine the relative ratios of mutant and wild-type sequences in mixed infections. Linearity and precision of the ddPCR assay were in agreement over a

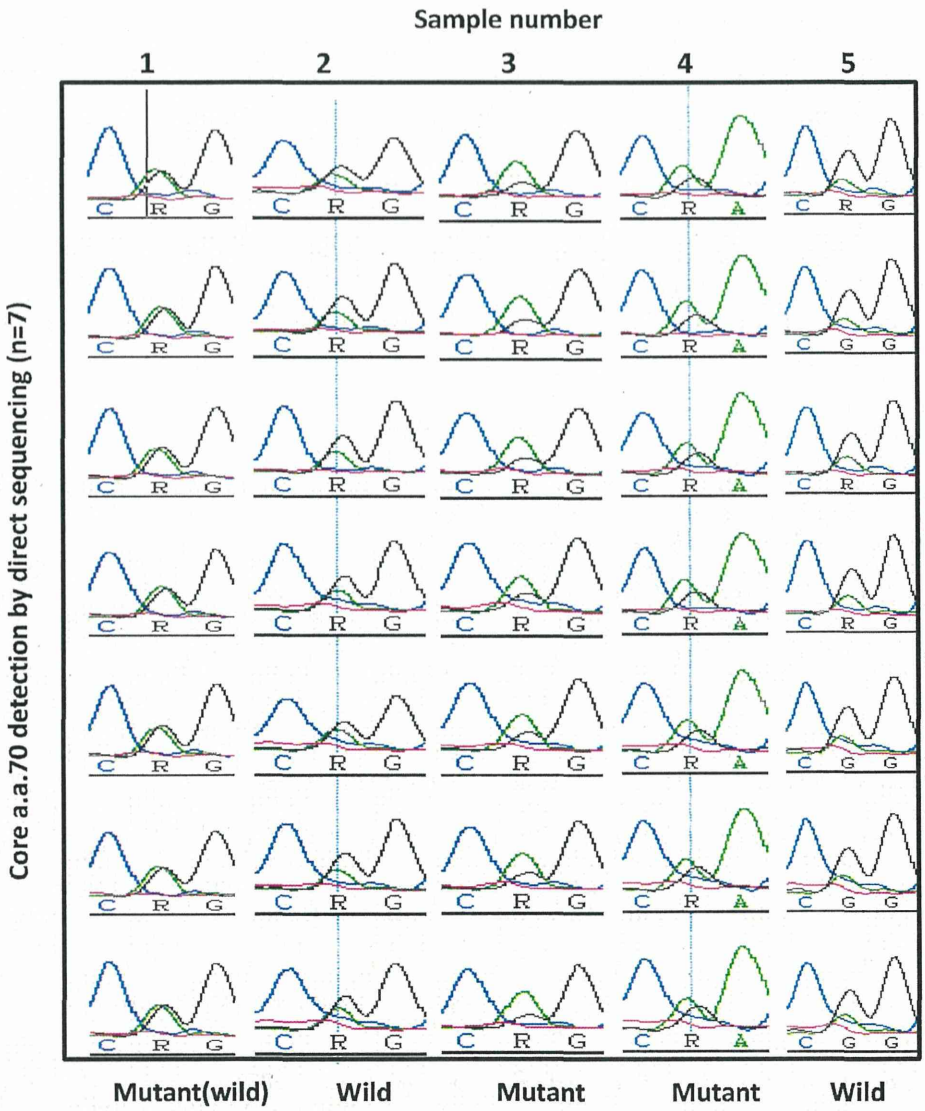

Fig. 1. The direct sequencing results are demonstrated by nucleotide sequence electropherograms. The HCV Core protein gene was amplified using nested PCR and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit. Core a.a.70 substitutions were analyzed as described by Akuta et al. (2005). Samples from 87 patients were analyzed. The elution profiles for five samples indicated the presence of more than one sequence. Even after repeating the analysis seven times, the samples represented two mutant sequences and two wild-type sequences; however, it was difficult to distinguish whether sample 1 was mutant.

range of 0–100% of mutant plasmids mixed with wild-type plasmids (Pearson's $R^2 = 0.99$, $p < 0.0001$) (Fig. 3A). When the mutant and wild-type plasmids were mixed in various ratios, 0.005% of the mutants were detected (Fig. 3B) in the presence of 100,000 copies of wild-type plasmid DNA per assay. The assay was linear when the proportion of the mutant plasmid was 0.005%–10% of the entire plasmid population (Pearson's $R^2 = 0.999$, $p < 0.0001$, Fig. 3B). The mutant plasmid was undetectable when present as less than 0.0025% of the total DNA. The observed and expected results were correlated using HCV standard plasmids (1.25, 2.5, 5, 10, 100, 1000, 10,000, 100,000, 160,000, and 180,000 copies) (Fig. 3C and D). The ddPCR results agreed with the calculated values and were in accordance with acceptance of trueness. The dashed lines perpendicular to the *x*- and *y*-axes indicate concentrations below the limit of detection (LOD; 2.5 copies/well) and the highest number of copies (100,000) tested. Furthermore, the assay was linear (Pearson's $R^2 = 0.999$, $p < 0.0001$) above LOD.

3.5. Repeatability (intra-assay variation) and reproducibility (inter-assay variation) of ddPCR

The repeatability and reproducibility (Table 2) of the ddPCR assay was determined by performing replicate measurements of 12 different samples (genotype 1b; 0.05–99.88% mutant DNA). Intra- and inter-assay variabilities were assessed. Sufficient volume for three aliquots of each sample was stored at 4 °C until use. For each sample, three replicates were tested in the same assay to evaluate intra-assay repeatability. Inter-assay reproducibility was evaluated by testing two additional aliquots, each on a separate day. The intra-assay averages ± standard deviation (SD) of the log number of copies/mL of the mutant, wild type, mutant rare %, and accepted droplets, respectively, were as follows: 4.36 ± 0.03, 5.32 ± 0.03, 26.41 ± 0.09, and 13,008 ± 769. The inter-assay averages ± SD were as follows: 4.36 ± 0.03, 5.32 ± 0.04, 26.43 ± 0.14, and 11,725 ± 1908, respectively. These results also suggested that the repeatability (intra-assay) variability of the assay was good, as

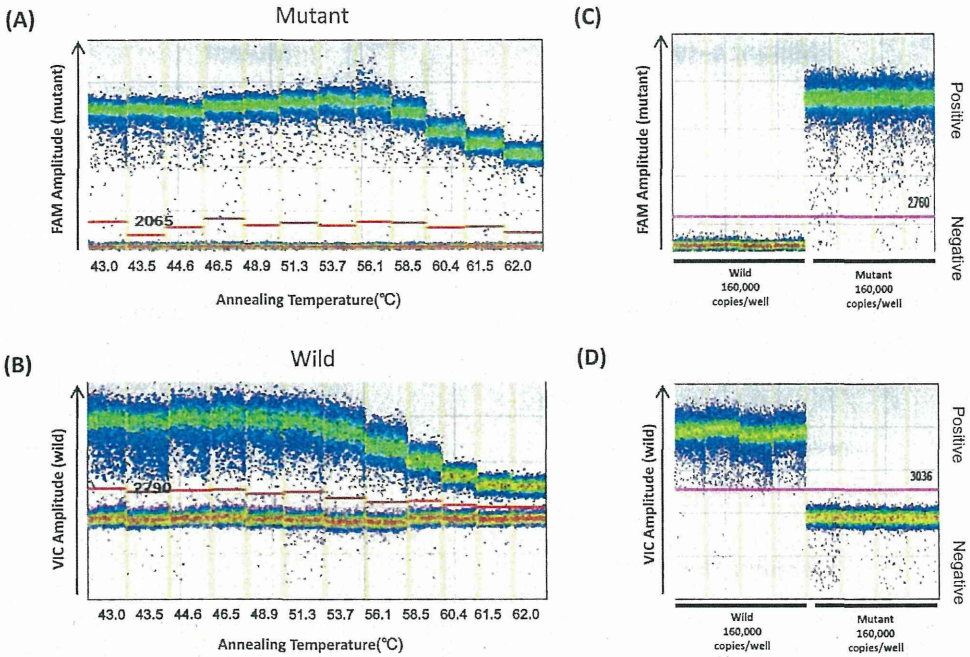

Fig. 2. Fluorescence amplitude plotted against annealing temperature. The assay was conducted across an annealing temperature gradient: 43.0, 43.5, 44.6, 46.5, 48.9, 51.3, 53.7, 56.1, 58.5, 60.4, 61.5, and 62.0 °C) (A and B). (A) FAM fluorescence amplitude of mutant plasmids (100,000 copies/well). (B) VIC fluorescence amplitude for wild-type plasmids (100,000 copies/well). (C) FAM fluorescence amplitude for mutant and wild-type plasmids (160,000 copies/each well). (D) VIC fluorescence amplitude for mutant and wild-type plasmids (160,000 copies/each well). The horizontal line indicates the threshold.

little variation was observed when samples were tested for reproducibility (inter-assay).

3.6. Correlation with the COBAS TaqMan® HCV RNA Test

The commercially available COBAS TaqMan® HCV RNA Test was used to confirm the accuracy of the quantitative results obtained using the ddPCR assay. The data generated by the analysis of clinical samples using the COBAS TaqMan® HCV Test were closely correlated with those of the ddPCR assay ($n = 87$, $p < 0.0001$, $y = 1.017x - 0.3111$, Pearson's $R^2 = 0.9120$, Fig. 4A). The arrows indicate a single point mutation, which was detected in 3 (3.45%) of the 87 samples (pattern 5, CTCGCCATCCCGA; pattern 6, YTCGCCGGCCCGA; and pattern7, CTCGCCGTCCCGA; Table 1). The data for the COBAS TaqMan® HCV Test and ddPCR assay were correlated for all samples, including each of the three samples. In addition, the results of quantitation of wild-type and mutant sequences obtained using ddPCR were compared with those obtained using the COBAS TaqMan® HCV Test. However, the data did not correlate for all quantified data of the mutant and wild-type sequences (Fig. 4B, $y = 0.4594x + 3.801$, Pearson's $R^2 = 0.4112$, $p < 0.0001$ and Fig. 4C, $y = 0.4584x + 4.076$, Pearson's $R^2 = 0.3673$, $p < 0.0001$, respectively). These data suggested that the novel assay could not only measure the frequency of mutations in Core a.a.70 but was also useful for the quantitation of mutations in the target gene in the polymorphic viral genome.

3.7. Correlation with direct sequencing

The results of ddPCR and direct sequencing were compared (Fig. 5). Direct nucleotide sequencing of the 87 patients with HCV infection was performed to determine mutation patterns in Core a.a.70. Wild-type and mutant sequences were found in 55 (63.2%) and 32 (36.8%) samples by direct sequencing, respectively. However, the ddPCR assay detected mutations in 85 (97.7%) of the 87 samples. The median frequencies were 0.262% ($n = 55$; range, 0–37.951%) and 99.687% ($n = 32$; range, 52.191–100%) for the wild-type and mutant sequences, respectively, by direct sequencing. These data indicated that mutations in Core a.a.70 existed in the wild-type sequences, although the frequencies were low.

3.8. Correlation with cloning

To address false positives, twenty clones in 32 clinical samples were picked up and analyzed the frequency of aa70 mutations by direct sequencing (Supplementary data 1). There are significantly correlated in the mutations between ddPCR and direct sequencing (Pearson's $R^2 = 0.975$, $p < 0.0001$). The three samples (nos. 1–3) showed less than 1% of the mutants by ddPCR. However, direct sequencing failed to detect mutant clones because of the differences in sensitivity and principles between the two methods. The error rates are thought to be reduced by removing the amplification efficiency reliance of qPCR and the data in Table 2 confirmed reproducibility. Further, no mutations were detected in HCV RNA negative samples from seven healthy patients (Supplementary data 2).

3.9. Validation analysis

Sixty-nine HCV-positive samples were prepared as validation sets. All mutations detected by direct sequencing were also detected by the ddPCR assay as first set measurements. To confirm whether the four patterns (nos. 1–4) of the seven probes in Table 1 were common among other HCV samples. The top 1–4 sequence patterns in Table 1 were found in 65 (94.2%) of the 69 patients. Therefore, the four sequences probes were common in HCV and sufficient to use for ddPCR (Supplementary data 3).

4. Discussion

In this paper, the development of a next-generation ddPCR assay for high-throughput, sensitive, and accurate quantitative

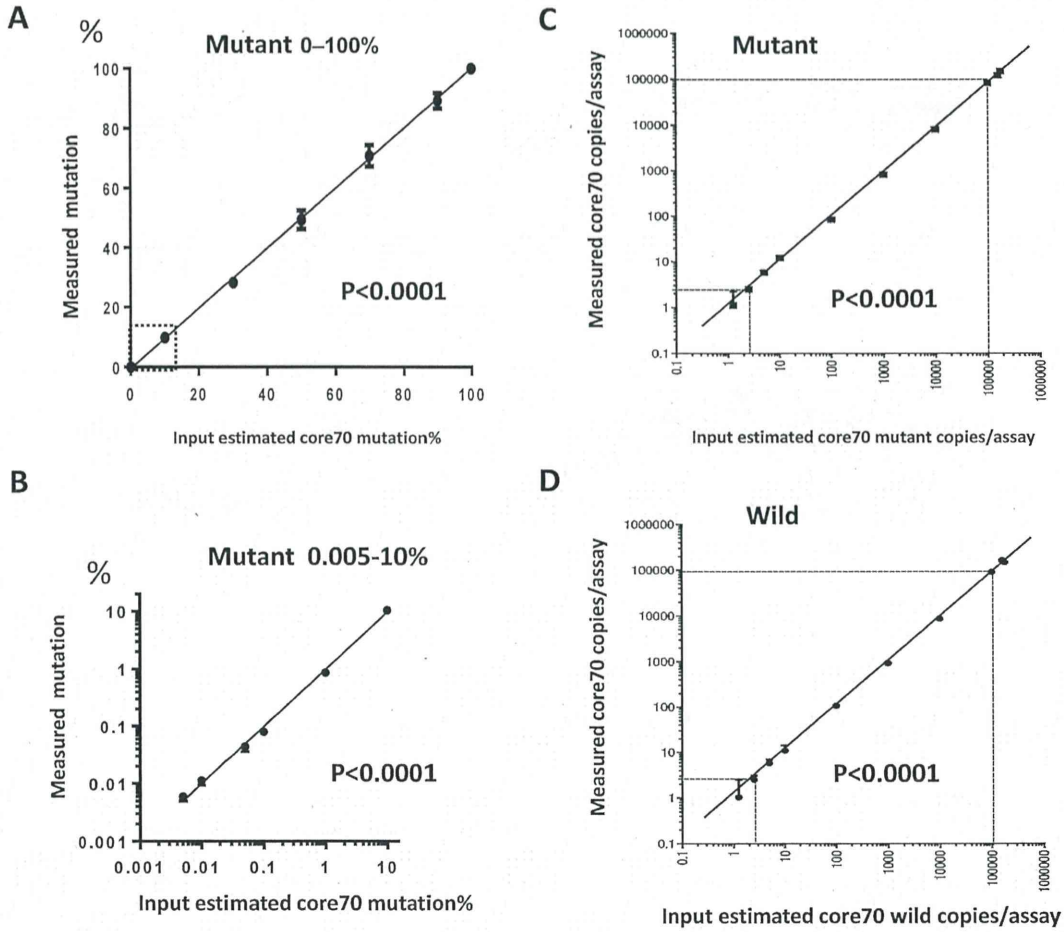

Fig. 3. Values are plotted against the estimated frequency (%) of the mutation in Core a.a.70. Error bars denote SD of the mutant ratio determined using ddPCR for each of five independent gravimetric dilutions. The number of plasmid DNA copies was 100,000 (A and B). (A) Plasmid DNA was mixed to 0, 10, 25, 50, 75, 90, and 100% of mutation (The square in A shows B). (B) Plasmid DNA was mixed so that the mutant sequence represented 0.0025%, 0.005%, 0.01%, 0.1%, 1.0%, and 10% of the population. The correlation between observed and expected results using HCV standard plasmids (1.25–180,000 copies/assay) was tested using the ddPCR assay (C and D). The dashed line indicates concentrations below LOD (2.5 copies/well) up to 100,000 copies (C and D). The assay was linear above LOD. (C) Mutant plasmid. (D) Wild-type plasmid.

detection of mutations in codon 70 of the HCV Core protein gene was described. This present study is the first to report that ddPCR is a powerful technique for the ultrasensitive and quantitative detection of single nucleotide polymorphisms within viral genomes. Furthermore, an important benefit of the ddPCR assay is that it can precisely quantitate the total amount of HCV RNA and determine whether mutations are present around the target point mutation of probes (Table 1, Fig. 4A).

The correlations between theoretical and experimental values were determined. To increase sensitivity and reproducibility, the novel assay employs a high-throughput automated instrument for preparing nucleic acids (Wenzel et al., 2010). Likewise, contamination, sensitivity, and reliability are key factors of any assay designed to detect mutations. The automated system incorporated an ultraviolet light decontamination system.

To clarify the effectiveness of the ddPCR assay, 87 clinical samples from patients with HCV infection were used for design purposes, and were analyzed for mutations in Core a.a.70 by direct sequencing and the ddPCR assay. All mutations detected by direct sequencing were also detected by the ddPCR assay. Moreover, the ddPCR assay detected mutant sequences present at extremely low frequencies. In particular, 32 (36.8%) of the 87 samples were identified as mutants for Core a.a. 70 by direct sequencing, while 85 (97.70%) of the 87 samples were identified as mutants by the ddPCR assay. Therefore, the ddPCR assay showed strong potential for highly precise quantitation of mutant HCV sequences in serum samples (CV%, intra-assay, 0.07–1.76; inter-assay, 0.04–1.76; Table 2). In intra- and inter-assays, CV% of a commercial HCV RNA quantitative assay (COBAS TaqMan®) ranged between 2.8% and 47.0% and 6.9–40.0%, respectively (Bossler et al., 2011). These results showed that the ddPCR assay is potentially more precise than the COBAS TaqMan® HCV Test, in accordance with the results of a recent report (Strain et al., 2013).

The HCV Core protein has oncogenic potential in transgenic mice and modifies the formation of reactive oxygen species (Korenaga et al., 2005; Moriya et al., 1998). Mutation in Core a.a.70 is an important primary factor that influences antiviral treatment efficacy, as well as the progression of liver disease and hepatocarcinogenesis (Okanoue et al., 2009; Akuta et al., 2012; Miura et al., 2013). However, the functional relevance of amino acid residue substitutions in the pathogenesis of hepatitis C is unknown. Therefore, it is important to develop more precise quantitative assays for the identification of mutants in order to understand the role of Core a.a.70. Currently available techniques, which are direct sequencing methods, are not sufficiently sensitive to detect mutations. Other methods, such as the TaqMan® amplification refractory mutation system and the Invader assay, have increased sensitivity to 1%. However, mutants are often present below this level, and LOD of

Table 2
Repeatability (intra-assay variation) and reproducibility (inter-assay variation) of ddPCR.

	No.	Mutant (log copies/mL)			Wild (log copies/mL)			Mutant (%)			Accepted droplets		
		Average	SD[1]	CV %[2]	Average	SD[1]	CV %[2]	Average	SD[1]	CV %[2]	Average	SD[1]	CV %[2]
Intra	1	3.53	0.06	1.76	6.92	0.01	0.16	0.05	0.01	13.02	14,167	866	6.11
	2	3.26	0.05	1.48	3.06	0.01	0.12	0.17	0.02	1051	1.1702	486	4.15
	3	3.04	0.01	0.47	5.34	0.01	0.09	0.27	0.01	4.39	14,049	1057	7.52
	4	3.53	0.05	1.59	5.75	0.07	1.27	0.59	0.02	3.89	14,064	501	3.53
	5	3.80	0.05	1.20	5.95	0.02	0.21	0.73	0.06	8.51	13,414	921	6.87
	5	3.90	0.01	0.35	5.81	0.16	6.06	1.32	0.03	2.80	13,644	1004	7.33
	7	4.71	0.02	0.49	8.08	0.01	0.21	4.42	0.22	5.32	12,776	481	3.76
	8	4.49	0.04	0.81	5.79	0.01	0.17	4.90	0.36	6.89	10,749	683	3.35
	9	4.69	0.01	0.16	5.94	0.01	0.12	5.65	0.11	1.73	13,792	925	6.70
	10	6.22	0.01	0.21	4.00	0.01	0.14	99.39	0.09	0.09	10,117	1209	11.95
	11	5.08	0.01	0.20	2.57	0.01	0.11	99.88	0.10	0.10	14,320	677	4.73
	12	6.02	0.00	0.07	3.38	0.05	1.47	9958	0.02	0.02	13,297	424	3.19
Total		4.36	0.03	0.73	5.32	0.03	0.84	26.41	0.09	4.78	13,008	769	6.02
Max		6.22	0.06	1.76	6.92	0.18	6.03	99.88	0.36	13.02	14,320	1209	11.95
Min		3.04	0.00	0.07	2.57	0.01	0.09	0.05	0.01	0.02	10,117	424	3.19
Inter	1	3.53	0.06	1.76	6.87	0.07	1.00	0.05	0.05	6.40	13,676	1820	13.30
	2	3.27	0.01	0.42	6.04	0.02	0.41	0.17	0.00	2.24	10,205	2116	20.74
	3	3.06	0.03	0.92	5.62	0.02	0.30	0.27	0.01	2.88	13,409	905	6.75
	4	3.52	0.02	0.70	5.75	0.02	0.26	0.59	0.01	1.95	12,041	2862	23.77
	5	3.81	0.02	0.60	5.92	0.04	0.60	0.80	0.09	11.90	12,157	1778	14.63
	6	3.87	0.04	111	5.79	0.03	0.54	1.18	0.20	16.74	11,509	3019	26.23
	7	4.71	0.00	0.04	6.07	0.02	0.37	4.28	0.22	5.27	10,263	3555	34.64
	8	4.51	0.03	0.71	5.78	0.01	0.15	5.08	0.26	5.21	9985	1081	10.83
	9	4.73	0.05	1.08	5.93	0.01	0.25	5.99	0.48	7.93	12,460	1884	15.12
	10	6.19	0.05	0.77	4.00	0.01	0.26	9955	0.06	0.06	9722	559	5.75
	11	5.09	0.01	0.18	2.62	0.08	3.09	99.65	0.07	0.07	13,258	1503	11.33
	12	6.06	0.06	1.04	3.50	0.21	5.88	99.70	0.17	0.17	12,013	1816	15.11
Total		4.36	0.03	0.78	5.32	0.04	1.09	26.42	0.14	5.05	11,725	1908	16.52
Max		6.19	0.06	1.76	6.87	0.21	5.88	99.70	0.48	16.74	13,373	3555	34.64
Min		3.06	0.00	0.04	2.32	0.01	0.15	0.05	0.00	0.06	9722	559	5.75

[1] Standard deviation.
[2] Coefficient of variation.

the ddPCR assay was 200-fold greater than that of the TaqMan® and Invader® assays (Nakamoto et al., 2009; Tadokoro et al., 2013). Another system, which was mutation-specific PCR, for detecting variants is not quantitative (Okamoto et al., 2007). Moreover, microfluidic chamber-based digital PCR was commercially available (Whale et al., 2013), however, it is expensive and the number of technical replicates that can be analyzed is limited.

UDPS techniques are prone to errors in base calling, alignment, and sequence data assembly (Nielsen et al., 2011). The error rate of UDPS techniques introduced by polymerase ranges from 0.01% to 1% (Noguchi et al., 2006), depending on the software used (Hoff, 2009), and UDPS has a higher error frequency than direct sequencing (Shendure and Ji, 2008). In addition, the sensitivity of the relative quantitative ddPCR assay was improved by 18.4-fold compared with a previously reported UDPS assay (Miura et al., 2013). Therefore, ddPCR was selected for the relative and absolute quantitative detection of mutations.

The ddPCR assay is ultrasensitive and offers high-throughput analysis, and therefore presents a potentially new assay to identify biomarkers of HCV-induced liver disease. Unfortunately, no report has yet provided statistical analysis of the relationship between the progression of liver disease and the rate of mutation in Core a.a.70. However, a new cut-off frequency of mutants to predict the prognosis of HCV-induced liver disease may be useful in future clinical studies.

The results of this study indicated that the ddPCR assay can be used for analysis of HCV mutations, although total quantitation of hepatitis viral sequences has not been reported. In general, the ddPCR assay performed well to identify single nucleotide polymorphisms in the human genome. The mutation rate of RNA viruses is higher than that of human genome (Holland et al., 1982). The ddPCR assay was thought to be unsuitable for the quantitation of

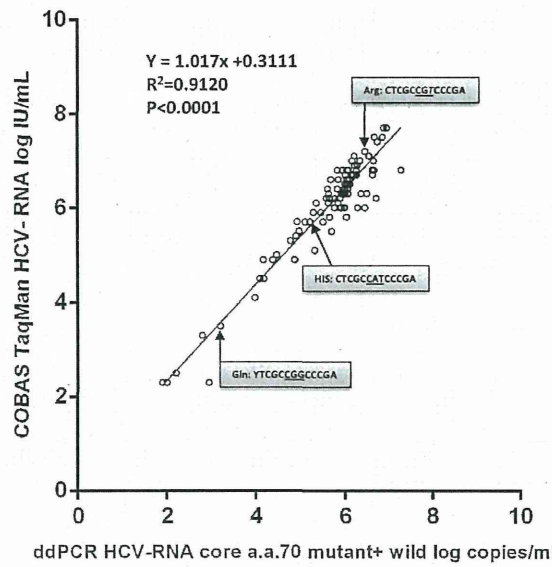

Fig. 4. Correlation between the results of 87 clinical specimens analyzed using the COBAS TaqMan® HCV Test and ddPCR assay combined with sample processing using a MagNA Pure LC instrument ($y = 1.017x + 0.3111$, Pearson's $R^2 = 0.9120$, $p < 0.0001$). Arrows point to the correlation of the results of the COBAS TaqMan® Test and ddPCR assay of three samples with a single point mutation complementary to the probe. The italic letters of nucleic acid sequence show the mutations and the underlines indicate at the codon of Core a.a. Total HCV RNA (mutant + wild-type copies/mL) was determined using the ddPCR assay (horizontal line) and COBAS TaqMan® HCV Test (vertical line).