ORIGINAL ARTICLE

Degenerate polymerase chain reaction strategy with DNA microarray for detection of multiple and various subtypes of virus during blood screening

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BACKGROUND: The risk of transferring blood-borne infections during transfusion is continually increasing because of newly emerging and reemerging viruses. Development of a rapid screening method for emerging viruses that might be transmitted by transfusion is required to eliminate such pathogens during blood donor screening. Owing to increased use of human materials in organ transplants and cell therapy, the risk of donor-transmitted viral infections is also increasing. Although nucleic acid amplification technology (NAT) is dedicated to blood screening, a small, convenient detection system is needed at the laboratory and hospital level.

STUDY DESIGN AND METHODS: We developed a new pathogen detection system that can detect multiple viruses simultaneously, using originally designed degenerate polymerase chain reaction primers to amplify a wide range of viral genotypes. Amplified samples were identified using a DNA microarray of pathogen-specific probes.

RESULTS: We detected very low copy numbers of multiple subtypes of viruses, such as human hepatitis C virus (HCV), human hepatitis B virus (HBV), human parvovirus B19 (PVB19), and West Nile virus (WNV), using a single plate. We also detected all genotypes of human immunodeficiency virus (HIV) but sensitivity was less than for the other viruses.

CONCLUSION: We developed a microarray assay using novel primers for detection of a wide range of multiple pathogens and subtypes. Our NAT system was accurate and reliable for detection of HIV, HBV, HCV, PVB19, and WNV, with respect to specificity, sensitivity, and genotype inclusivity. Our system could be customized and extended for emerging pathogens and is suitable as a future NAT system.

Quality and safety in blood products are major public health concerns. In addition to general quality control (QC) testing, introduction of good manufacturing practice and routine screening of blood material and products have assured consistency and quality in production and increased blood transfusion safety in recent decades. Newly developed serologic tests and nucleic acid technology (NAT) have markedly reduced the risk of transmitting human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) from infected blood.[1] Currently, several Food and Drug Administration (FDA)-licensed NAT assays are available to screen blood donors for HIV, HCV, HBV, and West Nile virus (WNV). However,

ABBREVIATIONS: DLC-chip = diamond-like carbon-coating microarray chip; dPCR = degenerate polymerase chain reaction; IC = internal control; NIBSC = National Institute for Biological Standards and Control; OE-PCR = overlap-extension polymerase chain reaction; PVB19 = parvovirus B19; TMA = transcription-mediated amplification; WNV = West Nile virus.

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TRANSFUSION **;**:**-**.

TABLE 1. Pathogen-specific dPCR primers for microarray detection*

Pathogens	Primer sequence (5′-3′)	GenBank Accession Number	Position (NT)	Gene name	Amplicons (bp)
HIV	RARAGGGGGGATTGGGGGGTA	NC_001802	4336-4356	Integrase	129
	YTGTCYCTGWAATAAACCCGA		4444-4464		
HCV	GAAAGCGYCTAGCCATGGCGT	D90208	59-327	5′-UTR	269
	TGCACGGTCTACGAGACCTCC		307-327		
HBV	AYTAYCAAGGTATGTTGCCCG	X70185	450-470	S	266
	GGAAAGCCCKRCGMACCACTG		695-715		
PVB19	AGTGGTGGTGAAAGCTCTGAA	NC_000883	2148-2168	NS1	122
	TCTCCTGAACTGGTCCCG		2252-2269		
WNV	GGHTGTTGGTATGGNATGGA	NC_009942	3451-3590	NS1	141
	CTCCTGGGTGRCCAAGAAC		3573-3591	NS2A	
IC	TCGAAGACGATCAGATACCGT	M10098	1147-1157	18S rRNA	129
	ATACTCCCCCCGGAACC		1259-1275		

* Code base description: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; B, C/G/T; N, A/T/C/G.

the continuous development of a highly sensitive screening system is a challenging task for NAT. The focus is mainly on assay sensitivity rather than the range and diversity of viral species detected; therefore, the current NAT systems only ensure detection of a restricted range of viruses and their subtypes and not newly diverged, emerging, or reemerging viruses.[2] It has been reported that the NAT sensitivity for HCV detection differs slightly in relation to virus subtype.[3] Recent advances in organ transplantation and cell therapy have also increased the risk of donor-transmitted viral infections, such as cytomegalovirus, Epstein–Barr virus, WNV, and lymphocytic choriomeningitis virus.[4] Further development of multiple virus detection systems is required to increase coverage of a range of virus strains and subtypes. We have experienced pandemics, such as WNV in the United States in 2003 and the chikungunya virus on Reunion Island in 2006; thus, there is a need to develop a rapid virus detection system that uses a more flexible blood-testing platform and meets the safety requirements for transfusion.

HBV is one of the most geographically widespread viruses and is subdivided into eight main genotypes (A–H),[5,6] causing liver cirrhosis and hepatocellular carcinoma. Although most infectious blood units are removed by screening for hepatitis B surface antigen (HBsAg), there is clear evidence that transmission by HBsAg-negative components occurs during the serologically negative window period and late stages of infection.[7] In addition to the window period of infection, HBV blood screening is required to detect all virus genotypes. Similar to HBV, several false-negative results in minipool NAT screening were reported after the introduction of WNV NAT because of the low viral load. Moreover, WNV continues to diverge rapidly from the originally isolated strain.[8,9] Multiplex NAT assays have become the modern method for detecting several viruses, and in conjunction with automated systems, they have the potential to improve processes that ensure blood safety. Candotti and colleagues[10] have reported the feasibility of a multiplex real-time quantitative reverse-transcriptase polymerase chain reaction (PCR) for HBV, HCV, and HIV-1, suggesting that simultaneous amplification of multiple pathogens is an effective approach for improving pathogen detection methods. The flexibility provided by multiplex PCR is limited, however, because the PCR primers are designed in commonly preserved regions of the viral genomes. To increase the detectable range for multiple pathogens, PCR using degenerate primers has been developed. Recently, bioinformatics has strongly improved the design of degenerate primers, allowing the coverage of a wide range of virus subtypes. We have developed a new method for designing degenerate primers.[11] Here, we used the diamond-like carbon-coating microarray chip (DLC-chip) to reduce background noise and increase the detection sensitivity of the system.[12,13] We combined two newly developed technology platforms for a multiple pathogen detection system using a degenerate PCR–based NAT system (dPCR-NAT).

MATERIALS AND METHODS

Design of dPCR primers and microarray oligoprobes

We designed dPCR primers that hybridized with HIV, HCV, HBV, human parvovirus B19 (PVB19), and WNV genomic sequences (Table 1). We used the CoCoMo (Coordination of Common Motifs) algorithm (www.geneknot.info/cocomo)[14] for dPCR primer design. CoCoMo determines primer regions in commonly conserved nucleotide regions in the assembled nucleotide sequences of virus strains. In each case, all viral sequences were identified from GenBank and EMBL, and a low degeneracy primer set was selected as a candidate using the CoCoMo algorithm. We collected data on 2072 HIV-1 nucleotide sequences and selected 14 complete genome sequences that corresponded to each genotype of HIV-1. We designed dPCR primer sets for detecting 14 complete genome sequences, resulting in approximately 3897

TABLE 2. Oligonucleotide probe sequences of DNA microarray assay

Virus	Probe name	Sequence (5′-3′)	Tm (°C)*
HIV	IR1-1	ACTATTCTTTCCCCTGCACTGTACCCCCCAATCC	78
	IR1-2	TCTGTTGCTATTATGTCTACTATTCTTTCCCC	66
	IR1-3	CTTTAGTTTGTATGTCTGTTGCTATTATGTCTAC	63
	IR1-4	GTAATTTGTTTTTGTAATTCTTTAGTTTGTATGTCTG	66
	IR3-1	GGGATTGTAGGGAATTCCAAATTCCTGCTTGATT	76
	IR3-3	CTTTAATTCTTTATTCATAGATTCTACTACTCCTTGACTTTG	69
HCV	CF1-1	AACCGGTGAGTACACCGGAATTGCCAGGAC	77
	CF1-2	TTTCTTGGATCAACCCGCTCAATGCCTGGAGATTTGGGCG	88
	CF1-3	TGCCCCCGCAAGACTGCTAGCCGAGTAGTGTTGGG	85
	CF2-1	AGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGA	86
	CF2-2	CTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTG	81
	CF2-3	GCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCT	82
	CR2-1	TCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCT	86
HBV	BF4-1	CTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGG	75
	BF4-2	CTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCCTC	78
	BF4-3	TGTCCTCTAATTCCAGGATCAACAACAACCAGTAC	73
	BF4-4	ATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACC	84
	BF4-5	CCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTC	84
	BF4-6	GTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAG	80
	BR4-1	CCAGAAGAACCAACAAGAAGATGAGGCATAGCAG	75
PVB19	PVB19F-1	GGCGCCTGGAACACTGAGACCCCGCGCTCTAGTAC	85
	PVB19F-2	GGCGCCTGGAACACTGAAACCCCGCGCTCTAGTAC	84
	PVB19F-3	GAACTCAGTGAAAGCAGCTTTTTCAACCTCATCACTCC	78
	PVB19R-1	GTACTAGAGCGCGGGGTCTCAGTGTTCCAGGCGCC	85
	PVB19R-2	GTACTAGAGCGCGGGGTTTCAGTGTTCCAGGCGCC	84
	PVB19R-3	GGAGTGATGAGGTTGAAAAAGCTGCTTTCACTGAGTTC	78
WNV	WNVF-1	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-2	ATGATTGACCCTTTTCAGTTGGGCCTTCTGGTCG	80
	WNVF-3	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-4	ACGCCGACATGATTGATCCTTTTCAGTTGGGCCT	81
	WNVR-1	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-2	CGACCAGAAGGCCCAACTGAAAAGGGTCAATCAT	80
	WNVR-3	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-4	AGGCCCAACTGAAAAGGATCAATCATGTCGGCGT	81
IC	IC-1	GTCGTAGTTCCGACCATAAACGATGCCGACCGG	81
	IC-2	GGCGATGCGGCGGCGTTATTCCCATGACCC	86
	IC-3	CCGCCGGGCAGCTTCCGGGAAACCAAAGTCTTTG	87
	IC-4	TCGAAGACGATCAGATACCGTCGTAGTTCCGACC	78
QC	QC	TTGGCAGAAGCTATGAAACGATATGGG	69

* The melting temperature (Tm) was calculated using NetPrimer (PREMIER Biosoft International, http://www.premierbiosoft.com/).

primer sets. For HCV, we collected 978 sequences and selected 167 complete genome sequences to design dPCR primer sets, generating 31 primer sets. For HBV, 1461 sequences were collected and 1344 complete genome sequences were selected to generate the dPCR primer sets, generating approximately 29 primer sets. For WNV, we collected 17,172 sequences and used 111 complete genome sequences to design the dPCR primer sets, generating 1649 primer sets. For PVB19, we collected 1145 sequences and selected seven complete genome sequences to design the dPCR primer sets, generating 2517 primer sets. Selected primers are listed in Table 1. The sequences of the oligonucleotide detection probes on the DLC-chip are indicated in Table 2. The probes were manually designed from regions amplified by the degenerate primers. Thirty to 42 oligomers that had a GC content between 50 and 60% were selected. The resultant melting temperature values were 62 to 88°C. The hybridization stability of the PCR fragments was biased according to strand; therefore, we designed probes on each strand of the PCR products (sense strand—same sense as forward primer).

Synthesis of genotype panel oligomers for screening primers

Genotype panel oligomers of HIV-1, HBV, PVB19, and WNV were prepared by overlap-extension PCR (OE-PCR;[15] Fig. 1A). Regions for OE-PCR were selected according to the nucleotide sequences amplified with our primers for each virus genotype. The nucleotide sequences of HBV subtypes B and C panel oligomers had the same sequence. The joining oligonucleotides listed in Supplemental Table S1 (available as supporting information in the online version of this paper) were designed using DNA works (http://helixweb.nih.gov/dnaworks/).[16] The nucleotide sequences of each genotype panel oligomers are listed in Supplemental Table S2 (available as supporting information in the online version of this paper). OE-PCR

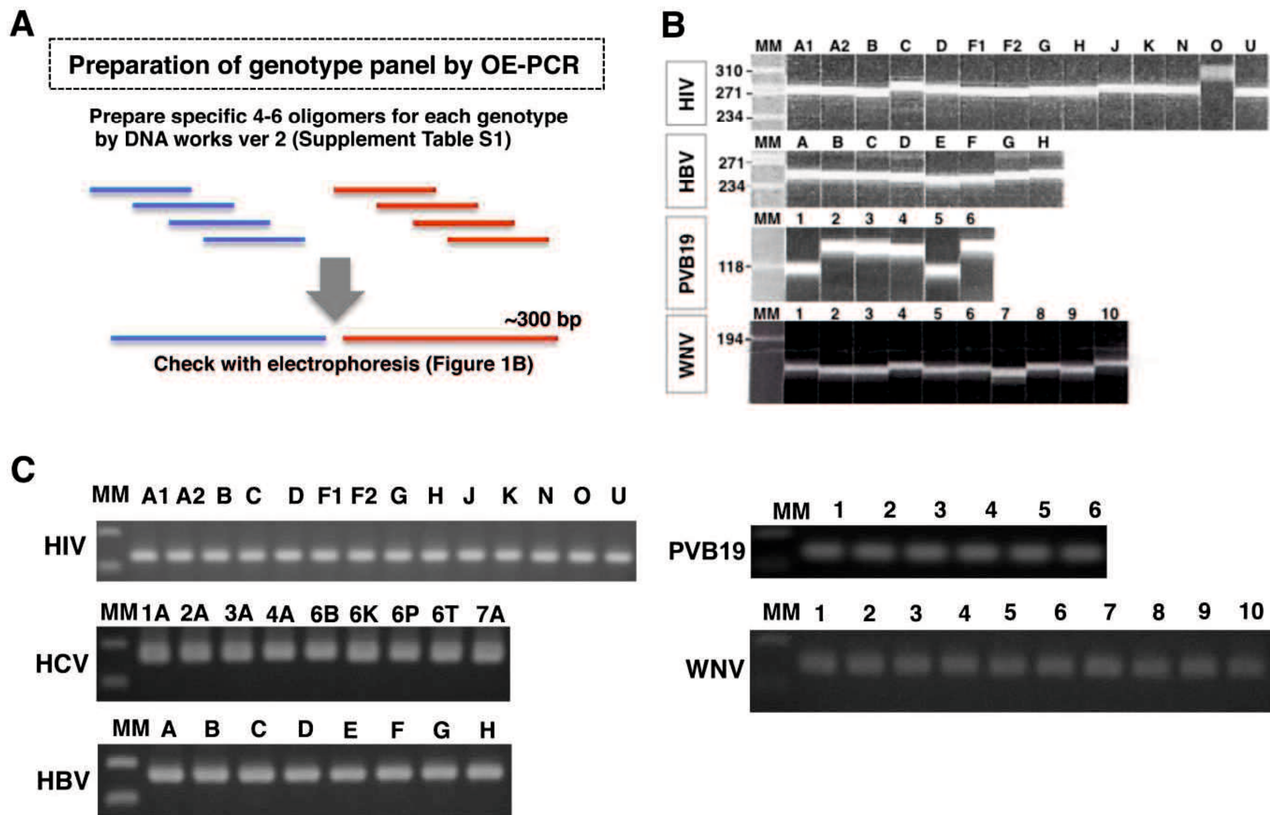


Fig. 1. Synthesis of genotype panel oligomers for HIV, HCV, HBV, PVB19, and WNV. (A) Preparation of genotype panel oligomers by PCR-based gene synthesis, OE-PCR. dPCR primers were validated with genotype panel oligomers. (B) Genotype panel oligomers were synthesized against 14 subtypes of HIV-1, eight genotypes of HBV, nine genotypes of HCV, six genotypes of PVB19, and 10 genotypes of WNV. (C) Each of the HIV-1 subtype oligomers was amplified by dPCR primers for HIV-1. The top panel shows the agarose gel electrophoresis analysis of PCR products to identify the 14 HIV-1 subtypes. Similar results were obtained from HCV, HBV, PVB19, and WNV genotype panel oligomers with specific dPCR.

was carried out according to a two-step reaction method by using a PCR kit (Prime Star, Takara-bio, Otsu, Japan; Fig. 1). The first reaction was carried out using a mixture of OE-PCR oligomers in 30 cycles of 98°C for 15 seconds, 55°C for 10 seconds, and 72°C for 15 seconds. One microliter of the first PCR products was transferred to the second PCR solution, which included 5 pmol/μL each of the 5′- and 3′-end primers. The second reaction consisted of 30 cycles at the temperature conditions used in the first reaction. The molecular weights of the OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu, Kyoto, Japan; Fig. 1B). HCV genotype panel oligomers were synthesized and obtained from Invitrogen (Carlsbad, CA; custom DNA oligonucleotide synthesis service).

Viral samples for dPCR-based NAT

For more accurate analysis, we purchased PVB19 NAT-based assays genotype panel (First International Standard; Category Number, 09/110 National Institute for Biological Standards and Control [NIBSC], UK); HIV-1 RNA genotype panel (Category Number 08/358 NIBSC); HCV RNA genotype panel (Category Number 08/264 NIBSC); HCV for NAT (Fourth WHO International Standard; Category Number 06/102 NIBSC); HBV for NAT (Third WHO International Standard; Category Number 10/264 NIBSC); and PVB19 DNA NAT assays (Second International Standard; Category Number 99/802 NIBSC). To evaluate the specificity of our dPCR-NAT system, we diluted each genotype panel with defibrinated plasma (Basematrix 53, SeraCare BBI Diagnostics, Milford, MA) to give a final concentration of 151 to 9722 copies/mL (HIV), 500 to 1500 IU/mL (HCV), 5754 to 123,027 IU/mL (HBV), and 870,964 to 954,933 IU/mL (PVB19), respectively. To evaluate the sensitivity of our dPCR-NAT system, we diluted each international standard with defibrinated plasma to give a final concentration of 1 to 10,000 IU/mL. Samples containing the New York strain of WNV RNA (NY 2001-6263; NATtrol, Category Number NATWNV-0005,

1 mL, 50,000 copies/mL) were purchased from ZeptoMetrix (Buffalo, NY) and were diluted with defibrinated plasma to give a final concentration of 1 to 1000 copies/mL. Blood specimens from healthy volunteer donors who were confirmed as negative for HCV, HBV, and HIV were provided from Japan Red Cross and used as a negative control.

DNA and RNA extraction, reverse transcription, and PCR amplification

Each viral DNA and RNA was extracted from 200 μL of diluted sample with a viral nucleic acid kit and a viral RNA kit (High Pure, Roche, Basel, Switzerland). Total RNA of HCV, HIV-1, and WNV were all reverse transcribed with a cDNA synthesis kit (Superscript III RT, Invitrogen) according to the manufacturer's protocol. Twenty-microliter cDNA samples were prepared for PCR amplification of each virus. The PCR was carried out with PCR mixture (GoTaq, Promega, Madison, WI). In the PCR mixture, diluted nucleic acid and 50 μmol/L of each degenerate primer were included. The reaction consisted of 50 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. We used 5 ng of human cDNA fragment as an internal control (IC).

Microchip fabrication

We purchased 3-mm^2 silicon DLC-chip from Toyo Kohan (Tokyo, Japan). Each probe was spotted by Spotarray 72 (Perkin-Elmer, Waltham, MA) with a 250-μm spot distance and 100-μm-diameter spots. Spotted probes were baked for 60 minutes at 80°C. We made DNA chips to evaluate probe sensitivity and for detection of viral samples, including WHO International Standards and WHO genotype panels. Each sequence for detection probes on the chip is listed in Table 2.

Synthesis and hybridization of fluorescently labeled DNA samples

PCR amplification of extracted DNA or cDNAs was performed for fluorescent labeling using polymerase (GoTaq, Promega) with Cy-5 dCTP. The PCR mixture included template DNA or cDNA, 50 μmol/L primers, 0.5 μL of Cy-5 dCTP (Perkin-Elmer), 1 μL of dNTP mixture (2.5 mmol/L each, 0.25 mmol/L dCTP), 5 μL of 5× PCR buffer (GoTaq, Promega), and 0.25 μL of polymerase (GoTaq, Promega). The 50-cycle PCR profile was 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Amplification was carried out in a PCR system (GeneAmp 9700, Applied Biosystems, Foster City, CA). Two microliters of the PCR-amplified reaction mixture was hybridized with the chip for 30 minutes at 50°C. The hybridized chip was washed with saline-sodium citrate buffer and scanned with a fluorescent scanner (FLA-8000, Fujifilm, Tokyo, Japan). Geographic origin was estimated from the obtained fluorescence patterns, thereby indicating specific genotypes.

Transcription-mediated amplification assay

To validate our assay sample preparation including viral DNA or RNA, we performed transcription-mediated amplification (TMA) assays for HBV, HCV, and HIV-1 by using an assay kit (Ultrio, Novartis Pharma, Tokyo, Japan) according to the manufacturer's protocol.

RESULTS

Synthesis of genotype panel oligomers by OE-PCR

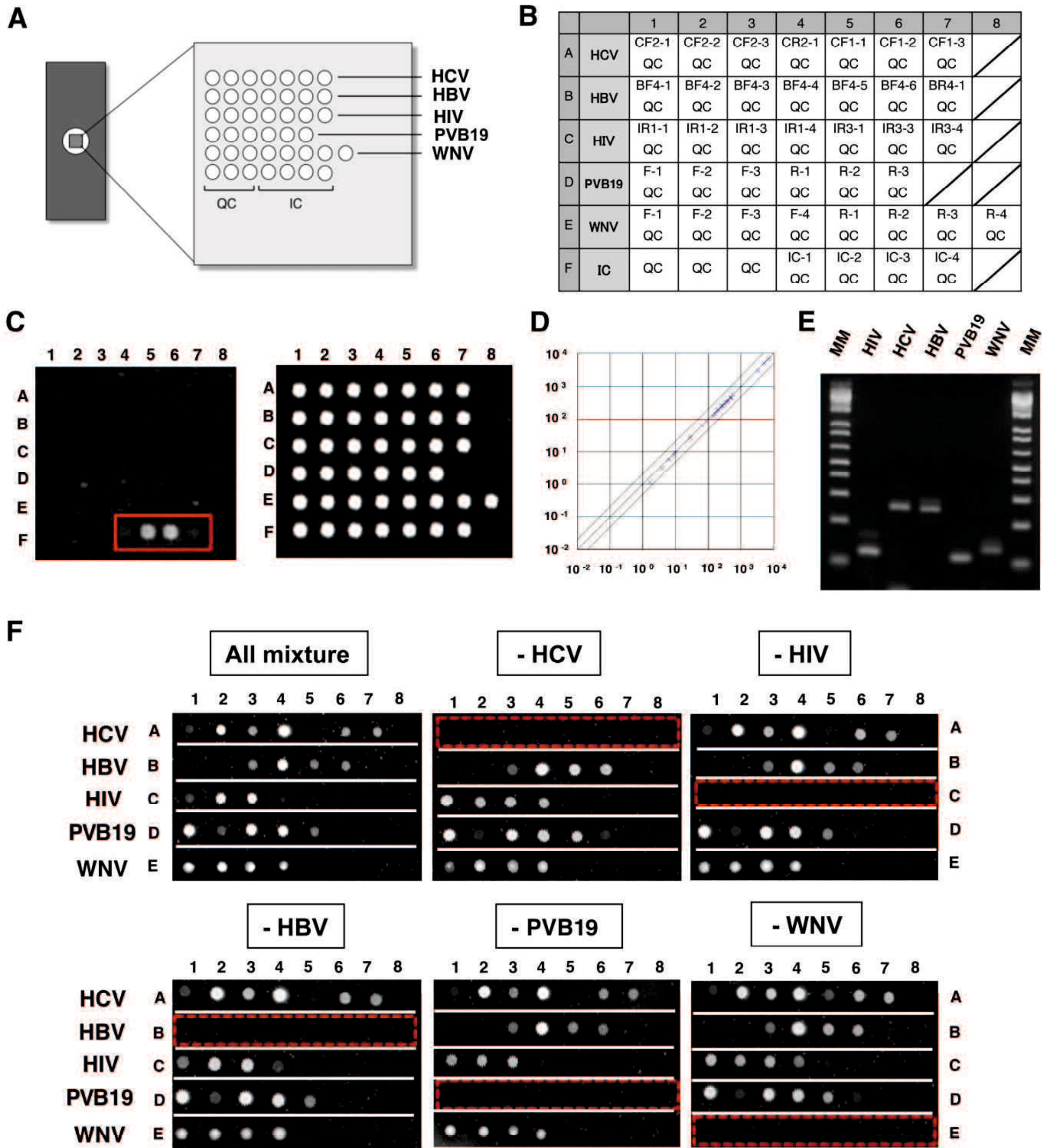
To verify our designed dPCR primers, we prepared genotype panel oligomers (100-300 bp) as viral genotype reference materials for HIV (A1, A2, B, C, D, F1, F2, G, H, J, K, N, O, and U), HBV (A–H), WNV (1-10), and PVB19 (1-6) made by OE-PCR (Fig. 1A). The target regions for OE-PCR were selected according to the nucleotide sequences of the standard strain for dPCR and the DLC-chip detection system (Supplemental Table S2). The joining oligonucleotides were designed using an online computer program (DNAWorks, Version 2, http://helixweb.nih.gov/dnaworks/). The nucleotide sequences of each genotype panel oligomer are listed in Supplemental Table S1. OE-PCR was carried out according to a two-step reaction method. The OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu). As expected, we could detect our OE-PCR products at appropriate molecular size (Fig. 1B). For HCV (1A, 2A, 3A, 4A, 6B, 6K, 6P, 6T, and 7A), genotype panel oligomers were designed and made by custom service (Invitrogen).

Validation of dPCR primer for detection of virus genotype panel oligomers

We confirmed whether our designed primers could specifically amplify each virus subtype by PCR. The PCR products for all HIV, HCV, HBV, PVB19, and WNV subtypes were detected at the expected size by gel electrophoresis (Fig. 1C).

QC of our pathogen detection DNA microarray system

To evaluate specificity of each probe in our DLC-chip, the detection ability of our probes was analyzed. We selected 36 specific probe sets that had high detection abilities (>50% of genotypes) from the 53 originally designed evaluated probes (data not shown) and then spotted them on DLP-Chip (Fig. 2A, B). DNA microarray images were



		1	2	3	4	5	6	7	8
A	HCV	CF2-1 QC	CF2-2 QC	CF2-3 QC	CR2-1 QC	CF1-1 QC	CF1-2 QC	CF1-3 QC	
B	HBV	BF4-1 QC	BF4-2 QC	BF4-3 QC	BF4-4 QC	BF4-5 QC	BF4-6 QC	BR4-1 QC	
C	HIV	IR1-1 QC	IR1-2 QC	IR1-3 QC	IR1-4 QC	IR3-1 QC	IR3-3 QC	IR3-4 QC	
D	PVB19	F-1 QC	F-2 QC	F-3 QC	R-1 QC	R-2 QC	R-3 QC		
E	WNV	F-1 QC	F-2 QC	F-3 QC	F-4 QC	R-1 QC	R-2 QC	R-3 QC	R-4 QC
F	IC	QC	QC	QC	IC-1 QC	IC-2 QC	IC-3 QC	IC-4 QC	

captured by an image analyzer (FLA-8000, Fuji Photo Film, Tokyo, Japan). Hybridized slides were inserted in the FLA-800, and the scan conditions were set as 10 μm resolution, standard scan mode, and photomultiplier tube high-value 100% laser. Saved DNA microarray images were analyzed, and fluorescent intensity of each spot was measured using a computer program (ArrayGauge Software, Fuji Photo Film). After removing the background signal, we defined the positive signal standard as radio intensity more than five times higher than background. The signal intensity was calculated as the total pixel value minus the global background. The signal intensities were then normalized to the mean for all the spots in the array.

To determine the specificity of the new system, we applied two different QCs: an IC probe (oligonucleotide complementary to the Cy5-labeled amplicon of the 18S

Fig. 2. (A) Schematic design of DLC-chip, including HIV, HCV, HBV, WNV, and PVB19 probes (B) and their relative position in the slide layout. Spotted probes of seven subtypes of HIV-1, seven genotypes of HCV, seven genotypes of HBV, six genotypes of PVB19, and eight genotypes of WNV were selected. All of the probes were spotted together on the chip, along with the QC probes. (C) Evaluation of the detection system using the IC (left panel) and QC (right panel). Anti-IC probes specifically hybridized to and amplified 18S rRNA PCR product from human DNA. (D) QC by using same amplified sample. Correlation coefficients using scatter plot indicated that each DLC-chip was highly reproducible, with a correlation of 0.99905. (E) Multiple detection system for five virus genomes. Agarose gel electrophoresis analysis of PCR products to detect HIV-1, HCV, HBV, PVB19, and WNV. These PCR products were positive on the microarrays. (F) Multiple detection system for five viruses by DLC-chip. The PCR products of five viruses were mixed at the following concentrations: HCV, 10 IU/mL; HBV, 10 IU/mL; HIV-1, 10,000 IU/mL; PVB19, 10 IU/mL; and WNV, 10 copies/mL. Each panel used a mixture of the five viruses that lacked HIV-1, HCV, HBV, PVB19, or WNV. The red line indicates the row where excluded virus was not detected. Cross-hybridization between the five viruses was not confirmed.

rRNA gene; Fig. 2C, left) and a QC probe (oligonucleotide complementary to the Cy3-labeled QC probe; Fig. 2C right). This allowed us not only to monitor the spot uniformity, but also to detect potential irregularities during the hybridization process. To evaluate the reproducibility of our DLC-chip, we hybridized the same PCR-amplified samples to different DLC-chips and measured each signal intensity. Correlation coefficients using scatter plots indicated that each DLC-chip was highly reproducible, showing a correlation of 0.99905 (Fig. 2D).

Multiple detection of five viruses in one test

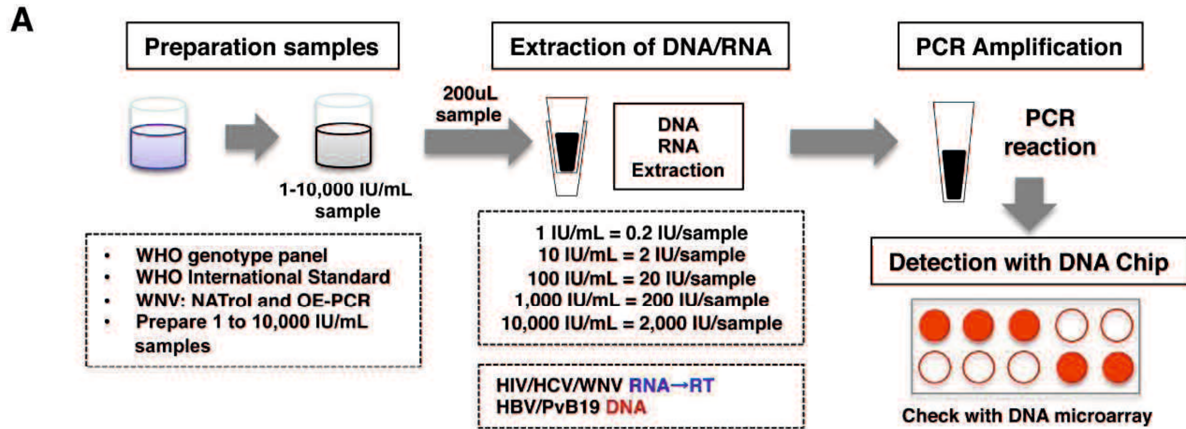
We determined whether our designed probes could detect the PCR products of all the HIV genotypes. On the DLC-chip, seven different HIV-1–specific probes were aligned. After being labeled with Cy5, PCR products were detected by hybridization to HIV-specific probes on the DLC-chip. We considered that a sample was positive if at least two different probes showed a positive signal. These HIV-1–specific degenerative primer and probe sets detected all of the following genotypes: A1, A2, B, C, D, F1, F2, G, H, J, K, N, O, and U (data not shown). Similar to the HIV detection system, different probe sets detected all the HCV, HBV, PVB19, and WNV genotypes (data not shown). To determine the ability to detect multiple viruses on one DNA chip, we separately performed virus-specific genome amplification (Fig. 2E) and mixed each PCR product in one tube and hybridized the PCR products onto the DLC-chip. The mixed viral PCR product (HIV, HCV, HBV, PVB19, and WNV) was readily detected as a hybridized spot on the DCL-chip (Fig. 2F). We prepared a mixed sample minus one virus amplicon as a negative control for cross-hybridization and nonspecific binding. We confirmed that no cross-hybridization occurred with HIV-1, HCV, HBV, PVB19, and WNV.

Specificity and sensitivity of our dPCR-NAT system by using WHO genotype panels and international standards

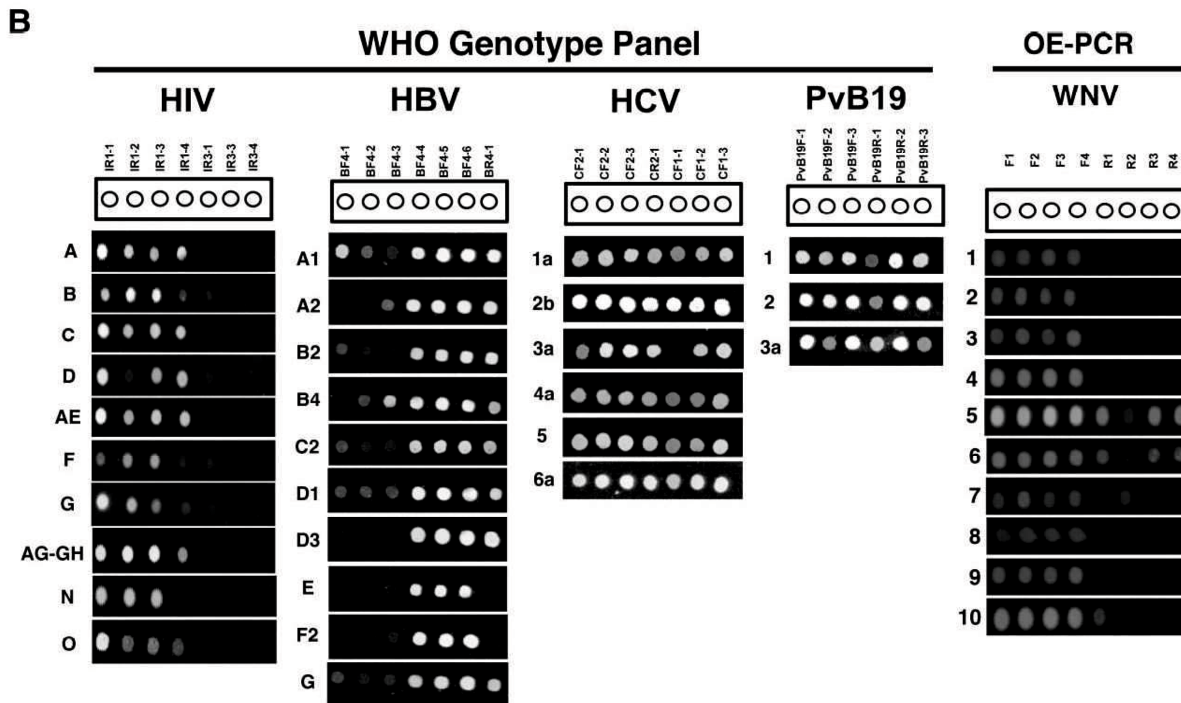
To determine the specificity of our DNA microarray system to detect each virus genotype, we prepared the WHO genotype panel samples for each virus. For WNV, we used the genotype panel oligomer described in Figure 1 as an NAT genotype panel because there was no commercially available panel. We extracted DNA or RNA from each genotype panel for HIV, HCV, HBV, PVB19, and WNV. Each template DNA or cDNA was amplified with each dPCR primer listed in Table 1. The amplified PCR products for all genotypes of each virus were confirmed on 3% agarose gels and DNA chips. We detected all the HIV, HCV, HBV, PVB19, and WNV genotypes (Fig. 3B). Our DNA microarray data are summarized in Supplemental Table S4 (available as supporting information in the online version of this paper). To validate the sensitivity of our NAT system, we used WHO International Standards as reference materials. To check our reference samples, we performed FDA-licensed NAT assays using a TMA assay (Supplemental Table S3, available as supporting information in the online version of this paper) before analysis. For sensitivity assay, we prepared HCV RNA, HBV, and PVB19 from NIBSC. These materials were used as international standards for NAT quality assurance. We prepared each sample to give a final concentration of 1 to 10,000 IU/mL and isolated DNA or RNA from 200-μL samples. Thus, each sample was assumed to contain 0.2 to 2000 IU virus if extraction efficiency was 100%. DNA and RNA were extracted. RNA samples were all reverse transcribed with a cDNA synthesis kit (Superscript III RT, Invitrogen), and all cDNA samples were used for PCR. We detected 1 IU/mL HCV, 1 IU/mL HBV, 1 IU/mL PVB19, and 1 copy/mL WNV (Fig. 3C). Similar results were obtained from at least three independent experiments. For HIV, we estimated the detection limit in at least five independent tests. We detected 10,000 IU/mL for 100%, 1000 IU/mL for 77%, 100 IU/mL for 7%, 10 IU/mL for 0%, and 1 IU/mL sample for 0%. Thus we conclude that our system could detect 1000 IU/mL equivalent to 200 IU/PCR sample for HIV. Detection limits for each virus are listed in Table 3.

DISCUSSION

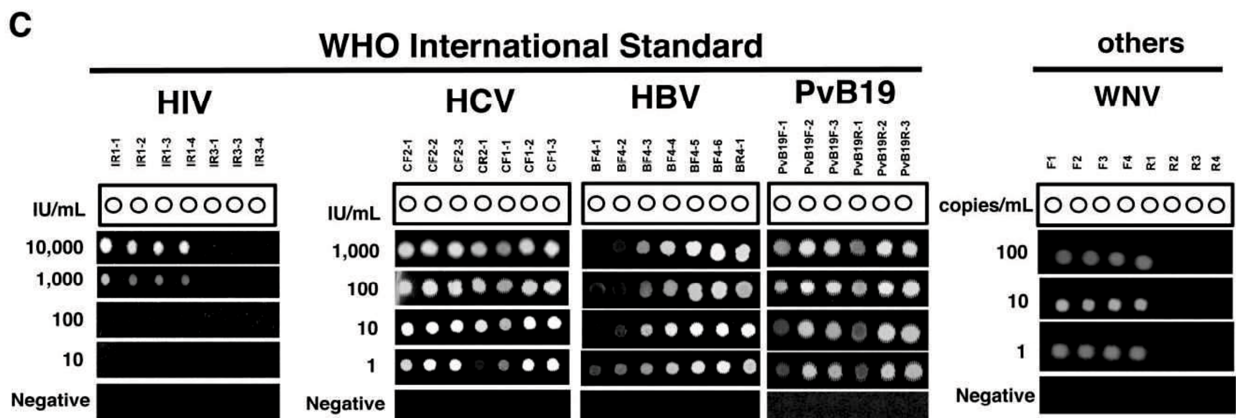
We investigated the performance of the new NAT system using dPCR primers and a DLC-chip. We showed that our NAT system was specific for HIV-1, HCV, HBV, PVB19, and



A
Preparation samples
Extraction of DNA/RNA
PCR Amplification
200uL sample
1-10,000 IU/mL sample
DNA RNA Extraction
PCR reaction
WHO genotype panel
WHO International Standard
WNV: NATrol and OE-PCR
Prepare 1 to 10,000 IU/mL samples
1 IU/mL = 0.2 IU/sample
10 IU/mL = 2 IU/sample
100 IU/mL = 20 IU/sample
1,000 IU/mL = 200 IU/sample
10,000 IU/mL = 2,000 IU/sample
HIV/HCV/WNV RNA→RT
HBV/PvB19 DNA
Detection with DNA Chip
Check with DNA microarray



B
WHO Genotype Panel
OE-PCR
HIV
HBV
HCV
PvB19
WNV
IR1-1 IR1-2 IR1-3 IR1-4 IR3-1 IR3-3 IR3-4
BF4-1 BF4-2 BF4-3 BF4-4 BF4-5 BF4-6 BR4-1
CF2-1 CF2-2 CF2-3 CR2-1 CF1-1 CF1-2 CF1-3
PvB19F-1 PvB19F-2 PvB19F-3 PvB19R-1 PvB19R-2 PvB19R-3
F1 F2 F3 F4 R1 R2 R3 R4
A B C D AE F G AG-GH N O
A1 A2 B2 B4 C2 D1 D3 E F2 G
1a 2b 3a 4a 5 6a
1 2 3a
1 2 3 4 5 6 7 8 9 10



C
WHO International Standard
others
HIV
HCV
HBV
PvB19
WNV
IR1-1 IR1-2 IR1-3 IR1-4 IR3-1 IR3-3 IR3-4
CF2-1 CF2-2 CF2-3 CR2-1 CF1-1 CF1-2 CF1-3
BF4-1 BF4-2 BF4-3 BF4-4 BF4-5 BF4-6 BR4-1
PvB19F-1 PvB19F-2 PvB19F-3 PvB19R-1 PvB19R-2 PvB19R-3
F1 F2 F3 F4 R1 R2 R3 R4
IU/mL
10,000
1,000
100
10
Negative
IU/mL
1,000
100
10
1
Negative
copies/mL
100
10
1
Negative

Fig. 3. (A) Schematic illustration of sensitivity analysis of our detection system, including extraction process. Detection of the isolated virus genomes from human plasma sample. Samples containing HIV-1, HCV, and HBV were diluted with defibrinated plasma (Basematrix 53; SeraCare) at 1 to 10,000 IU/mL. (B) For more accurate analysis, we prepared a WHO genotype panel for HIV-1, HBV, HCV, and PVB19. We detected all genotypes by using our designed degenerated primer. (C) For sensitivity analysis of our NAT system, we prepared WHO International Standards for HIV-1, HCV, HBV, and PVB19. We diluted these materials with negative sera at 1 to 10,000 IU/mL and extracted DNA and RNA from 200-μL samples. All RNA samples were transcribed with Superscript III. We detected HIV at 1000 IU/mL, HCV at 1 IU/mL, HBV at 1 IU/mL, and PVB19 at 1 IU/mL.

TABLE 3. Detection limit of our dPCR-NAT system*

IU/mL	HIV (%)	HCV (%)	HBV (%)	PVB19 (%)
10,000	100	100	100	100
1,000	77	100	100	100
100	7	100	100	100
10	0	100	100	100
1	0	50	25	75

* % positive: Reactive/Tested (Percent Reactive). The measurement obtained in each specimen was tested with two sample lots in two independent test assays. For HIV, we tested five sample lots in five independent test assays.

WNV at low viral loads. In addition, we showed that our system detected various virus genotypes. Degenerate primers are useful not only for detecting unknown genes, but also for the simultaneous amplification of mutated genes.[17] In the case of viruses, many mutated strains appear in a particular geographical area and at a specific time.[18] Recently, we used CoCoMo primers, a fully automatic design pipeline for PCR primers, according to the CodeHop primer design strategy, by which others can analyze the oligonucleotide motif incidence.[14] The CoCoMo program is available online (www.geneknot.info/cocomo). We utilized CoCoMo primers to design the primer sequences in this study. The algorithm-designed primers were confirmed to fit most subtypes or genotypes of the target viruses (Table 1) and enabled efficient detection of a wide range of viruses. In general, PCR procedures with the degenerate primers had lower sensitivity than that of the specific primers. To overcome this disadvantage, we used fluorescence detection on the DLC-chip, which provided higher sensitivity.[12] Additionally, the degenerate primer set was designed to detect the polymorphic region of the viral genome; therefore, subtypes or genotypes could be discriminated on the DLC-chip. The combination of the primers and DLC-chips was therefore validated. These results suggest that the combination of dPCR and DLC-chips is beneficial for blood-borne virus detection. To increase the safe use of the system, automation of our detection system will be required in the future.

A low level of HBV may proliferate in transfused recipients who are immunocompromised or immunosuppressed. In addition, the window period of HBV is relatively long, but the presence of HBV DNA without detectable HBsAg outside the window period, known as occult HBV infection, has been reported.[7] This suggests that the development of a highly sensitive detection system for HBV is particularly important. Although the current TMA sensitivity corresponds to 1 to 5 IU/mL at the reproductive level, using the same viral samples in this study we detected 1 to 10 IU/mL HBV. These data suggest that the sensitivity of our analysis system is equivalent to that of the TMA assay (Fig. 3C). Thus far, HBV Genotype C is the most prevalent genotype in Japan (85%), while the prevalence of Genotypes A and D is 1.7 and 0.4%, respectively.[19-21] Currently, the level of Genotype A is increasing in the younger generation because of horizontal infection.[11,22,23]

For HIV-1 detection and quantification, various methods have been developed, but most real-time techniques involve their sensitivity to point mutations within primer and probe target sequences. Our dPCR-NAT system could detect a wide range of HIV genotypes by using dPCR primers. Despite the wide range of genotype detection, sensitivity was not high. We could detect 200 IU HIV/PCR procedure. Improvement is needed for HIV dPCR sensitivity. Similarly for HBV and HCV, the next-generation virus detection system must be able to cope with this situation, namely, by possessing a wide detectable viral genotype range and a low detection level. With our detection system, all of the virus genotypes were detected at 1 to 1000 IU/mL sample. Previous studies by Hsia and coworkers[24] combined multiplex PCR and DNA chips and detected three different viruses in a single sample. Our mixed PCR product data (Fig. 2F) indicated that our system could simultaneously detect five different viruses in one DNA chip. These data suggest that our system is suitable for multiple pathogen testing.

In conclusion, the dPCR-NAT system is an accurate and reliable test for HIV, HBV, HCV, PVB19, and WNV detection with respect to specificity, sensitivity, and genotype inclusivity and a reproducible assay for the detection of multiple blood-contaminating pathogens.

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CONFLICT OF INTEREST

All authors concur with submission of this manuscript, and we affirm that the material submitted has not previously been reported, and is not under consideration for publication elsewhere. We do not have any conflicting financial interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Sequence of each oligomer set for preparing genotype panel oligomers.

Table S2. Sequence of each genotype panel oligomers.

Table S3. Validation of our sample using current NAT system.

Table S4. Summary of DLC-chip analysis of genotype panels and international standards.