Agric. Chem. Biotechnol. **49**(4), 143-147 (2006)

Article

A Rapid PCR-based Assay for Detecting Hepatitis B Viral DNA Using GenSpector TMC-1000

Bum Huh*, Youngju Ha, Jaetak Oh, Eunha Park, Jinsu Park and Haejoon Park

Mogam Biotechnology Research Institute, Guseong-Gu, Yongin 449-913, Korea

Received November 18, 2006; Accepted December 1, 2006

A rapid PCR-based assay for detecting hepatitis B viral DNA (HBV DNA) in serum and plasma was developed using a new PCR instrument named GenSpector (TMC-1000, Samsung electronics). PCR was carried out using a chip-based platform, which enabled 50 PCR cycles with internal controls, and melting-curve analysis in 30 minutes. Verification of the amplified HBV DNA product and the internal control was based on specific melting temperatures (Tm) analysis, executed by the GenSpector software. Primers were designed within the region conserved through HBV genotypes A to F. The lower limit of detection was 840 copies/ml serum, conducted with serial dilutions of a HBV DNA positive control (ACCURUN 325 series 700, Boston Biomedica Inc.). The assay was also compared to another assay for HBV DNA (Versant HBV DNA 3.0 assay, Bayer HealthCare) for 200 samples (each 100 clinical negative and positive samples). The sensitivity and specificity were 100% matched. This rapid PCR-based assay is specific, reproducible, and enables qualitative detection of HBV DNA.

Key words: *HBV, hepatitis B virus, chip-based PCR, molecular diagnosis, rapid detection*

Hepatitis B virus (HBV) is a highly infectious virus that is transmitted primarily through blood and blood-derived fluids. Approximately 2 billion people worldwide have been infected with the HBV, and infection is persistent in over 350 million individuals.[1] Because it is often non-symptomatic, chronic carriers are at high risk for the development of serious liver diseases, including cirrhosis and hepatocellular carcinoma.[2] Since screening for HBV began in 1969, the rate of infection through blood transfusions has decreased greatly. But the rate of HBV transmission through blood transfusion is still 1 out of every 137,000 units of blood. One reason for this is that currently available blood screening technologies detect core antibodies or surface antigens, which do not appear until 84 days after infection.[3,4] Over the past decade, nucleic acid testing (NAT) methods for the detection of HBV DNA have been developed. NAT combines the advantage of direct, highly sequence-specific detection of HBV DNA with an analytical sensitivity that is several orders of magnitude higher than assays for core antibody or surface antigen.[5]

The GenSpector (TMC-1000, Samsung Electronics, Yongin, Korea) is a rapid, real-time thermal cycler used for identifying DNA from prepared biological samples. By automating much of the testing process, and making each reaction individually programmable, it represents the fast and easy to use method for PCR analysis. The GenSpector delivers highly accurate and consistent test results in 30 minutes. Independently programmable 6 PCR modules perform one-color, real-time fluorometric detection. Samples are amplified and measured on a GenSpector chip, a shielded lab-on-a-chip device, which is designed to optimize rapid thermal transfer and optical sensitivity, as well as reduce the possibility of amplicon contamination. In the current study, we describe a sensitive, simple NAT assay for the detection of HBV DNA in serum.

*Corresponding author
Phone: 82-11-553-4511; Fax: 82-31-260-9838
E-mail: nosmoki2@snu.ac.kr

Abbreviations: DFIC, dimmer forming internal control; HBV, hepatitis B virus; NAT, nucleic acid testing; PCR, polymerase chain reaction.

Materials and Methods

Clinical specimens. To test the specificity of the GenSpector PCR assay, one hundred HBV DNA-negative serums were obtained from Nabi Diagnostics (Raton Boca, FL, USA), and 100 HBV DNA-positive human serums were obtained from individual donors (individuals in Korea attending patients with hepatitis) (Greencross Reference Laboratory, Yongin, Korea). The HBV DNA assays were performed using the VERSANT HBV DNA 3.0 Assay (bDNA) (Bayer HealthCare, NY, USA).[6] All samples were stored in aliquots at −70°C until use.

Control specimens. Control specimens were purchased from BBI Diagnostics, MA, USA including HBV, HCV, HIV, Parvovirus, West Nile Virus, and 6 genotypes of HBV from A to F. The names and quantities of the viruses used are listed in Table 1.

Isolation of viral DNA . HBV DNA was isolated from 200 μL of serum using the QIAamp MinElute Virus Spin kit (Qiagen, CA) according to the manufacturer's instruction. Viral nucleic acids were eluted using 50 μL of nuclease-free water and stored at −70°C until use.

Table 1. Names, quantities of virus used as control specimens (purchased from BBI Diagnostics, MA, USA), and results of GenSpector Assays

		Roche Molecular Systems Raritan, NJ	Bayer Diagnostics Tarrytown, NY	BBI Biotech Research Labs Gaithersburg, MD	GenSpector HBV DNA real-time PCR Assay	GenSpector HBV DNA real-time PCR Assay Mixed with HBV
ACCURUN 325 Hepatitis B Virus DNA Positive Control Series 700		Amplicor HBV COBAS 8.1×10^6 copies/m*l*	Versant HBV DNA 3.0 9.1×10^6 copies/m*l*		+	
ACCURUN 306 HCV RNA Positive Control Series 500		COBAS AMPLICOR HCV Monitor 2.0 9.1×10^5 IU/m*l*	Versant HCV RNA 3.0 Assay (bDNA) 3.9×10^5 IU/m*l*		-	+
ACCURUN 350 CMV DNA Positive Control Series 300		COBAS AMPLICOR CMV Monitor 1,900 copies/m*l*			-	+
ACCURUN 315 HIV-1 RNA Positive Control Series 200		Roche Amplicor HIV-1 Monitor Test version 1.5 1,600 IU/m*l*	Versant Branched DNA (bDNA) Assay 500 copies/m*l*		-	+
ACCURUN 355 Parvovirus B19 DNA Positive Control Series 600		LightCycler Parvovirus B19 DNA Quantification Kit 9.4×10^5 IU/m*l*			-	+
ACCURUN 365 West Nile Virus RNA Positive Control Series 200				West Nile Virus Taqman Probe Assay 5×10^2 copies/m*l*	-	+
Hepatitis B Virus DNA Genotype Performance Panel PHD201E	A	Amplicor HBV COBAS 2.2×10^4/m*l*	Versant HBV DNA 3.0 5.5×10^4/m*l*		+	
	B	Amplicor HBV COBAS 1.5×10^4/m*l*	Versant HBV DNA 3.0 6.7×10^3/m*l*		+	
	C	Amplicor HBV COBAS 1.5×10^5/m*l*	Versant HBV DNA 3.0 1.5×10^4/m*l*		+	
	D	Amplicor HBV COBAS 2×10^4/m*l*	Versant HBV DNA 3.0 3.7×10^4/m*l*		+	
	E	Amplicor HBV COBAS 2.8×10^4/m*l*	Versant HBV DNA 3.0 2.2×10^4/m*l*		+	
	F	Amplicor HBV COBAS 2.4×10^4/m*l*	Versant HBV DNA 3.0 3.2×10^4/m*l*		+	

+: positive, -: negative.

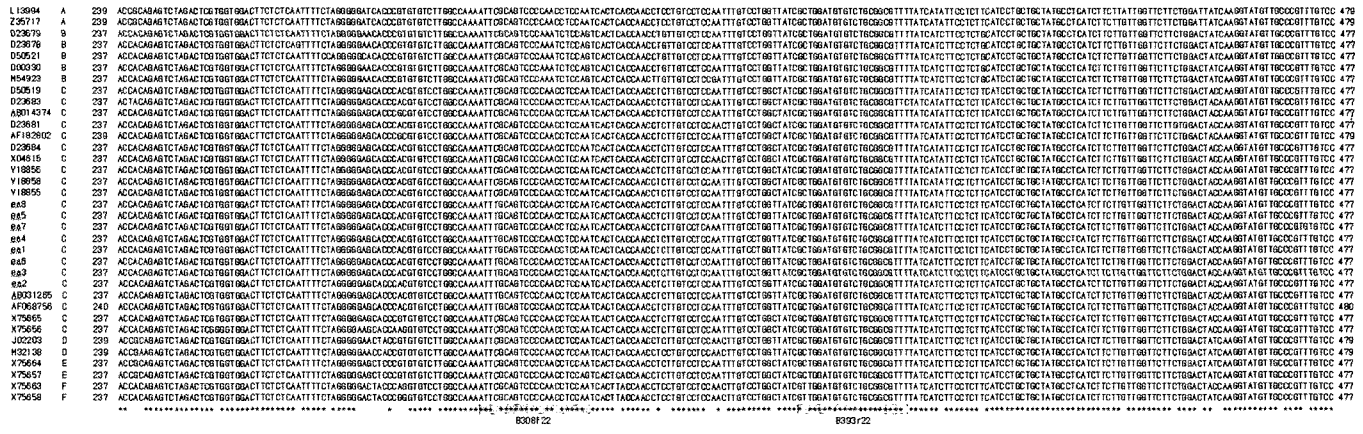

Fig. 1. Representative HBV sequences (35 total), including those from genotypes A to F. The primers used in this study were selected from the candidate sequences, and corresponded to highly conserved regions among the genotypes.

Primers. To design primers that would detect most of the HBV genotypes, 35 representative HBV sequences derived from phylogenetic analysis, including those from genotypes A to F, were aligned.[7,8] Primer B308f22 (sense primer) and primer B393r22 (antisense primer) corresponded to regions in the HBV polymerase gene, and were highly conserved among all genotypes (Fig. 1.). Other primer candidates which were not shown in this report were also tested, but they were not in the specific self-hybridizing temperature range masked by the internal control. Because the peak in other range could cause diagnostic error and confusion in making decision, these candidates were eliminated. The primers DFICf1 and DFICr1 (dimer forming internal control) were derived from the ribosomal 30 s gene from plants. The internal control primers had 9 nucleotides of complementary sequence at their ends, which acted as reciprocal priming sites, and were used to control for inhibition of PCR by unexpected factors. Table 2 shows the sequences of the primers used in these studies.

Real-time PCR. HBV DNA was amplified by real-time PCR using the home-brew PCR reagent kit supplied with the GenSpector TMC-1000. Each reaction volume was a total of 1 μl, including 0.5 μl of sample and 0.5 μl of reaction mixture containing 0.5 X of SYBR green I (Sigma, MO, USA), 200 μM dNTPs, 1x *Taq* Pol buffer, 0.04 U/μl of D-*Taq* Polymerase (Vivagen, Korea), 100 nM HBV primers, and 200 nM internal control primers. Cycling conditions included an initial denaturation step of 120 s at 94°C, followed by 50 cycles of 2 s at 94°C and 15 s at 52°C (30 s at 52°C in the last cycle). At the end of each cycle, the fluorescent signal at 530 nm was measured by the GenSpector instrument. Qualitative analysis of the PCR products was performed using melting-curves and the melting-curve-analysis program of the GenSpector.[9] Briefly, following the last amplification cycle, the reaction temperature was rapidly increased to 60°C and then slowly increased to 90°C at a rate of 0.1°C/s, with continuous fluorescence monitoring.

Contamination control. To minimize template contamination, the testing laboratory was divided into 3 sections. The first section was for reagent mixing, the second was for specimen extraction and loading, and the third was for carrying out the PCR reaction. The first section contained an aseptic clean bench, and in the second, all procedures were carried out in a fume hood. Both sections were irradiated with UV when not in use to reduce potential sources of contamination.[10]

Results

Assay characteristics. The specific melting temperature (Tm) was determined from serial dilutions of several control specimens (ACCURUN 325). The Tms of HBV-positive samples (100 positives from the VERSANT HBV DNA 3.0 Assay, bDNA) and internal controls were then measured, and a representative result using the GenSpector analysis software is displayed in Fig. 2. The mean Tm was 83.5 ± 2.5°C for the HBV-positive samples. The internal control provided with the assay master mix had a mean specific Tm of 72.5 ± 2.5°C. The variation of 2.5°C covered all peaks generated, and no other peaks in these ranges were observed. Peak areas were calculated automatically by the analysis software. Areas over 0.100 for HBV, 0.010 for the internal controls were considered

Table 2. The sequences of the primers used in these studies

Name	Sequence	Use
B308f22	ATT CGC AGT CCC CAA CCT CCA A	Sense primer
B393r22	AAA CGC CGC AGA CAC ATC CAG C	Antisense primer
DFICf1	CGA GCG TTT ATA TGA TTC TTT GAT AGA AAG	Internal control sense
DFICr1	AAC ATA CCC CTT TTG TGA TTT CTT TCT ATC	Internal control antisense

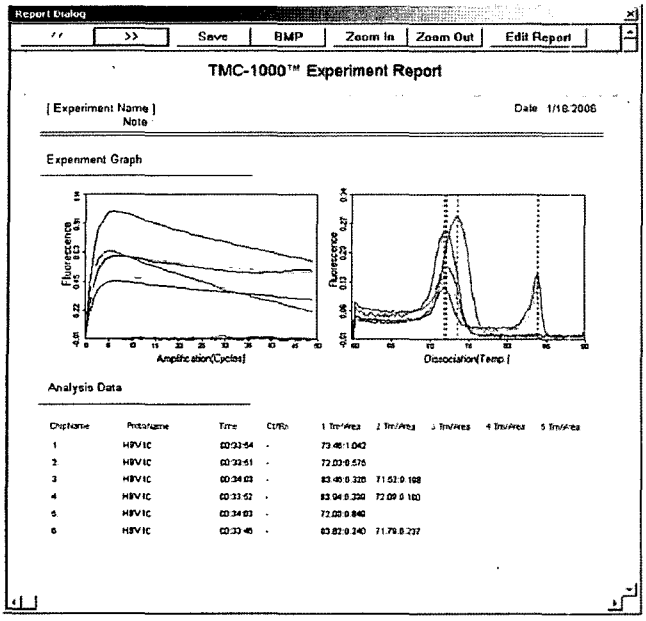

Fig. 2. The data report (print-out) generated by the GenSpector software. Included in the report are the chip name (designated by the user), protocol, amplification curves, melting curves, melting temperatures, and corresponding areas of the each peaks.

to be above background, and designated as positive peaks. All specimens were tested in triplicate. A specimen was considered positive if it was positive more than twice.

Detection limit. The detection limit of the GenSpector HBV DNA real-time PCR assay was determined by testing serial dilutions of samples from the ACCURUN 325. We prepared 3-fold serial dilutions of the positive control, using negative human serum, and extracted using a Qiagen DNA extraction kit. The dilution and extraction procedure was repeated 5 times on different days, and 5 different serial serum dilutions were tested each time. The lower limit of detection for HBV DNA was 1.2 copies per reaction, which corresponded to 840 copies per m*l* serum, with a 95% detection rate (Table 3).

Clinical sensitivity and specificity. One hundred HBV DNA positive serums (Greencross Reference Laboratory, Yongin, Korea) were assayed using both the VERSANT HBV DNA 3.0 Assay (bDNA) and the GenSpector HBV DNA real-time PCR assay. Concordant results were obtained for all 100 samples. Clinical specificity was evaluated by analysis of samples from Nabi Diagnostics that were non-reactive in the VERSANT HBV DNA 3.0 Assay (bDNA). Using the GenSpector HBV DNA real-time PCR assay, no HBV DNA was detected in any of these 100 samples, indicating a clinical specificity of 100%.

Detection of viral variants. To assess the ability of the GenSpector HBV DNA real-time PCR assay to detect the entire range of HBV genotypes, samples from the HBV DNA Genotype Performance Panel PHD201E (BBI Diagnostics, MA, USA) were tested. All genotypes from the panel were detected with similar efficiencies (Table 1).

Other Virus Detection. Reactivity to other viruses, which could generate false positive result, was tested. The BBI panel of viruses and mixed specimens of HBV with other viruses was examined. The names of the viruses and results are shown in Table 1.

Discussion

In this report, we described a rapid new real-time PCR assay for detecting HBV. The special features of the GenSpector real-time PCR assay include speed (analysis time of 30 minutes) and sensitivity (detection of 840 copies/m*l* serum). The GenSpector assay was comparable to that of a commercially available NAT assay for HBV (Roche COBAS Amplicor HBV Monitor and Bayer HBV bDNA 3.0 Assay). The GenSpector assay also has a DFIC internal control system, similar to other real-time PCR assays, as an internal amplification control.[11,12]

Compared to commercial real-time assays, GenSpector is much more rapid, even taking into account extraction time (30 minutes). Its individual modules can analyze each individual specimens regardless of their number, so that there is no need to gather fixed number of specimens. Moreover, use of a real-time assay minimizes template contamination, since a positive control is not needed, and the melting temperature analysis required no post-PCR processing of the samples. Although this melting temperature analysis cannot quantify DNA levels, as can be done in the other commercial detection system, it does clearly distinguish infected from non-infected specimens. We favor use in a small private hospital for primary diagnosis, to take advantage of the rapid analysis time, specificity and feasibility to analyze a small number of specimens.

Table. 3. Detection limit of GenSpector HBV DNA real-time PCR assay. Final detection limit was 840 copies/m*l* serum (NTC: no template control)

HBV concentration (copies/m*l* serum)	Repeats (Positives / Tests)					Detection rate (%)
	Day 1	Day 2	Day 3	Day 4	Day 5	
NTC	0 / 5	0 / 5	1 / 5	1 / 5	2 / 5	16
84	2 / 5	3 / 5	3 / 5	0 / 5	5 / 5	52
280	4 / 5	2 / 5	2 / 5	3 / 5	5 / 5	64
840	4 / 5	5 / 5	5 / 5	5 / 5	5 / 5	96
2,800	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5	100
7,700	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5	100

Some limitations of the GenSpector assay exist. In spite of the rapid analysis time, the instrument processes a maximum of 6 specimens at a time. The 6 modules function independently, which means that the data from each module cannot be compared directly, making quantitative comparisons of DNA levels problematic. And last, as with other NAT assays, the GenSpector assay had a high false positive rate of 16%. But by triplicated tests of each specimens, we reduced the probability of false positive to about 6.9% and no false positive was found in 100 clinical negative specimens.

In conclusion, the GenSpector HBV DNA real-time PCR assay provides a rapid, sensitive and cost-effective method for qualitative HBV DNA detection in blood. To provide a system for high throughput blood screening, further work is needed to develop and upgrade the GenSpector to handle more modules and multiple fluorescence detectors. But, as a new rapid method of a chip-based diagnosis system, the possibility of a further development is expected.

Acknowledgments

GenSpector rapid real-time PCR machine was kindly donated by Greencross Medical Science.

References

1. WHO. (1996) The World Health Report 1996, pp. 33-35, WHO, Geneva, Switzerland.
2. Lee, W. M. (1997) Medical Progress: Hepatitis B Virus Infection. *N. Engl. J. Med.* **337**, 1733-1745.
3. Campillo, M. L., Quiroga, J. A., Bartolomé, J., Moraleda, G., Castillo, I. and Carreño, V. (1992) Protein composition of the hepatitis B virus e antigen in the natural course of disease and following interferon therapy. *J. Clin. Microbiol.* **30**, 1256-1261.
4. Schreiber, G. B., Busch, M. P., Kleinman, S. H. and Korelitz, J. J. (1996) The risk of transfusion-transmitted viral infections. *N. Engl. J. Med.* **334**, 1685-1690.
5. Meng, Q., Wong, C., Rangachari, A., Tamatsukuri, S., Sasaki M., Fiss E., Cheng L., Ramankutty T., Clarke D., Yawata H., Sakakura Y., Hirose T. and Impraimi C. (2001) Automated Multiplex Assay System for Simultaneous Detection of Hepatitis B Virus DNA, Hepatitis C Virus RNA, and Human Immunodeficiency Virus Type 1 RNA. *J. Clin. Microbiol.* **39**, 2937-2945.
6. Yao, J. D. C., Beld, M. G. H. M., Oon, L. L. E., Sherlock, C. H., Germer, J., Menting, S., Thoe, S. Y. S., Merrick, L., Ziermann, R., Surtihadi, J. and Hnatyszyn, H. J. Multicenter (2004) Evaluation of the VERSANT Hepatitis B Virus DNA 3.0 Assay. *J. Clin. Microbiol.* **42**, 800-806.
7. Alestig, E., Hannoun, C., Horal, P. and Lindh, M. (2001) Phylogenetic Origin of Hepatitis B Virus Strains with Precore C-1858 Variant. *J. Clin. Microbiol.* **39**, 3200-3203.
8. Odgerel, Z., Nho, K. B., Moon, J. Y., Kee, S. H., Park, K. S., Song, K. J., Baek, L. J., Yeon, J. E., Byun, K. S., Less, C. H. and Song, J. W. (2003) Complete Genome Sequence and Phylogenetic Analysis of Hepatitis B Virus (HBV) Isolates From Patients With Chronic HBV Infection in Korea. *J. Med. Virol.* **71**, 499-503.
9. Boivin, G., Co^te´ S., De´ry, P., De Serres, G. and Bergeron, M. G. (2004) Multiplex Real-Time PCR Assay for Detection of Influenza and Human Respiratory Syncytial Viruses. *J. Clin. Microbiol.* **42**, 45-51.
10. Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Kaczmarski, E. B. and Fox, A. J. (2000) Contamination and Sensitivity Issues with a Real-Time Universal 16S rRNA PCR. *J. Clin. Microbiol.* **38**, 1747-1752.
11. Konnick, E. Q., Eralim, M., Ashwood, E. R. and Hillyard, D. R. (2005) Evaluation of the COBAS Amplicor HBV Monitor Assay and Comparison with the Ultrasensitive HBV Hybrid Capture 2 Assay for Quantification of Hepatitis B Virus DNA. *J. Clin. Microbiol.* **43**, 596-603.
12. Shyamala, V., Arcangel, P., Cottrell, J., Coit, D., Medina-Selby, A., McCoin, C., Madriaga, D., Chien, D. and Phelps, B. (2004) Assessment of the Target-Capture PCR Hepatitis B Virus (HBV) DNA Quantitative Assay and Comparison with Commercial HBV DNA Quantitative Assays. *J. Clin. Microbiol.* **42**, 5199-5204.