Title	Sensitive Assay for Quantification of Hepatitis B Virus Mutants by Use of a Minor Groove Binder Probe and Peptide Nucleic Acids
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Citation	Journal of Clinical Microbiology, 48(12), 4487-4494 https://doi.org/10.1128/JCM.00731-10
Issue Date	2010-12
Doc URL	http://hdl.handle.net/2115/45538
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Type	article (author version)
File Information	JCM48-12_4487-4494.pdf

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Title

Sensitive assay for the quantification of hepatitis B virus mutants by a minor groove binder probe and peptide nucleic acids

Running title

Sensitive assay for HBV mutants by MGB probe and PNA

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Abstract

Lamivudine is the first nucleoside analogue that was shown to have a potent effect on hepatitis B virus (HBV). However, the emergence of resistant or cross-resistant mutants to nucleos(t)ide analogues remains as a serious problem. Several assays for the detection and quantification of antiviral resistant mutants have been reported, but it has been difficult to measure the amounts of mutants accurately, especially when the target strain is minor in the mixed population. It has been shown that accurate measurement of a minor strain is difficult as long as matching reaction with a single probe was included in the assay. We developed a new method for quantification of lamivudine-resistant strains in a mixed virus population by real-time PCR using minor groove binder probes and peptide nucleic acids, and we achieved a wide and measurable range from 3 to 10 $\log_{10}$ copies/ml and a high sensitivity with a discriminative limit of 0.01% to the predominant strain. The clinical significance of measuring substitutions of not only M204 but also L180 residues of HBV polymerase was demonstrated by this method. This assay increases the versatility of a sensitive method for the quantification of a single nucleotide mutation in a heterogeneous population.

1. Introduction

Chronic liver diseases due to hepatitis B virus (HBV) infection are still serious problems worldwide and many patients suffer from liver cirrhosis and hepatocellular carcinoma (2, 7, 21). Nucleos(t)ide analogues have been introduced into the treatment of chronic hepatitis B, and inhibition of disease progression has been achieved (20). However, as several analogues have been developed and have come into wide use, the risk of the emergence of resistant or cross-resistant mutations is increasing. This is a serious problem, as it may lead to the virological and biochemical breakthroughs that result in the loss of therapeutic effects (18).

Lamivudine is the first nucleoside analogue that was demonstrated to have a potent effect on HBV (8, 17). The results of many studies on lamivudine-resistant mutants have been reported, and the impact of substitution from M204 to I204 or V204 as YMDD mutations was initially investigated (20). Thereafter, the clinical significance of other regions of HBV polymerase was clarified. We reported the significance of dual mutations of the YMDD motif and LLAQ motif in which the L180 residue was included for the severity of breakthrough hepatitis (29). Recently, substitution of the L180 residue has been noted as one of the most crucial changes for the emergence of entecavir-resistant mutants.

It is important to detect mutants as early as possible to confirm genotypic or phenotypic resistance followed by virological breakthrough and rebound. Several methods using polymerase chain reaction (PCR) or hybridization have been reported for the qualitative detection of antiviral-resistant mutants.

Some assays have been proposed as being sensitive methods for the quantitative measurement of mutant strains (33), and we have reported a quantification assay using type-specific minor groove binder (MGB) probes (40). However, we found that it was

difficult to measure the amounts of mutants accurately, especially when the target strain was minor in the mixed population.

In this study, we developed a new sensitive assay for the quantification of hepatitis B virus mutants by introducing peptide nucleic acids (PNAs) to real-time PCR with MGB probes. PNAs are DNA mimics and bind to their complementary nucleic acid sequences with high specificity and have PCR clamping effects (30, 31). We evaluated the efficacy of this assay and demonstrated the clinical significance of estimating the quantitative follow-up of dual mutants.

2. Materials and methods

2-1. Serum samples

HBs antigen-positive samples were obtained from chronic hepatitis B patients and stored at -20°C. DNA was extracted and purified from the serum by a spin column method using a QIAamp DNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. The study was conducted with approval of our institute's Ethics Committee and written informed consent was obtained from all patients.

2-2. Construction of minor groove binder (MGB) probes

MGB probes for the YMDD motif were designed to hybridize with HBV containing rt204 in domain C of the polymerase region in order to be complementary to the wild type (rt204M) and mutant strains (rtM204I and rtM204V). For the YIDD motif, two kinds of MGB probes were prepared because of the different kinds of codons for the same amino acid: YIDD1 for codon ATC and YIDD2 for codon ATT. Sequences of primers and MGB probes used were as follows: forward primer, 5'-GGGCTTTCCCCCACTGTT-3'; reverse primer, 5'-AAAGGGACTCAAGATGTTGTACAGACT-3'; YMDD probe, 5'-CTTTCAGTTATATGGATGATGTG-3'; YIDD1 probe,

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5'-CTTTCAGTTATATCGATGATGTGG-3';   YIDD2   probe,

5'-CTTTCAGTTATATTGATGATGTGG-3';   YVDD   probe,

5'-CTTTCAGTTATGTGGATGATGT-3'.

MGB probes for the LLAQ motif (rt179-182) in domain B were also designed to be complementary to the wild type (rt180L) and a mutant (rtL180M). Sequences of primers and MGB probes used were: forward primer, 5'- CCTATGGGAGTGGGCCTC-3'; reverse primer, 5'- AACAGTGGGGGAAAGCCCT-3'; LLAQ probe, 5'-CTCCTGGCTCAGTTTA-3'; LMAQ probe, 5'-TTCTCATGGCTCAGTTTACTA-3'. Those probes were purchased from Applied Biosystems Japan Ltd. (Tokyo, Japan).

2-3. Construction of PNAs

PNA was designed to exactly match the sequence of each MGB probe and was provided by Greiner Bio-One (Tokyo, Japan). Sequences of PNAs were as follows: PNA-YMDD,   5'-GTTATATGGATGATGTG-3';   PNA-YIDD1, 5'-GTTATATCGATGATGTGG-3'; PNA-YIDD2, 5'-GTTATATTGATGATGTGG-3'; PNA-YVDD,   5'-AGTTATGTGGATGATGT-3';   PNA-LLAQ, 5'-TCCGTTTCTCCTGGCTC-3'; PNA-LLAQ, 5'-CCGTTTCTCATGGCTCA-3'.

2-4. Construction of HBV plasmids for the standard

HBV plasmids were prepared for construction of a standard for quantification. PCR products that contained both YMDD and LLAQ motifs obtained from HBV-positive patients were cloned and *Escherichia coli* X L-1 cells as competent cells were transformed by each of the mutant-specific DNA fragments. Finally, plasmid DNA corresponding to each MGB probe was constructed.

2-5. Real-time PCR

Real-time PCR with the MGB probe and PNAs was performed using an ABI Real-Time

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PCR System 7300 (Applied Biosystems, Foster City, CA, USA). A sample was measured with an MGB probe that is complementary to a target strain and with PNAs that are complementary to each strain of the motif except for the target one (Fig. 1).

The reaction was carried out in a final volume of 50 μl in each well of a plate containing 10 μl of serum sample, 400 nM of forward and reverse primers, 250 nM of MGB probe, 2 μM of PNAs and 1×TaqMan ^{TM} Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Optimal concentrations of MGB probes and PNAs were determined by our own examinations as described later.

The conditions of the PCR were as follows: 10 minutes at 95°C for the initial denaturation followed by 50 cycles at 95°C for 15 seconds for denaturation and 71°C for 1 minute for PNA annealing and 60°C for 1 minute for primer extension.

A TaqMan probe has a reporter dye and a non-fluorescent quencher at the 5'and 3' ends, respectively. The reporter dye emission is quenched when the probe remains intact. When an MGB probe hybridized with a complementary strain, the fluorescence of a reporter dye became detectable (Fig.2a).

When PCR was performed with a complementary PNA and a non-complementary MGB probe to the target nucleotide, the extension and amplification was blocked by the preferentially bound PNA (Fig. 2b).

Real-time PCR was performed separately with each MGB probe and non-complementary PNAs for all sets of target strains. After the exclusion of background signals by subtracting signals of early cycles of PCR, a threshold was set in the linearly increasing region of accruals of fluorescence signals. Then the threshold cycle (Ct) at which the signal reached the threshold was measured. The amount of the target strain was calculated from Ct number and a linear regression curve for standards of each strain (Fig.3).

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3. Results

3-1. Standard curve for the measurement of a single strand using an MGB probe

HBV plasmids that had been quantitatively adjusted beforehand were used for 10-fold serial dilution from $10 \log_{10}$ copies/ml to $1 \log_{10}$ copies/ml; from this, the standard curves were generated. A strong inverse correlation between the logarithmic concentration of samples and Ct values was obtained, and the linearity was confirmed within a wide range from 3 to $10 \log_{10}$ copies/ml.

3-2. Measurement of a target mutant in the mixed strains

The accuracy of quantification of a single HBV strain was determined in a mixture of multiple strains by real-time PCR with a type-specific MGB probe without PNA. HBV plasmids serially diluted 10-fold within a range of $10 \log_{10}$ copies/ml to $4 \log_{10}$ copies/ml were used for quantification and under the condition of coexistence of $6 \log_{10}$ copies/ml of another strain in the same motif. Correct measurement of the target strain was achieved when the target strain predominated over another strain in their concentration, and the measurement error was estimated to be less than twice the amount theoretically calculated. However, measurements were overestimated when the concentration of the target strain was lower than that of another strain (Fig. 4).

3-3. Clamping effects of PNA on PCR

PNAs were introduced into the assay system to reduce quantitative errors due to mismatching of MGB probes. Beforehand, the clamping effects of PNAs were investigated to determine the concentration at which the non-complementary PNAs had a negative impact.

Six $\log_{10}$ copies/ml of HBV plasmids were quantified together with the MGB probe and PNA that were complementary to the target strain. Delays in the Ct value by different

concentrations of PNA were measured (Fig. 5). The delay correlated with PNA concentration, and a clamping effect of 1/100 to 1/1000 was observed when 100 pmol of PNA was used. When real-time PCR was performed with a complementary MGB probe and with all sets of the PNAs that are complementary to each strain in the same motif except for the target, a delay in the Ct value was observed; that was when there were high concentrations of non-complementary PNA. However, the delays were modest when the concentration of PNA was less than 100 pmol. The concentration of PNA for the assay was determined from this result.

3-4. Measurement using both MGB probes and PNA

For confirmation of the proper method of determination by this assay, the amount of HBV strain was measured in a mixture of two different strains of the same motif using a new method of real-time PCR with the MGB probe and PNA. At first, 10-fold diluted samples of target HBV plasmids from 9 $\log_{10}$ copies/ml to 3 $\log_{10}$ copies/ml were measured under the condition of coexistence of 3 $\log_{10}$ copies/ml of another strain. There were no significant differences in the values of the target strain regardless of the presence of the mixed strain. Next, the amount of a minority strain was determined in presence of a predominant strain. A 10-fold serial dilution of target HBV plasmids ranging from 9 $\log_{10}$ copies/ml to 3 $\log_{10}$ copies/ml were measured under the condition of coexistence of 9 $\log_{10}$ copies/ml of a counter strain. A linear correlation with the Ct values and sample concentrations was observed in the range from 9 $\log_{10}$ copies/ml to 5 $\log_{10}$ copies/ml. However, a target strain less than 5 $\log_{10}$ copies/ml could not be quantified accurately. Therefore, the detection limit of a non-dominant strain was determined to be 0.01% of the predominant one (Fig. 6).

3-5. A clinical case

The clinical and virological course of a case of breakthrough hepatitis after the emergence of lamivudine-resistant mutants is described below (Fig. 7a).

A 42-year-old male was administered 100 mg of lamivudine q.d. in 1999. At first, HBV DNA reduced steadily and serum ALT level also decreased to the normal range after the start of treatment. About 16 months later, viral breakthrough and mild elevation of ALT were observed. Despite the fact that HBV DNA and HBe antigen returned to relatively high levels, ALT levels remained almost within the normal range. Thereafter, the ALT level suddenly increased despite the fact that HBV DNA remained at the same level. When we measured mutants against lamivudine by a direct sequencing method, changes in ALT and HBV DNA were thought to be related to the changes in mutant patterns over time. However, we could not predict the time or the severity of breakthrough hepatitis by this limited information, especially when HBV DNA loads did not change.

Precise examination of changes in HBV mutants was performed by real-time PCR with the MGB probe and PNA (Fig. 7b).

ALT levels were stable, whereas total levels of HBV DNA remained high. An LLAQ/YIDD strain was dominant in the earlier phase. Next, an LLAQ strain became measurable, and when an YVDD strain emerged and overcame the YIDD levels, breakthrough hepatitis occurred. This case is a good example that shoes the clinical significance of measuring the amounts of HBV mutants of not only the YMDD motif but also the LLAQ motif by this new assay.

4. Discussion

Inhibition of disease progression for patients with chronic HBV infection has been achieved by treatment with nucleos(t)ide analogues (20). Treatment with these analogues has reduced not only the incidence of hepatic decompensation but also the risk of

occurrence of hepatocellular carcinoma (27). The same effect on recurrence has also been reported (4). However, the most serious issue is the emergence of viruses with resistance against nucleos(t)ide analogues, as this leads to the virological and biochemical breakthroughs. Therapeutic effects were lost in patients who developed the resistant mutations (18).

From a resistance or a cross-resistance perspective, nucleos(t)ide analogues are classified into three groups based on their structural characteristics (35, 42): L-nucleoside analogues (lamivudine, telbivudine, clevudine, and emtricitabine), alkyl phosphonates (adefovir and tenofovir) and the D-cyclopentane group (entecavir). Resistance rates after 5 years of treatment have been reported to be 80% for lamivudine, 29% for adefovir and 1.2% for entecavir. Although the rate of early-period resistance has been reduced as new drugs are introduced, the long-term results have not been yet confirmed. Moreover, the resistance rate becomes higher for patients with a lamivudine-resistant mutant, up to 20% within 1 year for adefovir and 51% after 5 years for entecavir.

It is important to detect and quantify these mutant strains, and various methods have been developed for such measurements. The most common method for detection of mutant viruses is direct sequencing after PCR amplification. However, the detection limit of a minor strain in the heterogeneous virus population is about 20%. Recently, a new method named ultra-deep pyrosequencing has been developed (26, 36). This sequencing relies on the detection of DNA polymerase activity by measuring the pyrophosphate (PPi) released by the addition of a deoxyribonucleoside monophosphate (dNMP) to the 3' end of a primer. The detection limit increased up to 1% by this technique.

There are several methods for hybridization-based genotyping: line probe assay (LiPA) (12, 37), mixed hybridization-sequencing-PCR (mini-sequencing) (15) and

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oligonucleotide tip assay (13). The sensitivity is 5 to 10% by LiPA and 2 to 10% by mini-sequencing assay. The sensitivity of the high-density DNA chip reported by Tran et al. was 30 to 50%, but it was able to detect 245 mutations, 20 deletions and 2 insertions at 151 positions (39). Honh et al. reported that mass spectrometry is another method for detection of mutants. Matrix-assisted laser desorption / ionization time of flight mass spectrometry (MALDI-TOF MS) had a detection limit of 100 copies/ml and a sensitivity of 1% (10).

Some methods modified with real-time PCR have been developed for the quantification of mutant viruses: PCR with molecular beacons (32), PCR with a minor groove binder (MGB), PCR with an LNA-mediated probe (19) and ARMS-PCR (33).

MGB can fit snugly into the minor groove, which is the deep narrow space between the two phosphate-sugar backbones of a double stranded DNA helix.

Afonina et al. reported that DNA probes with a tripeptide [1,2-dihydro-(3H)-pyrrolo [3,2-e]indole-7-carboxylate (GDPI$_3$)] which is conjugated to the 5'-end of short oligodeoxynucleotides (ODNs) formed unusually stable hybrids with complementary DNA and can be used as a PCR primer (1). This MGB probe has a higher melting temperature (Tm) and has increased specificity compared to that of ordinary DNA probes, especially when a mismatch is in the MGB region of the duplex (6, 16). Zhao et al. reported a real-time PCR method using a TaqMan-MGB probe for measurements of total amounts of HBV DNA (41), and we have reported quantification of lamivudine-resistant mutants by the type-specific TaqMan MGB probe assay (40).

Several authors have reported similar sensitivity of 10% for the detection of minor variants (9, 11, 25). Intra-experimental variability was reported to be 4.9% by Lole et al. and 1.0 to 2.2% depending on the type of mutants by our own evaluation (40).

However, our study revealed that the accuracy of quantification was reduced in the case of measurements of minor strains in a mixed population. Therefore, we introduced peptic nucleic acids (PNAs) to be used in combination with the MGB probes.

PNAs are DNA mimics in which the deoxyribose phosphate backbone of DNA has been replaced by N-(2-aminoethyl) glycine linkages (30). They recognize and bind to their complementary nucleic acid sequences with high thermal stability and specificity (31). PNAs cannot function as primers for DNA polymerase and can be used to block a PCR amplification process in a sequence-specific manner. This PCR clamping allows for direct analysis of single base mutations by PCR.

Kirishima et al. (14) reported a sensitive method for the detection of a lamivudine-resistant mutant by PNA-mediated PCR clamping with RFLP, and Mori et al. performed semiquantitative measurement of mutants using PNA (28).

Under the condition of real-time PCR in our assay, one reaction cycle started at 95°C for denaturation and finished at 60°C for extension. The melting temperature (Tm) of PNA was designed to be 3°C higher than that of an MGB probe. Because of the gap in this temperature, PNA is expected to clamp a non-complementary strain first and then an MGB probe will hybridize to the target strand thereafter. As a result, the assay was able to achieve a significant reduction in mismatches. However, amplification delay was observed with an excessive amount of non-complementary PNA. No matter how well an assay is constructed, mismatches cannot be completely avoided as long as the reaction includes a matching process. However, it is thought that the actual measurement can be performed correctly by setting the appropriate concentration of the reaction mixture. As for the limit of detection, we showed that 0.01% of the minority strain could be measured by our assay. However, more robust analysis is required to determine the true analytical

sensitivity by measuring a number of clinical samples.

This requires a precise knowledge of the sequence of the mutants prior to the determination with this assay; as such, it cannot be construed as a general method for the study of viral quasispecies where mutants and emerging mutants will have an unknown sequence. However, it seems ideally suited for the approach of measuring a known mutant virus such as HBV-resistant mutants against nucleos(t)ide analogues.

Although the risk of breakthrough hepatitis is thought to correlate with the duration of infection of mutant viruses (23), not only the duration but also the change in strains of the mutants is important for the occurrence of hepatitis. We reported that patterns of the YMDD motif / LLAQ motif often changed from YMDD/LLAQ to YIDD/LLAQ, YIDD/LMAQ and YVDD/LMAQ in that order and that the degree of liver damage increased as the mutation accumulated (29). As demonstrated by the case presented in this report, breakthrough hepatitis seems to have a relationship with the specific type of mutants and the duration from emergence of a new mutant. The degree of viral increase after the emergence of dual mutations was smaller than that after emergence of the first mutation despite the fact that the level of liver dysfunction was higher with the latter mutation. This result suggested that breakthrough hepatitis closely correlated with changes of viral characters. Das et al. (5) reported differences in inhibitory effects on HBV DNA polymerase by different lamivudine-resistant mutants. The inhibitory effects were measured by recombinant HBV DNA polymerase expressed in a baculovirus transfer vector. The fold changes of inhibition to the DNA polymerase were 8.0 for M204I, 15.2 for L180M+M204I and 25.2 for L180M+M204V, compared to the wild type. The results of our clinical case showed a tendency similar to that of their evaluations and similar to the results of a recent molecular modeling study. Sharon et al. demonstrated

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that binding energy was significantly different between dCTP and 3TC in mutant HBV polymerases and that 3TC lost the binding affinity with the mutants in comparison to the n atural substrate dCTP (34). L180 residues did not interact directly with 3TC, but the M180 mutant caused loosening of the hydrophobic pocket formed by the residues A87, F88, P177, L180 and M204. Consequently, dual mutations L180M+M204I or L180M+M204V were more likely to show steric clash between I204 or V204 and 3TC. Despite the high risk of multi-drug resistance by lamivudine, it has been widely used mainly due to its low cost. Additive substitution of any residue of T184, S202 and M250 on M204V and L180M is necessary in the case of entecavir-resistant mutation. Therefore, it is important to observe the change of the L180 substitution prior to M204V emergence even for cases where a drug other than lamivudine has been administered.

In conclusion, we developed a new method for the accurate quantification of lamivudine-resistant strains in a mixed virus population. This is the first report on the combined use of an MGB probe and PNAs for the quantification of viral amounts using real-time PCR. This methodology should prove immensely useful to better understand the dynamics of the emergence of HBV-resistant mutants. Moreover, this assay has the versatility to be applied as a sensitive method for the quantification of a single nucleotide mutation in a heterogeneous population of not only viruses but also other nucleic acids.

References

1. **Afonina, I., M. Zivarts, I. Kutyavin, E. Lukhtanov, H. Gamper, and R. B. Meyer.** 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. Nucleic Acids Res. **25:**2657-2660.

2. **Chen, C. J., H. I. Yang, J. Su, C. L. Jen, S. L. You, S. N. Lu, G. T. Huang, U. H. Iloeje, and R.-H. S. Grp.** 2006. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. J.A.M.A. **295:**65-73.

3. **Chieochansin, T., S. Chutinimitkul, S. Payungporn, A. Theamboonlers, P. Tangkijvanich, P. Komolmit, and Y. Poovorawan.** 2006. Rapid detection of lamivudine-resistant hepatitis B virus mutations by PCR-based methods. Tohoku J. Exp. Med. **210:**67-78.

4. **Chuma, M., S. Hige, T. Kamiyama, T. Meguro, A. Nagasaka, K. Nakanishi, Y. Yamamoto, M. Nakanishi, T. Kohara, T. Sho, K. Yamamoto, H. Horimoto, T. Kobayashi, H. Yokoo, M. Matsushita, S. Todo, and M. Asaka.** 2009. The influence of hepatitis B DNA level and antiviral therapy on recurrence after initial curative treatment in patients with hepatocellular carcinoma. J. Gastroenterol. **44:**991-999.

5. **Das, K., X. F. Xiong, H. L. Yang, C. E. Westland, C. S. Gibbs, S. G. Sarafianos, and E. Arnold.** 2001. Molecular modeling and biochemical characterization reveal the mechanism of hepatitis B virus polymerase resistance to lamivudine (3TC) and emtricitabine (FTC). J. Virol. **75:**4771-4779.

6. **de Kok, J. B., E. T. G. Wiegerinck, B. A. J. Giesendorf, and D. W. Swinkels.** 2002. Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). Hum. Mutat. **19:**554-559.

7. **de Franchis, R., G. Meucci, M. Vecchi, M. Tatarella, M. Colombo, E. Delninno, M. G. Rumi, M. F. Donato, and G. Ronchi.** 1993. The natural history of asymptomatic hepatitis B surface antigen carriers. Ann. Intern. Med. **118:**191-194.

8. **Dienstag, J. L., E. R. Schiff, T. L. Wright, R. P. Perrillo, H. W. L. Hann, Z. Goodman, L. Crowther, L. D. Condreay, M. Woessner, M. Rubin, N. A. Brown, and U. S. L. I. Grp.** 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. N. Engl. J. Med. **341:**1256-1263.

9. **Geng, H. F., B. Hua, H. Wang, Y. H. Cao, Y. Sun, and A. R. Yu.** 2006. Dual-probe assay for detection of lamivudine-resi stance hepatitis B virus by real-time PCR. J. Virol. Methods **132:**25-31.

10. **Hong, S. P., N. K. Kim, S. G. Hwang, H. J. Chung, S. Kim, J. H. Han, H. T. Kim, K. S. Rim, M. S. Kang, W. Yoo, and S. O. Kim.** 2004. Detection of hepatitis B virus YMDD variants using mass spectrometric analysis of oligonucleotide fragments. J. Hepatol. **40:**837-844.

11. **Hua, R., Y. Tanaka, K. Fukai, M. Tada, M. Seto, Y. Asaoka, M. Ohta, T. Goto, F. Kanai, N. Kato, H. Yoshida, T. Kawabe, O. Yokosuka, and M. Omata.** 2008. Rapid detection of the hepatitis B virus YMDD mutant using TaqMan-minor groove binder probes. Clin. Chim. Acta **395:**151-154.

12. **Hussain, M., S. Fung, E. Libbrecht, E. Sablon, C. Cursaro, P. Andreone, and A. S. F. Lok.** 2006. Sensitive line probe assay that simultaneously detects mutations conveying resistance to lamivudine and adefovir. J. Clin. Microbiol. **44:**1094-1097.

13. **Jang, H., M. Cho, J. Heo, H. Kim, H. Jun, W. Shin, B. Cho, H. Park, and C. Kim.** 2004. Oligonucleotide chip for detection of lamivudine-resistant hepatitis B virus. J. Clin. Microbiol. **42:**4181-4188.

14. **Kirishima, T., T. Okanoue, Y. Daimon, Y. Itoh, H. Nakamura, A. Morita, T. Toyama, and M. Minami.** 2002. Detection of YMDD mutant using a novel sensitive method in chronic liver disease type B patients before and during lamivudine treatment. J. Hepatol. **37:**259-265.

15. **Kobayashi, S., K. Shimada, H. Suzuki, K. Tanikawa, and M. Sata.** 2000. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). Hepatol. Res. **17:**31-42.

16. **Kutyavin, I. V., I. A. Afonina, A. Mills, V. V. Gorn, E. A. Lukhtanov, E. S. Belousov, M. J. Singer, D. K. Walburger, S. G. Lokhov, A. A. Gall, R. Dempcy, M. W. Reed, R. B. Meyer, and J. Hedgpeth.** 2000. 3 '-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. **28:**655-661.

17. **Lai, C. L., R. N. Chien, N. W. Y. Leung, T. T. Chang, R. Guan, D. I. Tai, K. Y. Ng, P. C. Wu, J. C. Dent, J. Barber, S. L. Stephenson, D. F. Gray, and G. Asia Hepatitis Lamivudine Study.** 1998. A one-year trial of lamivudine for chronic hepatitis B. N. Engl. J. Med. **339:**61-68.

18. **Lai, C. L., J. Dienstag, E. Schiff, N. W. Y. Leung, M. Atkins, C. Hunt, N. Brown, M. Woessner, R. Boehme, and L. Condreay.** 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin.Infect. Dis. **36:**687-696.

19. **Latorra, D., K. Campbell, A. Wolter, and J. M. Hurley.** 2003. Enhanced allele-specliflic PCR discriminationin SNP genotypling using 3 ' locked nucleic acid (LNA) primers. Hum. Mutat. **22:**79-85.

20. **Liaw, Y. F., J. J. Y. Sung, W. C. Chow, G. Farrell, C. Z. Lee, H. Yuen, T. Tanwandee, Q. M. Tao, K. Shue, O. N. Keene, J. S. Dixon, D. F. Gray, J. Sabbat,**

and M. Cirrhosis Asian Lamivudine. 2004. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N. Engl. J. Med. **351:**1521-1531.

21. **Liaw, Y. F., D. I. Tai, C. M. Chu, and T. J. Chen.** 1988. The development of cirrhosis patients with chronic type B hepatitis – a prospective study. Hepatology **8:**493-496.

22. **Libbrecht, E., J. Doutreloigne, H. Van De Velde, M. F. Yuen, C. L. Lai, F. Shapiro, and E. Sablon.** 2007. Evolution of primary and compensatory lamivudine resistance mutations in chronic hepatitis B virus-infected patients during long-term lamivudine treatment, assessed by a line probe assay. J. Clin. Microbiol. **45:**3935-3941.

23. **Lok, A. S. F., C. L. Lai, N. Leung, G. B. Yao, Z. Y. Cui, E. R. Schiff, J. L. Dienstag, E. J. Heathcote, N. R. Little, D. A. Griffiths, S. D. Gardner, and M. Castiglia.** 2003. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology **125:**1714-1722.

24. **Lole, K. S., and V. A. Arankalle.** 2006. Quantitation of hepatitis B virus DNA by real-time PCR using internal amplification control and dual TaqMan MGB probes. Journal of Virological Methods **135:**83-90.

25. **Malmstrom, S., C. Hannoun, and M. Lindh.** 2007. Mutation analysis of lamivudine resistant hepatitis B virus strains by TaqMan PCR. Virol. Methods **143:**147-152.

26. **Margeridon-Thermet, S., N. S. Shulman, A. Ahmed, R. Shahriar, T. Liu, C. L. Wang, S. P. Holmes, F. Babrzadeh, B. Gharizadeh, B. Hanczaruk, B. B. Simen, M. Egholm, and R. W. Shafer.** 2009. Ultra-Deep Pyrosequencing of Hepatitis B Virus Quasispecies from Nucleoside and Nucleotide Reverse-Transcriptase Inhibitor (NRTI)-Treated Patients and NRTI-Naive Patients. J. Infect. Dis. **199:**1275-1285.

27. **Matsumoto, A., E. Tanaka, A. Rokuhara, K. Kiyosawa, H. Kumada, M. Omata, K. Okita, N. Hayashi, T. Okanoue, S. Iino, K. Tanikawa, and G. Inuyama Hepatitis**

Study. 2005. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: A multicenter retrospective study of 2795 patients. Hepatol. Res. **32:**173-184.

28. **Mori, K., M. Minami, T. Kirishima, K. Kunimoto, M. Okita, M. Nakayama, A. Makiyama, J. Yamaoka, T. Nakajima, K. Yasui, Y. Itoh, and T. Okanoue.** 2006. Prediction of breakthrough hepatitis due to lamivudine-resistant hepatitis B virus by a sensitive semiquantitative assay using peptide nucleic acids. Intervirology **49:**274-280.

29. **Natsuizaka, M., S. Hige, Y. Ono, K. Ogawa, M. Nakanishi, M. Chuma, S. Yoshida, and M. Asaka.** 2005. Long-term follow-up of chronic hepatitis B after the emergence of mutations in the hepatitis B virus polymerase region. J. Viral Hepat. **12:**154-159.

30. **Ohishi, W., H. Shirakawa, Y. Kawakami, S. Kimura, M. Kamiyasu, S. Tazuma, T. Nakanishi, and K. Chayama.** 2004. Identification of rare polymerase variants of hepatitis B virus using a two-stage PCR with peptide nucleic acid clamping. J. Med. Virol. **72:**558-565.

31. **Ørum, H., P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, and C. Stanley.** 1993. SINGLE-BASE PAIR MUTATION ANALYSIS BY PNA DIRECTED PCR CLAMPING. Nucleic Acids Res. **21:**5332-5336.

32. **Pas, S. D., S. Noppornpanth, A. A. van der Eijk, R. A. de Man, and H. G. M. Niesters.** 2005. Quantification of the newly detected lamivudine resistant YSDD variants of Hepatitis B virus using molecular beacons. J. Clin. Virol. **32:**166-172.

33. **Punia, P., P. Cane, C. G. Te, and N. Saunders.** 2004. Quantitation of hepatitis B lamivudine resistant mutants by real-time amplification refractory mutation system PCR. J. Hepatol. **40:**986-992.

34. **Sharon, A., and C. K. Chu.** 2008. Understanding the molecular basis of HBV drug resistance by molecular modeling. Antiviral Res. **80:**339-353.

35. **Shaw, T., A. Bartholomeusz, and S. Locarnini.** 2006. HBV drug resistance: Mechanisms, detection and interpretation. J. Hepatol. **44:**593-606.

36. **Solmone, M., D. Vincenti, M. C. F. Prosperi, A. Bruselles, G. Ippolito, and M. R. Capobianchi.** 2009. Use of Massively Parallel Ultradeep Pyrosequencing To Characterize the Genetic Diversity of Hepatitis B Virus in Drug-Resistant and Drug-Naive Patients and To Detect Minor Variants in Reverse Transcriptase and Hepatitis B S Antigen. J. Virol. **83:**1718-1726.

37. **Stuyver, L., C. Van Geyt, S. De Gendt, G. Van Reybroeck, F. Zoulim, G. Leroux-Roels, and R. Rossau.** 2000. Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. J. Clin. Microbiol. **38:**702-707.

38. **Sun, Z., L. F. Zhou, H. Y. Zeng, Z. Chen, and H. H. Zhu.** 2007. Multiplex locked nucleic acid probes for analysis of hepatitis B virus mutants using real-time PCR. Genomics **89:**151-159.

39. **Tran, N., R. Berne, R. Chann, M. Gauthier, D. Martin, M. A. Armand, A. Ollivet, C. G. Teo, S. Ijaz, D. Flichman, M. Brunetto, K. P. Bielawski, C. Pichoud, F. Zoulim, and G. Vernet.** 2006. European multicenter evaluation of high-density DNA probe arrays for detection of hepatitis B virus resistance mutations and identification of genotypes. J. Clin. Microbiol. **44:**2792-2800.

40. **Yoshida, S., S. Hige, M. Yoshida, N. Yamashita, S. I. Fujisawa, K. Sato, T. Kitamura, M. Nishimura, M. Chuma, M. Asaka, and H. Chiba.** 2008. Quantification of lamivudine-resistant hepatitis B virus mutants by type-specific TaqMan minor groove binder probe assay in patients with chronic hepatitis B. Ann. Clin. Biochem. **45:**59-64.

41.   **Zhao, J.R., Y. J. Bai, Q. H. Zhang, Y. Wan, D. Li, and X. J. Yan.** 2005. Detection of

hepatitis B virus DNA by real-time PCR using TaqMan-MGB probe technology. World J.

Gastroenterol. **11;**508-510.

42.   **Zoulim, F., and S. Locarnini.** 2009. Hepatitis B Virus Resistance to Nucleos(t)ide

Analogues. Gastroenterology **137:**1593-1608.

Figure legends

Fig. 1. Measurements of viral amounts by real-time PCR with the MGB probe and PNAs.

Patterns of the combination of the MGB probe and non-complementary PNAs are shown

in the table.

Fig. 2 (a). PCR with a complementary MGB probe.

During the extension phase with primers, the DNA polymerase cleaves the reporter dye

from the probe and the dye emits its characteristic fluorescence. When a PNA added to

the reaction mixture is non-complementary to the target sequence, it does not affect

amplification with a complementary MGB probe.

NFQ: non-fluorescent quencher.

Fig. 2 (b). PCR with a complementary PNA.

The mismatch of a non-complementary MGB probe to the target sequence is blocked by

the PNA that is preferentially bound by the higher melting temperature than the MGB

probes.

Fig. 3. Calculation of values from the results obtained by real-time PCR.

The threshold was set in the linearly increasing region of accruals of fluorescence signals

(ΔRn) and the threshold cycle (Ct) was measured. The value of the target strain was

calculated from Ct number and a standard curve for each strain. The measurement was

performed separately with each MGB probe and non-complementary PNAs for all sets of target strains.

Solid lines: 10-fold serial diluted standards. Dotted line: a sample of the target strain.

Fig. 4. Measurement of a target strain in mixed strains.

Samples of a 10-fold serial dilution of the YVDD strain were measured under the condition of coexistence of 6 $\log_{10}$ copies/ml of the YIDD2 strain. Line (A): MGB for YVDD. Line (B): MGB for YVDD with PNAs for YMDD, YIDD1 and YIDD2. The linearity was maintained even at the lower concentration range.

Fig. 5. Clamping effects of PNA on PCR.

Delays in the Ct value by different concentrations of complementary (solid line) and non-complementary (dotted line) PNAs were measured with the MGB probe for the LLAQ strain (closed circle) and the LMAQ strain (open triangle). PNAs used were (A) complementary to the LLAQ strain (—●—), (B) non-complementary to the LLAQ strain (---●---), (C) complementary to the LMAQ strain (—△—) and (D) non-complementary to the LMAQ strain (---△---). Delay in the Ct value was observed even with non-complementary PNAs when the concentration was high. The differences in delays between (A)/(B) and (C)/(D) are similar.

Fig. 6. Real-time PCR with MGB probe and PNAs.

Samples of a 10-fold serial dilution of the YIDD1 strain were measured under the condition of coexistence of 9 $\log_{10}$ copies/ml of YMDD strain with an MGB probe complementary to YIDD1 and PNAs complementary to the YMDD motif other than YIDD1 (YMDD, YIDD2 and YVDD).

ΔRn: accruals of a fluorescence signal.

Fig. 7 (a). Course of a clinical case.

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HBV DNA was measured by a transcriptional-mediated amplification assay [logarithmic genome equivalent (LGE) /mL].

L, M, I and V of rt180 or 204 represent the amino acid: leucine, methionine, isoleucine and valine, respectively.

Fig. 7 (b). Changes in viral amounts of the LLAQ and YMDD motif.

ALT levels were stable while the LLAQ/YIDD strain was dominant. When the LMAQ/YVDD strain became dominant over preexisting strains, breakthrough hepatitis occurred.

Reaction mixture

serum sample (or standard)		10 µl
forward and reverse primer	(20 pmol/µl)	1 µl each
target MGB probe *	(2.5 pmol/µl)	5 µl
non-complementary PNAs**	(50 pmol/µl)	2 µl each
2 × TaqMan PCR mix		25 µl
DW		
		(final volume : 50 µl)

Mixture pattern		1	2	3	4
target strain		YMDD	YIDD1	YIDD2	YVDD
* MGB probe		YMDD	YIDD1	YIDD2	YVDD
** PNA	YMDD	—	○	○	○
	YIDD1	○	—	○	○
	YIDD2	○	○	—	○
	YVDD	○	○	○	—

Real-time PCR

95℃ for 10 min. : initial denaturation
50 cycles
    95℃ for 15 sec. : denaturation
    71℃ for 60 sec. : PNA annealing
    68℃   (MGB probe hybridization)
    60℃ for 60 sec. : primer extension

Measurement of Ct value (sample and standards)

generation of a standard curve

Calculation of viral amounts

Fig. 1. Measurements of viral amounts by real-time PCR with the MGB probe and PNAs. Patterns of the combination of the MGB probe and non-complementary PNAs are shown in the table.

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During the extension phase with primers, the DNA polymerase cleaves the reporter dye from the probe and the dye emits its characteristic fluorescence. When a PNA added to the reaction mixture is non-complementary to the target sequence, it does not affect amplification with a complementary MGB probe. NFQ : non-fluorescent quencher.
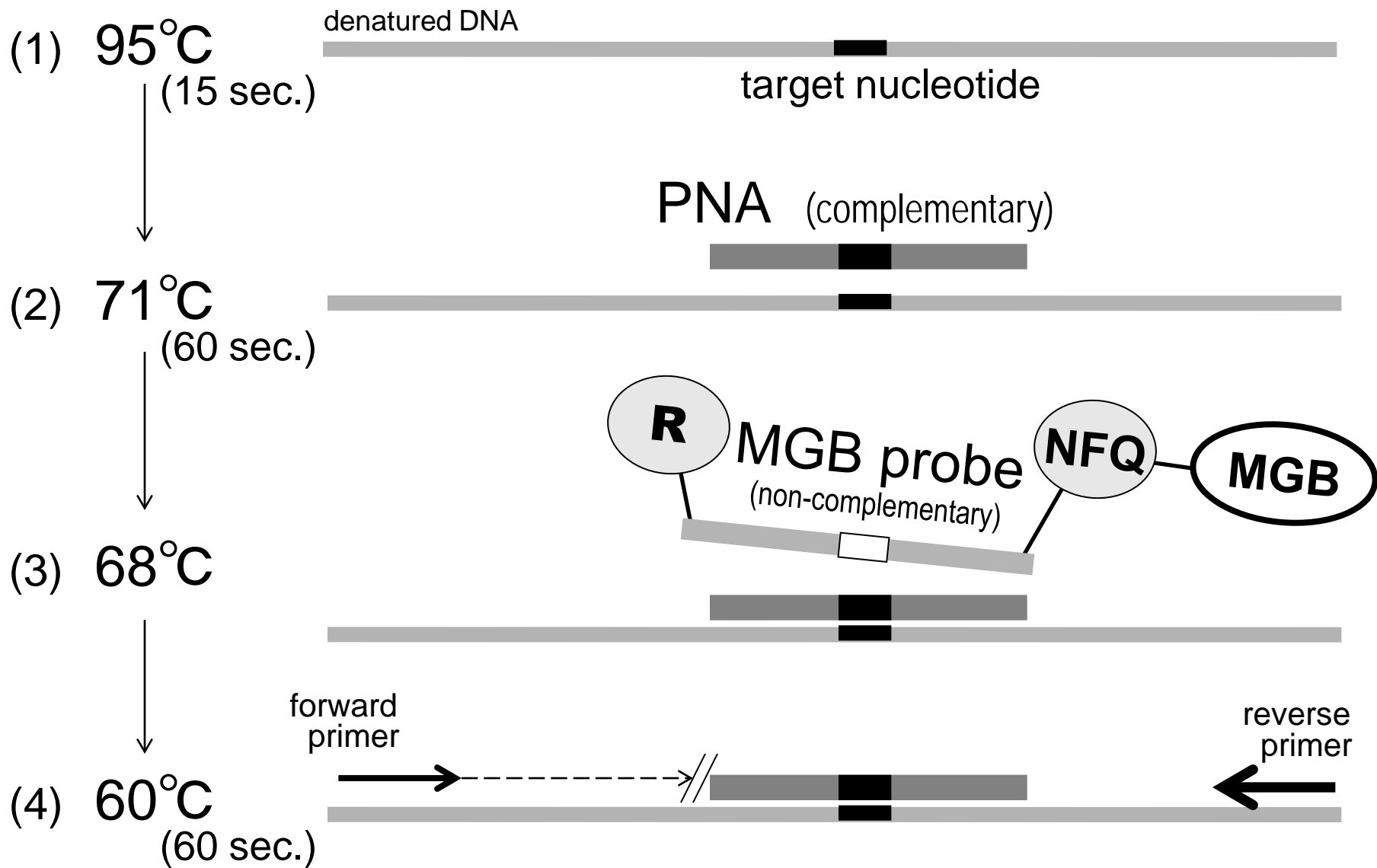
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Fig. 3. Calculation of values from the results obtained by real-time PCR.
The threshold was set in the linearly increasing region of accruals of fluorescence signals (ΔRn) and the threshold cycle (Ct) was measured.
The value of the target strain was calculated from Ct number and a standard curve for each strain. The measurement was performed separately with each MGB probe and non-complementary PNAs for all sets of target strains.
Solid lines: 10-fold serial diluted standards. Dotted line: a sample of the target strain.

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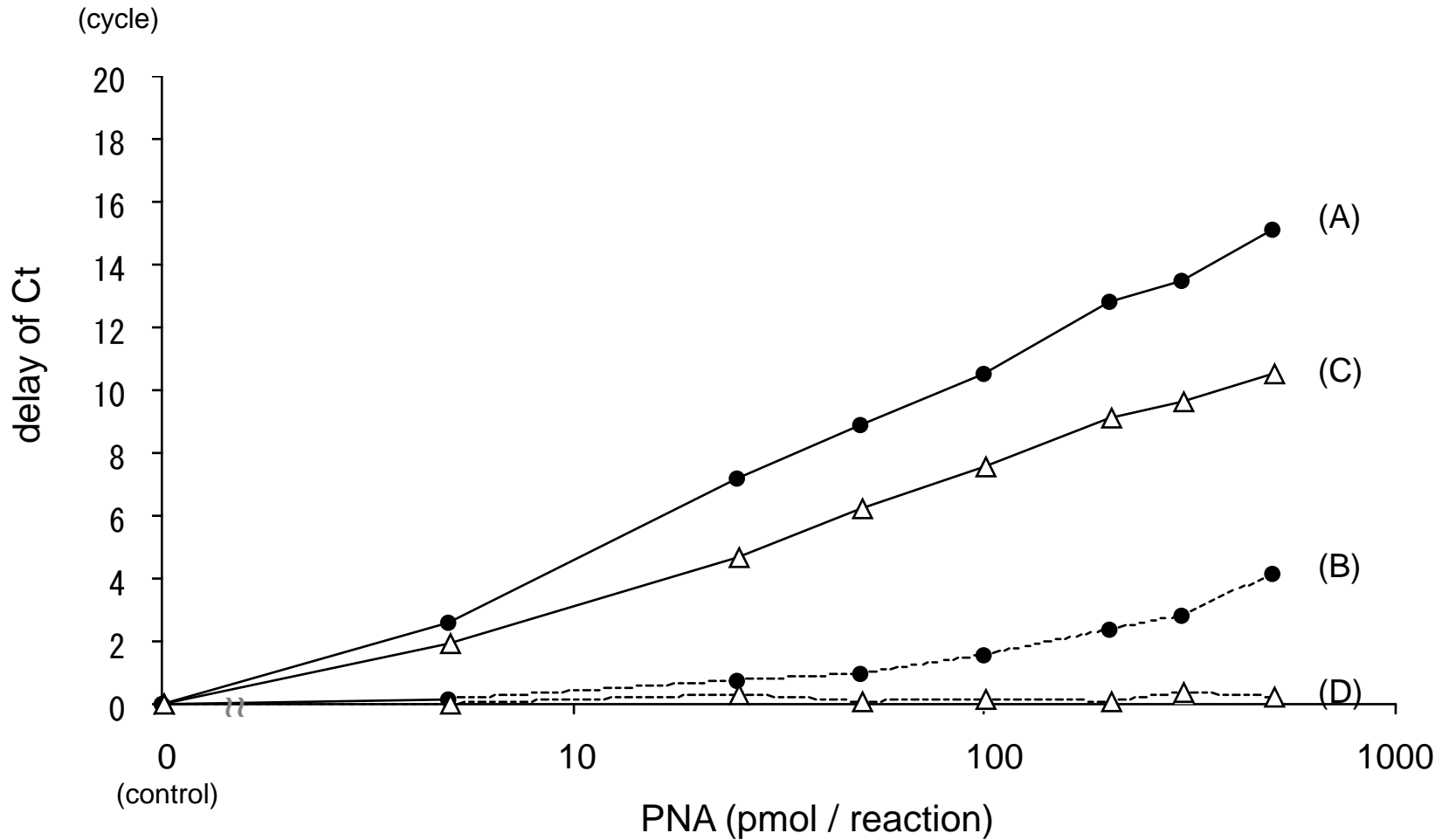The linearity was maintained even at the lower concentration range.

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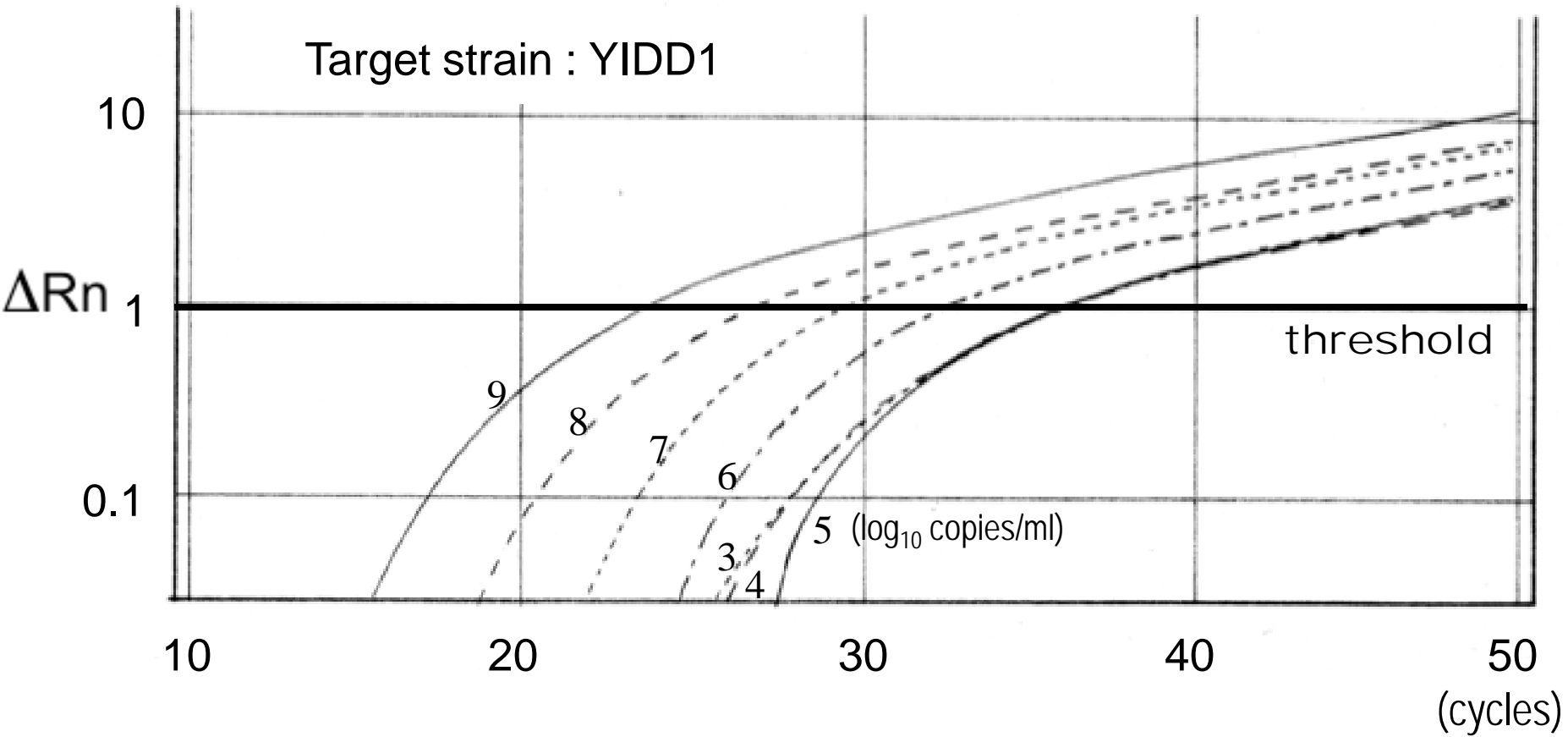
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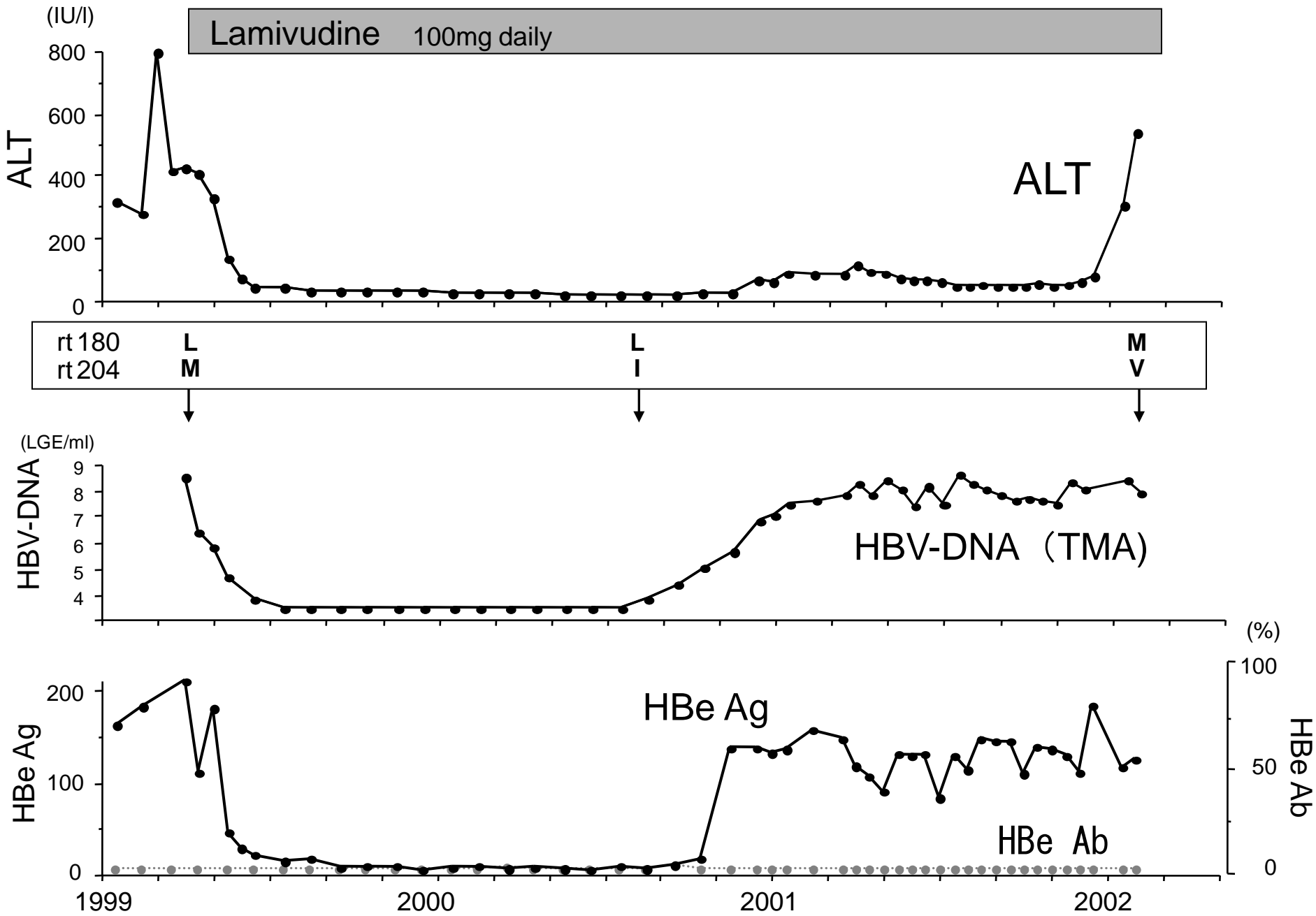
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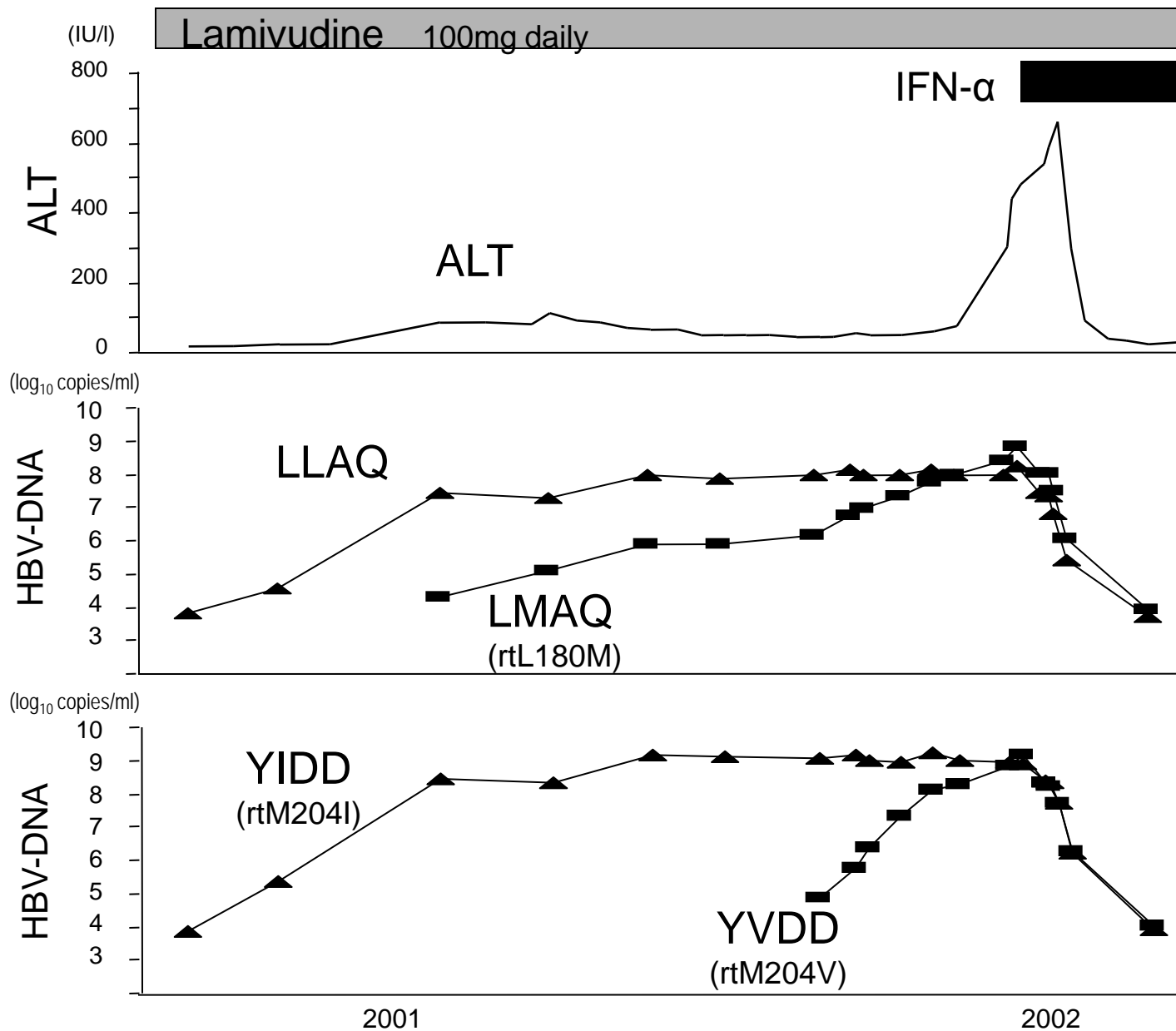
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