Electronic Supplementary Information

Quantitative detection of single base mutation by combining PNA hybridization and MALDI-TOF mass analysis

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Experimental details

Oligonucleotides and PNA probes. For amplification of HBV wild-type and mutant DNA that possessed the YMDD motif sequences of the 225-bp region by PCR, a forward primer (5′-TTGCACTTGTATTCCCATCC-3′) and 5′-biotinylated reverse primer (5′-biotin-CAAAAGAAAATTGGTAATAGAGGTA-3′) were synthesized (Cosmo Genetech, Seoul, Korea). The PNA probes shown in Fig. 1 were synthesized from PANAGENE Inc. (Daejeon, Korea). Internal control PNA probe contained a sequence in common with the wild- and mutant-type template DNA. Wild-type PNA (WT-PNA) and mutant-type PNA (Mut-PNA) were designed to distinguish a point mutation in the YMDD motif sequence (bold-faced character in the PNA sequence, Fig. 1A). PNA oligomers were quantified using 260 nm absorbance measurements.

PCR amplification of DNA. DNA was isolated from the whole blood of individuals who harbored HBV DNA with or without YMDD mutation polymorphisms as described previously (Ref. 5). PCR amplification was performed using buffer containing 2.5 mM dNTPs, 70 ng of DNA, 50 pmol of forward primer, 50 pmol of 5′-biotinylated reverse primer, and 2.5 U of *pfu* DNA polymerase (Bio solution, Suwon, Korea). PCR was performed on a MJ Mini™ Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA) using a program starting with an initial denaturation of 5 min at 94°C and followed by 30 cycles of 30-s denaturation at 94°C, 30-s annealing at 52°C, 30-s extension at 72°C, and a final extension of 7 min at 72°C. The PCR products were purified using a multiple elution kit (Bio-solution, Suwon, Korea).

PNA probe hybridization and nuclease digestion. An empty DNA synthesis column (Glen

Research, Sterling, VA, USA) was filled with 6 mg/ml (300 μM) streptavidin Sepharose High Performance beads (GE Healthcare Life Sciences, Piscataway, NJ, USA) and then washed with Dulbecco's Phosphate Buffered Saline (DPBS) (WelGene Biopharmaceuticals, Daegu, Korea). The biotinylated 225-bp DNA template mixtures were loaded onto the column and washed with DPBS. Denaturation of double-stranded DNA with 0.2 M NaOH was then performed to release the free single-stranded (ss)DNA. The biotin-labeled ssDNA that was bound to the streptavidin beads was washed with water and re-suspended in SSPE buffer (150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA). For PNA probe hybridization, 1 μM of Mut-PNA and WT-PNA in SSPE buffer were added to the immobilized ssDNA and the mixture was incubated at 69°C for 30 min. The beads were loaded onto the column and washed with DPBS 3 times to remove unbound PNA probes. Then, 12.5 U of benzonase (Merck Millipore, Darmstadt, Germany) was added to the re-suspended beads in benzonase buffer (50 mM ammonium bicarbonate, pH 8.0, 1 mM $MgCl_2$) and incubated at 37°C for an hour. After 1 h of incubation, the reaction product was desalted using a Pierce C18 spin column (Thermo Fisher Scientific, Waltham, MA, USA) and dried in a CentriVap® DNA Centrifugal Concentrator (LABCONCO, Kansas City, MO, USA). Samples were kept at -70°C until MALDI-TOF MS analysis.

MALDI-TOF Mass Spectrometry. First, 10 mg/ml α-cyano-4-hydroxycinnamic acid (Bruker Daltonik GmbH, Bremen, Germany) in 70% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA) solution was prepared as a matrix solution. Matrix solution and desalted samples containing PNA probes were then mixed at a 1:5 ratio (v/v) and 1 μl of the mixture was placed on a μFocus MALDI plate (Hudson Surface Technology, Inc., Fort Lee, NJ, USA). Thereafter, PNA probes were detected using an AB SCIEX 4800 MALDI TOF/TOF™ Analyzer (AB SCIEX, Foster city, CA, USA) in positive reflectron mode.

Supplementary Data

MALDI-TOF MS analysis of PNA probes obtained from DNA samples spiked in blood serum

Blood serum (200 μl) was spiked with mixture of DNA samples (total of 625 ng) with different mixing ratios between the mutant and wild-type DNA templates (mutant : wild-type = 0.60:0.40 for sample #1 and 0.15:0.85 for sample #2). The blood serum samples were subject to the same procedure for PCR amplification and PNA probes hybridization as stated above. MALDI-TOF analysis of remaining PNA probes shows that the predefined mixing ratio was exactly reflected by the mass peak intensities for each mutant PNA probe with 59.3 % and 14.7 % for sample #1 and #2, respectively (**Fig. S1**). The result indicates that this analytical method can be applied to actual clinical samples, such as blood or blood serum containing various amounts of mutant-type HBV DNA.

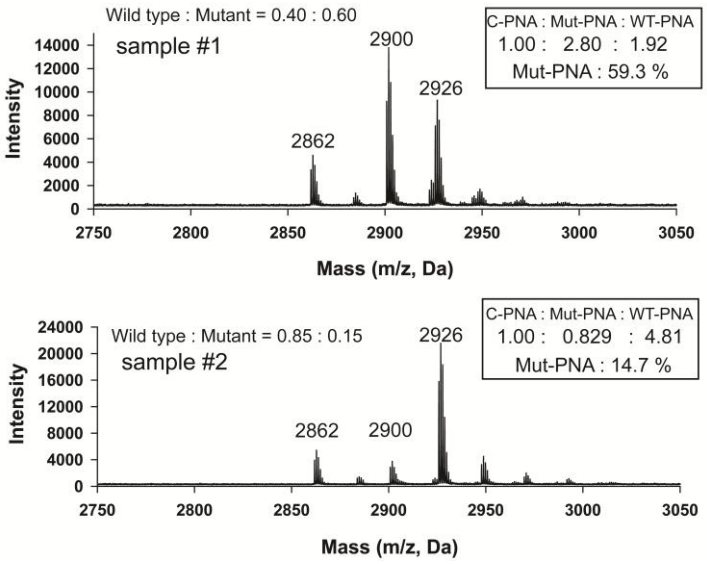

Fig. S1. MALDI-TOF MS analysis of PNA probes obtained following hybridization of PCR-amplified DNA templates from DNA samples (mutant : wild-type = 0.60:0.40 and 0.15:0.85 for sample #1 and #2, respectively) spiked in blood serum. Normalized mass peak intensities for each PNA probe and calculation of the relative amount of mutant PNA are denoted in the box.

Determination of lower limit of mutant DNA detection by MALDI-TOF MS

LLOD (lower limit of detection) is defined by showing the measurement probability for the substance at the blank. In general, its value is given as probability density function with 1% confidence level. LLOD can be calculated based on the standard deviation of the response (SD) and the slope of the standard curve (S) at levels approximating the limit of detection, which could be a blank signal according to the formula: LLOD = 3.3*(SD/S). For the LLOD determination, we obtained the standard curve for mass peak intensity ratio by performing repeated quantification of various relative molar amounts of mutant HBV DNA template mixed with wild-type DNA template. Using the values of SD and S from standard curve (SD = 0.0868 and S = 0.0646) obtained by six repeated measurements for several points of wild-type and mutant DNA mixture ratios (**Fig. S2**), LLOD for the detection of mutant DNA ratio was calculated as 4.4 % ratio according to the mentioned formula. It represents that our analytical method can detect the mutant DNA with a mixing ratio as low as 4.4 %. To validate the determined LLOD value, we further carried out the experiment with blood serum samples containing low mixing percentages (2.0 and 5.0%) of mutant DNA. The quantitative analysis of 2.0 and 5.0 % of the mutant DNA samples exhibited percentage of 6.8 and 7.8%, respectively (data not shown), indicating that the LLOD of our quantitation method is valid for the analysis of mutant DNA as low as 5 % of the mixing ratio. In addition, using the LLOD value the lower limit of detection amount for the mutant DNA is calculated to be 27.5 ng in the blood serum (625 ng DNA × 4.4 %) before the PCR amplification.

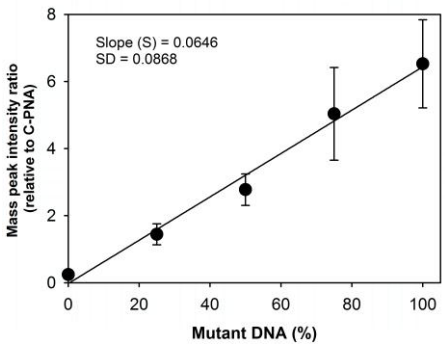

Fig. S2. Standard curve for LLOD determination for mutant DNA detection by MALDI-TOF MS. A linear correlation was plotted with lines for mass peak intensity of mutant PNA.