Background: Antiviral therapy by nucleoside/nucleotide analogues (NAs) effectively reduces HBV replication in chronic hepatitis B (CHB) patients. Because long-term NA treatments will eventually select for drug-resistant mutants, early detection of mutants and frequent monitoring of viral loads is crucial for successful NA therapy. Because no efficient test for one-tube quantification and qualification of various HBV-resistant mutants exists, we propose to use high-resolution melting (HRM) analysis in combination with real-time PCR to achieve this unmet need.

Methods: We developed a single amplicon for detecting HBV mutants resistant to lamivudine (LMV), adefovir (ADV) and entecavir (ETV), which are commonly used for CHB treatment. Our design consists of two steps: real-time PCR for viral quantification, and hybridization probe HRM analysis for detection of specific drug-resistant mutants.

Results: Assay quantification was accurate ($R^2=0.98$) for viral loads from $10^3$ to $10^9$ copies/mL. HRM analysis produced distinct melting temperatures that clearly distinguished the mutants, rtM204V/I (LMV), rtA181V and rtN236T (ADV), and rtT184G and rtM250V (ETV), from their respective wild types. The assay detected mutants at only 10–25% of the HBV population. The clinical applicability of this assay was tested in a pilot study with serial samples from patients receiving LMV treatment.

Conclusions: Flexibility, speed and cost-efficiency are additional benefits unique to our assay. The clinical sample results further support the feasibility of applying our design to frequent and long-term monitoring of CHB patients receiving NA treatments in the clinical setting.

Of the estimated 350 million people who suffer from persistent HBV infection, 15–40% are expected to develop liver cirrhosis, liver failure or hepatocellular carcinoma [1–3]. Orally administered nucleoside/nucleotide analogues (NAs) have been shown to control and prevent progression of liver disease in patients with chronic hepatitis B (CHB) infections [4]. By blocking the active site of the HBV reverse transcriptase (RT), NAs terminate DNA synthesis and thus inhibit viral replication. Although effective in preventing disease advancement, NAs cannot affect covalently closed circular DNA to eradicate existing viruses. As a result, long-term usage of NAs will eventually select for drug-resistant mutants with specific mutations in the viral RT domain [5].

At present, the most commonly used NAs for HBV are lamivudine (LMV), adefovir (ADV) and entecavir (ETV), each with unique mutation patterns for drug resistance. LMV, a type of L-nucleoside, displays resistant mutations at rtM204V/I (LMV), rtA181V and rtN236T (ADV), and rtT184G and rtM250V (ETV), from their respective wild types. The assay detected mutants at only 10–25% of the HBV population. The clinical applicability of this assay was tested in a pilot study with serial samples from patients receiving LMV treatment.

Conclusions: Flexibility, speed and cost-efficiency are additional benefits unique to our assay. The clinical sample results further support the feasibility of applying our design to frequent and long-term monitoring of CHB patients receiving NA treatments in the clinical setting.
domain and rtN236T in the D domain. ETV, a potent cyclopentane, displays two complex mutation patterns: rtM230V ±rtI69T+rtM204V+rtL180M, and rtT184G+rtS202I+rtM204V+rtL180M [5].

The absence of proofreading functions in HBV RT contributes to the formation of diverse viral quasispecies and the subsequent emergence of drug-resistant mutants [6]. Early detection of these mutants during the course of therapy is vital for two reasons. First, NA therapies have limited therapeutic effect after the emergence of drug resistance [7]. Because genotypic resistance leads to viral breakthrough, viral rebound, alanine aminotransferase elevation and liver disease progression, early mutant detection allows for treatment modifications to avoid the development of drug resistance [8]. Secondly, early mutant detection prevents high viral loads, which require more time to suppress to undetectable levels. The longer the time required to reach complete viral suppression, the higher the risk of developing drug resistance [9]. Therefore, regular monitoring of viral loads and early detection of drug resistance mutants is essential to successful NA-based HBV therapy.

Current assays for detecting and monitoring drug resistance include viral load assays, DNA sequencing, DNA hybridization, INNO-LiPA and so forth [8,10]. However, these methods share a major shortcoming: the inability to determine viral load and detect NA-resistant mutants in a single-tube assay, which makes these methods labour-intensive and expensive. A new high-throughput screening and monitoring method that eliminates this shortcoming is an unmet clinical need.

We proposed to use high-resolution melting (HRM) analysis in combination with real-time PCR to achieve this goal. The technique uses the DNA property of heat separation. When this property is used in real-time PCR instruments with saturation dyes that fluoresce in the presence of double-stranded DNA, HRM becomes a powerful tool for mutation scanning [11–15]. When hybridization probes especially designed to anneal to specific target sites are added, hybridization probe-based HRM is capable of type-specific qualitative differentiation of mutants in virology [16–20]. Because saturation dyes used for HRM can also be used for real-time quantification analysis, we aimed to integrate real-time PCR and probe HRM to develop a one-tube assay for determining HBV viral load and identifying drug-resistant mutants. Our results supported the application of our one-tube assay for viral load determination and LMV, ADV and ETV resistant mutation detection in a single amplicon. Our platform demonstrates great potential for clinical application owing to its flexibility, specificity, speed and cost-efficiency in screening and quantifying LMV, ADV and ETV drug-resistant mutants in genotype B and C HBV.

Methods

Saturation dye and assay design
The design principles of our assay are outlined in Figure 1. Our assay consists of two successive steps completed in the same reaction tube: step 1 for viral quantification and step 2 for the mutant detection. The dye used in this study for both real-time PCR and probe HRM was High Resolution Melting Dye (Roche Diagnostics Applied Science, Mannheim, Germany), which is a fluorescence dye that binds to double-stranded DNA in a saturated manner. This saturation binding quality enables the detection of single base pair changes in DNA fragments up to 400 bp by melting curve analysis. Therefore, the use of this saturation dye is crucial for our assay design, which aims to identify drug resistance mutants by their nucleotide changes. The fluorescence was detected for real-time DNA quantification in step 1 and for probe HRM analysis in step 2.

Primer and probe set design
In this study, we focused on genotype B and C HBV for their prevalence in Asia and Taiwan [21]. For accurate quantification of genotype B and C HBV, primers were carefully designed on the basis of genetic data compiled from 175 genotype B and 275 genotype C HBV (from the NCBI database). To ensure adequate amplification, primers (ConsF and ConsR) were designed to anneal to consensus regions with >90% sequence conservation in genotype B and C HBV. Sequence variations between genotype B and C were overcome by incorporating degenerate nucleotides into the primers. The resulting amplicon was 532 bp and included all the primary mutation sites for LMV, ADV and ETV (Table 1 and Figure 2A).

The melting profile of a PCR product depends on intrinsic qualities such as GC content, length and sequence. Therefore, the melting temperature ($T_m$), the temperature at which fluorescence is 50% of the maximum, is also characteristic of the sequence. For most human genes, subtle genetic changes within a PCR product with less than 400 bp can be easily detected by analysing melting peaks generated by HRM analysis with saturation dyes. However, in the case of our large (532 bp) HBV genome amplicon that contains complex genome variations, additional hints are required to discern specific mutants from their respective wild types. The use of hybridization probes, 30–40 bp oligonucleotides with 3′-phosphorylation, was the solution [22]. In hybridization probe HRM, melting profiles are dictated by probes, which can be designed to specifically cover primary mutation sites corresponding to NA-resistant HBV mutants. This is advantageous because only variations within the annealing region contribute to the $T_m$ change; this bypasses
the complex melting profiles owing to genome variation in different HBV genotypes. Therefore, probes used for HRM can be tailored to show distinctive $T_m$ profiles for mutant detection (Figure 1, step 2).

The principles used in primer design were also applied to probe design. To simplify melting profiles, inosines were incorporated to mask sequence polymorphisms between genotypes B and C in the hybridizing region.

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**Figure 1. Overview of the principles and methods used in the design of the assay**

Details described in Methods. The design comprises two successive steps. (Step 1) Asymmetric real-time PCR for amplification and DNA quantification by comparison with standards (Stds) ranging from $10^3$ to $10^9$ copies/ml. (Step 2) Hybridization probe-based high-resolution melting (HRM) analysis to generate melting profiles with characteristic melting temperature ($T_m$) values, the temperature at which fluorescence is 50% of the maximum, for type-specific mutant identification. Saturation dyes that fluoresce in the presence of double-stranded DNA were essential for both steps. $-(dF/dT)$, the negative derivative of fluorescence with respect to temperature.
The underlines represent the mutation sites for specific drug resistance. The 3' ends of the probes were phosphorylated to prevent probe elongation by Taq polymerase during PCR. Polymorphic nucleotides for mutation detection are labelled in bold. Nucleotide positions are derived from the sequence of HBV subtype ayw (GenBank accession number NC_003977). Degenerate nucleotides (W=A/T transversion; R=A/G transition) and inosines (I) were used in primers and probes, respectively, to mask genotypic variations. Note that AD236 was designed to match the mutant.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Sequence (5'–3')</th>
<th>Position, nucleotides</th>
<th>Length, bp</th>
<th>$T_m$, °C</th>
</tr>
</thead>
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<tr>
<td>Forward primer (ConsF)</td>
<td>5'-CAAAACCTWCGAGCGGAACTG-3'</td>
<td>571–592</td>
<td>22</td>
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<tr>
<td>Reverse primer (ConsR)</td>
<td>5'-TGCGAGAGGATRAAGGGCTG-3'</td>
<td>1022–1082</td>
<td>21</td>
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<td>LM204 (probe)</td>
<td>5'-GCTTTCCCACATGTCAGTATATGGATGATGTTG-3' (rtM204V: AGG→GTC; rtM204I: AGG→ATT)</td>
<td>703–750</td>
<td>44</td>
<td>Wild type: 72.8</td>
</tr>
<tr>
<td>AD181 (probe)</td>
<td>5'-GGAGTGGGCTGCTAGTTTCATAGTACCCCT-3' (rtA181V: GCA→GTG)</td>
<td>641–685</td>
<td>45</td>
<td>Mutant type: 75.6</td>
</tr>
<tr>
<td>AD236 (probe)</td>
<td>5'-ACCAATITTCTGTCCTTGGATATCACGTTAACCT-3' (rtN236T: AAT→ACC)</td>
<td>800–838</td>
<td>39</td>
<td>Wild type: 67.0</td>
</tr>
<tr>
<td>ET184 (probe)</td>
<td>5'-GGAGTGGGCTGCTAGTTTCATAGTACCCCT-3' (rtT184G: ACT→GTC)</td>
<td>641–685</td>
<td>45</td>
<td>Mutant type: 76.1</td>
</tr>
<tr>
<td>ET250 (probe)</td>
<td>5'-CTTAACCTCATGGGATATGATGGTAGTGGGAC-3' (rtM250V: AGT→GTC)</td>
<td>866–903</td>
<td>38</td>
<td>Mutant type: 66.1</td>
</tr>
</tbody>
</table>

The probes were designed with the flexibility to be used individually or collectively with other probes; for example, ET184 and ET250 could be used to simultaneously detect one of the two complex mutation patterns of ETV drug resistance. Although multiple probes can be applied at once, the simultaneous application of no more than three probes is recommended to avoid complicated melting profiles. In the case in which more than three probes are required for screening, the PCR product can be divided into aliquots exceeding the 6 μl minimum for detection of specific drug-resistant mutants by adding different probes.

The relative primer and probe annealing positions on the HBV genome are shown in Figure 2A and the DNA sequences are summarized in Table 1. Primers were synthesized by Mission Biotech (Taipei, Taiwan) and the probes were synthesized by Prisma Biotech (Taipei, Taiwan).

**DNA template**

As a pilot study to test the concept of our assay, we constructed a representative drug resistance mutant that included the following LMV, ADV and ETV mutations: rtT184I and rtM250I. Plasmid pB10-5 was chosen as the DNA template to optimize experimental conditions, assess quantification accuracy and test probe and primer design. pB10-5 was constructed by cloning a monomer of the genotype B HBV genome (nucleotides 1–3182) into vector pGEM3Z [24]. The drug-resistant mutations were introduced into pB10-5 using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manual instructions. For rtM204VI, nucleotides GTG (Val) and ATT (Ile) were respectively substituted for ATG (Met). For rtA181V, nucleotides GGT (Gly) were substituted for ACT (Thr). For rtT184I, nucleotides CAT (His) were substituted for CAG (Gln). For rtN236T, nucleotides ACC (Thr) were substituted for AAA (Lys). For rtT184I, nucleotides GTT (Val) were substituted for TAT (Ile). For rtM204I, nucleotides GCT (Ala) were substituted for ACT (Thr). For rtM204I, nucleotides GCT (Ala) were substituted for ACT (Thr). The results of site-directed mutagenesis were confirmed by sequencing.

**Drug-resistant mutant quantification and differentiation**

Our design contained two successive steps. The first step was real-time PCR for viral quantification and the second step was probe HRM analysis for type-specific
Figure 2. Detection of HBV mutants resistant for lamivudine, adefovir and entecavir by HRM

(A) Relative primer and probe annealing positions in the pB10-5 amplicon. Representative results from high-resolution melting (HRM) probe analysis to identify (B) lamivudine (LMV)-, (C) adefovir (ADV)- and (D) entecavir (ETV)-resistant HBV mutants in a single amplicon. The presented melting peaks had sufficiently different melting temperature values, the temperature at which fluorescence is 50% of the maximum, to clearly differentiate drug-resistant mutants from wild-type HBV. nt, nucleotide; -(dF/dT), the negative derivative of fluorescence with respect to temperature.

mutation identification (Figure 1). Both steps were completed with LightCycler® 480 (Roche Diagnostics Applied Science). To facilitate mutant identification in HRM analysis, unequal primer concentrations were used to generate the probe annealing strand in excess during asymmetric PCR in step 1. The 20 μl PCR reagent in DNA quantification included: 4 μl DNA template, 10 μl LightCycler® 480 Master Mix, which contained FastStart Taq DNA Polymerase, dNTP mix and High Resolution Melting Dye (Roche Diagnostics Applied Science), 3.2 μl of 25 mM MgCl₂, 2 μl H₂O, 0.4 μl of 2 μM forward primer and 0.4 μl of 10 μM reverse primer. Empirical trials were conducted to determine the optimum primer ratio (forward:reverse =0.04:0.2 μM) that yielded good PCR efficiency in step 1 without jeopardizing melting peak resolution in step 2. The following PCR protocol was used for DNA quantification: preincubation at 95°C for 10 min; and amplification at 95°C for 10 s, 60°C for 15 s and 72°C for 25 s for 40 cycles. Fluorescence was detected at the end of each cycle for DNA quantification.

After DNA quantification in step 1, the reagent concentrations were adjusted to 2 mM MgCl₂ and 0.25 M probe for HRM analysis in step 2. The following
protocol was used to generate the melting peaks for type-specific mutant identification: DNA denaturation at 95°C for 1 min; probe annealing at 40°C for 1 min; and probe and DNA denaturation from 65 to 95°C at 0.02°C/s ramp rate. Fluorescence was detected continuously from 65 to 95°C to generate melting peak data, which were plotted as the negative derivative of fluorescence with respect to temperature (−dF/dT) as shown in melting profiles in the figures.

The risks of contamination while adjusting the MgCl2 and adding probes for the qualification in step 2 were minimized by: centrifuging the multi-tube reaction plate for 2 min at 1,500×g before removing the LightCycler® 480 sealing foil to ensure that the reaction plate for 2 min at 1,500×g was at the bottom of each of the 200 µl v-shaped reaction tube; including negative controls throughout the assay; and renewing the sealing foil before probe HRM. Another intrinsic guard against DNA contamination was the lack of DNA amplification in the probe HRM qualification step of our protocol. Without amplification, it is unlikely that relatively minute foreign DNA could significantly alter qualification results.

Sensitivity of drug-resistant mutation detection

Assay sensitivity in detecting the presence of minor mutant populations among wild types is important because drug resistance begins with the appearance of the first drug-resistant mutant. To determine the sensitivity of our assay, mutant and wild-type pB10-5 were mixed in the following proportions: 50% (1:1), 25% (1:3) and 10% (1:9). Probe HRM was used to determine the lowest proportion of mutants required to display a clear melting peak for mutant identification.

Clinical samples

To determine the clinical applicability of this study, we first assessed the accuracy of DNA quantification. Using a well-established protocol previously developed in this laboratory [25], we obtained 124 samples of genotype B and C HBV sera DNA extracts. Quantification of these extracts was completed using the aforementioned primers and protocol. The quantification data were then correlated to viral load data determined by the established quantitative real-time PCR assay [25].

Next, we tested the clinical applicability of our assay concept and the efficacy of our probes and primers by monitoring viral loads and screening drug-resistant mutants in clinical samples. Four LMV-treated patients (Patients A–D) were enrolled for our pilot study. Patients A, B and C were hepatitis B e-antigen-negative whereas patient D was hepatitis B e-antigen-positive. Patients A and B were diagnosed with liver cirrhosis whereas Patients C and D were diagnosed with CHB. We collected sera samples from these patients at different time points throughout their LMV monotherapy. To determine the potential of our assay to be used for frequent and long-term monitoring of the development drug-resistant HBV in patients, we processed these samples using our assay, which included real-time PCR DNA quantification and LM204 probe HRM for LMV-resistant mutant detection.

To further test the applicability of our proposed assay on clinical samples, we included 22 additional patient serum samples for probe HRM testing. Twelve samples were obtained from patients that had not received any NA treatment, and thus displayed wild-type sequences for all residues related to drug resistance. The remaining 10 mutant samples contained five rtM204V and five rtM204I mutants; they were included to test the applicability of our HRM in drug-resistant mutant detection.

Results

Drug-resistant mutant detection by HRM analysis with hybridization probes

To determine the efficacy of the designed probes for type-specific mutation differentiation, we applied the probes associated with each drug simultaneously to wild-type and mutant HBV constructs derived by site-directed mutagenesis of pB10-5 (rtM204V/I, rtA181V and rtN236T, rtT184G and rtM205V). For LMV in Figure 2B, probe LM204 showed distinct Tm values for rtM204I (70.1°C), rtM204V (70.7°C) and wild type (72.8°C). For ADV in Figure 2C, probes AD181 and AD236 clearly distinguish mutants rtA181V (75.4°C) and rtN236T (68.9°C) from their respective wild types (Tm=78.8°C and 67.0°C). For ETV in Figure 2D, probes ET184 and ET250 also clearly distinguished mutants rtT184G (76.1°C) and rtM250V (66.1°C) from their respective wild types (Tm=78.9°C and 69.0°C). Overall, mutants were clearly distinguishable from their respective wild types because of sufficient differences between their Tm values, which ranged from 1.9 to 3.4°C as summarized in Table 1.

Assay sensitivity in detecting mutant among wild type

Assay sensitivity is determined by the lowest proportion of mutants among wild type to produce a clear mutant melting peak. This sensitivity is related to the efficacy of HRM as a method of mutation screening. To test the sensitivity of our probes, we applied them to pB10-5 constructs containing different proportions of mutants and wild type. Probes LM204 (Figure 3A and 3B), AD181 (Figure 3C), ET184 (Figure 3E) and ET250 (Figure 3F) confirmed the presence of mutants at concentrations as low as 10% of the HBV population; probe AD236 (Figure 3D) detected the presence of mutants at 25% of the population. Overall, this assay can clearly
identify the presence of mutants at only 10–25% of the viral population.

Accuracy of DNA quantification

One of the benefits of our assay is speed because of its ability to qualify and quantify mutant HBV in a one-tube assay. To determine the accuracy of DNA quantification of this assay, a standard curve was first generated by serial dilution of pB10-5 from $1 \times 10^3$ to $1 \times 10^{11}$ copies/ml as shown in Figure 4A and 4B. The amplification efficiency was found to be 1.70 with the slope -4.34 and the linear range is from $10^3$ to $10^{11}$ copies/ml.

For further verification of assay quantification accuracy, we correlated viral load data obtained using our assay to the data obtained by the well-established and widely used real-time PCR quantification assay [25]. The correlation of quantification data for these 124 genotype B and C clinical samples is displayed in Figure 4C. The viral titres determined by these two methods showed high correlation of $R=0.98$, which confirmed the quantification accuracy of our assay.

Clinical applications

To test the clinical applicability of our assay, we conducted a pilot test with sera samples collected from liver cirrhosis and CHB patients receiving LMV monotherapy. Following real-time PCR amplification for quantification, we used probe HRM to complete LMV-resistant mutant screening. The viral loads and melting peak results of the three sera samples from each of the four participating patients are displayed in Figure 5. The sera samples were collected at serial time points during their treatment: samples in Figure 5A were collected before treatment; samples in Figure 5B were collected during treatment before the development of drug resistance; and samples in Figure 5C were collected after LMV-resistant mutants were detected. The viral loads were also assessed by the aforementioned

Figure 3. Assay sensitivity for detecting lamivudine, adefovir and entecavir mutants among wild-type HBV

A

rtM204V

C

rtA181V

E

rtT184G

B

rtM204I

D

rtN236T

F

rtM250V

Mutant proportion

0%  10%  25%  50%  100%

(A) Probe LM204, (C) probe AD181, (D) probe AD236, (E) probe ET184 and (F) probe ET250 are shown. Overall, assay sensitivity was high as mutants comprising only 10–25% of the population produced clearly distinguishable melting peaks. wt, wild type; -(dF/dT), the negative derivative of fluorescence with respect to temperature.
well-established quantification assay; these results are included in parentheses in Figure 5.

Overall, viral loads in Figure 5A were higher than their respective samples in Figure 5B. Following this initial decrease, viral loads from samples in Figure 5B were observed to rebound to their respective Figure 5C values. Furthermore, the viral rebound observed in Figure 5C corresponds to the first-time detection of drug-resistant mutants since the start of LMV monotherapy as indicated by the presence of mutant melting peaks with lower $T_m$ values. The presence of drug-resistant mutants was each confirmed by DNA sequencing. Sequencing results indicated the presence of mutant rtM204I (ATG→ATT) in Patients A, B and D, and mutant rtM204v (ATG→GTG) in Patient C. In addition to the rtM204I mutation, the mutants in Patient B were also discovered to have two non-specific nucleotide changes (ntG707A and ntA731T) in the probe annealing region. This explained the $T_m$ drop observed in Figure 5C for Patient B. Overall, the corresponding DNA quantification and DNA sequencing results strongly supported the possibility of applying our assay to the clinical setting as a method of frequent and long-term HBV monitoring and mutant screening.

Furthermore, we included 22 additional clinical specimens to test the applicability of our proposed assay for clinical use. The probe LM204 results showed that the $T_m$ values for the 12 wild-type samples closely matched that of the wild-type plasmid control (Additional file 1). Although each of the five samples containing rtM204V or rtM204I showed minor $T_m$ shifts from their respective plasmid controls, their $T_m$ values were still clearly distinguishable from that of the wild-type plasmid control (Additional file 1). Therefore, probe LM204 clearly and accurately identified these 22 clinical samples as wild type or mutants. Moreover, the results from testing the ADV and ETV probes on the 12 wild-type specimens also showed $T_m$ values that mostly overlapped with $T_m$ values of the plasmid controls (Additional file 2). Although minor $T_m$ shifts were detected in a few samples, most of the clinical wild-type $T_m$ values remained clearly differentiable from the $T_m$ of the mutant plasmid being scanned for by the probe.
Discussion

The primers designed in this study produced a 532 bp amplicon containing the common mutation sites for LMV, ADV and ETV resistance. These primers were equally effective when applied to clinical samples for viral DNA quantification. With the exception of probe AD236, the hybridization probes designed for this study produced mutant melting peaks with \( T_m \) values that were between 1.9 and 3.4°C lower than their respective wild types. Because probe AD236 was designed to match the mutant, the mutant \( T_m \) was 1.9°C higher than the wild type \( T_m \) (Figure 2B, 2C and 2D, and Table 1). These \( T_m \) differences were sufficient to allow clear differentiation of mutants from their respective wild type, which not only supported the concept of using probe HRM for type-specific mutant detection, but also the effectiveness of our designed probes. In addition to accurate mutant screening, the sensitivity of mutation screening of our assay was high because it is able to qualify mutants comprising only 10–25% of the HBV population (Figure 3). Furthermore, the accuracy of DNA quantification for this assay was also high as evidenced by the \( R=0.98 \) correlation of quantification datasets from our assay to the standard quantification assay in Figure 4C. These results indicate our assay to be an effective, sensitive and accurate method for single-tube quantification of viral loads and qualification of LMV, ADV and ETV resistant HBV mutants.

Similarly promising results were also observed when our assay was applied to clinical samples. For all four patients in this study, mutant screening results by our assay corresponded to sequencing results, which verified the accuracy of probe HRM for mutant HBV screening. Additionally, viral load quantification results obtained using our assay not only corresponded to quantification data obtained from a well-established quantification essay (value in parentheses), but also mirrored changes in the patients’ NA monotherapy and disease progression: in response to initial LMV monotherapy, the viral load decreased (Figure 5A and 5B); following continuous LMV treatment, the viral load decreased further (Figure 5C and 5D).
Table 2. DNA sequence variation in the primer annealing regions of type A–D HBV

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position, nucleotides</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ConsF</td>
<td>571–592</td>
<td>C A A A A C T W C G G A C G G A A A C T G A_{137} T_{132} T_{139}</td>
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<tr>
<td>Type A (140 sequences)</td>
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<td>Type B (175 sequences)</td>
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<td>Type C (275 sequences)</td>
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<td></td>
</tr>
<tr>
<td>Type D (153 sequences)</td>
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</tr>
<tr>
<td>ConsR</td>
<td>1102–1082</td>
<td>T G G C G A G A A A G T R A A A G C C T G A_{71} A_{170} G_{265} G_{142}</td>
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<td>Type A (140 sequences)</td>
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<tr>
<td>Type B (175 sequences)</td>
<td></td>
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<tr>
<td>Type C (275 sequences)</td>
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<td></td>
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<tr>
<td>Type D (153 sequences)</td>
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<td></td>
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</tbody>
</table>

aCompiled from 140 type A, 175 type B, 275 type C and 153 type D sequences bNucleotide positions are derived from the sequence of HBV subtype ayr (GenBank accession number NC_003977). cNucleotides with mismatch rate >20% for each HBV genotype are marked in bold. Subscripts indicate the number of samples with that mismatch. R, A/G degenerate nucleotides; W, A/T degenerate nucleotides.

In this study, the probes were added after the quantification step to achieve good melting profiles. Our reason for separately adding the probes was to avoid aberrant melting profiles obtained in optimization trials in which all reagents were loaded together. A possible explanation for these irregular melting profiles is incomplete or unstable 3′-phosphorylation on the probes. Therefore, improved probe blocking, with amino-modified C6, inverted dT, or C3 spacer blockers, could allow the separate quantification and qualification steps in this study to be merged into one [28]. This combination would further streamline our assay to one that is the gold standard for mutant detection because of its ability to detect all mutations in the HBV genome. However, this comprehensiveness is achieved at the expense of speed and sensitivity because DNA sequencing is a lengthy process that cannot detect minor species comprising less than 20–30% of the population. Other detection methods include the line probe assay, DNA chip technology, peptide nucleic acid cramping, fluorescent bprobe hybridization and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based genotyping [10]. Although these methods are more sensitive than DNA sequencing and possibly more sensitive than our assay, they are too expensive and time consuming to be routinely applied for standard clinical monitoring. Therefore, the potential clinical value of our assay is even more evident because it bypasses the shortcomings that prevent other methods from being regularly used in the clinical setting.

A potential limitation of our assay in mutant detection needs to be taken into consideration: non-target nucleotide variation of the HBV genome within the probe annealing region may result in unpredictable $T_m$ (such as that shown for patient B in Figure 5). In this case, sequence analysis needs to be conducted to determine the exact sequence of the detected mutation.

In this study, the probes were added after the quantification step to achieve good melting profiles. Our reason for separately adding the probes was to avoid aberrant melting profiles obtained in optimization trials in which all reagents were loaded together. A possible explanation for these irregular melting profiles is incomplete or unstable 3′-phosphorylation on the probes. Therefore, improved probe blocking, with amino-modified C6, inverted dT, or C3 spacer blockers, could allow the separate quantification and qualification steps in this study to be merged into one [28]. This combination would further streamline our assay to one...
Table 3. DNA sequence variation in the probe annealing regions of type A–D HBV

<table>
<thead>
<tr>
<th></th>
<th>Position, probe nucleotides</th>
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<td>ET184</td>
<td>G G A G T G G G C C T G T C G T T C T C I T G G C C T C A G T T T A C T A</td>
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</tr>
<tr>
<td>Type B</td>
<td>C160</td>
<td></td>
</tr>
<tr>
<td>Type C</td>
<td>T265</td>
<td></td>
</tr>
<tr>
<td>Type D</td>
<td>T125</td>
<td></td>
</tr>
<tr>
<td>AD236</td>
<td>A C C A A T T T T T C T T T G T C T T T G G T A A C A T T T I A C C C C C T</td>
<td></td>
</tr>
<tr>
<td>Type A</td>
<td>A138</td>
<td>A40</td>
</tr>
<tr>
<td>Type B</td>
<td>A156</td>
<td>A75</td>
</tr>
<tr>
<td>Type C</td>
<td>G136</td>
<td>A75</td>
</tr>
<tr>
<td>Type D</td>
<td>A152</td>
<td>A153</td>
</tr>
<tr>
<td>ET250</td>
<td>C T T A A C T T C A T G G G A T A T G T A A T T G G I A G T T G G G G I A C</td>
<td></td>
</tr>
<tr>
<td>Type A</td>
<td>A93</td>
<td>T99</td>
</tr>
<tr>
<td>Type B</td>
<td>A143</td>
<td>G146</td>
</tr>
<tr>
<td>Type C</td>
<td>A136</td>
<td>C144</td>
</tr>
<tr>
<td>Type D</td>
<td>T112</td>
<td>A137</td>
</tr>
</tbody>
</table>

*Compiled from 140 type A, 175 type B, 275 type C and 153 type D sequences. *Nucleotide positions are derived from the sequence of HBV subtype ayr (GenBank accession number NC_003977). *Nucleotides with mismatch rate >20% for each HBV genotype are marked in bold. Subscripts indicate the number of samples with that mismatch. R, A/G degenerate nucleotides; W, A/T degenerate nucleotides.
of even higher throughput, and thus make it an even more practical option for use in the clinical setting.

Although the focus of this study was on genotype B and C HBV for their prevalence in Asia, we investigated the possibility of expanding our assay design to include genotype A and D HBV. After comparing nucleotide variability in the primer and probe regions of 743 full-length HBV sequences, we discovered relatively high sequence conservation in all annealing regions. The following results are summarized in Tables 2 and 3. The forward primer used in this study showed two mismatches to genotype A (nucleotides 584 and 590) and genotype D (nucleotides 572 and 590); the reverse primer showed no mismatches. The probes used in this study showed the following mismatches: probe LM204 mismatched genotype A at one nucleotide (nucleotide 733); probe ET184 and AD181 mismatched genotype D at one nucleotide (nucleotide 653); probe ET250 mismatched genotype A at two nucleotides (nucleotides 868 and 880), and genotype D at nine nucleotides (nucleotides 866, 868, 871, 880, 886, 893, 897, 898 and 902). Because probe AD236 was designed to perfectly match the mutant instead of the wild type, it mismatched all genotypes at one nucleotide (nucleotide 834). Therefore, in theory, only minor modifications are needed to apply our assay in the form of primer and probe design in order apply our proposed assay to genotype A and D HBV.

Although the probes designed for our assay can only detect resistance to LMV, ADV and ETV, which represent all three classes of NAs and only three of five pathways of drug resistance development, the concept of our assay has much broader applications. By applying our principles of probe and primer design, and conducting more optimization trials, our proposed assay can theoretically be applied to screen for both known and new drug-resistant mutants. Even though our proposed assay may require DNA sequencing to decode unknown mutation patterns, our assay still has much clinical usefulness for the following reasons: the emergence of mutants can be rapidly identified upon the detection of any previously absent melting peaks; and as a tool for long-term monitoring, patient history will probably limit the number of possible mutants for screening, which would limit the occasions requiring DNA sequencing. In conclusion, our study supports the feasibility of using real-time PCR and probe HRM for quantifying and qualifying multiple HBV viral mutants in a single amplicon. The flexible, specific, rapid and inexpensive qualities of our assay further underscore its importance as a practical method for frequent and long-term monitoring of NA-based CHB therapy.

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Disclosure statement
The authors declare no competing interests.

Additional files
Additional file 1: Probe LM204 high-resolution melting (HRM) results for wild-type and lamivudine-mutant clinical samples can be found online at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2072_Hsiao_Add_file1.pdf

Additional file 2: Results from screening 12 wild-type clinical samples with adefovir and entecavir probes can be found online at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2072_Hsiao_Add_file2.pdf

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One-tube HBV quantification and drug-resistant mutant detection


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