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Combination of Allele-Specific Detection Techniques to Quantify Minority Resistance Variants in Hepatitis B Infection: a Novel Approach

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Abstract

Detection of minor variant viral quasispecies of the rtV173L+rtL180M+rtM204V combination mutation in the hepatitis B virus (HBV) polymerase mediating both lamivudine resistance and vaccine escape is potentially important for tracking the development and evolution of resistance within both individuals and populations. A highly sensitive and specific assay to quantitate HBV genomes was developed with this mutation combination directly from viral DNA in serum using allele-specific quantitative PCR with locked nucleic acid primers and a minor groove binder probe. This combination of primers and probe yields a linear detection range down to 150 copies. This strategy has 100% specificity even in mixtures of predominately wild type genomes. The assay accurately detected 3×10^2 copies of the triple mutant spiked into 3×10^8 copies of the wild-type genomes (0.0001%), while maintaining 100% specificity. This approach was validated using serum from a subject infected with known lamivudine-resistant HBV. The triple mutant viral population was quantitated at 2.86×10^8 copies/mL within a total viral concentration of 1.03×10^{10} copies/mL of serum (2.8%). This quantitative allele-specific PCR strategy therefore is a useful method for highly sensitive and specific detection of point mutation combinations that are clinically important in the pathogenesis of drug resistance and/or immune escape.

Keywords

HBV; drug resistance; lamivudine; vaccine escape; quantitative PCR; minority variant

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1.0 INTRODUCTION

The detection of drug resistant minority variants is an important method for monitoring response to therapy for several viral diseases, including HIV, hepatitis B Virus (HBV), and hepatitis C Virus (HCV). In HIV infection, minority variant drug resistant viruses present at the initiation of therapy are predictors of subsequent treatment failure (Li et al., 2011). Recent data suggests a threshold of 2000 copies/mL of the K103N mutation predicts failure of efavirenz containing regimens (Goodman et al., 2011). Similarly, baseline HBV minority variant drug resistant viruses may predict treatment failure during HBV treatment. In addition to assessing the frequency of minority variants with specific point mutations, it is important to measure quasispecies of point mutation combinations within the same genome. Such combinations of mutations may provide compensatory changes that increase the fitness of drug resistant viruses or may otherwise provide an additional survival advantage. For example, the triple mutation in HBV polymerase rtV173L+rtL180M+rtM204V, associated with lamivudine drug resistance, confers changes to the envelope gene because of overlapping reading frames that result in an HBV vaccine escape phenotype while compensating for the loss in viral fitness (Delaney et al., 2003; Torresi et al., 2002a).

Having a straightforward, inexpensive assay to detect minor viral quasispecies with specific combinations of mutations is a priority for monitoring HBV drug resistance. Chronic HBV infection affects 400 million people globally and is an important cause of cirrhosis, liver cancer, and death (Dienstag, 2008). Efficacy of antiviral therapy for hepatitis B is limited by the emergence of drug resistant mutants with cumulative resistance to lamivudine, an important treatment for HBV, occurring at 14–20% a year (Shaw et al., 2006). The primary lamivudine resistance mutation, rtM204V in the HBV polymerase, reduces replicative capacity, but additional mutations rtV173L and rtL180M are associated with compensated fitness (Delaney et al., 2003; Nakanishi et al., 2005). Furthermore, this combination of mutations also alters the overlapping envelope reading frame causing surface antigen (HBsAg) mutations E164D and I195M, (Cooley et al., 2003; Santos et al., 2004; Torresi, 2002) which confer a vaccine escape phenotype (Torresi et al., 2002b). This mutant raises significant public health concerns of potential transmission of drug resistant HBV to previously HBV-vaccinated individuals, although this has not yet been observed in human populations. The ability to detect minority viral quasispecies carrying drug resistance and the concomitant vaccine escape phenotype will thus be an important method for monitoring HBV infection within individuals as well as within populations. Although there have been several different approaches for measuring minority variants in HBV drug resistance, to date, none has the ability to measure quickly and easily the triple mutant combination within the same genome.

Currently available techniques to assess HBV drug resistant minority variants have several limitations. Consensus sequencing of the entire viral population is insensitive for minority variants, detecting only mutations that represent at least ~20% of the total viral population, with no information about whether multiple mutations are present within the same genome. Line probe assays, such as LiPA DR (Innogenetics), are more sensitive than consensus sequencing with a sensitivity of 5–10% of typical viral concentrations $>10^4$ copies/mL, i.e. between 500–5000 copies of mutant virus. However, they have higher false-negative and false-positive rates, sensitivity is different for different mutations, and they cannot determine whether combination of mutations are in the same genome. Pyrosequencing can provide numerous clonal sequences and thus is highly sensitive for detecting minor mutations in combination, but its routine use is limited due to short amplicon reads, high error rate (Huse et al., 2007) and expense relative to PCRbased methods.

Given the current limitations of existing minority variant assessment techniques and the importance of identifying of multiple mutations on the same genome, it was important to create a highly sensitive and specific, rapid, and inexpensive assay to detect minority variant viral quasiespecies of the drug resistant/vaccine escape triple mutant rtV173L+rtM204V+rtL180M directly from clinical samples. An allele-specific quantitative PCR (qPCR) using a novel combination of modified oligonucleotides was developed. Beyond the detection of HBV drug resistant mutations, this novel approach may be applied to any situation in which the detection of minor variants carrying multiple mutations on the same template within a mixed viral population is required.

2.0 MATERIALS AND METHODS

2.1 Preparation of wild type (WT) and mutant (rtV173L+rtL180M+rtM204V) plasmid standards

Standard human serum samples with HBV (genotype A) were obtained from AcroMetrix (Life Technologies, Benicia, CA, USA). Viral DNA was extracted using the Ultrasense Viral Isolation Kit (Qiagen, Valencia, CA, USA). HBV *polymerase* was amplified with MasterTaq using forward primer 5' CCA CAG GCC ATG CAG TGG AA and reverse primer 5' GGC AGC ACA SCC TAG CAG CCA TGG with PCR cycling conditions of 94°C for 5 minutes followed by 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72 °C for 1 minute 15 seconds. The amplified polymerase gene (GenBank accession number, submitted and pending) was TOPO cloned into the pCR 2.1 TOPO vector (Invitrogen), yielding the “Wild Type (WT)” plasmid. Site directed mutagenesis using the Stratagene Quickchange II XL kit (Agilent Technologies La Jolla, CA, USA) was used to create rtV173L (G>C), rtL180M (T>A), and rtM204V (A>G) mutations alone or in combination in *polymerase* (see figure 1) to serve as “Mutant” plasmids. The presence of these mutations was confirmed by sequencing.

2.2 Design of real-time PCR primers and probe for total HBV genome quantitation

In order to determine the percentage of mutant viruses within the entire viral population it is necessary first to quantify the total HBV serum/plasma concentration. Therefore qPCR primers and a probe targeting a highly conserved nucleotide sequence within *polymerase* were designed. Multiple HBV polymerase genes representing all of the major genotypes A–H (Kramvis et al., 2005) were aligned and a highly conserved region was identified. An optimal primer-probe combination in this region was designed with the assistance of Primer Express software (Applied Biosystems, Foster City, CA, USA) as well as considering distance between the primers, compatible T_m, and avoidance of sites of known resistance mutations. The primer and probe sequences (nt positions relative to gta reference sequence X02763) are forward primer (nt 599–621): 5' - TGT ATT CCC ATC CCA TCG TCC TG -3', reverse primer (nt 801-778): 5' - AAC AGC GGT ATA AAG GGA CTC ACG - 3', and probe (nt 698–732) 5' - FAM TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TC - BHQ - 3' (Integrated DNA Technologies, San Diego, CA, USA). Quantitation of total viral concentration with this primer-probe combination was validated by a comparison with the AcroMetrix Optiquant DNA Quantification Panel (Life Technologies, Benicia, CA, USA), HBV samples of known quantity which have been calibrated with the WHO 1st International Standard for HBV DNA (see 2.4 below).

2.3 Design of modified real-time allele-specific PCR primers and probe for triple mutant HBV genome quantitation

In order to detect rtV173L, rtL180M and rtM204V simultaneously within a single template, a combination of oligonucleotide binding specificity-enhancing technologies was employed in a qPCR assay. For increased single nucleotide discrimination, a minor groove binding

(MGB) probe (Applied Biosystems, Foster City, CA, USA) and locked nucleic acid (LNA) primers (Exiqon, Vedbaek, Denmark) were designed to work in combination. Allele-specific LNA forward and reverse primers were designed to detect the rtV173L and rtM204V mutations, respectively. An allele-specific MGB probe was designed to detect the rtL180M mutation. The length of the primers as well as the number and position of the nucleic acids locks were determined empirically (data not shown). Subsequently, the rtL180M specific MGB probe was designed to have a compatible T_m for use with the LNA primers using Primer Express software (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences are (see also Figure 1, nt positions are relative to gtA reference sequence X02763): MGB rtL180M probe (nt 658–674) 5' - 6FAM CCG TTT CTC ATG GCT CA MBGNFQ (Applied Biosystems, Foster City, CA, USA), LNA rtV173L forward primer (nt 629–647) 5' GCAAATACCTATGGGA+CT and, LNA rtM204V reverse primer (nt 756–739) 5' CAATACCACATCATCC+AC, with LNA bases denoted as +C, +A, respectively.

2.4 Quantitative PCR standard curves and conditions

WT and rtV173L+rtL180M+rtM204V triple mutant plasmids were then 10-fold serially diluted from 10^9 to 10 copies/ μ L, plus an extra point at 50 copies/ μ L, in order to generate standard curves. The WT standard curve was validated against the AcroMetrix Optiquant HBV DNA Quantification Panel (Life Technologies, Benicia, CA, USA) which had been calibrated with the WHO 1st International Standard for HBV DNA. Additional specificity reactions of each single mutant template, rtV173L, rtL180M, and rtM204V, and the clinically significant double mutant rtM204V+rtL180M were also testing for potential false amplification. Reactions were set up using 10^8 copies/ μ L of the single mutants and at 10^4 and 10^5 copies/ μ L of the double mutant as these concentrations approximate highest likely concentration ranges in vivo. Quantitative PCR reactions were set up in a total volume of 10 μ L containing 3 μ L of purified, eluted HBV DNA template, 5 μ L of TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.25 μ L (250 nM) of each primer and probe. Cycling conditions for the triple mutant qPCR were as follows: 98°C for 30 seconds followed by 40 cycles at 95°C for 15 seconds and 58.5°C for 1 minute. Cycling conditions for the total viral concentration were the same except with an annealing temperature of 60.0°C. Reactions were run on a StepOne Plus real-time PCR machine (Applied Biosystems, Foster City, CA, USA). All samples were run in triplicate and mean cycle threshold values (Ct) were used to create standard curves using SDS 2.2.2 software (Applied Biosystems, Foster City, CA, USA).

2.5 Mixing studies with WT and mutant plasmids

In order to determine the performance characteristics of the triple mutant qPCR when detecting minor variants within large heterogeneous viral populations mixing studies were performed. Mixtures of the WT and mutant plasmids were prepared with 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% of resistant variants relative to WT and used as template for HBV triple mutant genome qPCR. The mixing studies were repeated in duplicate over a range of representative total viral concentrations of 10^4 , 10^6 , and 10^8 copies/ μ L, which are typical for HBV plasma concentrations seen in infected individuals.

2.6 Normalization of quantification using an external standard

Prior to extraction all serum samples were spiked with 25 pg of a plasmid containing the murine Heat Stable Antigen (HSA, or murine CD24) gene, for use as an external standard to control for differences in extraction efficiency and pipetting errors. HSA was quantified according to a previously published qPCR protocol, slightly modified to run in a smaller reaction volume (Ali and Yang, 2006). Briefly, HSA sequence was quantified using the following primers and probe: forward primer: 5' – GCA CCG TTT CCC GGT AAC CAG

AAT AT – 3', reverse primer: 5' – AGA GAG AGA GAG AGC CAG GAG A – 3', probe: 5' FAM – TCC CTG CAG TCC ACA GCT GG -3' Tamra (Integrated DNA Technologies, San Diego, CA, USA). Reactions were set up in 10µL containing 3 µl of template, 5 µl of TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.25 µl (250 nM) of each primer and probe and run on a StepOne Plus real-time PCR machine with the following cycle conditions: 98°C for 30 seconds followed by 40 cycles at 95°C for 15 seconds and 60.0°C for 1 minute.

2.7 Validation using serum from an HBV infected individual

Serum from a lamivudine experienced HIV/HBV genotype A co-infected subject whose personal health information had been de-identified was obtained. Two hundred fifty microliters of serum was first spiked with 25 pg of HSA as an external control for normalization. HBV and HSA DNA were extracted from serum using the Ultrasense Viral Isolation Kit and eluted in 60 µL according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). The sample was diluted 1:1000 prior to amplification. The total HBV viral concentration was measured using the qPCR described above and validated using the commercially available COBAS Amplicor HBV viral load test. In parallel, the triple mutant HBV viral concentration was quantified from the same sample using the allele-specific qPCR described above. Copies of HSA were measured in parallel for each sample, and total viral concentration and triple mutant viral concentration measurements were normalized to HSA copy number. All samples were tested in triplicate. Normalized mean C_t numbers of the triple mutant qPCR results were used to determine both the absolute copy number and percent of the total viral concentration based on the WT and triple mutant DNA standard curves using SDS 2.2.2 software (Applied Biosystems, Foster City, CA, USA).

3.0 Results

3.1 Design of allele-specific primers and probes

A combination of nucleic acid modification technologies was employed in a quantitative allele-specific amplification in order to detect simultaneously 3 HBV polymerase drug resistance mutations with maximum specificity without loss of sensitivity. Allele-specific amplification capitalizes on the principle that there must be a perfect match between primer and template at the final 3' primer position in order for nucleotide addition to occur efficiently. However, a mismatch at the 3' primer may inefficiently block polymerase extension thereby leading to decreased specificity of the allele-specific amplification. Primers using "locked nucleic acid" (LNA)- enhanced nucleotides were used to improve the specificity of the forward and reverse primers specific for the drug resistance mutations at positions rt173 and rt204, respectively (Figure 1). The length of the primers as well as the number and position of the nucleic acids locks were determined empirically (data not shown). The highest specificity and sensitivity of amplification was achieved by incorporating a single LNA nucleoside one position away from the 3' end (penultimate position) of the primer.

Subsequently, an allele-specific rtL180M probe was designed to have a compatible T_m for use with the LNA primers in a TaqMan probe-based qPCR. A minor groove binding modification was used to enhance the specificity of the probe designed to bind the rtL180M T to A mutation, which lies between the rt173 and rt204 mutations (Figure 1). The addition of the MGB modification permitted the use of a relatively short allele-specific probe while maintaining a sufficiently high T_m for use in the qPCR. Lastly, the optimal annealing temperature for the qPCR was determined using a temperature gradient.

3.2 Sensitivity and Specificity of the triple mutant qPCR assay

Once the LNA primers and MGB probe had been designed and the optimal annealing temperature established, the sensitivity and specificity of the qPCR assay to detect the triple mutant combination was determined using dilutions of plasmid standards containing either the wild type (WT) HBV polymerase gene or the triple mutant, created by site-directed mutagenesis of the WT plasmid. The sensitivity of the quantitative triple mutant assay was determined using 3 μ L per reaction of serial 10 fold dilutions, plus an extra point at 50 copies/ μ L, of rtV173L+rtL180M+rtM204V triple mutant plasmid DNA. A range from 3×10^9 copies to 3×10^1 copies was tested, as this represents the range of viral concentrations typically seen in infected individuals (Figure 2A and G). The qPCR assay reliably detected as few as 150 copies in the linear range of amplification (Figure 2A, squares, $R^2=0.9993$). Standard deviations among the triplicate samples ranged from 0.04–0.27 cycles for detection. Next, the specificity of the assay was measured by determining whether the allele-specific reagents were able to amplify the WT template. There was no amplification in the range of 3×10^9 – 3×10^1 copies (Figure 2, circles, note a value of 0 equates to a result of Ct = “undetermined”). In contrast to other methods for detection of HBV drug resistance genotypes, this assay maintained 100% specificity even at high copy numbers of the WT template. Additional specificity testing was performed on single mutant templates (rtL180M, rtM204V, or rtV173L) and the double mutant template rtM204V and L180M, the most common clinically relevant double mutant. There was no amplification at 3×10^8 copies of the single mutants rtL180M, rtM204V, or rtV173L (Figure 2B–D). There was also no amplification of the rtM204V+rtL180M double mutant at concentrations of 3×10^4 or 3×10^5 copies (Figure 2E–F). Some false amplification of the double mutant template above the threshold of detection was detected at 10^6 and higher copy numbers.

3.3 Validation of sensitivity and specificity using mixed viral populations

Previous approaches to measure minor viral populations of drug resistance mutants have suffered from loss of sensitivity and specificity when WT virus is present in great excess relative to the mutant virus. In order to determine whether the same sensitivity and specificity were maintained when both WT and mutant templates were present in heterogeneous viral populations, a series of mixing studies were performed. Mixtures of WT and mutant plasmids were prepared with 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% of resistant variants relative to WT and used as template for the triple mutant qPCR. Three different total copy numbers were tested, 10^4 , 10^6 , and 10^8 copies/ μ L. The sensitivity and specificity of the triple mutant qPCR assay were not significantly altered by using mixtures of WT and mutant templates (Figure 3). Specifically, there was no change in sensitivity of detection of the mutant template in the mixtures of 10^4 , 10^6 total copy number, while there was up to a 0.3 log decrease in sensitivity with the 10^8 total copy number mixtures. However, given the small standard deviations and more reliable detection at higher copy numbers this apparent decrease in sensitivity was most likely due to inaccurate dilutions rather than an actual change in sensitivity of detection. The assay was able to detect 3×10^2 copies/ μ L of the mutant template in total concentrations of 3×10^4 , 3×10^6 , and 3×10^8 copies/ μ L, although in each case this dilution was just at the limit of reliable detection. This level of detection corresponds to minor variant viral populations of 1%, 0.01%, and 0.0001%, respectively. Even with gross excess of WT template in mixed viral populations the triple mutant qPCR maintained specificity. No non-specific amplification above the 3×10^2 copies/ μ L limit of detection was detected at any of the proportions or total template concentrations tested.

3.4 Quantitation of triple mutation viral population from a subject with known lamivudine resistance

The triple mutant allele-specific quantitative PCR was then validated on a serum sample from a subject with known lamivudine resistance. Previous consensus sequencing characterized the sample as containing HBV genotype A with the rtM204V mutation, but the additional rtV173L and rtL180M mutations were not detected (data not shown). Both the total HBV viral concentration and the triple mutant viral concentration were determined in parallel using the qPCR assays described above (see Figure 4). Samples were tested in triplicate and results were normalized to an external control template, HSA, to correct for possible errors introduced through extraction or pipetting. Between 2.5–3% variability was observed between replicates with HSA mean C_t values of 24.659 ± 0.612 and 23.303 ± 0.699 for total and triple mutant samples, respectively. The final absolute viral concentration was calculated by first normalizing to the external HSA control then converting to copies/mL of serum. The total HBV measurement was 1.03×10^{10} copies/mL (Figure 4A), equivalent to 1.77×10^9 IU/mL, which was similar to the viral concentration determined using the COBAS Amplicor HBV viral load test (1.24×10^9 IU/mL). The triple mutant viral concentration was 2.86×10^8 copies/mL, representing 2.8% of the total viral population (Figure 4B). This proportion of the triple mutant relative to the total viral concentration is well below the standard threshold of detection by standard genotyping assays that use consensus sequencing (~20 %).

4.0 Discussion

The allele-specific quantitative PCR assay described here is a marked improvement on existing techniques for the detection of multiple simultaneous HBV drug resistance mutations present in minor viral populations. A combination of primers and probes with different nucleic acid modifications, i.e. - locked nucleic acids (LNAs) and minor groove binders (MGBs), was utilized to increase the specificity of detection of the triple mutation rtV173L+rtL180M+rtM204V in HBV polymerase without reducing the sensitivity. Locked nucleic acid nucleosides contain a methylene bridge connecting the 2' oxygen of ribose with the 4' carbon of the ribose ring resulting in increased melting temperatures necessary for allele-specific discrimination (Braasch and Corey, 2001; Latorra et al., 2003). Similarly, minor groove binder ligands at the 3' end of TaqMan probes increase the annealing temperature of the probes, allowing for a shorter probe with enhanced specificity (Afonina et al., 1997). This study appears to be the first application of the combination of LNA primers with an MGB probe to detect multiple mutations within the same genome. Using this approach it was possible to detect with 100% specificity minor viral populations of the triple mutant resistant genotype at 1%, 0.01%, and 0.0001%, in 10^4 , 10^6 , and 10^8 copies/ μ L, respectively. This assay was then used on a clinical specimen to give an absolute quantification of the circulating minor variant triple mutant virus population in vivo. Additionally, unlike methods that measure the mutations independently of each other or rely on an initial PCR amplification step, which is prone to PCR-induced recombination, this method requires all three mutations to be present simultaneously on the same template molecule, ensuring accurate direct quantitation of genomes carrying the triple mutant combination.

Multiple techniques for the detection of minor variant populations of HBV drug resistance mutations have been described, but none is able to quantitate directly the presence of multiple simultaneous mutations within the same genome and maintain specificity and sensitivity. Consensus sequencing of the HBV polymerase gene is utilized clinically to detect drug resistance mutations, but only detects minority variants representing at least ~20% of the total viral population and cannot determine if multiple mutations are present on the same template (Cooley et al., 2003; Zhang et al., 2003). Line probe assays have improved

sensitivity of ~5% of the total viral population when viral concentrations are at least 10^4 copies/mL (i.e. absolute sensitivity between 500–5000 copies) but have higher false positive and false negative rates, and also cannot detect linked mutations (Degertekin et al., 2009; Libbrecht et al., 2007). Several allele-specific quantitative PCR methods have been published for the quantitation of multiple HBV drug resistance mutations, but again cannot detect specific combinations of mutations (Fang et al., 2009; Geng et al., 2006; Punia et al., 2004; Wen and Li, 2007). Furthermore, several of these methods require initial non-allele-specific PCR amplification before a quantitative nested PCR reaction, making these assays only semi-quantitative (Fang et al., 2009; Punia et al., 2004). The reported sensitivities of these assays range from about 100 to 1000 copies of mutant template in the presence of excess wild type (Fang et al., 2009; Geng et al., 2006; Wen and Li, 2007). In most cases the specificities decrease at low concentrations of mutant virus relative to wild type, yielding false-positive results (Fang et al., 2009; Geng et al., 2006; Wen and Li, 2007). Both LNA (Fang et al., 2009) and MGB (Geng et al., 2006) modifications have been used previously to increase oligonucleotide specificity, but they have never been used in combination to detect specific combinations of mutations. More recently pyrosequencing has been used to characterize minor quasispecies of HBV drug resistance mutations (Margeridon-Thermet et al., 2009; Solmone et al., 2009). Although this approach is highly sensitive in theory, in practice technical difficulties with data analysis and a high sequencing error rate reduce the overall sensitivity of this approach to about 1 to 2% when HBV DNA concentrations are at least 10^4 IU/mL (i.e. – an absolute sensitivity of approximately 600 to 1200 copies), and it is more expensive relative to qPCR. Another recently published approach for detection of multiple HBV drug resistance minor variant populations is the PCR Invader method (Tadokoro et al., 2011). Although this method can detect multiple mutations, they are measured independently with only a sensitivity of 2% (20 copies in 1000). As with pyrosequencing, clonal HBV sequencing (via either cloning of PCR products or limiting dilution PCR) theoretically has unlimited sensitivity, but its routine use is limited by the number of clones that must be sequenced to detect minor mutants.

Although this approach has many advantages over existing assays, it does also have a few limitations. Although the total HBV viral concentration qPCR reagents were designed to detect all genotypes, the allele-specific primers and probes were designed to work best with genotype A and D sequences, genotypes common in cohorts found in Africa and the United States. The performance characteristics of the allele-specific primers and probe on other genotypes have not been determined. Other assays have absolute sensitivities lower than the 150 copies/ μ L, but generally those assays also have a higher false-positive rate in the low range of detection (Fang et al., 2009; Wen and Li, 2007). Another general limitation of this approach is the fact that the mutations of interest must be relatively close together (i.e. within several hundred nucleotides) for real-time PCR to work well. Therefore, this approach works best for the detection of multiple mutations in close proximity, as is the case for the combination rtV173L+rtL180M+rtM204V in HBV.

The detection of minor viral populations with multiple linked lamivudine-resistance mutations within the HBV polymerase gene has important clinical implications. When the three mutations rtV173L+rtL180M+rtM204V are present simultaneously, there is an associated vaccine epitope immune escape phenotype due to associated changes in the overlapping *envelope* reading frame. Therefore being able to determine that all three mutations are within the same genome is critical for monitoring the development of the vaccine escape phenotype. An additional benefit of this approach is that theoretically the allele-specific primers and probe can be exchanged with their wild type equivalents to adapt the assay to detect any combination of these three mutations. The flexibility of the method allows for the potential for detailed characterization of the longitudinal development of first single, then double, and finally triple mutant minor variant populations within an infected

individual over time. This type of detailed molecular epidemiology will help track the development of the vaccine escape triple mutant genotype both within individuals and populations and may inform HBV treatment practices.

In summary, the allele-specific quantitative PCR described here, which uses a combination of locked nucleic acid primers and a minor groove binder probe, is a highly sensitive, specific, rapid, and inexpensive method for directly measuring minor viral quasiespecies of the triple combination mutation rtV173L+rtL180M+rtM204V within one HBV genome. Beyond the specific use of this approach to detect HBV mutations, this methodology can be used more broadly in any situation where the measurement of multiple mutations within the same template molecule is necessary. For example, this approach can be applied to the simultaneous detection of combinations of multiple drug resistance or immune escape mutations in HIV or hepatitis C infection or to identify multiple simultaneous combinations of mutations associated with oncogenesis.

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> Measurement of point mutation combinations on the same viral genome is important. > An allele-specific assay was developed for the HBV triple mutant rtV173L+rtL180M+rtM204V > This combination of primers and probe yields a linear detection range down to 150 copies. > This strategy has 100% specificity even in mixtures of predominately wild type genomes.

Figure 1. Location and design of allele-specific primers and probe

A stretch of genotype A HBV *polymerase* between nt 609–781 (relative to gtA reference sequence X02763) shows the location of 3 drug resistance mutations, rtV173L (G>C), rtL180M (T>A), and rtM204V (A>G). A forward primer specific for the rtV173L (G>C) mutation was designed with a locked nucleic acid (LNA) at the penultimate position. A reverse primer specific for the rtM204V (A>G) mutation also contains a nucleic acid lock at the penultimate position. Note that for the forward rtV173L primer the lock is at the mismatch, but for the reverse rtM204V primer the lock is adjacent to the mismatch. A TaqMan probe specific for the rtL180M (T>A) mutation was designed with a minor groove binding (MGB) modification. Both the LNA and MGB modifications were designed to optimize specificity without decreasing sensitivity.

Figure 2. Sensitivity and specificity of the allele-specific triple mutant quantitative PCR
 A.) Sensitivity was determined using 3μL each of serial 10-fold dilutions, plus an extra point at 50 copies/μL, of the triple mutant template from 10⁹-10 copies/μL (squares). Linear detection was achieved reliably down to 150 copies ($R^2=0.9993$). Standard deviations of C_t values for triplicate samples were very small (0.04–0.27). Specificity was determined by performing the allele-specific qPCR on serial dilutions of the WT template from 10⁹-10 copies/μL (circles). Non-specific amplification of the WT template did not occur at any of the concentrations tested (note a value of 0 equates to a result of C_t = “undetermined”), i.e. 100% specificity within the range tested. Additional specificity testing was performed on single (panels B–D, 10⁸ copies) and double mutant templates (panels E–F, 10⁴ or 10⁵ as

marked). The genotype of the template is marked in the upper right corner. Amplification of the triple mutant template (10^9 - 10^2 copies) under the same conditions as the single and double mutant templates is shown in G.

Figure 3. Sensitivity and specificity of the allele-specific triple mutant quantitative PCR to detect minor viral populations within mixed viral populations of various sizes

Mixtures of triple mutant and WT plasmids were prepared with 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% mutant relative to WT. Three different total template amounts were tested in duplicate experiments, 10⁴, 10⁶, and 10⁸. Mean and standard deviation of the total copy number of the triple mutant detected in each mixture is shown relative to the limit of detection.

Figure 4. Determination of total HBV and triple mutant viral concentrations from a serum sample from an HBV-infected subject with known lamivudine resistant HBV infection
 Viral DNA was extracted from HSA plasmid-spiked serum, diluted 1:1000, and then used directly as template for both total viral concentration (A) and triple mutant viral concentration (B) determination. The sample was tested in triplicate, and the amplification plots used to determine the mean C_t number are shown. The final absolute viral concentration was calculated by first normalizing to the external HSA control and then converting to copies/ μ L of serum.