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Quantification of complex precore mutations of hepatitis B virus by SimpleProbe real time PCR and dual melting analysis

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Abstract

Background—Hepatitis B virus (HBV) precore G1896A mutation is associated with Hepatitis B e antigen (HBeAg) seroconversion. This mutation and the adjacent G1899A mutation also appear to associate with increased risk of hepatocellular carcinoma. Quantitative mutant dynamics may help determine the potential of these mutants as clinical biomarkers. However, a reliable method to quantify either mutant is not available, partly because the viral genome has polymorphisms in general and the precore mutations are complex.

Objectives—(1) To develop a reliable and ultrasensitive assay for the quantification of HBV G1896A and/or G1899A mutants. (2) To obtain preliminary data on the quantities of the precore mutants in patients.

Study Design—A SimpleProbe real time PCR assay was developed to quantify the HBV precore mutants. Dual melting analysis and a primer-probe partial overlap approach were used to increase detection accuracy. A wild-type selective PCR blocker was also developed to increase mutant detection sensitivity.

Results—The assay correctly identified the precore sequence from all 62 patient samples analyzed. More than 97% of precore sequences in the GenBank can be recognized. Mutant detection sensitivity reached 0.001% using a wild type-selective PCR blocker. At least one precore mutant can be detected from all 20 HBeAg-positive individuals who were negative for precore mutations by DNA sequencing.

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Conflict of interest
The authors declared no conflict of interest with respect to this manuscript.

Conclusions—The reliability of this ultrasensitive mutation quantification assay was demonstrated. The same approaches may be useful for the detection of other clinically significant mutations. Evolution of the precore mutants warrants further studies.

Keywords

mutation; HBV; precore; quantification; SimpleProbe; qPCR

1. Background

Chronic infection by hepatitis B virus (HBV) is one of the major risk factors for liver cirrhosis and hepatocellular carcinoma (HCC) [1–3]. Disease progression is not fully understood, but involves complex interplay between the host antiviral response and the evolving virus.[4–7] It was shown that the appearance of G1896A or G1899A mutation in the precore region correlated with increased risk of HCC,[8, 9] although inconsistent results were also reported.[10] Supporting this correlation, these mutants also appeared to be associated with more severe liver inflammation or acute-on-chronic liver failure.[11–14] The G1896A mutation in particular creates a premature stop codon in the hepatitis B e antigen (HBeAg) open reading frame, thereby abolishing HBeAg production.[11] This mutation was frequently associated with HBeAg seronegativity. However, about 9% HBeAg-positive patients had G1896A mutant as dominant species.[15]

Previous precore mutation studies mostly relied on DNA sequencing; the quantitative dynamics of the mutant viruses during chronic infection is largely unknown. For example, it is not clear when the mutant begins to appear, how quickly it becomes dominant, what factors contribute to its dominance, and whether the mutant percentage would change in response to treatment. Clarification of these questions will help understand the significance of the precore mutations in disease progression and its potential as a biomarker. However, this requires a quantitative and highly sensitive mutation detection assay which is not yet available.

2. Objectives

Develop a quantitative PCR assay that can reliably recognize the precore mutations. Increase the mutation detection sensitivity so that the assay can be used to monitor mutant development from early on.

3. Study Design

3.1. General molecular biology protocols and patient samples

HBV nucleotide (nt) 1406–1935 was cloned from a patient by PCR and various precore mutations were constructed by site-directed mutagenesis. They were used for assay development and as standards for quantification and melting curve analysis. Real time PCRs were carried out in the LightCycler 480 instrument (Roche Applied Sciences). Patient serum samples were collected with signed informed consent from the ethnic Asian communities in the Philadelphia area.[16–18] Viral DNA was isolated from 200 μl serum samples using the QIAamp DNA Blood Mini kit (Qiagen).

3.2. Analysis of the target precore mutation site and assay design

A BLAST analysis of GenBank sequences was performed to obtain mutation patterns in and around the target precore site. Polymorphisms within 10 nucleotides up-or down-stream of the target precore site (nt 1896–1899) were limited. However, the precore site itself was a hotspot for mutations (Fig. 1A). G1896A and G1899A occurred frequently either separately

or together as a G1896A/G1899A double mutation. A detailed analysis of the precore hotspot is in Figure 1B. In view of the complex mutation patterns in the target precore site, we chose SimpleProbe PCR for its "self-evaluating" melting curve analysis capability. Two probes were designed (Fig. 1C), one (SPC1) for mutant quantification and differentiation, and the other (SPC2) for additional mutant differentiation power. A wild-type (WT)-selective PCR blocker was also designed (Fig. 1C) to increase the mutation detection sensitivity.

3.3. Precore G1896A and G1899A quantification assay

A two-step assay was developed for the quantification of G1896A and/or G1899A mutants with high sensitivity. The first step PCR was designed to suppress the amplification of the WT DNA and allow the mutant DNA to be amplified. The 15-μl reaction contained 0.5 μM each of F1 and R1 primers, 5 μl of the purified patient sample DNA as template, and 2 μM WT-selective PCR blocker. Amplification was carried out at 95°C for 10 s, 59°C for 10 s and 72°C for 5 s, for a total of 16–20 cycles. The first step PCR products were diluted 1:32 and used as templates in the second step real time PCR which was composed of LightCycler480 Genotyping Master Mix, 0.1 μM primer F1, 0.5 μM primer R1, 3 mM $MgCl_2$, and 0.1 μM SPC1 or SPC2 (sequences see Fig. 1C). The real time PCR was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 66°C for 10 s (with fluorescence acquisition), and 72°C for 5 s, and then followed by a melting curve analysis from 30°C to 80°C. Plasmids carrying WT precore or precore mutations were included as melting peak standards. Serial diluted plasmids carrying G1896A were included in the first step PCR so that a standard concentration curve can be generated. Quantification calculations were performed using the LightCycler480 SW1.5 software. The two-step design separated the probe from the WT-blocker which would otherwise inhibit the real time PCR signals. The second step PCR could also be used as a stand-alone assay but the mutant detection sensitivity will be in the normal range (~5–10% mutant) rather than 0.001% for the two-step assay.

When confirmation of mutant detection by DNA sequencing was needed, the R1 primer was replaced with R2 primer (5'-aggctgtaggcataaattgg-3', corresponding to nt 1778–1797) to generate a longer amplicon (150 bp) suitable for direct sequencing.

3.4. HBV total viral load measurement

This assay was virtually the same as the precore mutation assay except the PCR blocker was omitted. The thermal program for the SimpleProbe PCR was modified slightly as 38 cycles of 95°C 10 sec, 55°C 10 sec (with fluorescence acquisition), and 72°C 5 sec. This assay is only used in conjunction with our mutant assay for the purpose of calculating mutant percentage.

4. Results

4.1. Use of SimpleProbe SPC1 for mutant and total viral load quantification

SimpleProbe emits fluorescence signals only when bound to the specific template.[19] SPC1 had higher melting temperatures with the three targeted mutants (G1896A, G1899A and G1896A/G1899A) than with the WT and others (Fig. 2A). At the annealing temperature (also for fluorescence acquisition) of 66°C, the three targeted mutants, but not the others, could bind to the probe and generate amplification signals (Fig. 2B and 2C). Mutant amplification signals could be suppressed by the co-existed WT DNA (Fig. 2E), but the threshold cycle was not changed significantly, thus mutant quantification was still achievable. When the WT DNA was >10-fold of the mutant DNA, however, the mutant was barely detectable (Fig. 2E and 2F).

When the annealing temperature was reduced to 55°C, all the precore variants could bind to the probe and generate signals during amplification (Fig. 2D), thus the "total" viral titer could be measured.

4.2. Dual melting to enhance mutant differentiation

When small amount of G1896A or G1899A co-existed with the WT, it was difficult to distinguish between G1896A and G1899A by SimpleProbe SPC1 (Fig. 2F, lines 5–7). Thus, SimpleProbe SPC2 was designed to give unambiguous differentiation of these two mutations (Fig. 3A and 3B). In the case when G1896A and G1899A were present as a mixture, their "total" titer could be quantified using the probe SPC1. The titer for each mutant could be estimated by the relative height of the melting peaks in the SPC2 melt (Fig. 3B). Under this condition, the assay became semi-quantitative.

4.3. Further evaluation of probe performance

Sixty-two patient samples were analyzed by PCR sequencing (Fig. 4; GenBank accession numbers HQ907993-HQ908054). Consistent with the BLAST analysis (Fig. 1A), no significant nucleotide polymorphisms were observed within the amplified region. Mutations at nt 1896–1899 were limited to G1896A, G1899A, G1896A/G1899A or their mixtures, with the exception of 2 cases in which G1898A was detected. Not surprisingly, the SimpleProbe melting patterns from the patient samples matched with those from the plasmid standards, as well as with DNA sequencing results in all 62 cases. A unique melting pattern from a patient sample, showing a mixture of G1899A and G1896A/G1899A, is shown in Figure 5A.

Because of the overlap between the SPC1 probe and the primer F1 (Fig. 1C), any nucleotide polymorphism in the region covered by the primer F1 would be converted to the primer sequence after amplification and therefore would not change the melting curves. The portion of the probe that would respond to mutations (the effective length) was thus reduced to 10 nucleotides corresponding to nt 1890–1899, of which a detailed BLAST analysis is shown in Fig. 5B. The first six sequence patterns accounted for 97.4% of all the mutant precore sequences in GenBank; they, and the WT, can be identified correctly using our probes. Some of the precore mutations may still be recognizable even when they were accompanied by extra mutations/polymorphisms (patterns 10 and 17, Fig. 5C and 5D). Other patterns (7–9, 11–16) did not possess G1896A or G1899A mutation; they had melting temperatures lower than that of the WT (not shown) thus would not interfere with the quantification of the three target mutations.

4.4. Ultrasensitive quantification of the precore G1896A and G1899A mutants

To increase mutation detection sensitivity, a WT-selective PCR blocker was developed to selectively suppress the amplification of the WT DNA. The PCR blocker was designed to match the WT sequence and had locked nucleic acids (LNAs) to increase the binding affinity [20] to the WT DNA (Fig. 1C). The mutants have mismatch(es) to the PCR blocker, thus primer extension on the mutants was not affected under optimal conditions. As a result of WT DNA suppression, minute amount of mutant undetectable by conventional methods became readily detectable by DNA sequencing (data not shown) or SimpleProbe PCR. Shown in Figure 6A and 6B, the mutant was detectable in the presence of 1,000,000-fold excess of the WT DNA. In the absence of the WT blocker, however, mutant was barely detectable at 5–10% (Fig. 2E and 2F).

We measured G1896A and/or G1899A mutations in 20 HBeAg-positive patient samples which had no detectable precore mutants by manual sequencing reading. Our assay revealed

the presence of G1896A in 19/20 patients and G1899A in 16/20 samples. The percentage of mutants was in the range from 0.001 – 5.326% (Table 1).

5. Discussion

Detection of HBV mutations using hybridization-based assays, such as real time PCR and Line Probe assay, can be unreliable mainly because the viral genome tends to have nucleotide polymorphisms. The complex mutation patterns in the target precore site add further difficulties in mutation differentiation. Therefore, any probe-based mutant detection method must be evaluated thoroughly. Quantification of HBV G1896A mutant was reported previously using a TaqMan probe.[21] The probe matched with the G1896A sequence, being almost identical to our SPC2 probe except complementary to each other. This probe could be used to quantify the G1896A single mutation but not G1896A/G1899A because the amplification signals of the double mutation could not be separated from those of the WT DNA (Fig. 3A). This could cause significant underestimation of the amount of G1896A.

Unlike TaqMan PCR, the SimpleProbe PCR includes a melting analysis after the amplification to examine which sequence variant(s) has been amplified and which variant(s) is generating the amplification signals. In addition to rational probe design, we used a primer-probe partial overlap approach and dual melting analysis to improve mutation differentiation capability. As a result, >97% of HBV sequences in GenBank can be correctly identified using our assay. Consistently, the melting patterns completely matched with the sequencing results in all 62 patient samples.

Compared with DNA sequencing, melting curve analysis has the advantage of distinguishing whether G1896A and G1899A are separate (single mutations) or in the same genome (double mutation). The double mutation may have important biological meanings as both A1896 and A1899 can enhance the stability of the secondary structure of the pre-genome encapsidation signal by pairing with T1858 and T1855, respectively. [22, 23] It will also be useful in evolutionary studies to distinguish the single from the double mutation.

We also developed a LNA-containing WT-selective PCR blocker which improved the mutation detection sensitivity of SimpleProbe PCR to 0.001%. Successful LNA-based selective PCR blocker and quantification of HBV mutants with ultrasensitivity have not been reported before. Using this novel assay, we found that G1896A, previously reported to be associated with high virulence, replication advantage and HBeAg seronegativity,[11, 24–26] is present in 95% of HBeAg-positive patients. It will be interesting to see how the mutant evolves quantitatively and becomes selected over time in chronically infected patients. The evolutionary pattern(s) will shed light on the virological features of the mutant and the relation to disease progression.

Abbreviations

HBV	hepatitis B Virus
HBeAg	hepatitis B e antigen
HCC	hepatocellular carcinoma
LNA	locked nucleic acid
nt	nucleotide
TVL	total viral load
WT	wild type

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A

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          1896  1899
                |                              entries     %
GCCTTGGGTGGCTTTGGGGCATGGACATTGACCCTTATA  8395
.....................................  2271  27.1
.....................................G....  1928  23.0
..............A..................G....   591   7.0
.......A.....................................   273   3.3
..............A..............................   260   3.1
.................................T......   249   3.0
..............A..A..........................   235   2.8
.....................................A....   194   2.3
..............A..............T.......   168   2.0
............................C........   150   1.8
..............A............C.....C....   131   1.6
..............A..A..............G....   128   1.5
..............A..A.............T.......   118   1.4
..............A............C........   105   1.3
..............A..................A.G....   102   1.2
..................A..............G....    93   1.1
.....................................A.G....    87   1.0
..................A..........................    82   1.0
```

B

```
1896  1899
      |  entries    %
GGGG   2735
A...   1720  62.9
A..A    668  24.4
A..C      1   0.0
A.AA      1   0.0
A.TA      1   0.0
AA..      1   0.0
C...      1   0.0
T...     59   2.2
.A..     13   0.5
.A.A      1   0.0
.C..      1   0.0
.T..      1   0.0
..A.     17   0.6
..AA      1   0.0
..T.      1   0.0
...A    245   9.0
...C      2   0.1
...T      1   0.0
```

C

Figure 1. Target mutation site and assay design

A non-contiguous megablast was performed using a query sequence of HBV nucleotide 1867–1926. A total of 8395 sequences were retrieved and the sequence patterns for the nucleotide 1881–1919 were compiled and sorted according to their number of entries and percentage representation (***A***). ***B***, mutation patterns at nucleotide 1896–1899. ***C***, assay design. Capital letters indicate LNAs. The 3'-end "PH" stands for phosphorylation. The nucleotide with fluorescence label was highlighted.

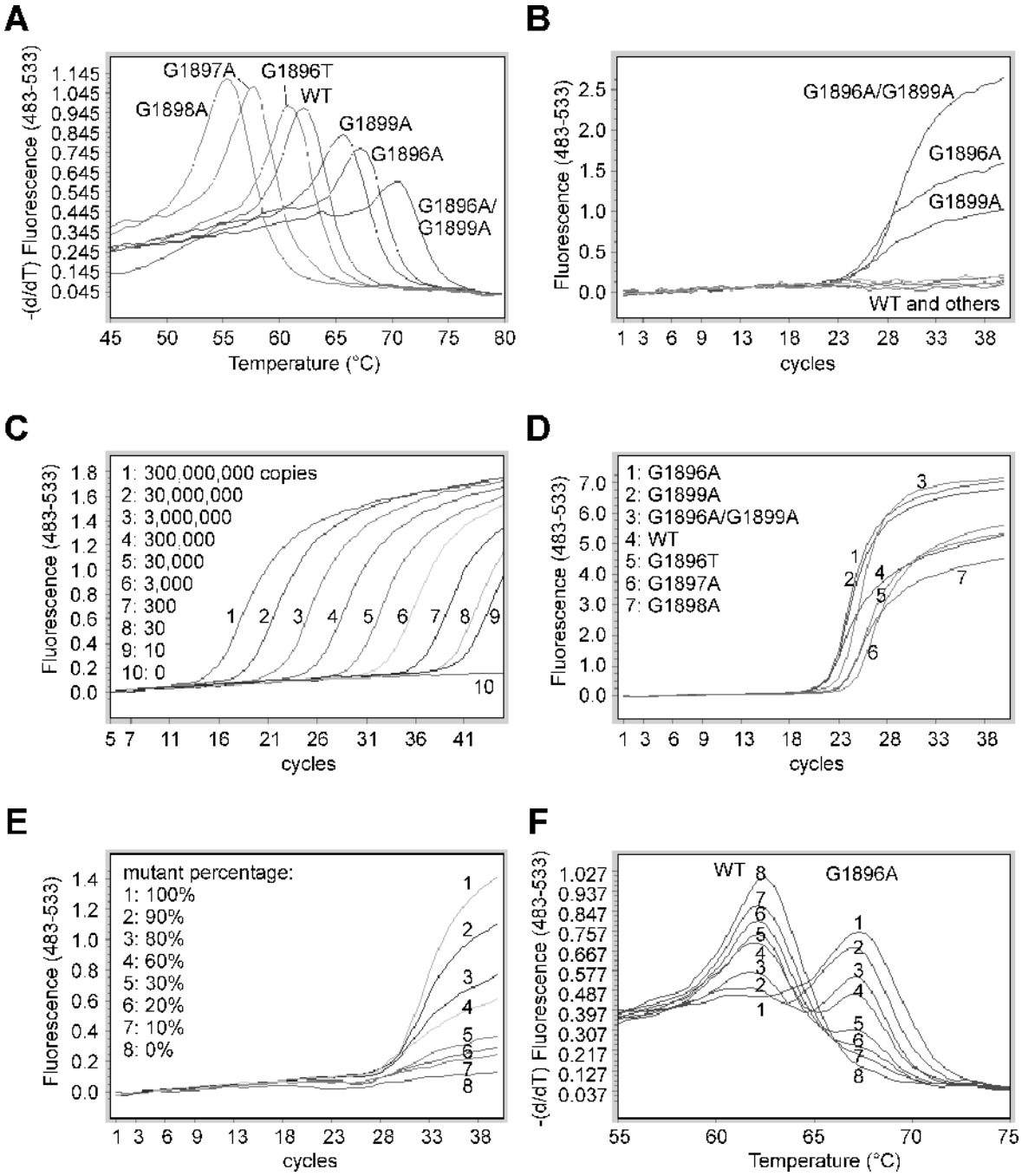

Figure 2. SimpleProbe PCR using probe SPC1

A, Melting curves of different precore variants. Amplification curves of precore variants at the annealing temperature of 66°C (***B***), or 55°C (***D***). Serial diluted G1896A plasmids were amplified (***C***) to obtain a concentration standard curve. Amplification (***E***) and melting (***F***) of G1896A in the presence of WT DNA. The labels in ***F*** are the same as in ***E***.

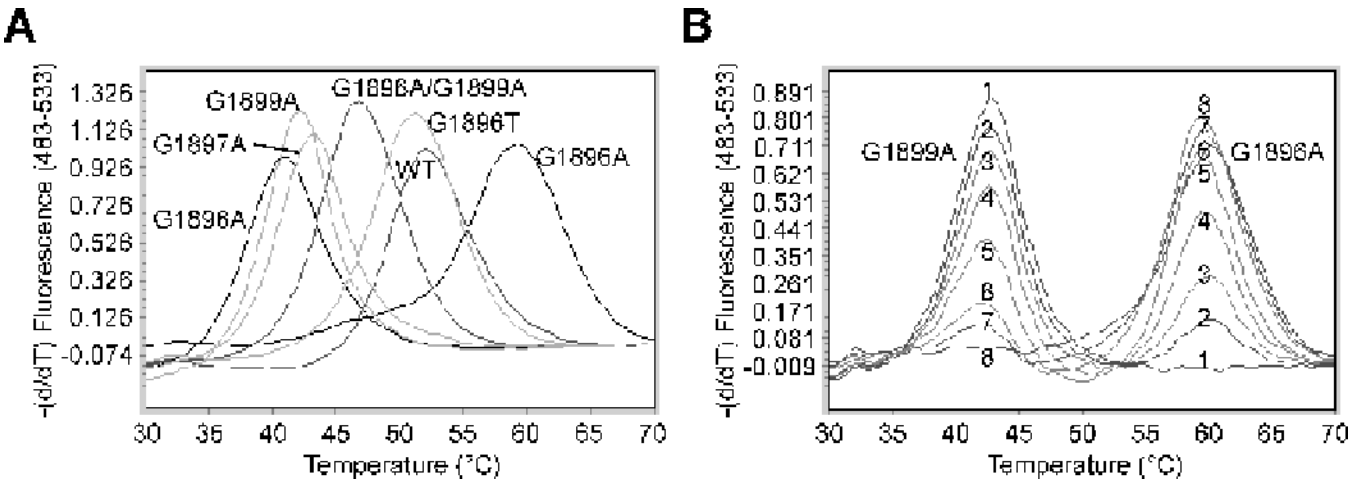

Figure 3. Melting analysis using the probe SPC2

A, melting patterns of seven precore variants. Estimation of relative quantities of G1896A and G1899A by melting peaks (***B***). Numbers 1–8 represent a G1899A:G1896A ratio of 1:0, 9:1, 7.5:2.5, 6:4, 4:6, 2.5:7.5, 1:9 and 0:1, respectively.

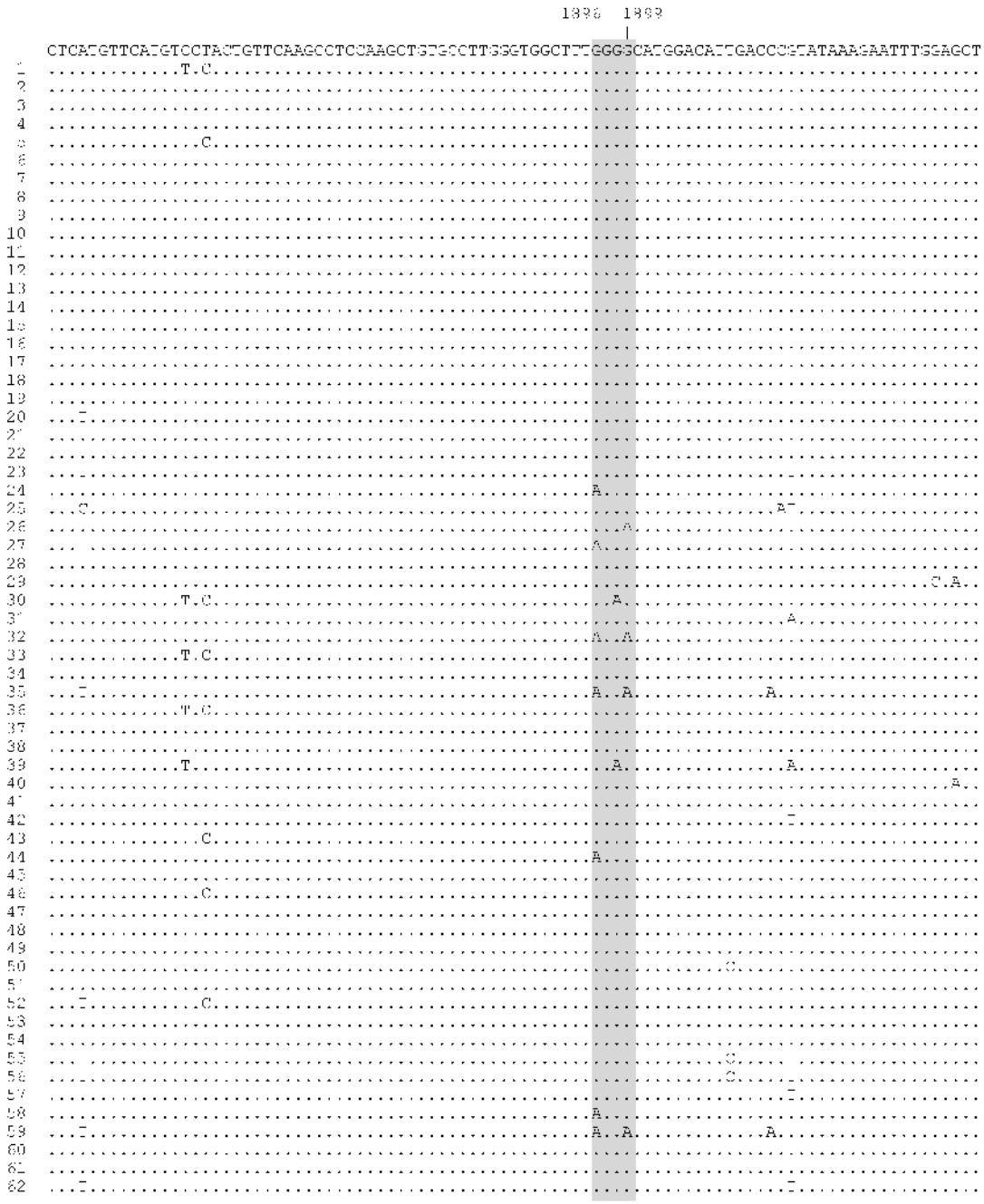

Figure 4. Analysis of HBV precore area by direct sequencing

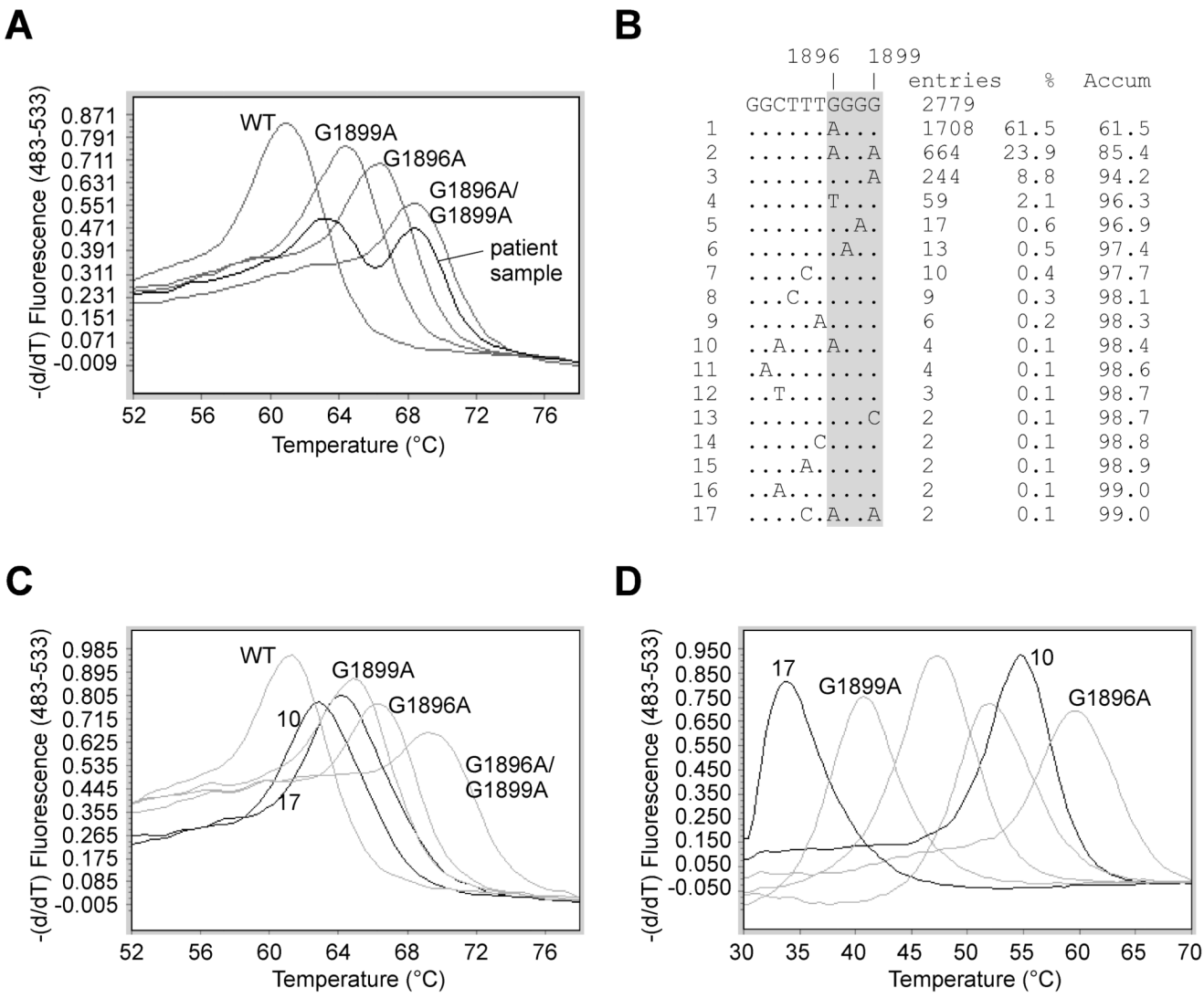

Figure 5. Reduced probe effective length increases probe performance

(***A***) An example of melting curve analysis in a patient sample showing a mixture of G1899A and G1896A/G1899A. (***B***) A non-contiguous megablast analysis of HBV nucleotide 1890–1899 sequence patterns. Patterns were sorted according to their total number of entries in GenBank, together with their percentage and accumulative percentage. Sequences identical to the top consensus sequence were not listed. Patterns 10 and 17 were analyzed using SPC1 (***C***) and SPC2 (***D***) probes.

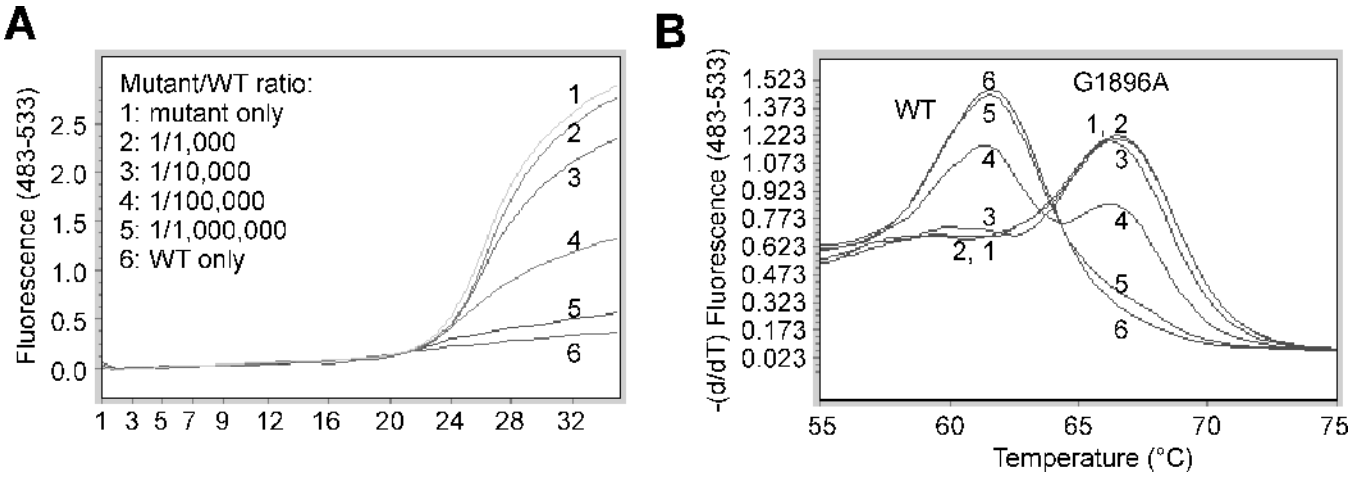

Figure 6. WT-selective PCR blocker increases mutant detection sensitivity

Plasmid templates containing 300 copies of G1896A only (1), or with WT plasmids at different ratios (2–5), or 3×108 copies of WT plasmid (8) were used as templates. SPC1 amplification curves (***A***) and melting curves (***B***) are shown.

Table 1

Ultrasensitive quantification of HBV precore G1896A and G1899A mutations from 20 HBeAg-positive patient samples.

No.	TVL*	G1896A*	G1899A*	G1896A%	G1899A%
1	4.52E+08	-	8.20E+03	-	0.002
2	2.29E+09	2.08E+06	5.21E+05	0.091	0.023
3	3.17E+08	6.29E+06	3.31E+05	1.986	0.105
4	8.81E+08	1.30E+07	2.30E+06	1.479	0.261
5	1.13E+09	3.23E+04	-	0.003	-
6	2.00E+09	4.45E+06	-	0.222	-
7	1.53E+09	8.17E+07	9.08E+06	5.326	0.592
8	8.67E+07	1.63E+05	7.00E+04	0.188	0.081
9	4.42E+09	1.36E+07	9.06E+06	0.308	0.205
10	7.18E+08	2.68E+06	1.15E+06	0.373	0.160
11	1.90E+08	6.83E+05	1.71E+05	0.360	0.090
12	8.13E+07	5.13E+05	-	0.631	-
13	1.81E+09	1.01E+07	2.52E+06	0.560	0.140
14	4.78E+07	6.09E+05	2.61E+05	1.273	0.546
15	5.73E+08	3.15E+06	3.50E+05	0.550	0.061
16	1.23E+09	1.36E+06	7.15E+04	0.110	0.006
17	2.97E+06	2.31E+01	-	0.001	-
18	1.29E+09	2.09E+06	5.23E+05	0.162	0.040
19	5.72E+06	4.08E+03	1.02E+03	0.071	0.018
20	6.32E+08	1.38E+06	7.27E+04	0.219	0.012

* TVL (total viral load), G1896A and G1899A are in copies/ml. G1896A is the “total” G1896A mutation (G1896A + G1896A/G1899A). Likewise, G1899A is the total G1899A whether or not associated with G1896A. For example, a mixture of 10% G1896A and 20% G1896A/G1899A indicates 30% G1896A and 20% G1899A.