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**A HIGH THROUGHPUT DETECTION METHOD FOR  
HEPATITIS B VIRUS MUTATIONS AMONG THE  
CHINESE POPULATION**

**Investigators**

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## **SUMMARY**

**BACKGROUND** Hepatitis B is a major disease which causes serious public health problems worldwide. Hepatitis B virus (HBV) genome is composed of four open reading frames, i.e. C, S, P and X gene. The presences of single nucleotide polymorphisms (SNPs) on these genes are associated with hepatocellular cirrhosis and carcinoma. The aim of this study is to apply Arrayed Primer Extension (APEX) as a simultaneous and high-throughput screening platform for the detection of SNPs in HBV genome, monitoring of disease development, patients population study, drugs or vaccines development and hepatocarcionogenesis study.

**STUDY DESIGN AND METHODS** We have optimized APEX for detecting thirty SNPs in the HBV genome. HBV obtained from 33 infected Chinese patients were investigated in this study. Single-stranded HBV DNA fragments were allowed to hybridize with the oligonucleotides corresponding to the sites of SNPs immobilized on a glass surface, followed by incorporation of different fluorescently labeled dideoxynucleotides.

**RESULTS** APEX allows fast and unequivocal discriminations between wild type and mutant genotypes with high dideoxynucleotides incorporation efficiency, sensitivity and specificity. The coexistences of both genotypes were also detected by APEX, which was not possible by routine DNA sequencing method.

**CONCLUSIONS** The simultaneous and high-throughput screening of SNPs in HBV genome by APEX enables large-scale and diagnostic analysis, which is a possible alternative genotyping method to DNA sequencing.

**IMPLICATIONS** As HBV infection is the major cause of hepatocellular carcinoma that ranked as the third cancer killer in Hong Kong, the application of APEX as a simultaneous and high-throughput genotyping platform for HBV SNPs genotyping may be beneficial to the development of treatment strategies for this expanding

chronic hepatitis B patient population. The APEX technology may also be extended to other SNPs-based applications.

## INTRODUCTION

Hepatitis B virus (HBV) is the infective agent for the widespread liver disease in humans known as hepatitis B. According to the World Health Organization, about 2 billion people have been infected with the HBV worldwide. More than 350 million people have been chronically infected; three-quarters of these are Asian<sup>1</sup>. Chronic infection has been associated with a high risk for development of liver cirrhosis and hepatocellular carcinoma (HCC)<sup>2,3</sup>, killing one million people each year<sup>1</sup>.

The HBV genome is a 3.2 kb, circular, partially double-stranded DNA molecule containing four open reading frames (ORFs) i.e. core C, polymerase P, surface S and X gene. The C gene codes for the nucleocapsid/ core protein (HBcAg). Hepatitis B e antigen (HBeAg) is transcribed from precore/ basal core promoter region. The P gene is responsible for producing reverse transcriptase. The S gene is responsible for producing the three envelope proteins (pre-S1, pre-S2 and S) and the X gene codes for the X protein (HBx).

Evidences have been accumulating that chronic HBV infection are associated with the development of point mutations or single nucleotide polymorphisms (SNPs) on HBV genome. Studies also showed that the development of SNPs on HBV genome is correlated with HCC development. Therefore, different methods have been used for HBV SNPs detection for monitoring the disease development. Conventional methods for detecting mutations are generally not practical for detecting multiple SNPs accurately. Also, for clinical and diagnostic analysis, simple techniques with high-throughput potential, promised reproducibility, sensitivity and specificity are required.

Here we describe an enzyme assisted hybridization-based method for rapid detection of multiple mutations in a microarray format, known as arrayed primer extension (APEX)<sup>4</sup>. Based on the hybridization of short single-stranded DNA

templates with an array of immobilized oligonucleotide probes on a glass surface and the incorporation of different fluorescently labeled dideoxynucleotides mediated by polymerase (Fig. 1), detection of known SNPs is possible in a single reaction by designing hundreds to thousands of oligonucleotides spotted accordingly on the same chip. One of the advantages of using APEX to detect known SNPs is its high signal-to-noise ratio.

#### **AIMS AND OBJECTIVES**

To apply APEX as a simultaneous and high-throughput genotyping platform to detect the SNPs present on HBV genome.

## **METHODS**

### ***SELECTION OF PATIENTS IN THE CHINESE POPULATION AND HBV DNA EXTRACTION***

The blood samples from HBV-infected patients with HBV DNA level equal to or higher than  $1 \times 10^4$  copies/ml were selected and the viral DNA were extracted. The DNA amount and quality was quantified and checked.

### ***PCR AMPLIFICATION***

The four open reading frames of HBV was amplified from serum HBV-DNA using nested PCR. The outer primer pairs and inner primer pairs for specific open reading frames were listed in Table 1. C gene was separately amplified by 2 sets of primers. The sequences of second PCR products were determined by DNA sequencing.

### ***PREPARATION OF OLIGONUCLEOTIDES AND ARRAY PRINTING***

According to the genomic sequence of hepatitis B virus (GenBank accession no. NC\_003977), oligonucleotides for detecting the SNPs on the open reading frames were designed as listed in Table 2. The 25-mer oligonucleotides with 12-carbon amino linkers at their 5' end were spotted in triplicates on an enhanced aminosilane-coated microarray slides. Salmon sperm DNA was also spotted on each array as negative controls. After DNA immobilization, the free active groups on the slides were blocked by ammonia. The quality of the spotted arrays was randomly determined by SYBR<sup>®</sup> Green II fluorescent staining<sup>5</sup>.

### ***TEMPLATE PREPARATION FOR APEX***

The second purified PCR products were fragmented by thermolabile uracil N-glycosylase (UNG) and the unincorporated dNTPs were inactivated by shrimp alkaline phosphatase (sAP) treatment. For fragmentation control, 1  $\mu$ l of the reaction mixtures were subject to agarose gel electrophoresis.

***ARRAYED PRIMER EXTENSION (APEX)***

The PCR products were heat-denatured. Then each fluorescently labeled dideoxynucleotides (Cy3-ddCTP, Cy5-ddUTP, FITC-ddGTP and Texas Red-ddATP), Thermo Sequenase, Sequenase Version 2.0 DNA Polymerase were added immediately to the denatured templates. APEX reaction mix was applied onto the SAL slides for 2 hours at 58°C. A standard APEX reaction without HBV PCR product was prepared for each lot of slides to check if there were self-extending oligonucleotides and non-specific background.

***IMAGE RECORDING AND ANALYSIS***

The slides were scanned using a confocal laser scanner at 550 nm for Cy3-ddCTP, 650 nm for Cy5-ddUTP, 497 nm for FITC-ddGTP and 593 nm for Texas Red-ddATP. The spot intensity for each spot on the microarray was quantified and subsequently analyzed. Each HBV PCR products were sequenced by commercial DNA sequencing service (Tech Dragon Limited). The DNA sequencing data were checked manually to ensure there were no overlapping sequences. By comparing the APEX and DNA sequencing data, specificities and sensitivities of each oligonucleotides were calculated according to the following equations<sup>6</sup>:

$$\text{Sensitivity} = 100 \times (\text{true positives}) / (\text{true positives} + \text{false negatives})$$

$$\text{Specificity} = 100 \times (\text{true negatives}) / (\text{true negatives} + \text{false positives})$$

## **RESULTS**

### **APEX SNPs genotyping**

In this study, the four open reading frames of eighty HBV-infected patients were subject to PCR. Thirty-three patients with HBV viral load equal to or more than  $1 \times 10^4$  copies/ml and all four open reading frames PCR positive were selected for downstream APEX experiments. The results were compared with the 'gold standard' DNA sequencing data for method validation. Typical comparisons of APEX SNP genotyping and DNA sequencing on different nucleotides were shown in Fig. 2. Each SNP can be detected by either sense or anti-sense primers. Only either one sense or anti-sense oligonucleotide was listed in Table 2. Among 30 the SNPs present on the whole HBV genome, the specificities ranged from 100% to 65.5% while the sensitivities ranged from 100% to 59.4%.

### **Coexistence of wild-type and mutant in one SNP**

The coexistences of both wild-type and mutant for one SNP site in a single individual were successfully genotyped by APEX, however the same cases were left undetected by DNA sequencing (Fig. 2). These cases happened most frequently on SNP G1896A on C gene (9 out of 33 patients). The other SNPs showed no significant amount of coexistences of wild type and mutant genotype.

### **Prevalence Study by APEX**

According to the APEX result, the most frequent mutation was G1896A on C gene (23 out of 33). All the patients carried G1896A mutant SNP, which creates a TAG stop codon in codon 28 of precore region, also showed T1858C wild type SNP. The other 'hotspots' are A1762T and G1764A double mutations present in the precore region, comprised of 22 and 20 patients respectively.

Besides, some frequent mutations are also present in C gene which are A1979G (codon 27), A2159G (codon 87), A2189C (codon 97) and C2288A (codon 130) which

comprised of 18%, 27%, 27% and 30% respectively,

For P gene, the SNPs A739G and G741T leading to lamivudine resistance in the YMDD motif are only found in 3 and 2 patients respectively. For S gene, only 1 patient expressing A530G and 1 patient carrying G546A mutation, indicating that these are minor mutations. For X gene, eight nucleotides deletion (nt 1768 -1775) that is commonly present in the COOH terminal are not found in our pool of samples. However, 33 percent of patients (11 out of 33) are suffered from T1464C/G mutation.

## DISCUSSION

Liver cancer ranks the third cancer killer in Hong Kong. HBV infection is one of the major causes of liver cancer. Many studies showed that SNPs present on the HBV genome are correlated with liver cancer development. Therefore, large scale population studies to determine the prevalence of SNPs, its natural course, and response to treatment are urgently needed. The strategy of using APEX to detect mutations in HBV genome will be applicable in the regard, as APEX allows multiplex screening of different SNPs in a single experiment of both hybridization and extension. By designing sequence-specific oligonucleotides, different mutations in the HBV genome could be genotyped accordingly. In some occasion, destabilization of hybrids may occur if a mismatched nucleotide is present on the flanking nucleotide sequence<sup>7</sup>. Moreover, approximately 10% of the oligonucleotides form stable secondary structures, leading to self-extension and loss of specificities during APEX<sup>8</sup>. These factors caused the low specificities and sensitivities in some oligonucleotides. Thus, the design of redundant set of oligonucleotides against the same SNPs is the possible solution to ensure the highest statistical significance and the correct interpretation of signals<sup>9</sup>. After fragmentation of uracil-containing PCR products by uracil-N-glycosylase to increase the hybridization efficiency and functional inactivation of unincorporated dNTPs by shrimp alkaline phosphatase in single-tube reactions, the fragmented DNA templates are hybridized with the oligonucleotides in a sequence-specific manner. In the presence of DNA polymerases, the 3' ends of different oligonucleotides are specifically incorporated with complementary, fluorescently labeled ddNTPs corresponding to the SNPs. The advantage of APEX is that all the SNPs present in an open reading frame could be detected in a single-step reaction.

According to our result, the most common coexistence of wild-type and mutant

SNPs within individual patients are A1762T, G1764A and G1896A of the C gene. However, these coexistences of both genotypes are usually undetectable by DNA sequencing. This is due to the ambiguity in base-calling by the instrument during the presence of double peaks. This limitation minimizes the reliability of direct sequencing for the identification of nucleotides in these SNPs, at least under automated conditions<sup>10</sup>. The coexistence of wild-type and mutant genotypes may imply the transition stage of chronic infection or one viral species is dominant over the others<sup>11</sup>. Thus, the detection of both dominant and non-dominant viral species by APEX, which can be missed by DNA sequencing, might be significant for monitoring the disease development.

The APEX results also showed that A1762T, G1764A and G1896C are the most frequent SNPs present in the precore/ basal core promoter region as well as the HBV whole genome. The presence of G1896A mutation causes a premature TAG stop codon at codon 28 of HBeAg, leading to suppressed HBeAg secretion<sup>12</sup>. As HBeAg is a major humoral and cellular target, HBeAg negativity is regarded as an indicator of immunological clearance of HBV and rapid viral replication. The A1762T and G1764A double mutations suppress the transcription of precore mRNA and stop HBeAg production. Therefore, the detection of this double mutation can also monitor the disease development.

Besides, some frequent mutations are also present in C gene, i.e. A1979G (codon 27), A2159G (codon 87), A2189C (codon 97) and C2288A (codon 130) that are the epitopes of cytotoxic T lymphocyte (CTL) epitope or B-cell. Because HBcAg is an important target for CTL attack, changes in core gene sequence and thus disturb the level of HBcAg expression, and/or structure of HBcAg epitopes may have more direct impact on the activity of HBV-induced liver disease due to evasion of immune clearance<sup>13</sup>. As a result, the genotyping of these SNPs are necessary for detecting

HBV immunological escape.

For S gene, no significant number of SNPs could be detected in our samples. However, the conformational epitopes of the *a* determinant is controlled by the sequence of S gene. Due antibodies induced by current vaccines do not recognize critical changes in the surface antigen domain<sup>14</sup>, SNPs present in this region can allow replication of HBV in vaccinated persons. Thus, the detection of SNPs present on S gene will be beneficial for vaccines development.

For P gene, although there is no significant number of SNPs were detected in our samples, the detection of SNPs present on the P gene are crucial for the patients who receive lamivudine therapy during the course of HBV infection. Lamivudine is one of reverse-transcriptase inhibitors and has antiviral activity against HBV. However, recent studies reported the emergence of lamivudine-resistant HBV during treatment. Lamivudine-resistant HBV was revealed to have methionine (M) to isoleucine (I) or valine (V) substitutions in the tyrosine (Y), methionine (M), aspartate (D), aspartate (D) motif (YMDD motif) of the RNA-dependent DNA polymerase<sup>15</sup>. Therefore the detection of YMDD motif can be an alternative method for monitoring the development of lamivudine resistant of HBV.

For X gene, re-sequencing by APEX was used to detect the presence of HBx COOH-terminal in our study. The design of an APEX array for comparison sequencing is straightforward based on the complement of the gene of interest<sup>16</sup>. According to our result, none of the patients suffered from HBV X gene COOH-terminal deletions. However, studies demonstrated that the HBx COOH-terminal is responsible for HBx transactivation, critical in regulating its transcriptional activity, controlling cell viability and proliferation.<sup>17</sup> The deletion will eventually abolish the transactivation and suppressed HBV replication<sup>18</sup>. Besides, a prevalent HBV X gene mutant T1464C/G (HBx alanine-31) present in Taiwanese

patients with liver cirrhosis and HCC is also included in our system. Thirty-three percent of patients (11 out of 33) are suffered from T1464C/G mutation. This mutant is less potent in transactivating HBV viral genes, less effective in supporting HBV replication and less effective in enhancing TNF- $\alpha$  induced apoptosis. Development of this mutant might represent a strategy of the virus to escape immune surveillance<sup>19</sup>. So a reliable genotyping method will be beneficial for studying the process of multiple-step hepatocarcinogenesis.

## **CONCLUSIONS**

SNPs are the major sources of genetic variations in all organisms. The identification and mapping of SNPs are becoming more significant in the studies of population genetics, medical genetics and complex genetics disease. Thus, the technologies with high throughput, robotic, promised sensitivity and specificity are becoming more important and preferable. In this report, we demonstrated APEX is capable of satisfying these criteria for identification different SNPs of HBV genome. Such technology could also be applied for detecting heterogeneous alleles, deletions and insertions in all cases. Thus APEX might be the principal choice for large scale HBV patient population studies in the future.

## **IMPLICATION/RELEVANCE**

The combination of APEX to detect these SNPs accurately and new therapies for chronic hepatitis B may be beneficial to the development of treatment strategies for this expanding chronic hepatitis B patient population.

## **DISSEMINATION**

The application of APEX for HBV SNPs genotyping can be implemented in hospitals specialized in liver cancer diagnosis and therapy. All Hepatitis B carriers in Hong Kong are subject to population study during the course of HBV infection. Also, due to the robotic manipulation, satisfactory genotyping accuracy and simple design of APEX system, other SNPs-based diseases such as thalassaemia can also be genotyped by APEX in a simultaneous and high-throughput manner.

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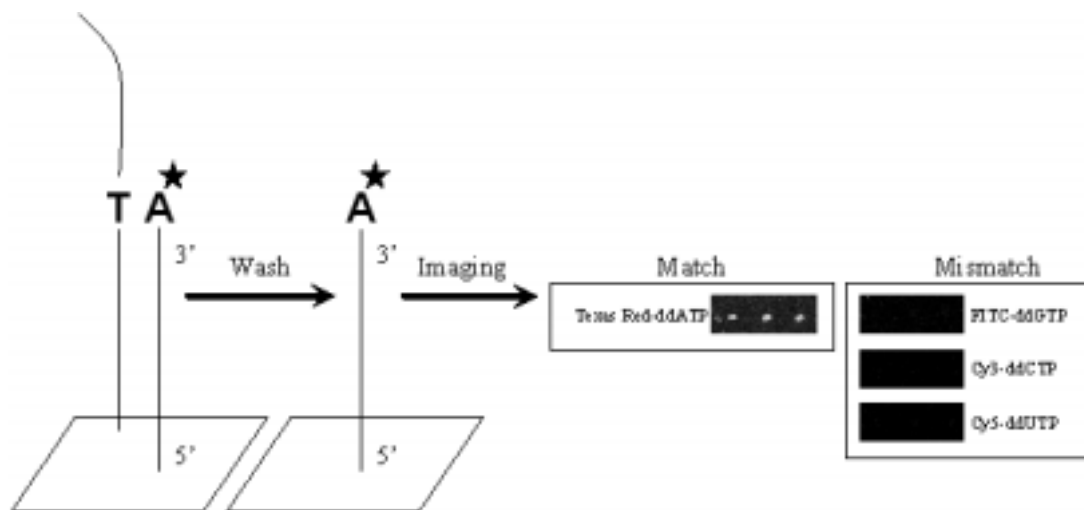
## APPENDIX

**Table 1.** The four open reading frames of HBV were amplified by nested PCR using different sets of primers. C gene was amplified separately by 2 sets of primers.

ORFs	Outer Primers (5' 3')	Sizes	Inner Primers	Sizes
X	gatccatactgcggaactcc (nt 1263-1282)	685 bp	gcggaactcctagcagcttg (nt 1273-1292)	620 bp
	gagtaactccacagaagctc (nt 1947-1928)		gccacccaaggcacagcttg (nt 1892-1873)	
C	catggagaccaccgtgaac (nt 1607-1625)	670 bp	tgcccaaggtcttacacaag (nt 1639-1658)	584 bp
	tccacactccaaaagacacc (nt 2257-2276)		ggcaggaatgtgaaaccac (nt 2203-2222)	
	catacagcactcaggcaagc (nt 2054-2073)	550 bp	tgtgtgggggtgagtgatg (2079-2098)	399 bp
	cagagggcccacatatgtt (nt 2603-2584)		tcccacctatgagtccaag (nt 2477-2458)	
P	tatgtgcccgtttgtcctc (nt 460-479)	533 bp	tggacggaaactgcacttg (nt 581-600)	290 bp
	tgacatactttccaatcaatagg (nt 992-970)		aaggagtagccccaacgtt (nt 870-851)	
S	tcctgctgctatgcctcatc (nt411-430)	485 bp	ccaggaacatcaactaccag (nt 485-504)	386 bp
	ttccaattacatatcccatg (nt 895-876)		aaggagtagccccaacgtt (nt 870-851)	

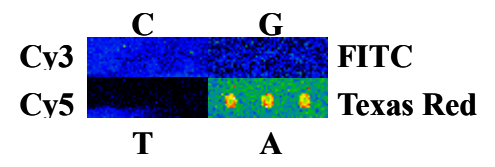
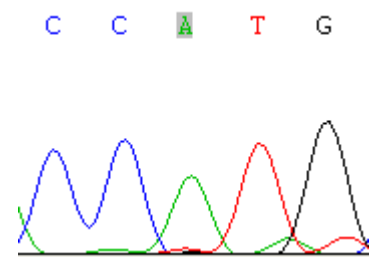
**Table 2.** The most common SNPs present on the 4 open reading frames of HBV were genotyped by the oligonucleotides. Among 30 SNPs present on the whole HBV genome, the specificities ranged from 100% to 65.5% while the sensitivities ranged from 100% to 59.4%.

Gene	No.	Name	Oligonucleotides sequence (5' → 3')	sensitivity	specificity
X	1	T1464G_S	CGC TGA ATC CCG CGG ACG ACC CGT C	70.0%	95.5%
	2	A1762T_S	GAG TTG GGG GAG GAG ATT AGG TTA A	100%	94.4%
	3	G1764A_AS	TGC CTA CAG CCT CCT AGT ACA AAG A	88.9%	100%
	4	1768_S	GGG GAG GAG ATT AGG TTA AAG GTC T	96.9%	78.1%
	5	1769_S	GGG AGG AGA TTA GGT TAA AGG TCT T	100%	97.0%
	6	1771_S	GAG GAG ATT AGG TTA AAG GTC TTT G	93.8%	93.5%
	7	1772_AS	CCA ATT TAT GCC TAC AGC CTC CTA G	80.6%	92.6%
	8	1773_AS	ACC AAT TTA TGC CTA CAG CCT CCT A	75.9%	86.4%
	9	1774_S	GAG ATT AGG TTA AAG GTC TTT GTA C	100%	78.1%
	10	1775_AS	AGA CCA ATT TAT GCC TAC AGC CTC C	93.5%	65.5%
C	11	A1762T_S	GAG TTG GGG GAG GAG ATT AGG TTA A	100%	94.4%
	12	G1764A_AS	TGC CTA CAG CCT CCT AGT ACA AAG A	88.9%	100%
	13	A1814C_S	ATT AAT TGG TCT GTT CAC CAG CAC C	94.4%	100%
	14	G1816T_S	AAA TTG GTC TGT TCA CCA GCA CCA T	94.4%	97.1%
	15	C1856T_S	CTG CCT AAT CAT CTC ATG TTC ATG T	100%	97.2%
	16	T1858C_S	GCC TAA TCA TCT CAT GTT CAT GTC C	100%	97.2%
	17	G1896A_AS	TTT ATA CGG GTC AAT GTC CAT GCC C	94.4%	91.2%
	18	G1899A_S2	AAG CTG TGC CTT GGG TGG CTT TAG G	91.7%	85.3%
	19	A1979G_AS	GAG GCG GTG TCG AGG AGA TCT CGA A	71.88%	82.61%
	20	A2075G/T_AS	TTC ATC AAC TCA CCC CAA CAC AGA A	72.73%	95.83%
	21	T2076C_S	CAC CAT ACA GCA CTC AGG CAA GCT A	96.97%	96.77%
	22	C2078G_S3	CCA TAC AGC ACT CAG GCA AGC TGT T	73.91%	80.77%
	23	A2159G_AS	AGG CCC ATA TTA ACA TTG ACA TAG C	79.17%	79.17%
	24	C2288A_AS	GGG GCA TTT GGT GGT CTG TAA GCG G	59.38%	95.0%
	25	G2357T_S	GGA AAC TAC TGT TGT TAG ACG ACG A	59.38%	84.21%
	26	C2444T_AS	TAT GAG TCC AAG GGA TAC TAA CAT T	100%	87.5%
S	27	A530G_AS2	AAC ATA GAG GTT CCT TGA GCA GGA G	96.9%	90.6%
	28	C546A_AS	ACA GCA ACA AGA GGG AAA CAT AGA G	90.9%	96.7%
P	29	A739G_AS	TTG GCC CCC AAT ACC ACA TCA TCC A	100%	93.9%
	30	G741T_S	CCC ACT GTT TGG CTT TCA GCT ATA T	66.7%	95.7%

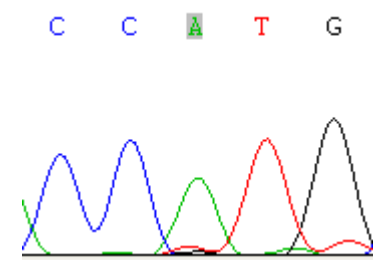


**Fig. 1.** Principle of arrayed primer extension. Single-stranded HBV PCR products anneal complementarily to the amino-modified 5' end oligonucleotides immobilized on enhanced amino-saline glass slides. In this case, the match Texas Red-ddATP will be correctly incorporated at the free 3' oligonucleotide end according to the base of the site of SNP catalyzed by DNA polymerases, giving positive signal. The other mismatch fluorescently labeled ddNTPs will be washed away, giving negative signals during imaging.

A:

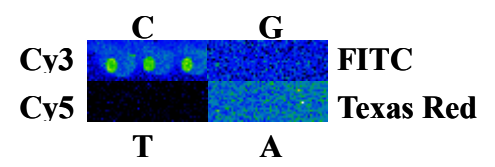


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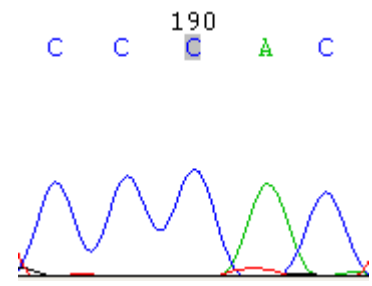


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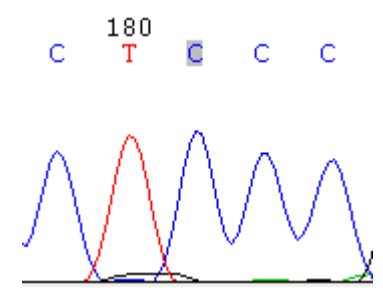
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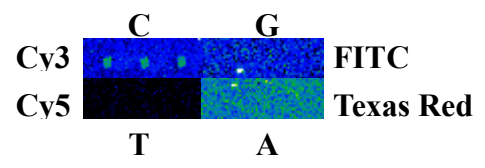
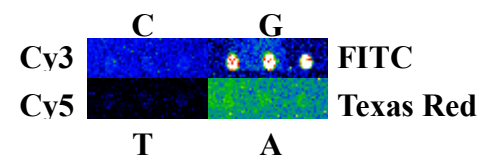
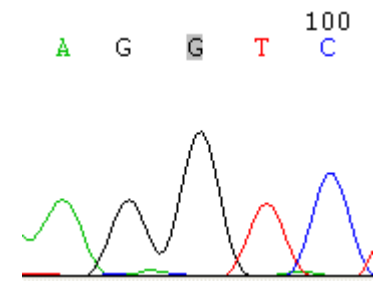
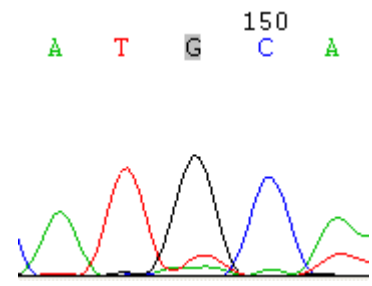
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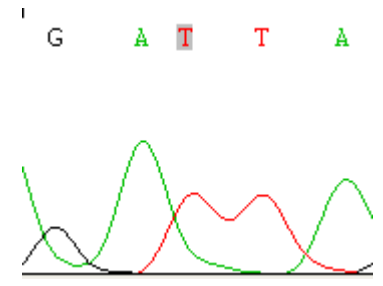
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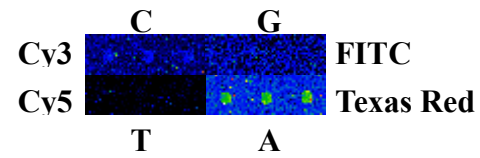
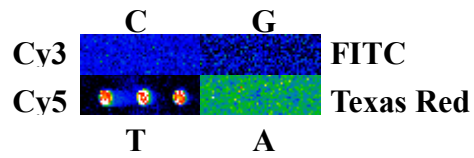
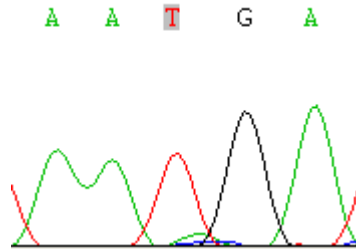


Sense

Anti-sense

T:

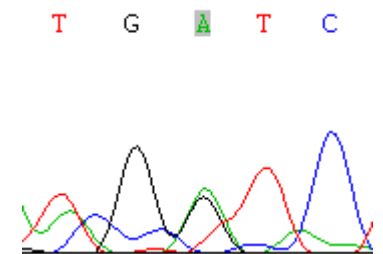
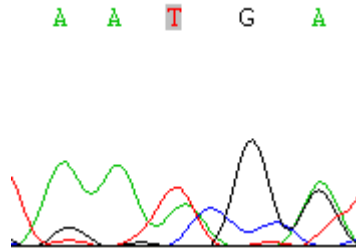


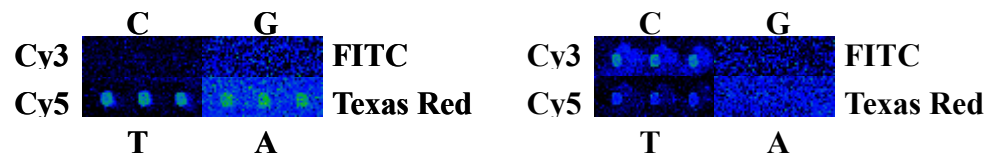


Sense

Anti-sense

Co-existence:





Sense: co-existence

Anti-sense: co-existence

**Fig. 2.** Examples of comparisons between DNA sequencing and APEX genotyping of different SNPs by sense and anti-sense oligonucleotides. The coexistences of wild-type and mutant in one SNP of a single individual were successfully genotyped by APEX, but the same cases were left undetected by DNA sequencing.