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Reliable and Fast Allele-Specific Extension of 3′-LNA Modified Oligonucleotides Covalently Immobilized on a Plastic Base, Combined with Biotin-dUTP Mediated Optical Detection

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In the present work, a convenient microarray SNP typing system has been developed using a plastic base that covalently immobilizes amino-modified oligonucleotides. Reliable SNP allele discrimination was achieved by using allelic specificity-enhanced enzymatic extension of immobilized oligonucleotide primer, with a locked nucleic acid (LNA) modification at the SNP-discriminating 3′-end nucleotide. Incorporation of multiple biotin-dUTP molecules during primer extension, followed by binding of alkaline phosphatase-conjugated streptavidin, allowed optical detection of the genotyping results through precipitation of colored alkaline phosphatase substrates onto the surface of the plastic base. Notably, rapid primer extension was demonstrated without a preliminary annealing step of double-stranded template DNA, allowing overall processes to be performed within a couple of hours. Simultaneous evaluation of three SNPs in the genes TGFB1, SOD2 and APEX1, previously investigated for association with radiation sensitivity, in 25 individuals has shown perfect assignment with data obtained by another established technique (MassARRAY system).

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Introduction

The importance of single nucleotide polymorphisms (SNPs) is increasingly recognized following completion of the sequencing of whole human genome.[1] SNPs are useful as genetic association markers for various human diseases as well as for prediction of individual responses to therapeutic treatment such as drugs[2,3] and ionizing radiation.[4-6] Currently, more than 10 million human SNPs are registered in the public database,[7,8] allowing large scale, genome-wide association studies to be practical. Therefore, many high throughput SNP genotyping technologies involving DNA chip technology,[9] bead technology,[10] mass spectrometry[11] and cleavage of fluorescent probe technology[12] have been established.

For routine molecular biology research and bedside clinical diagnosis, readily available technologies are required to genotype limited number of SNPs selected by previous large scale association studies. The allele-specific primer extension method has been considered an ideal choice for the above purpose, however, it has been hampered due to frequent undesirable extension of mismatched primers. Some mismatches such as T:G or C:A are clearly not refractory to extension.[13] Recently, reliable allele-specific extension has been adapted to microarray formats for SNP typing.[14,15] These procedures, however, require complicated strategies such as RNA templates and reverse transcriptase[14] or apyrase-mediated degradation of nucleotide substrates[15] to support sufficient allelic discrimination. Giusto and King recently developed two microarray systems involving an exonuclease-mediated single base extension (E-SBE) and a proofreading allele-specific extension (PRASE) genotyping,[16] respectively, taking advantage of the protecting ability of an LNA (locked nucleic acid) nucleotide located at the penultimate (L-2) position. LNA is a new class of bicyclic high-affinity DNA analogs, in which the fructose ring of the ribose sugar is chemically locked by introduction of an O2′,C4′-methylene bridge, resulting in unprecedented hybridization affinity toward complementary DNA molecules.[17] This modified nucleotide is able to protect the immobilized oligonucleotide primer against the 3′ → 5′ exonuclease activity of a proofreading DNA polymerase.[16] Recently, simple addition of an LNA nucleotide at the 3′-end of oligonucleotide primer has been shown to enhance the discrimination power of allele-specific real-time quantitative PCR assay.[18]

In this study, the 3′-end LNA modification has been successfully applied to a microarray-format SNP typing system. Enhancement of allelic discrimination in primer extension

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Table 1 Oligonucleotide sequences

SNP ID	Gene name	Type	Sequence	Product size/bp	Biotin site[g]
rs1800469	TGFB1	PCRF[a]	5′-ACGTTGGATGAAGAGGGTCTGTCAACATGG	124[e]	18
		PCRR[a]	5′-ACGTTGGATGTTCTTACAGGTGTCTGCCTC		
		Massxt[b]	5′-CCTCCTGACCCTTCCATCC		
		QPCRF[c]	5′-ACGTTGGATGAAGAGGGTCTGTCAACATGG(LNA)		
		QPCRR1[c]	5′-CTCCTGACCCTTCCATCCC(LNA)	97[f]	
		QPCRR2[c]	5′-CCTCCTGACCCTTCCATCCT(LNA)	98[f]	
		Opcxt1[d]	5′-(C6 Amino)CTCCTGACCCTTCCATCCC(LNA)		
		Opcxt2[d]	5′-(C6 Amino)CCTCCTGACCCTTCCATCCT(LNA)		
rs1799725	SOD2	PCRF	5′-ACGTTGGATGGGCTGTGCTTTCTCGTCTTC	93	7
		PCRR	5′-ACGTTGGATGTTCTGCCTGGAGCCCAGATAC		
		Massxt	5′-CCTGGAGCCCAGATACCCCAAA		
		QPCRF1	5′-TGGAGCCCAGATACCCCAAAG(LNA)	76	
		QPCRF2	5′-CTGGAGCCCAGATACCCCAAAA(LNA)	77	
		QPCRR	5′-ACGTTGGATGGGCTGTGCTTTCTCGTCTTC(LNA)		
		Opcxt1	5′-(C6 Amino)TGGAGCCCAGATACCCCAAAG(LNA)		
		Opcxt2	5′-(C6 Amino)CTGGAGCCCAGATACCCCAAAA(LNA)		
rs3136820	APEX1	PCRF	5′-ACGTTGGATGCACCTCTTGATTGCTTTCCC	129	13
		PCRR	5′-ACGTTGGATGAATTCAGCCACAATCACCCG		
		Massxt	5′-CCTTCCTGATCATGCTCCTC		
		QPCRF	5′-ACGTTGGATGCACCTCTTGATTGCTTTCCC(LNA)		
		QPCRR1	5′-CCTTCCTGATCATGCTCCTCC(LNA)	98	
		QPCRR2	5′-GCCTTCCTGATCATGCTCCTCA(LNA)	99	
		Opcxt1	5′-(C6 Amino)CCTTCCTGATCATGCTCCTCC(LNA)		
		Opcxt2	5′-(C6 Amino)GCCTTCCTGATCATGCTCCTCA(LNA)		
	Positive control		5′-(C6 Amino)ATATC(Biotin)		1
	Negative control		5′-GGAGGGTTATTGGACCCGG(C6 Amino)		0

a. Oligonucleotides used to amplify target sequence by PCR. b. Oligonucleotides for extension reaction of MassARRAY SNP typing. c. Oligonucleotides for real-time quantitative PCR synthesized with or without 3′-LNA modification. d. Oligonucleotides for on-plastic chip allele-specific primer extension. e. Product size of target sequence-amplification PCR. f. Product size of allele-specific quantitative PCR. g. Number of biotin-dUTP incorporation site in PCR product.

reaction was confirmed for the covalently immobilized LNA-modified oligonucleotide primers on the surface of a plastic base. This enabled highly selective and rapid incorporation of multiple biotin-dUTP molecules as substrates for extension of oligonucleotide primers that matched to template genotypes, providing reliable and sensitive optical detection of typing results.

Experimental

S-Bio PrimeSurface plastic base (75 × 25 × 1 mm, Sumitomo Bakelite, Tokyo, Japan) developed for covalent immobilization of amino-modified DNA was used in this study. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and Proligo (Paris, France).

SNPs and design of oligonucleotide primers

Three SNPs (Table 1), previously investigated for their association with cancer patients' radiation sensitivity,[4] were used. Oligonucleotides for amplification of the target sequences and for typing target SNPs by the MassARRAY system (Sequenom, San Diego, CA) were designed using MassARRAY Assay Design software (Sequenom, CA) and synthesized without any modification of nucleotides. For allele-specific real-time quantitative PCR, the oligonucleotides for typing target SNP by MassARRAY were used with some modifications as follows. The DNA sequence was designed to include the target SNP nucleotide at its 3′-end and then its length was varied by subtracting or adding corresponding nucleotides from/to 5′-end until the theoretical T_m was 60°C without considering the stabilization effect of LNA modification. One of the oligonucleotides to amplify target sequence for MassARRAY system, located with an opposite orientation to the discriminating oligonucleotides, was directly used for the reverse primer. All the oligonucleotides used for allele-specific real-time quantitative PCR were synthesized with an LNA modification at the 3′-end. For on-chip primer extension, the allele-discriminating oligonucleotides, used for real-time genotyping, were synthesized by adding a C6 amino modifier at the 5′-end and an LNA modification at the 3′-end; these were purified by HPLC after synthesis. All oligonucleotides used in this study are listed in Table 1.

Preparation of DNA templates

Human genomic DNAs were individually extracted using a Kurabo NA-2000 automated DNA extraction machine (Kurabo, Tokyo, Japan) from the blood of 25 healthy volunteers. Target sequences were amplified from the extracted genomic DNA by conventional PCR with HotStar Taq DNA polymerase (Qiagen, Valencia, CA) using standard reaction conditions (35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s) in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The amplified products were quantified using a Quant-iT PicoGreen dsDNA Quantitation Kit (Invitrogen, Carlsbad, CA). Products were then directly used as primer extension templates for MassARRAY SNP typing and on-chip primer extension without any further purification.

MassARRAY SNP typing

MassARRAY SNP typing[11] was carried out following the manufacturer's instructions. SNP genotypes of 25 healthy volunteers are shown in Table 2.

Table 2 Genotype of healthy volunteers

Patient number	rs1800469		rs1799725		rs3136820	
	MassARRAY	New method	MassARRAY	New method	MassARRAY	New method
1	CT	Heterozygote	GA	Heterozygote	CA	Heterozygote
2	CT	Heterozygote	AA	Allele 2	AA	Allele 2
3	CT	Heterozygote	AA	Allele 2	CA	Heterozygote
4	CT	Heterozygote	AA	Allele 2	CC	Allele 1
5	TT	Allele 2[b]	AA	Allele 2	CA	Heterozygote
6	CT	Heterozygote	AA	Allele 2	AA	Allele 2
7	CT	Heterozygote	AA	Allele 2	AA	Allele 2
8	CT	Heterozygote	AA	Allele 2	AA	Allele 2
9	CT	Heterozygote	GA	Heterozygote	AA	Allele 2
10	TT	Allele 2	AA	Allele 2	CC	Allele 1
11	CT	Heterozygote	AA	Allele 2	CA	Heterozygote
12	CC	Allele 1[a]	AA	Allele 2	CA	Heterozygote
13	CC	Allele 1	GA	Heterozygote	CA	Heterozygote
14	TT	Allele 2	GA	Heterozygote	CC	Allele 1
15	CC	Allele 1	AA	Allele 2	CC	Allele 1
16	TT	Allele 2	AA	Allele 2	CA	Heterozygote
17	CC	Allele 1	AA	Allele 2	CA	Heterozygote
18	CT	Heterozygote	AA	Allele 2	CA	Heterozygote
19	CT	Heterozygote	AA	Allele 2	CA	Heterozygote
20	TT	Allele 2	GA	Heterozygote	CA	Heterozygote
21	CT	Heterozygote	GA	Heterozygote	CA	Heterozygote
22	CC	Allele 1	GA	Heterozygote	AA	Allele 2
23	CT	Heterozygote	GG	Allele 1	CA	Heterozygote
24	TT	Allele 2	AA	Allele 2	CA	Heterozygote
25	CT	Heterozygote	GG	Allele 1	AA	Allele 2

a. Allele 1 homozygote. b. Allele 2 homozygote.

Allele-specific real-time quantitative PCR

Twenty microliters of reaction mixture contained 1 × iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 10 ng of genomic DNA and 0.2 μM of each PCR primer. The reaction mixtures were heated at 95˚C for 15 min and then subjected to 60 rounds of 2-step temperature cycling (95˚C for 10 s and 65˚C for 20 s) using a LightCycler 480 (Roche Diagnostics, Basel, Switzerland). The crossing point (CP) for each amplification curve was determined by the second derivative maximum method.

Oligonucleotide immobilization on S-Bio PrimeSurface plastic base

Immobilization of amino-modified oligonucleotides onto an S-Bio PrimeSurface plastic base was carried out following the manufacturer's instructions with some minor modifications. Oligonucleotides were prepared as 0.2 μM solution in 1 × S-Bio spot solution (Sumitomo Bakelite, Japan). Drops of approximately 50 nl were spotted onto the plastic base by a MassARRAY Nanodispenser (Sequenom, CA). The spotted base was heated at 80˚C for 1 h to fix the oligonucleotides then washed for 1 min in 0.1% Tween-20. The unreacted surface of the base was then inactivated by soaking the base with 1 × S-Bio blocking solution (Sumitomo Bakelite, Japan) containing 0.1% Tween-20 for 5 min. The plastic chip thus formed was thoroughly washed first in 1 × TBS-T (10 mM Tris, pH 7.6, 150 mM NaCl and 0.1% Tween 20) for 1 min, then in 0.1% Tween-20 solution for 1 min twice. Centrifuging the chip at 100*g* for 1 min efficiently removed the residual solution, the chip was stored at 4˚C until use.

On-plastic chip allele-specific primer extension reaction

One hundred microliters of a reaction mixture containing 1 × ThermoPol II (Mg-free) reaction buffer (New England Biolabs, Beverly, MA), a selected amount of PCR product mixture, 5 units of HotStar Taq DNA polymerase, 10 μM of biotin-modified dUTP (Fermentas, Hanover, MD), 10 μM each of normal nucleotides (dATP, dCTP and dGTP) and selected concentration of $MgCl_2$ were heated at 95˚C for 15 min. Heated reaction mixtures were cooled to room temperature for 5 min, then poured onto individual plastic chips. The chips were placed in a prewarmed, humidified plastic box. The box was sealed thoroughly with Saran wrap (AsahiKASEI Life and Living, Tokyo, Japan) and incubated at the appropriate temperature for the indicated periods of time. After incubation, the chips were washed for 1 min in 1 × TBS-T.

Detection of signals and data processing

The washed chip was exposed to 200 μl of alkaline phosphatase-conjugated streptavidin (1/100 dilution) (Bio-Rad Laboratories, CA) in 1 × TBS-T. It was incubated at room temperature for 10 min, then washed in 1 × TBS-T. Two hundred microliters of 5-bromo-4-chloro-3′-indolylphosphatase (BCIP)/nitro-blue tetrazolium chloride (NBT) (Perkin Elmer Optoelectronics, Fremont, CA) with 0.1% Tween-20 was poured onto the chip. Each chip was incubated at room temperature for 30 min, then washed in 0.1% Tween-20 solution for 1 min. Centrifuging the chip at 100*g* for 1 min efficiently removed the solution. The image on the chip was captured by a Nikon D70 digital camera (Nikon, Melville, NY); the 16-bit RAW format RGB files were converted to 8-bit grayscale TIFF format files by Adobe Photoshop Version 6.0 software (Adobe Systems, San Jose, CA). The brightness of all the images was adjusted to 40 arbitrary units and the contrast was adjusted to 60 arbitrary units. The spots in the TIFF images were quantitated by Quantity One Version 4.6 image analysis

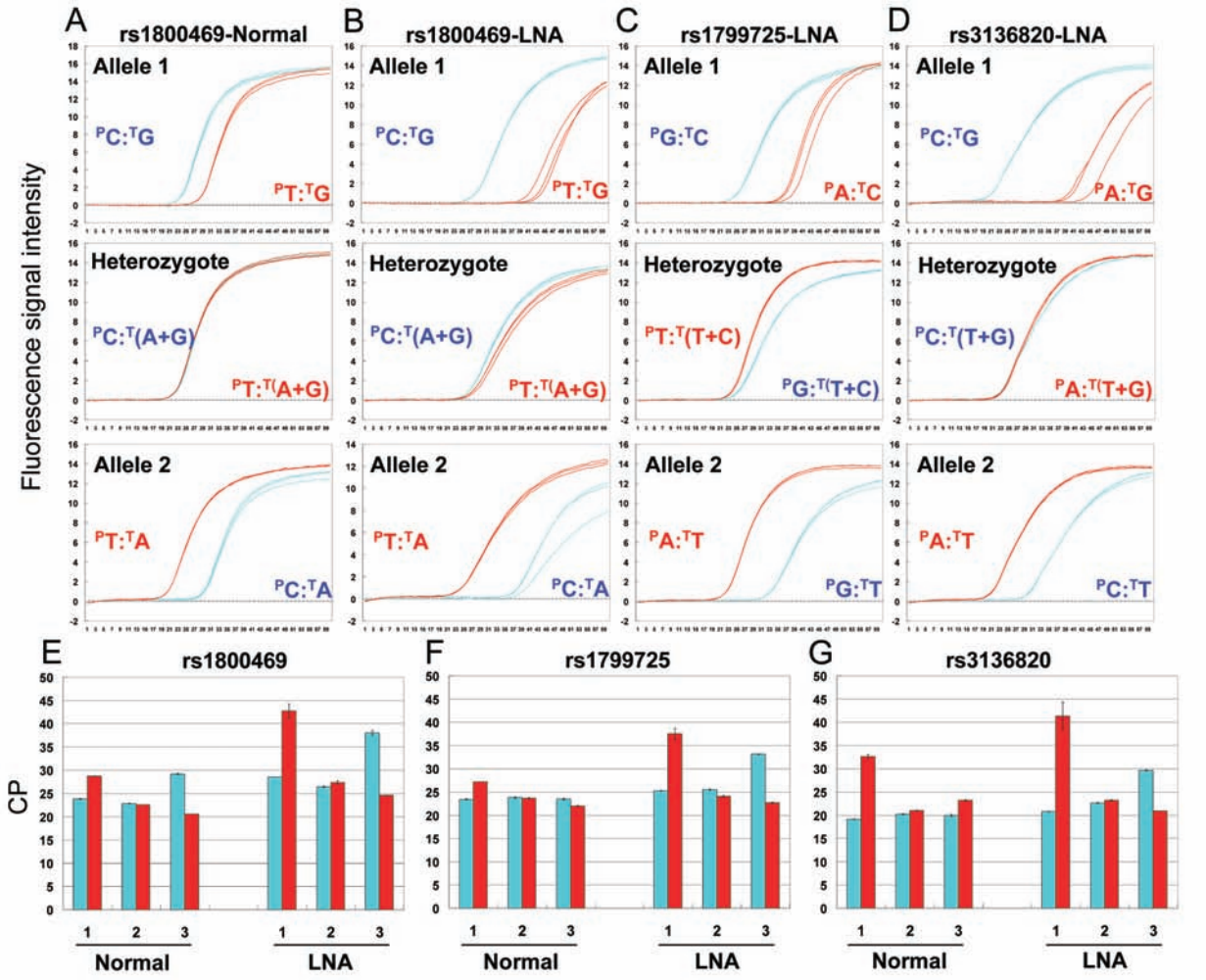

Fig. 1 Allele-specific real-time quantitative PCR. (A) Normal oligonucleotides for rs1800469 in TGFB1 gene. (B) 3′-LNA modified oligonucleotides for rs1800469. (C) 3′-LNA modified oligonucleotides for rs1799725 in SOD2 gene. (D) 3′-LNA modified oligonucleotides for rs3136820 in APEX1 gene. The blue line in each panel indicates an amplification curve of the allele 1 discriminating primer set shown in Table 1. The red line of each panel indicates the amplification curve of the allele 2 discriminating primer set. Template genotype was indicated in each panel. A typical pattern for each genotype template is shown in each panel. The combination between primer and template genotypes for each assay is shown by the trace. (E, F, G) Summary of normal and 3′-LNA modified oligonucleotide comparison. CP for each amplification curve was plotted on the *y*-axis. Blue bar, CP of allele 1 discriminating oligonucleotide sets; red bar, CP of allele 2 discriminating oligonucleotide sets. 1, Allele 1 homozygote template; 2, heterozygote template; 3, allele 2 homozygote template.

software (Bio-Rad Laboratories, CA). The relative signal intensity of spots for the allele-discriminating oligonucleotides was calculated using the signal intensity of the positive control spot as 100%.

Results and Discussion

Allele-specific real-time quantitative PCR by 3′-LNA modified primers

Real-time quantitative PCR was used to test whether LNA modification at the 3′-end of oligonucleotides could enhance discrimination of the three SNP alleles listed in Table 1. One of these SNPs, rs1800469 of TGFB1, forms primer/template T:G and C:A mismatches (shown as $^{P}T:^{T}G$ and $^{P}C:^{T}A$ in Fig. 1) that were reported to be not refractory to allele-specific primer extension.[13] On the other hand, rs1799725 of SOD2 forms opposite combinations of the same mismatches ($^{P}A:^{T}C$ and $^{P}G:^{T}T$). Comparison of normal oligonucleotide and 3′-LNA modified oligonucleotide, as shown in Figs. 1A, B and E, indicated that the LNA modification was effective to enhance delay in amplification of mismatched templates for both alleles. The delay of crossing point (ΔCP) for the normal oligonucleotide $^{P}T:^{T}G$ mismatch (CP = 28.91) against normal oligonucleotide $^{P}C:^{T}G$ match (CP = 23.82) was only 5.09, whereas that for the 3′-LNA oligonucleotide $^{P}T:^{T}G$ mismatch (CP = 28.51) against 3′-LNA oligonucleotide $^{P}C:^{T}G$ match (CP = 42.77) was 14.26. This allelic discrimination superiority of 3′-LNA modification was confirmed in all combinations of primer/template for the other 2 SNPs (Figs. 1C, D, F, G).

Immobilization of 5′-amino and 3′-LNA modified oligonucleotides on an S-Bio PrimeSurface plastic base

The 3′-end LNA modified oligonucleotides, that have been proven to have superior discriminating ability of the selected 3 SNPs were then resynthesized with an additional amino modification at the 5′-end. They were purified by HPLC and approximately 10 fmol (50 nl of 0.2 μM solution) of each was

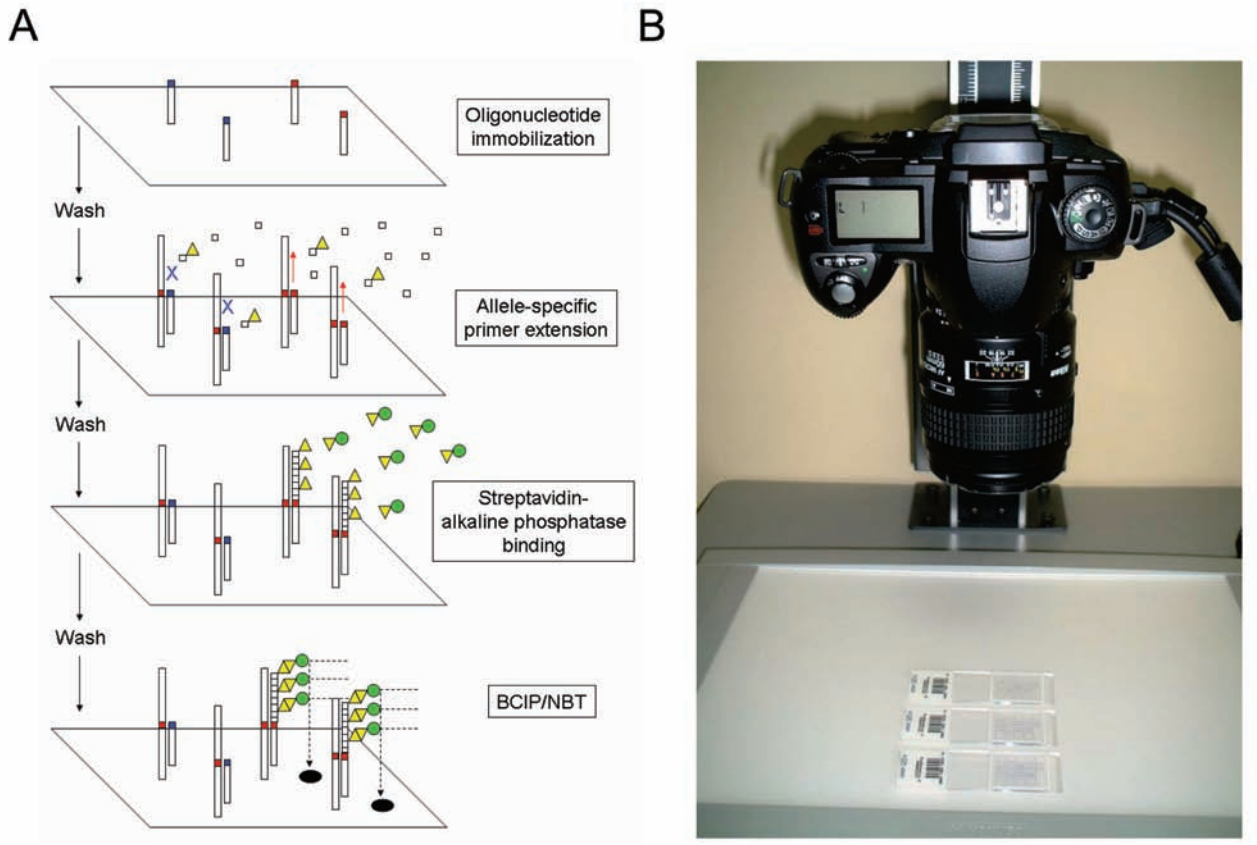

Fig. 2 Schematic representation of experimental procedures. (A) Chemical reactions on a plastic chip. Immobilized 3′-LNA modified oligonucleotides (vertical rectangle with red or blue color at top; the color reflects SNP allele) were incubated with a primer extension mixture containing template DNA (long vertical rectangle with red color at the middle), DNA polymerase and substrate deoxynucleotides (square) including biotin-dUTP (square with upward yellow triangle). LNA modification of 3′-end allowed selective extension of matched primer/template combination is indicated by the same color. The incorporated biotin-dUTPs were utilized to capture streptavidin-alkaline phosphatase (green circle with inverted yellow triangle) moving freely in a solution. Then visible precipitations (closed circle) were formed from BCIP/NBT by the catalytic activity of captured alkaline phosphatase. (B) Convenient optical acquisition of SNP typing data by a digital camera.

spotted and immobilized on an S-Bio PrimeSurface plastic base, as described in Experimental.

Biotion-dUTP mediated on-plastic chip optical detection system for primer extension

Allelic discrimination of the immobilized 3′-LNA modified oligonucleotides was then investigated using the system illustrated in Fig. 2. Incorporation of multiple biotin-dUTP molecules during allele-specific primer extension provided sufficient sensitivity for optical detection of typing results. Figure 3 shows typical patterns observed for rs1800469 of TGFB1 by this system. Biotin-dUTP was selectively incorporated into the immobilized Opcxt1 oligonucleotides when using template DNA from an individual with homozygous allele 1, as seen in Fig. 3A, and the signal intensities of spots for the allele-discriminating oligonucleotides relative to that of positive control were plotted on Fig. 3B. The signal intensities of Opcxt1 spots reached more than 30% relative to that of positive control spot at the upper right corner, however, those of Opcxt2 spots were negligible. On the contrary, signals appeared on Opcxt2 spots using template DNA from an individual with both alleles (Figs. 3C and D) or an individual with homozygous allele 2 (Figs. 3E and F). The Opcxt1 signals selectively disappeared at the latter mismatch case, as expected. These observations indicate that the enhanced allelic discrimination by 3′-end LNA modified oligonucleotides can be conserved even after immobilization on the plastic base.

Effect of $MgCl_2$ concentration and temperature on reaction efficiency

Factors that influence allelic discrimination on a plastic chip were systematically investigated. Firstly, the effects of magnesium concentration and temperature on primer extension were investigated; results are shown in Fig. 4. At 55°C (Figs. 4A and D), allele specific signals for Opcxt1 and for Opcxt2 were observed in the reaction mixture with 4 mM and above magnesium concentration. Increase of reaction temperature to 65°C (Figs. 4B and E) resulted in lowering minimum magnesium requirement to 1 mM without affecting allelic specificity. Further increase of reaction temperature was supposed to improve reaction efficiency by enhancing enzymatic activity of Taq DNA polymerase. However, at 72°C, the overall reaction efficiency was reduced, especially for Opcxt2. This should be caused by reduced thermal stability of 3′-end of primer/template base pair (^{P}T:^{T}A for Opcxt2 whereas ^{P}C:^{T}G for Opcxt1). Oligonucleotides for another two SNPs showed a similar tendency toward magnesium concentration and reaction temperature (data not shown). Thus, a magnesium concentration of 4 mM and a reaction temperature of 65°C were chosen to be used as standard conditions in further experiments.

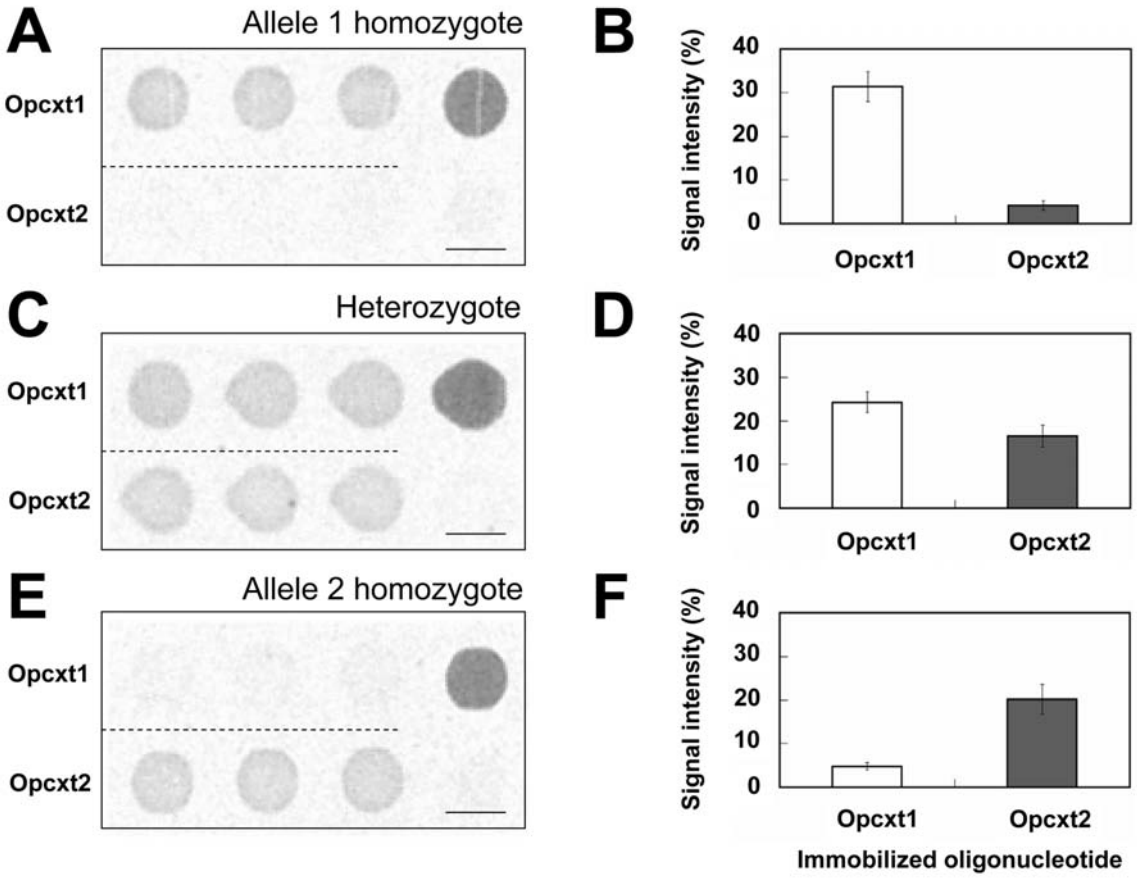

Fig. 3 Discrimination of rs1800469 SNP in TGFB1 by on-plastic chip allele specific primer extension. (A, C and E) Images of on-plastic chip allele-specific primer extension. Oligonucleotides for allele 1 discrimination (Opcxt1) of rs1800469 were spotted at upper side of the panel in triplicate and oligonucleotides for allele 2 discrimination (Opcxt2) of rs1800469 were spotted at lower side in triplicate. A spot at the upper right corner is of positive control oligonucleotides with one biotin molecule at the 3′-end. (A and B) Reaction with allele 1 homozygous template. Signal intensities of spots for the allele-discriminating oligonucleotides relative to that of positive control are plotted respectively on panel B. (C and D) Reaction with heterozygous template. (E and F) Reaction with allele 2 homozygous template. Primer extension reactions were carried out using a standard condition of 4 mM $MgCl_2$ with 15 fmol template at 65˚C for 30 min. Scale bars at the lower left side of panels A, C and E indicate 100 μm.

Kinetics of on-plastic chip allele-specific primer extension

Next, optimum amount of template and incubation time were investigated, as summarized in Fig. 5. The reaction mixtures containing three different amounts of template DNA amplified from heterozygote of rs1800469 SNP (15, 7.5 and 1.5 fmol, respectively) were heated in a reaction tube at 95˚C for 15 min to activate HotStar Taq DNA polymerase, cooled down to room temperature for 5 min, then poured onto the plastic chips with immobilized oligonucleotides (approximately 10 fmol). The plastic chips were incubated at constant temperature of 65˚C to allow primer formation and extension for various time periods. As shown in Figs. 5A and D, positive signals for both alleles were observed at 10 min of incubation when 15 fmol template was used. At this time point the signals already appeared to reach *quasi*-saturation value. Lowering the amount of template DNA reduced the overall signal intensity and the time to reach *quasi*-saturation value was prolonged (Figs. 5B, C, E and F). It is most likely that lowering the amount of template DNA reduced the frequency of immobilized oligonucleotides to meet template DNA; thus, prolonged time was necessary. Once primer formation occurs, the following initiation of replication and nucleotide extension reaction should proceed in a short time, since the *quasi*-saturation value for excessive template DNA amount (15 fmol) was only 10 min. Considering these observations, we decided to use 15 fmol template amount and 30 min incubation time as standard conditions.

The fast kinetics of primer extension without preliminary hybridization process observed here contradicts reports that showed a couple of hours were needed to reach *quasi*-saturation point for hybridization between immobilized DNA and free complementary double-stranded DNA.[19] Higher affinity toward complementary strands by LNA-modification of the primer oligonucleotides might be responsible for the rapid hybridization kinetics,[20] as well as providing genotype specificity. Interestingly, Taq DNA polymerase, the DNA polymerase whose derivative was used in this study, possesses a partial strand-displacement activity,[21] which might explain the above features. It should be noticed, that using this system, it takes less than 1 h to obtain SNP data after PCR amplification. The absence of a preliminary hybridization process and the fast kinetics of primer extension provided this rapidity. Remarkably, the overall process was performed within less than 3 h when we started from direct PCR of blood samples (data not shown).

SNP typing accuracy

Genotypes of three SNPs from a total of 25 individuals were

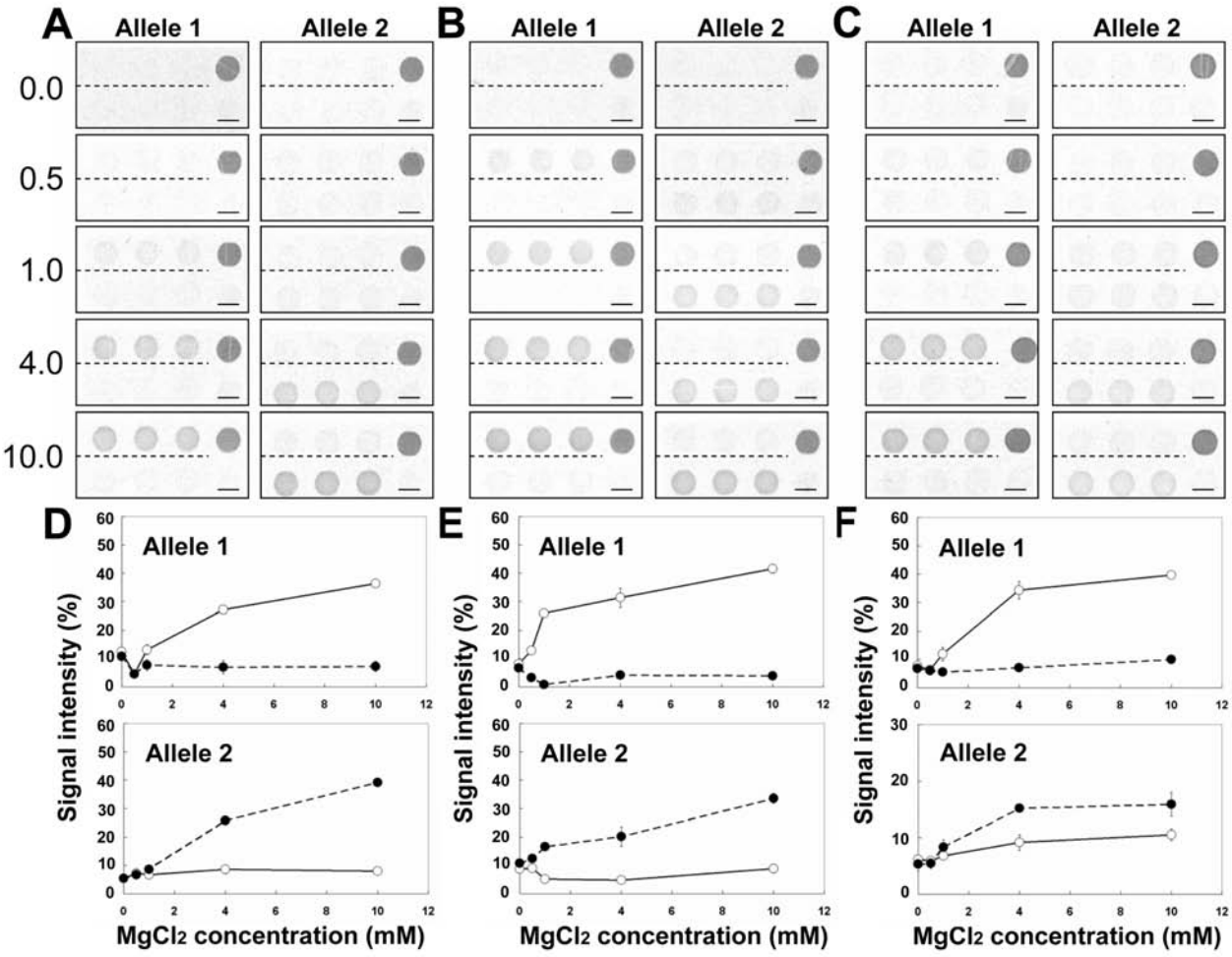

Fig. 4 Effect of magnesium concentration and reaction temperature on discrimination of rs1800469 SNP in TGFB1 gene by on-plastic chip allele-specific primer extension. Effects of different magnesium concentrations on genotyping specificity were investigated using template DNA with individual alleles at 55˚C (A and D), 65˚C (B and E) and 72˚C (C and F). (A, B and C) Images of on-plastic chip allele-specific primer extension. Spot design on the plastic chip was essentially the same as shown in Fig. 3. In each series of image panels, the magnesium concentration used in each assay increases from top to bottom as indicated at the left side of panel A. Genotype of template DNA used is indicated on top of each series of panels. Allele 1: allele 1 homozygous template DNA. Allele 2: allele 2 homozygous template DNA. Scale bars at the lower left side of panels A, B and C indicate 100 μm. (D, E and F) Plot of relative signal intensity at various magnesium concentrations shown in the panels A, B and C. The solid lines with open circles in panels D, E and F indicate the signal intensity of the allele 1 discriminating oligonucleotide relative to that of positive control. The dotted lines with filled circles indicate the signal intensity of the allele 2 discriminating oligonucleotide relative to that of positive control.

then simultaneously determined using the standard experimental condition (4 mM $MgCl_2$, 65˚C, 15 fmol template DNA, 30 min incubation time) established above. Results are plotted 2-dimensionally (*x*-axis: allele 1 relative signal intensity, *y*-axis: allele 2 relative signal intensity) in Fig. 6 and are summarized in Table 2. Excellent separation of three clusters of plots along the *x*-axis or the *y*-axis or of those at the intermediate place was observed for all three SNPs. Each cluster consisted only one genotype, as indicated by a color according to MassARRAY determination (blue, allele 1 homozygote; green, heterozygote; red, allele 2 homozygote), thus proving the accuracy of this system.

Advantages and limitations of on-plastic chip allele-specific primer extension method

The system developed in this study can easily be carried out at ordinary laboratories. Primer extension can be performed in an incubator that simply keeps constant temperature. Colored precipitation of BCIP/NBT at the final step enables visual detection of typing results, eliminating the need of an expensive fluorescent scanner, often used by other SNP typing methods, or a mass spectrometer. Since a rather high amount of template (15 fmol; approximately 1 ng for 120 bp fragment) was determined to be a standard condition, this system would be recommended to use for typing up to hundreds of predetermined marker SNPs rather than for genome-wide high throughput purpose. This limitation in the number of SNP typing is also restricted by spotting space availability on a plastic base. Currently it is possible to spot 768 (2 sets of 384) oligonucleotides on a single plastic base. Since 6 spots are necessary for typing 1 SNP (3 pots for allele 1 and 3 spots for allele 2), 128 SNPs should be considered as the maximum number for simultaneous determination. However, spotting area on the plastic base is not fully occupied with the current spot size of 100 μm; thus the spot number limitation will be relieved if a higher density spotter is available.

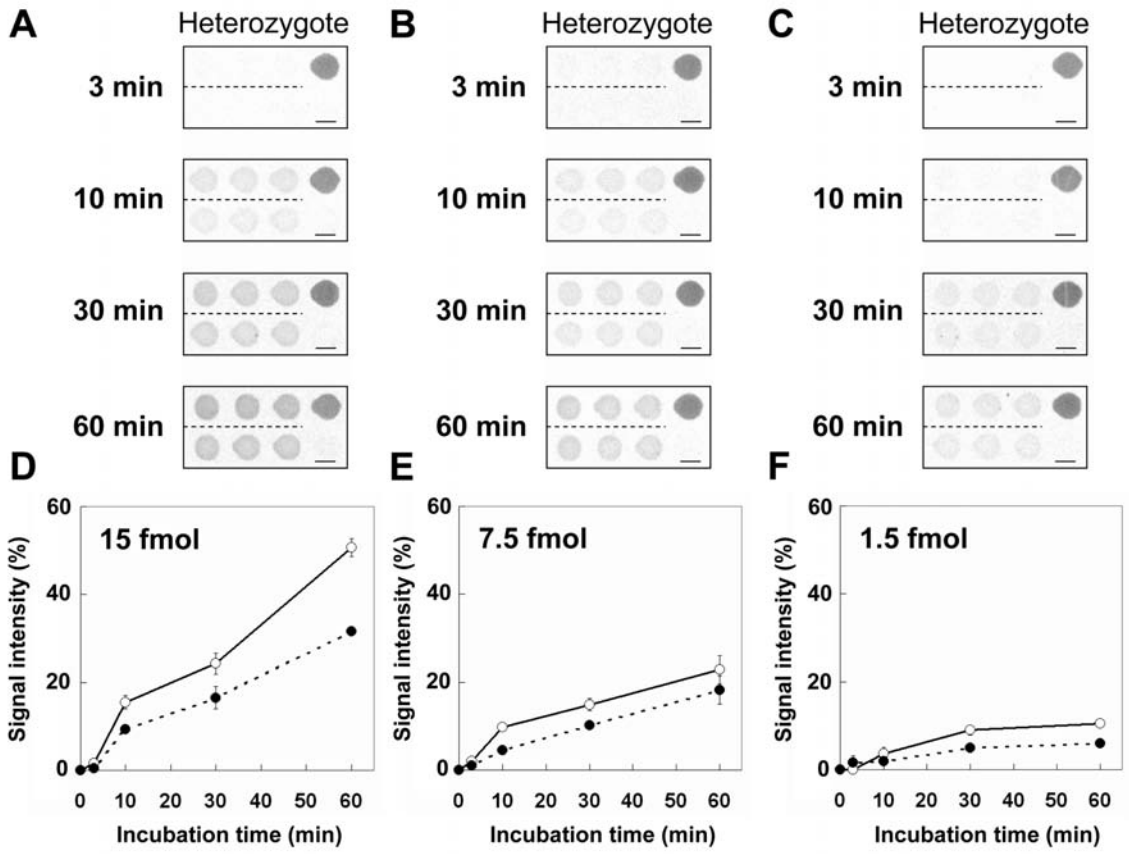

Fig. 5 Kinetics of on-plastic chip allele-specific primer extension for discrimination of rs1800469 SNP in TGFB1. Primer extension was terminated at various time points as indicated on the left side of each panel. Template DNA: A and D, 15; B and E, 7.5; C and F, 1.5 fmol. (A, B and C) Images of on-plastic chip allele-specific primer extension. Spot design on the plastic chip was essentially the same as that shown in Fig. 3. In each series of image panels, incubation time increases from top to bottom, as indicated at the left side of each panel. Genotype of template DNA used was derived from heterozygote as indicated on the top of each series of panels. Scale bars at the lower left side of panels A, B and C indicate 100 μm. (D, E and F) Plot of relative signal intensity at various incubation times shown in the panels A, B and C. The solid lines with open circles in panels D, E and F indicate signal intensity of the allele 1 discriminating oligonucleotides relative to that of positive control. The dotted lines with filled circles indicate the signal intensity of the allele 2 discriminating oligonucleotides relative to that of positive control.

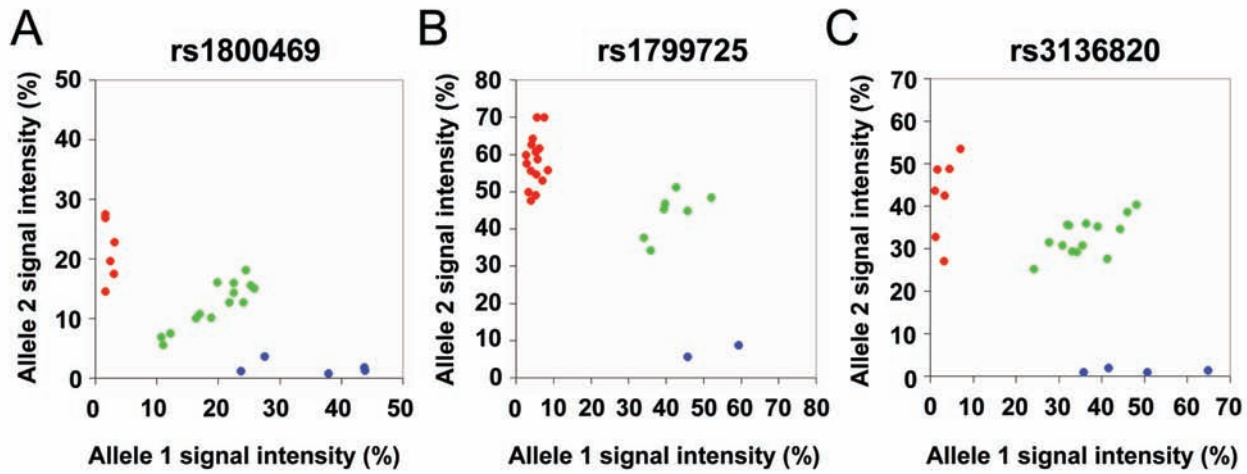

Fig. 6 Two-dimensional representation of allelic discrimination by the on-plastic chip primer extension system. (A) rs1800469 in TGFB1. (B) rs1799725 in SOD2. (C) rs3136820 in APEX1. On each panel, the signal intensities of allele 1-discriminating oligonucleotides relative to that of positive control for 25 individuals are plotted on the *x*-axis and signal intensities of allele 2-discriminating oligonucleotides relative to that of positive control are plotted on the *y*-axis, respectively. The color of each plot reflects the genotype determined by MassARRAY system (blue circle, allele 1 homozygote; green circle, heterozygote; red circle, allele 2 homozygote). Primer extension reactions were carried out using a standard condition of 4 mM $MgCl_2$ with 15 fmol template at 65˚C for 30 min.

Conclusion

Allele-specific primer extension reaction has been successfully applied to a microarray-format SNP typing system with support of 3′-end LNA modification. This system can be performed within a couple of hours without using a specific instrument for typing previously selected limited number of SNPs. All these benefits should meet the increasing demands in current molecular biology research and in bedside clinical diagnosis.

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