

Review

Visible Genotype Sensor Array

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Abstract: A visible sensor array system for simultaneous multiple SNP genotyping has been developed using a new plastic base with specific surface chemistry. Discrimination of SNP alleles is carried out by an allele-specific extension reaction using immobilized oligonucleotide primers. The 3'-ends of oligonucleotide primers are modified with a locked nucleic acid to enhance their efficiency in allelic discrimination. Biotin-dUTPs included in the reaction mixture are selectively incorporated into extending primer sequences and are utilized as tags for alkaline phosphatase-mediated precipitation of colored chemical substrates onto the surface of the plastic base. The visible precipitates allow immediate inspection of typing results by the naked eye and easy recording by a digital camera equipped on a commercial mobile phone. Up to four individuals can be analyzed on a single sensor array and multiple sensor arrays can be handled in a single operation. All of the reactions can be performed within one hour using conventional laboratory instruments. This visible genotype sensor array is suitable for "focused genomics" that follows "comprehensive genomics".

Keywords: Visible sensor, SNP, array, plastic, primer extension.

1. Introduction

Demands for convenient genotyping of previously characterized marker nucleotides in various organisms, including humans, have been increasing recently. Following human whole genome sequencing, the need for establishing high throughput genotyping methods with a potential for genome-wide association studies (GWAS) was recognized [1-5]. Several methods were then developed [6-10] which have provided fruitful results [11-15]. Now GWAS is carried out as a routine strategy in many research groups. GWAS successes have generated the need for convenient analysis of the resulting marker genotypes for research groups and therapeutic hospitals. Most of the methods used for GWAS are not directly applicable for groups whose interest is focused rather than comprehensive. Generally, high throughput methods are not convenient for analysis of a limited number of targets in terms of labor, cost and time. Thus, accompanying progress in GWAS there is an increasing demand for convenient genotyping methods optimized for a small number of marker nucleotides.

We recently developed a new SNP typing sensor device that allows visible inspection of genotyping results [16]. This device was used to investigate human radiation sensitivity-associated genes [17-18]. Previously, there was one chip-based genotyping method that had been reported to allow visual detection of typing results by naked eyes [19]. This method uses a commercially available biosensor chip that is capable of transducing specific molecular interactions into signals that can be visualized even by the naked eye. The chip (6 x 6-mm squares) is small enough to be placed in a 96-square-well reaction plate. Mass deposited on the thin-film surface by enzymatic catalysis alters the wavelength of light reflected by the optical layer resulting in a perceived color change on the surface. This method uses allele-specific ligation reaction of biotin-conjugated oligonucleotide probes for discrimination of genotypes. The biotin molecule in the probe is used for visualization of typing results.

On the contrary, our device is a microarray slide (75 x 25 mm) that uses enzymatic allele-specific primer extension reactions to discriminate genotypes. The array is composed of a commercially available plastic slide base with specific chemical modifications on its surface that allow covalent immobilization of amino-modified oligonucleotides [20]. Briefly, the surface of a cyclic olefin copolymer (COC) was coated by random copolymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC), *n*-butyl methacrylate (BMA), and *p*-nitrophenyloxycarbonyl polyethyleneglycol methacrylate (MEONP). In aqueous solution, BMA forms aggregates and becomes adsorbed onto a hydrophobic substrate surface of a COC. On the other hand, the hydrophilic properties of MPC provide suitable environment for DNA-DNA hybridization reactions and enzymatic activity such as that of DNA polymerase. MEONP works as an active ester unit to form covalent bonds with 5'-C6-amino-oligonucleotides. The immobilization-ready, post-functionalized plastic slide base is already commercially available from Sumitomo Bakelite Co. (Tokyo, Japan). Plastic bases can be handled safely and are less easily broken during operation and transportation than glass. Reliable genotype discrimination is achieved by enhancing allelic specificity in an enzymatic extension of immobilized oligonucleotide primers with a locked nucleic acid (LNA) modification at the 3'-end [21-22]. Selective incorporation of multiple biotin-dUTP molecules during the primer extension reaction, followed by binding of alkaline phosphatase-conjugated streptavidin allows visible detection of genotypes through precipitation of colored alkaline phosphatase substrates onto the surface of the plastic base.

Precipitation of colored substrates allows immediate inspection by the naked eye and images can be recorded by a digital camera equipped on a mobile phone as shown in Fig. 1. Unlike other SNP typing systems, this array does not need expensive instruments such as a fluorescent high resolution scanner or a mass spectrometer for detection. Furthermore, the overall processes can be carried out quite easily in a relatively short time period. It takes only one hour from primer extension to observation of typing results.

In this review article, details of the sensor array including design of allele-discriminating oligonucleotides, principles of SNP typing reaction chemistry, sensor array preparation and examples of operation are described.

Figure 1. Recording the visible genotype sensor array image using a digital camera equipped on a mobile phone. A: Image recording using a mobile phone. B: Recorded image on the mobile phone. Individual spots on the sensor array indicate target SNP allele types. Visibility of the genotyping spots allows immediate inspection of results.

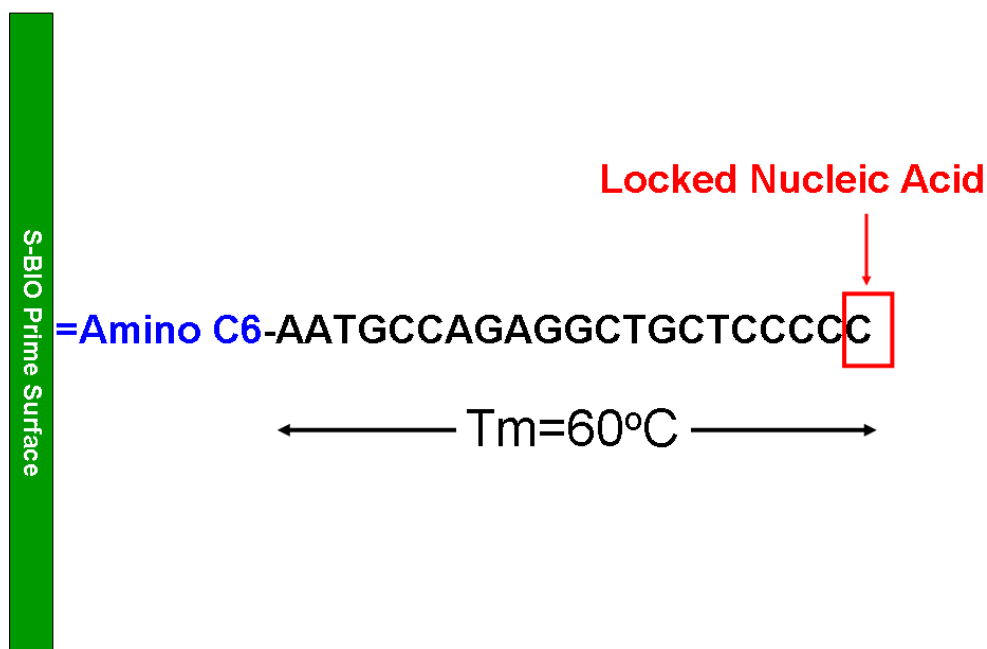


2. Design of allele-discriminating oligonucleotides

An example of an allele-discriminating oligonucleotide is shown in Figure 2. It should be noted that the 3'-end nucleotide opposing the target SNP nucleotide in the template DNA is LNA modified to enhance its allelic discrimination efficiency as reported in [21] and [22]. The T_m of the backbone oligonucleotide was set to be 60°C by adjusting the number of overall nucleotides while the SNP

nucleotide was always located at the 3'-end. Each oligonucleotide was synthesized with additional 5'-end amino C6 modification for covalent attachment to the plastic base.

Figure 2. Structure of an allele-discriminating immobilized oligonucleotide. The 3'-end nucleotide opposing the target SNP nucleotide in the template DNA is LNA modified to enhance allelic discrimination efficiency. The T_m of the backbone oligonucleotide is set to be 60°C. Amino C6 is attached to the 5'-end nucleotide for covalent immobilization to the surface of a plastic base.



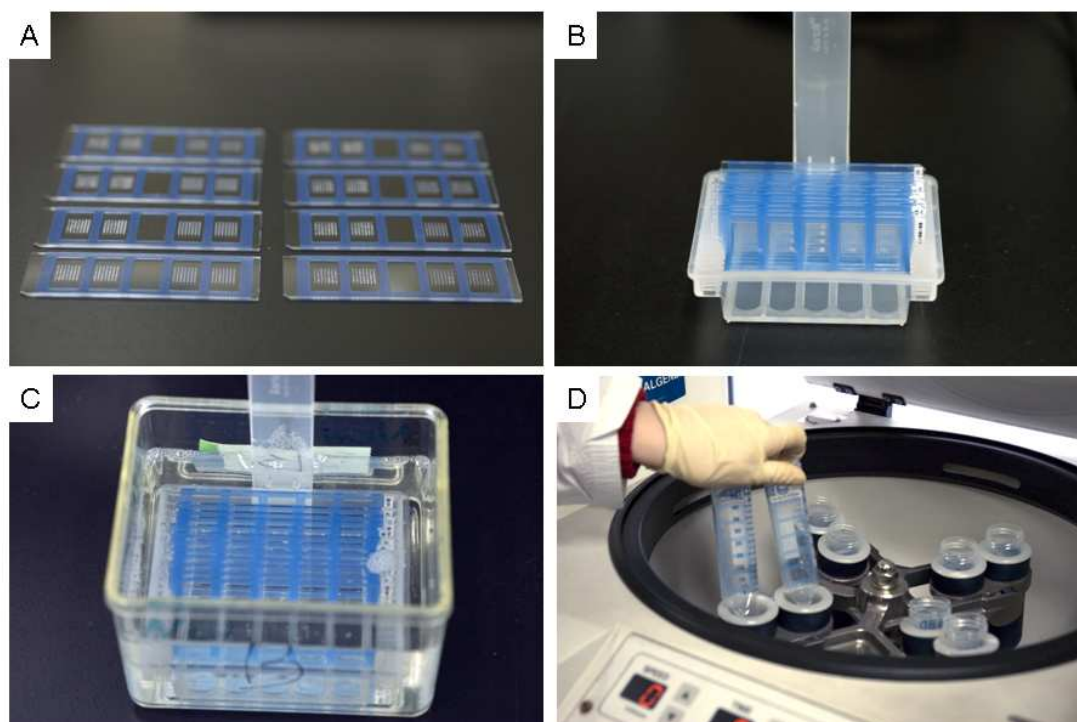
3. Preparation of genotyping array

Synthesized allele-discriminating oligonucleotides were immobilized as follows. First, the concentration of oligonucleotides was adjusted to be a 0.05 μ M solution in 1 x S-BIO spot solution (Sumitomo Bakelite, Tokyo, Japan). Drops of approximately 50 nL were spotted onto S-BIO Prime Surface plastic bases (75 x 25 x 1 mm, Sumitomo Bakelite, Tokyo, Japan) using a MassARRAY Nanodispenser (Sequenom, San Diego, CA) as shown in Figures 3A and 3B. The spotted plastic bases were heated at 80°C for 1 h to stimulate covalent immobilization of the oligonucleotides onto the surface of the plastic base (Figure 3C). White spots were clearly visible after heating as shown in Figure 3D. A Multiwell GeneFrame (19 x 10 mm x 5 wells/frame, ABgene House, Surrey, UK) was then placed carefully on each base (Figure 4A). The bases were washed in 0.1% Tween-20 at room temperature for 1 min as shown in Figures 4B and 4C. The white spots disappeared after this washing process. The bases were then soaked in 1 x S-BIO blocking solution (Sumitomo Bakelite, Tokyo, Japan) containing 0.1% Tween-20 at room temperature for 5 min. They were washed in 1 x TBS-T (10 mM Tris, pH 7.6, 150 mM NaCl and 0.1% Tween-20) at room temperature for 1 min, then in 0.1% Tween-20 at 80°C for 1 h to remove excess surface adhesive chemicals from the Multiwell GeneFrame and finally in 0.1% Tween-20 at room temperature for 1 min. They were centrifuged at 100 x g for 1 min as shown in Figure 4D and dried at room temperature for 10 min. The arrays thus prepared were placed in a desiccator and stored at 4°C until use.

Figure 3. Spotting of oligonucleotides on plastic bases. A: MassARRAY Nanodispenser. B: Spotting on a plastic base. C: Heating of the spotted plastic base. D: Comparison of original and spotted plastic bases.



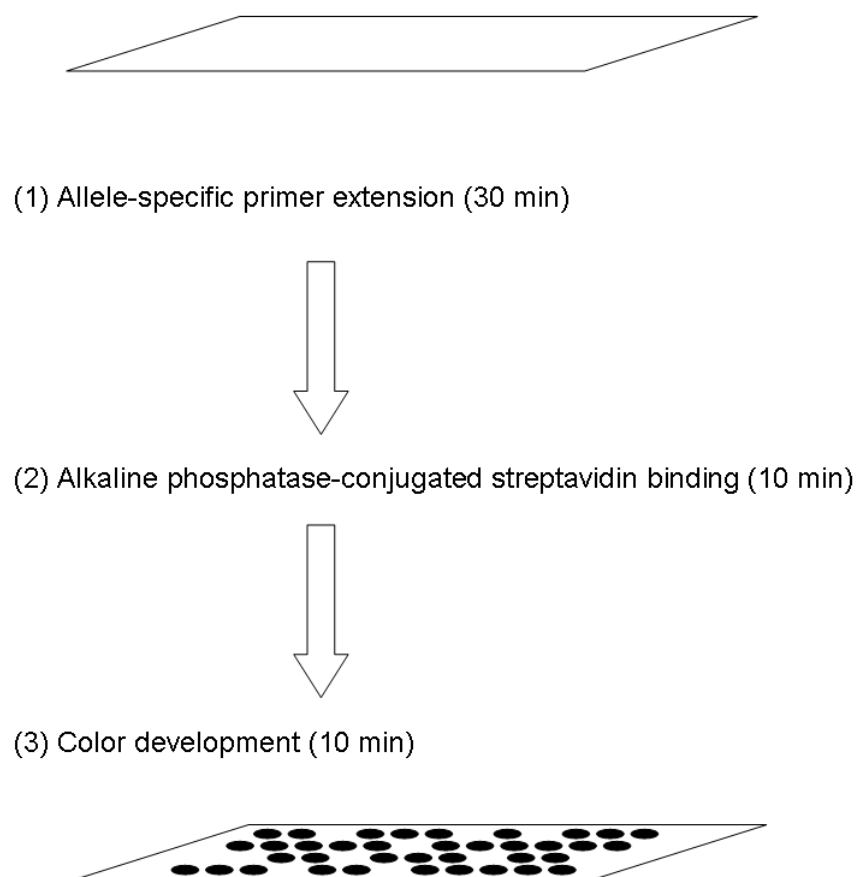
Figure 4. Post spotting processes in preparation of visible genotype sensor array. A: Attaching a Multiwell GeneFrame onto each plastic base. B: Simultaneous handling of multiple plastic bases. C: Masking unspotted surface of plastic bases by soaking in 1 x S-BIO blocking solution. D: Removal of surface solution by centrifugation.



4. Reaction chemistry

Overall reaction processes are illustrated in Figure 5. They consist of the following three steps: Step 1: Multiple allele-specific immobilized oligonucleotide primer extension. Step 2: Binding of alkaline phosphatase-conjugated streptavidin to biotin-dUTPs incorporated during primer extension process. Step 3: Visible color development. Overall reactions can be completed in one hour.

Figure 5. Scheme of reaction processes on the visible genotype sensor array. Only three reaction processes are necessary. These reactions can be performed by conventional laboratory instruments shown in Figure 9. Typing results can be visibly inspected within one hour.



4.1. Step 1: Allele-specific primer extension (Figs. 6, 9A and 9B)

Multiple immobilized oligonucleotides were extended simultaneously and selectively by the perfectly matched template DNAs (Figure 6). Typically, fifty microliters of a reaction mixture contained 1 x Mg-free ThermoPol II reaction buffer (New England Biolabs, Beverly, MA), 2.5 units of HotStar Taq DNA polymerase, 10 μ M biotin-modified dUTP (Fermentas, Hanover, MD), 10 μ M each of normal nucleotide (dATP, dCTP and dGTP), 4mM MgCl₂, and 1 μ L of PCR product mixture. The reaction mixtures were initially heated at 95°C for 15 min to activate the HotStar Taq DNA polymerase,

then cooled down for a couple of minutes and poured onto the wells on the array. The arrays were placed in prewarmed, humidified plastic boxes as shown in Figure 9A. The boxes were then sealed thoroughly with Saran wrap (AsahiKASEI Life and Living, Tokyo, Japan) and incubated at 65°C for 30 min as shown in Figure 9B. The biotin-dUTPs incorporated during the primer extension reaction are used as tags for sensitive visible detection of spots in subsequent steps. It is advisable to use PCR products with sizes up to 800 bp (based on unpublished observation). Longer template DNA seems to be introducing static and/or spatial hindrance and reduce the hybridization efficiency. Genomic DNA might be feasible to use if it is fragmented to short size and using fluorescence to detect signals, though we have not yet examined. HotStar Taq DNA polymerase has been used since it does not possess 3'-5' exonuclease activity. The 3'-5' exonuclease activity should be eliminated since it removes mismatched base pair at the SNP nucleotide and supports non-specific primer extension. Other DNA polymerase without 3'-5' exonuclease activity may be used. In this system, primer extension reactions, including initial hybridization between free-moving template DNAs and immobilized oligonucleotide primers, take place rapidly compared with other hybridization-only methods that usually require more than several hours. Ten minutes incubation might be sufficient to get enough signal strength as judged by ref. 16, however, we recommend using 30 minutes for stable and reliable genotyping.

Figure 6. Allele-specific primer extension reaction. The immobilized oligonucleotide (Allele 1) that is hybridized to perfectly matched template (Allele 1 template) can be extended according to the sequence of the template. Biotin-dUTPs in the solution are thus incorporated during the extension process. Extension of the immobilized oligonucleotide (Allele 1) hybridized to a mismatched template (Allele 2 template) is efficiently inhibited by the LNA modification at its 3' end.

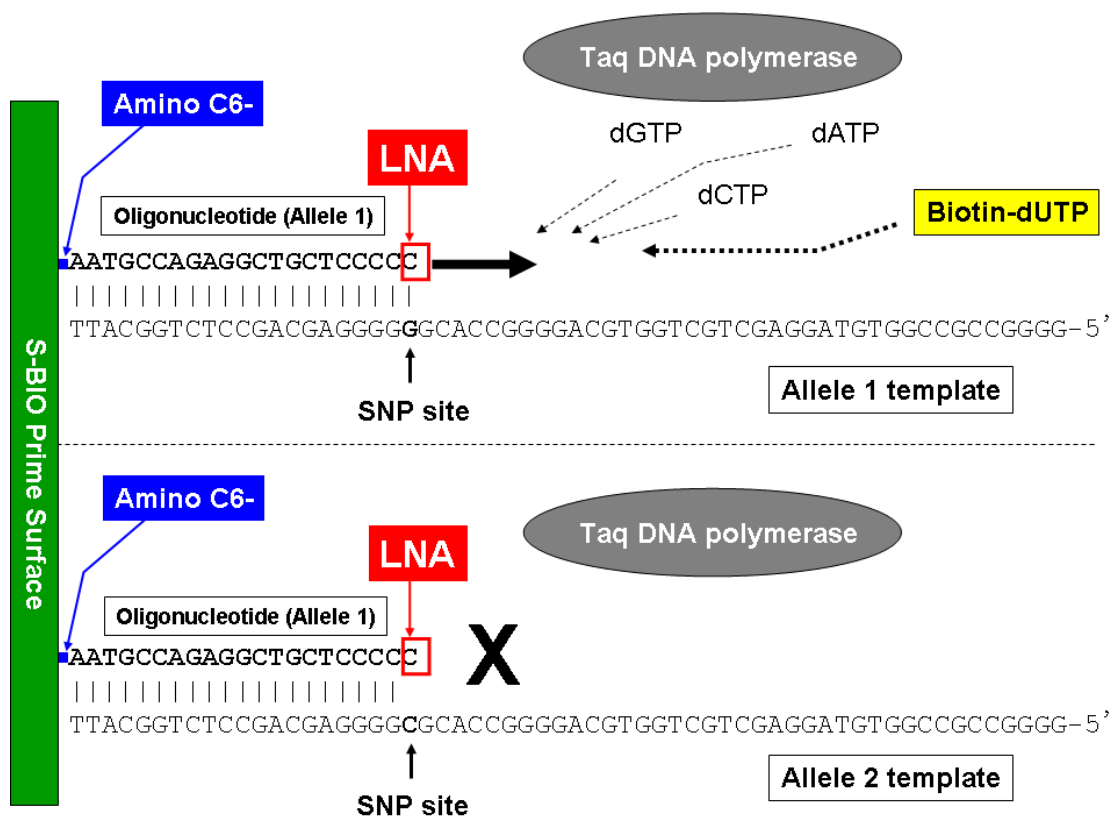


Figure 8. Colored substrate precipitation. Captured alkaline phosphatases catalyze conversion of soluble NBT into insoluble, colored NBT-formazon. The resulting NBT-formazon precipitate adheres to the surface of the plastic base, giving visible spots.

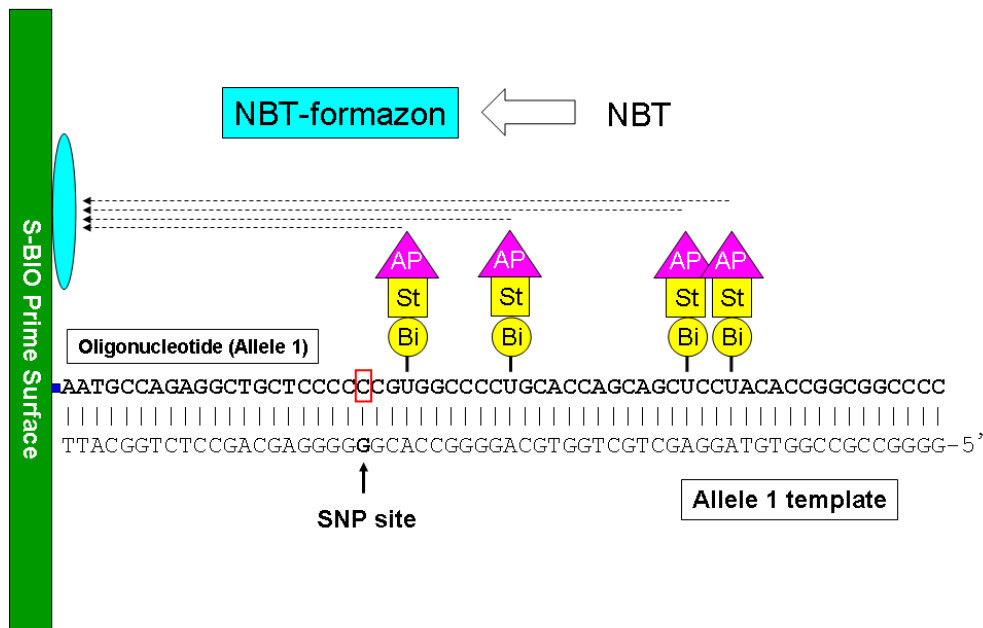


Figure 9. Overview of experimental set-up for reactions on the sensor array. A: Set-up of allele-specific primer extension. The reaction mixture was added to each well of the visible genotype sensor arrays then placed on a plastic tip case containing pre-warmed water. B: Allele-specific primer extension was performed in a constant temperature incubator at 65°C. The plastic tip case was wrapped with Saran wrap to preserve humidity. C: Incubation of alkaline phosphatase-conjugated streptavidin. D: Color development using BCIP/NBT.

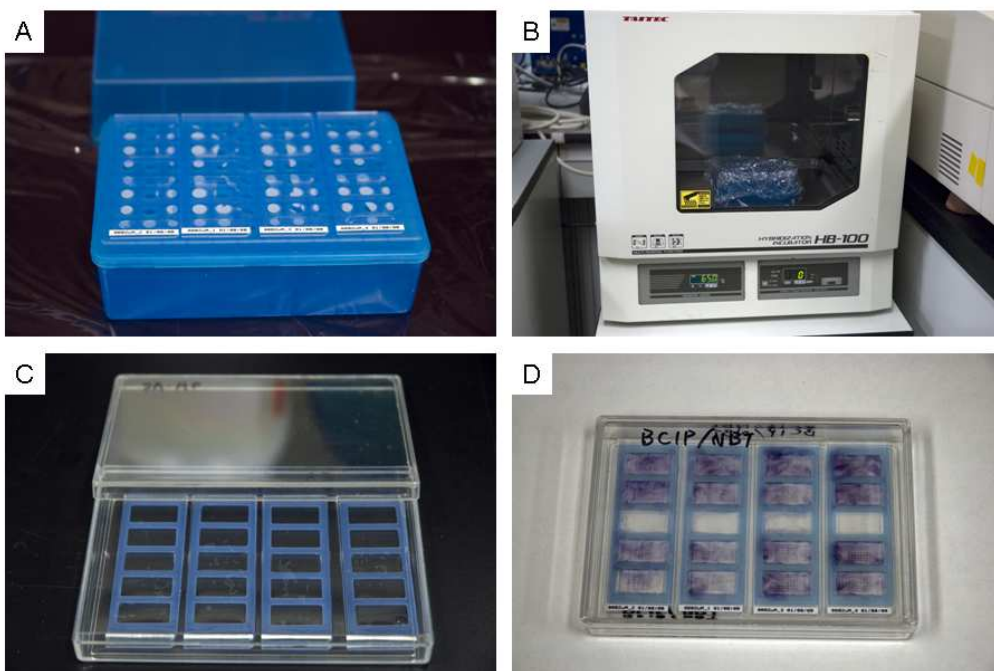
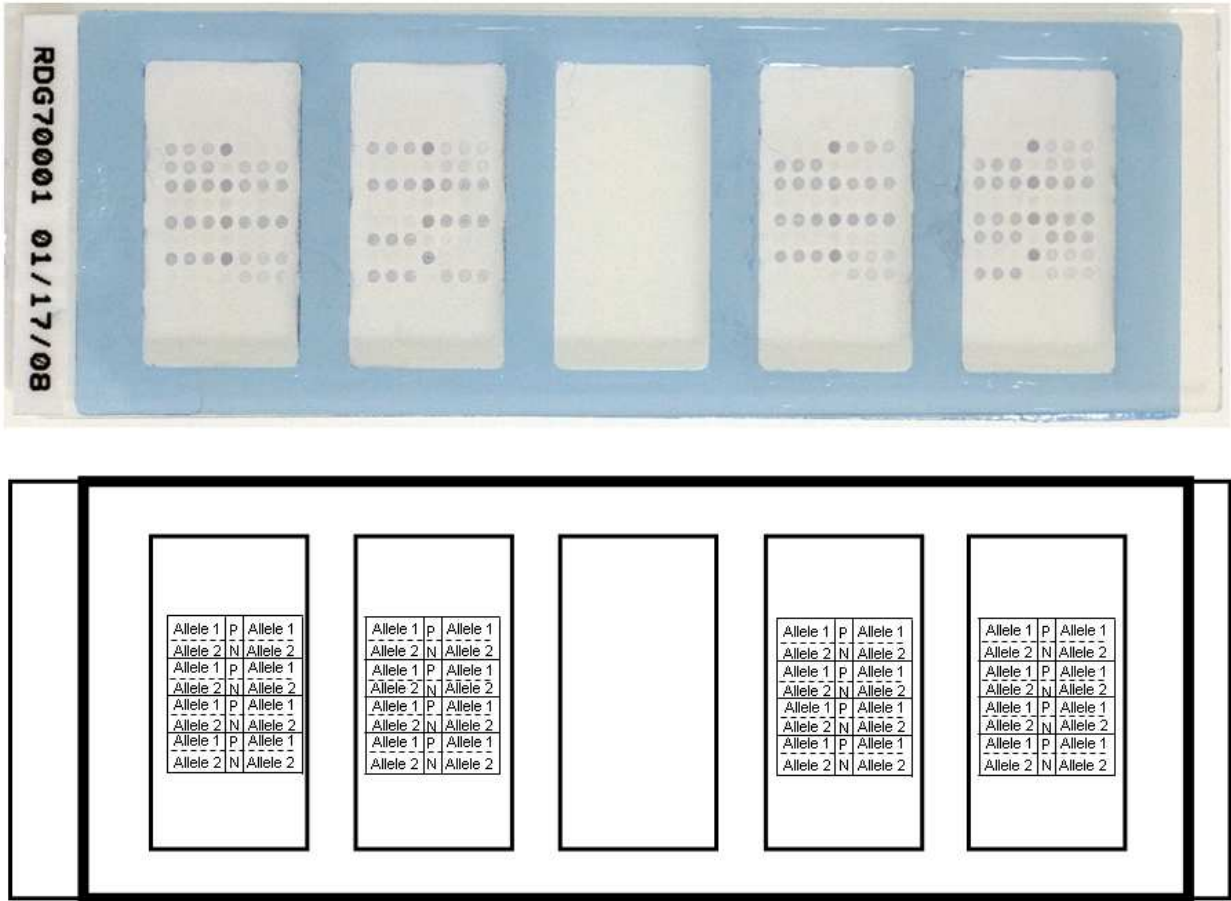


Figure 10. Examples of spot images from the visible genotype sensor array. Genotypes of four individuals were simultaneously analyzed on one sensor array. Allele 1: Allele-1 discriminating oligonucleotides spotted in triplicate. Allele 2: Allele-2 discriminating oligonucleotides spotted in triplicate. P: Positive control oligonucleotide [16]. N: Negative control oligonucleotide [16].

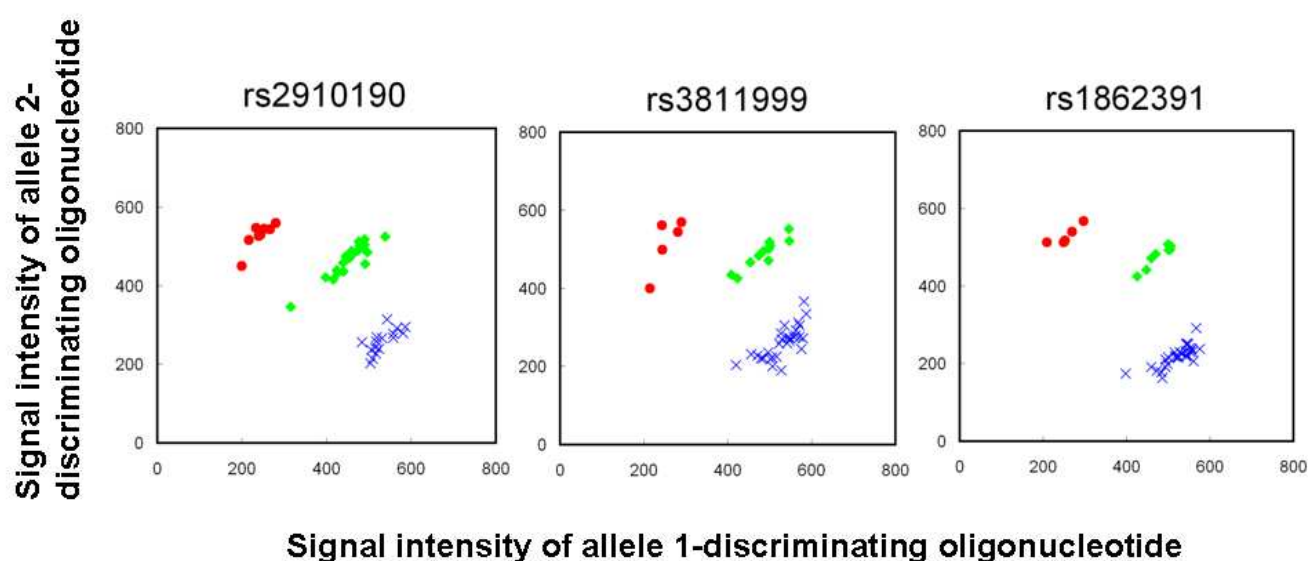


5. Recording images and data processing

Typing results can be immediately inspected by the naked eye and can be recorded by a digital camera equipped mobile phone (Figure 1). In our laboratory, for detailed quantitative assessments, the images of spots on the sensor array were recorded using a Nikon D70 digital camera (Nikon, Melville, NY) fixed on a tripod. The original 16-bit RAW format RGB files (shown in Figure 10 as an example) were then converted to 8-bit grayscale TIFF format files using Adobe Photoshop Version 6.0 software (Adobe Systems, San Jose, CA). The signal intensities of individual spots on the sensor array were then quantified using the Daredemo DNA Array Kaiseki software version 1.0 (Dynacom, Chiba, Japan). Typical scatter plots of signal intensities are shown in Figure 11. A total of forty-five individuals whose genotypes had been previously investigated by another established method (MassARRAY) were used to validate this sensor array. All data points of individual genotypes were clustered in well-separated regions. Detailed assessment of the allelic discrimination by this sensor array has been carried out by calculation of Silhouette scores according to [23]. All of the oligonucleotides

investigated were revealed to have a score beyond the cutoff value of 0.65, confirming reliability of allelic discrimination by this sensor array [18].

Figure 11. Scatter plots of signal intensities measured for three SNPs. X-axis: Signal intensity of allele-1 discriminating oligonucleotide. Y-axis: Signal intensity of allele-2 discriminating oligonucleotide. Data plots were of 45 individuals previously genotyped by another established method (MassARRAY). Blue crosses: Allele 1 homozygotes determined by MassARRAY. Green open diamonds: Heterozygotes determined by MassARRAY. Red closed circles: Allele 2 homozygotes determined by MassARRAY. Plots are modified from ref. 18.



6. Applications of the visible genotype sensor array

Increasing reports of success with GWAS in many fields have generated a need for convenient analysis of the selected marker genotypes by research groups and therapeutic hospitals. Furthermore, these techniques are increasingly in demand in developing countries to improve human healthcare [24]. The visible genotype sensor array described in this review article should be considered as a candidate to meet the above needs. The method of Zhong *et al.* [19] uses allele-specific ligation reaction of biotin-conjugated oligonucleotide probes to the immobilized oligonucleotides that hybridized to template PCR products. Ligation reaction has superior allelic discrimination ability to that of DNA polymerase. However, the biotin-conjugated oligonucleotide probe must be synthesized for individual SNPs and their cost cannot be ignored when analyzing substantial number of different SNPs. Our method, on contrary, uses biotin-conjugated dUTP that can be universally applied to any SNPs. In addition, multiple biotin-conjugated dUTP molecules can be incorporated into single immobilized oligonucleotide thus providing higher sensitivity to our method, providing strengthening of spot signals. This property has been essential for eliminating need of microscopical recording of detailed typing results used for the chip and enabled direct capturing of the images. It also provided

enlargement of device size from the chip to the microarray slide. The reactions on our sensor array can be easily performed with conventional laboratory instruments such as a constant temperature incubator as shown in Figure 9B. Typing results can be immediately inspected by the naked eye and can be recorded by a digital camera equipped on a mobile phone as shown in Figure 1. The overall time required is less than one hour from applying the template PCR products onto the array to getting the visible images. Up to four patients can be simultaneously analyzed using a single array and multiple arrays can be conveniently handled in a single operation. These features allow easy access to most research laboratories and therapeutic hospitals including those in developing countries.

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