ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Development of an Automated SNP Analysis Method Using a Paramagnetic Beads Handling Robot

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Received 13 November 2006; revised 25 January 2007; accepted 2 February 2007

Published online 2 March 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21380

ABSTRACT: Biological and medical importance of the single nucleotide polymorphism (SNP) has led to development of a wide variety of methods for SNP typing. Aiming for establishing highly reliable and fully automated SNP typing, we have developed the adapter ligation method in combination with the paramagnetic beads handling technology, Magtration®. The method utilizes sequence specific ligation between the fluorescently labeled adapter and the sample DNAs at the cohesive end produced by a type IIS restriction enzyme. Evaluation of the method using human genomic DNA showed clear discrimination of the three genotypes without ambiguity using the same reaction condition for any SNPs examined. The operations following PCR amplification were automatically performed by the Magtration®-based robot that we have previously developed. Multiplex typing of two SNPs in a single reaction by using four fluorescent dyes was successfully preformed at the almost same sensitivity and reliability as the single typing. These results demonstrate that the automated paramagnetic beads handling technology, Magtration®, is highly adaptable to the automated SNP analysis and that our method best fits to an automated in-house SNP typing for laboratory and medical uses.

Biotechnol. Bioeng. 2007;98: 420–428.

KEYWORDS: SNP typing; ligation; paramagnetic beads; automatic typing

Introduction

Analysis of DNA sequence variation has led to advances in the mapping of human disease genes. Identification of single nucleotide polymorphisms (SNPs) and the application of SNP data have been the focus for human genetics research and drug discovery (Landegren et al., 1988). The genome sequence and the initial analysis revealed that more than 3 million SNPs would be involved in the human genome (Cooper et al., 1985). Huge efforts, including HapMap project (The International HapMap Consortium, 2005) and the SNP consortium (TSC) intended to discover and map hundreds of thousands SNPs in the human genome and to develop a map of high density SNP markers (Marshall, 1999), have been made to find SNPs that cause or have close relation to diseases. The SNPs that were confirmed to be useful markers for particular diseases should be very useful for genetic analysis of common diseases, drug responsiveness and population differences. Thus, the need for high-throughput and cost effective genotyping technologies has increased to facilitate the use of SNPs to drug discovery or diagnosis of diseases. A number of different techniques have been developed for analyzing SNPs including single strand conformation polymorphism analysis (SSCP) (Orita et al., 1989), gel based restriction fragment length polymorphism analysis (RFLP) (Cooper et al., 1985), allele specific oligonucleotide (ASO) hybridization

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(Saiki et al., 1989), oligonucleotide ligation assay (Landegren et al., 1988) and so on. New technologies, such as DNA microarrays, mass spectrometry, and microbeads, have been developed and incorporated into the detection and readout of allele signals based on either hybridization or enzymatic discrimination (Buetow et al., 2001; Chen et al., 2000, 1997, 1999; Fan et al., 2006; Fu et al., 1998; Gilles et al., 1999; Livak et al., 1995; Matsuzaki et al., 2004; Tyagi et al., 1998).

One of the major focuses in the development of SNP typing method is to simplify the overall analysis protocol for quick, easy and automatic operation. Many efforts have been made to achieve simple one step reaction to date. Typical examples in this respect are TaqMan (Lee et al., 1993), Invader (Mein et al., 2000) and oligonucleotide ligation assay (OLA) methods (Tobe et al., 1996).

In this study, we have developed an accurate and automated SNP typing method to accelerate in-house SNP analysis and practical application of SNP genotyping to medical inspection. Our protocol utilizes sequence specific ligation between cohesive ends of DNA. After the PCR amplification, the sample DNA fragment was digested by type IIS restriction enzyme, producing a short protruding single stranded DNA that contained the nucleotide at the SNP site. Then, the sequence specific ligation was employed at the cohesive ends between the sample DNA fragment and the synthesized adapter DNA.

The protocol consists of several steps of reactions and requires removal excess fluorescent labeled adaptors. Thus, we have introduced the robotics equipped with a paramagnetic handling device, a Magtration® unit, for the automatic typing of SNP. Magtration® is a technology for automated paramagnetic beads handling (Obata, 2001). While paramagnetic beads are captured inside the vessels in a common technique, Magtration® devices capture the beads inside the liquid handling tips by applying an integrated magnetic device to outer surface of the tips. This remarkable feature of Magtration® has realized automated, reliable and cross-contamination free operation of paramagnetic beads (Sawakami-Kobayashi et al., 2003). We have developed the protocol fully compatible to the automated robot. Evaluation of the performance and accuracy of our method was addressed by the analysis of polymorphic regions on human genome.

Materials and Methods

This study was approved by the research ethics committees of National Institute of Advanced and Industrial Science and Technology (AIST), Tokushima University and Astellas Pharma Inc. We obtained written informed consent from all subjects. Genomic DNA for the SNP typing assay was prepared from voluntarily donated blood. After the nucleotide at SNP of interest was determined by direct sequencing methods, the genomic DNAs were subjected for the experiments as standards for validation. Oligonucleotides were chemically synthesized with modification by biotin or florescent dye when necessary (Table I).

Single SNP Typing Reaction

The procedure of the adapter ligation method is schematically shown in Figure 1. DNA fragments for the assay were prepared by two steps PCR. The DNA fragments of 500 bp to 1,000 bp in length were produced by the first PCR using 0.05 μg of genomic DNA as a template. The product was subjected to the second PCR using a pair of a biotinylated primer and a primer containing the *Bse*RI recognition sequence. The PCR amplification was performed in 20 μl of reaction containing 10 pmol each of the primers, 250 μM each of dNTPs and 5 units of ExTaq DNA polymerase (Takara Bio Co. Ltd. Siga, Japan) in 1 × Takara ExTaq buffer using 35 cycles at 95°C, for 20 s, at 55°C, for 30 and 60 s, 72°C for 60 s. The 10 μl of the amplified fragments from the second PCR were digested with 8 units of *Bse*RI in 20 μl of 1 × medium buffer (10 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) at 37°C for 1 h. The ligation reaction of the *Bse*RI digests and the fluorescently labeled adapters were carried out using 85 units of T4 DNA ligase in 25 μl of 1X T4 DNA ligase buffer (66 mM Tris–HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP) containing 40 pmol each of the annealed adapters labeled by FITC and Cy5, for the detection of A and G alleles, respectively, at 16°C for 1 h. After the ligation, the biotinylated DNA fragments were captured on 25 μg of streptavidin coated paramagnetic beads (Dynabeads M280; Dynal Biotech, Oslo, Norway) and followed by washing the beads three times with 1 × BW buffer (5 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 1M NaCl). The fluorescence from the beads was measured by fluoro-microplate reader (Corona Electric, Ibaraki, Japan), at appropriate excitation and emission wavelengths for each fluorescent dye.

Automated Single or Multiple SNP Typing Reaction

DNA fragments harboring SNP sites were amplified from the genomic DNA by two steps PCR with LA Taq DNA polymerase (Takara Bio Co. Ltd.) which showed higher fidelity than ExTaq in this work. Ten micro litres of the amplified DNA fragment was used for SNP typing reaction, which is performed by MagSNiPer FD (PSS Co. Ltd., Chiba, Japan, http://www.pss.co.jp/english/index.html), the automated robot, equipped with a 12-arrayed Magtration® unit, a thermal cycler unit and incubation blocks for microplate. The biotinylated DNA fragments were treated with 8 units of *Bse*RI at 37°C for 10 min in 20 μl of 1 × Medium buffer. The ligation reaction was carried out at room temperature for 20 min by the addition of 7.05 μl of ligation mix (1.8 × Ligation-Convenience Kit (Nippon Gene, Tokyo, Japan), 40 pmol each of Cy3- or Cy5-labeled adapters) to each well.

The ligated fragments were captured by the addition of 50 μg of the streptavidin beads suspended in 25 μl of 2 × BW buffer. The beads were washed three times with BW buffer supplemented with 0.05% of Adecanol (Asahi Denkakagyo, Tokyo, Japan) and were subjected to fluorescence measurement with

Biotechnology and Bioengineering, Vol. 98, No. 2, October 1, 2007

Table I. Oligonucleotides used in this study

SNP No	first PCR	second PCR	Adapters for typing reaction
	Forward primer Reverse primer Amplicon Length	Forward primer Reverse primer Amplicon Length	Adapter name for allel 1: Sequence Adapter name for allel 2: Sequence
ATase6	CTGCAGTATGATTTTAGTGG AAAGGAAATAAGTTTCATAG 576	Biotin-CTTTGGCTCAAGTCAAATGAGGATCC GCACCAATAGATAAGAAGCTACAAGATTCTGAAGAGAGGAGCCATCCT' 227	CC-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**CC** CT-FITC: TACAAGATTCTGAAGACACCACCCATCCTT**CT**
ATase8	GAACTAGATCTATATGAAGGTCG TTTCTCCTTAGAAATGTTGATGAG 432	Biotin- TTTCTCCTTAGAAATGTTGATGAG ATTTGGTTAGCACTGTTCCAGAATCTGCTACGCCTGAGGAGCTTGCTTA 159	CG-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**CG** TG-FITC: TACAAGATTCTGAAGACACCACCCATCCTT**TG**
SNP225	AAAATTCTTCAGTGGTCTGCCACTCT CCACCACACTCAGCCAACTTTCA 409	Biotin-GGTGATCTTAAGATCCCGTCCATG CTGTGCAAAAAGAATCTTATTGACAAACATAGTCCGAGGAGAACAGAAA 194	TA-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**TA** CA-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**CA**
SNP158	AGGGATTTAGAGACCACAGAACTC CTGTACTTAGCTTTTCTGTCTTAAGGG 403	Biotin-GTATCCCTGCAAGGATTAAATCCAGG GAATATTTTTGAACAACACAGCAAATGACTATTACGAGGAGGGATATTA 200	CT-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**CT** TT-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**TT**
A003	TCCTCTACACCGAATCACTGACTGTT ACGAGAACAGCATGAGAGTAACC 452	Biotin-TCTTTGCTTGTCCCATTCTCTCTC AGAGGGACAGAAGAAATGACATTGGAGCTGAACACGAGGAGAGGTATAG 121	TG-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**TG** TA-Cy5
A004	AGAAGAAAGTTATCCAGGTTGGTC ACAAGTTGCATGCTAGAAGGTGT 430	Biotin-AGAAGAAAGTTATCCAGGTTGGTC AATCAGTAGATTAAATGTTAATCTTCGCCCCAGTTGAGGAGGGGTTGAA 179	TG-Cy3 AG-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**AG**
SNP337	TACAAGACCAGCCTTGCCAGAGA GCCTTTTATGGTCTTGCCTCCCAA 453	Biotin-CTGAGGCAGGAGAATTGCTTCAAC GAATTCTGAGGACACATGGATCACTTTTTCTTGGGGAGGAGTGTGTGCC 190	AT-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**AT** GT-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**GT**
SNP331	CAGGATCTAAAACTACAACTGGTTC TGTGAGAAGTTCACAGTTTGTCC 410	Biotin-CAGGATCTAAAACTACAACTGGTTC AGAACATTTTGGGTTTTATGTTAGTCTGAATCCAGAGGAGCAGAGACAA 181	AG-Cy3 AC-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**AC**
SNP332	TTTAGCTGAGTGACTGCAGCACA GGTGAATTTTGTGGAAGACTATGTCTC 410	Biotin-ATTCCTCTAGTGCAGGCTGGACTG TAAAAAGCTAAAAAACGAAGCTTAAGAAAATGATGAGGAGCATTTCAGA 191	AA-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**AA** AG-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**AG**
SNP334	TACTCTTTCACTGTTGGCCAGAAC GGAGGTACAAGACTGAATACTCC 421	Biotin-TACTCTTTCACTGTTGGCCAGAAC ACAGAATAGTGTATTAAAGCCATTGAAAAAGCATGGAGGAGTTATCTAA 200	TA-Cy5 CA-Cy3
SNP336	ATTCCTTTCCCTTACATAGCATTT CAGGATGGTCTTGATCTCCTTATC 435	Biotin-TACACTACTACAACTAGTAACTGACA CCTTTTGAATTTTTGTTTTGTAAGGGACCGAGGAGACGACATA 193	AC-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**AC** TC-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**TC** AC-FITC: TACAAGATTCTGAAGACACCACCCATCCTT**AC** TC-TexasRed: TACAAGATTCTGAAGACACCACCCATCCTT**TC**
SNP657	ACTAACAAAGTGTGTTTGCTTCCCT CCTTGTTCTACTGTAGAACTTGGGTGTT 402	Biotin-ACTAACAAAGTGTGTTTGCTTCCCT ACCGGTGTCTGGAATTATAATATATGCACACTGAAGAGGAGTAACCCTG 237	CA-Cy3 GA-Cy5: TACAAGATTCTGAAGACACCACCCATCCTT**GA**
SNP909	TGTTACATGAAAATGTTCTTCTGGT GGGAATAGCGTGCATTTTATTCCAAG 437	Biotin-CTTGACATTATATACCTCTAGTGGC TTGTTAAACTCCTGCTAAATTGGTCTTTAGACTTAGAGGAGCATCACAT 185	TT-Cy3 AT-Cy5

DOI 10.1002/bit

SNP531	ACAAGAGGTGTAATGAGCTCTTACT	Biotin-ACAAGAGGTGTAATGAGCTCTTACT	AG-Cy3
	CCTGTTAATTTGTTCTGCTATCCTC	GCGGCTGTGTCCTGGGCAGGCAAAGGGACCTAGGGAGGAGGCAGGTCCA	AC-Cy5
	369	129	
SNP678	GTGCCTTTAGGAAGACACTACTGTTC	Biotin-CCTCGCAGGCTCACTCCCTCTGCAC	TC-Cy5
	AAGACCTAGGCTCATGCCTGGTTCTC	TATCCCAGCTCCATTAGGGAGATAAGTCATCTGATGAGGAGTTATGTCA	CC-Cy3: TACAAGATTCTGAAGACACCACCCATCCTT**CC**
	369	129	
			Adapter up: AAGGATGGGTGGTGTCTTCAGAATCTTGTA

The *Bse*RI recognition sequences are underlined. Nucleotides on the adapters corresponding to each allele at the SNP sites are indicated bold characters. The length of amplified fragment with each primer sets are indicate. The sequence of the adapter used for typing more than one SNP are indicated where it appears first in the table. The adapter for each SNP was prepared by annealing one of the oligonucleotides indicated for each SNP and the complementary "Adapter up" oligonucleotide, which were used commonly for all the SNPs.

microplate reader (Molecular Devices, Menlo Park, CA). For the multiplex SNPs typing, 5 μl of the PCR reactions for SNP334 and SNP336 derived from the same individual were mixed together and were used for the typing reaction in the same procedure as that for the single SNP typing except for the usage of four adapters labeled with Cy3, Cy5, FITC and Texas-Red. The overall SNP typing reaction for 12 individuals was performed in about 60 min by using MagSNiPer FD.

Results

Single SNP Typing by Adapter-ligation

In order to validate the present method, we examined SNPs on exon 6 (at 20 nt at 57,183,902 by NCBI Build 34) and exon 8 (at 119 nt, at 57,182,585) of the amidophosphoribosyltransferase (E.C. 2.4.2.14, ATase) gene, locating on chromosome 4q12 at the genomic position from 57,175,073 to 57,217,235 with the genome size of 42,163 including 11 exons**.** These two SNPs had G/A and C/T diallelic polymorphisms, respectively. Genomic DNAs from 50 individuals were extracted from blood samples and were used for the analysis. The results of the typing with manual operation are shown in Figure 2. A plot of fluorescence intensity from the allele 1 against that from allele 2 showed clear discrimination of three genotypes (AA, GG and AG) for exon 6 and two genotypes (CC and CT) for exon 8 (Fig. 2A and B), which were completely consistent to those determined by the direct sequencing and the TaqMan methods (data not shown). There are no individuals that show TT allele in the present samples. Control experiment using a cloned DNA fragments containing the T allele showed the expected result (data not shown). The average of the ratio of intensities from the two fluorescent dyes (I_{FITC}/I_{Cy5}) for exon 6 were 0.30 ± 0.06 and 9.73 ± 0.77 for alleles 1 and 2 homogygotes, respectively, and 1.27 ± 0.08 for heterozygotes. The ratio of those for exon 8 were 0.59 ± 0.06 for allele 1 homogygotes and 1.56 ± 0.14 for heterozygotes. Although the discrimination for exon 8 appeared less reliable than for exon 6, clear discrimination without any overlap was achieved for both SNPs. The palindromic sequence at the overhang (-GC-3′) produced from the C allele might cause dimerization of the sample DNA fragments, resulting in reduction of ligation efficiency between the sample and the reference (adapter) DNAs. Immobilization of the sample DNA with the two bases palidromic overhang on the beads before ligation resulted in extremely low ligation efficiency between the sample and the adaptor DNAs. The enrichment of the sample DNAs with a palindrome on the surface of the beads appeared to enhance the ligation efficiency between themselves. Thus, the present method uses the ligation in solution and the high concentration of the adaptor DNAs. Calling SNP genotypes was achieved simply by calculating the ratio between the two fluorescence intensities at each wave length. References for each SNP allele analyzed in parallel with samples may

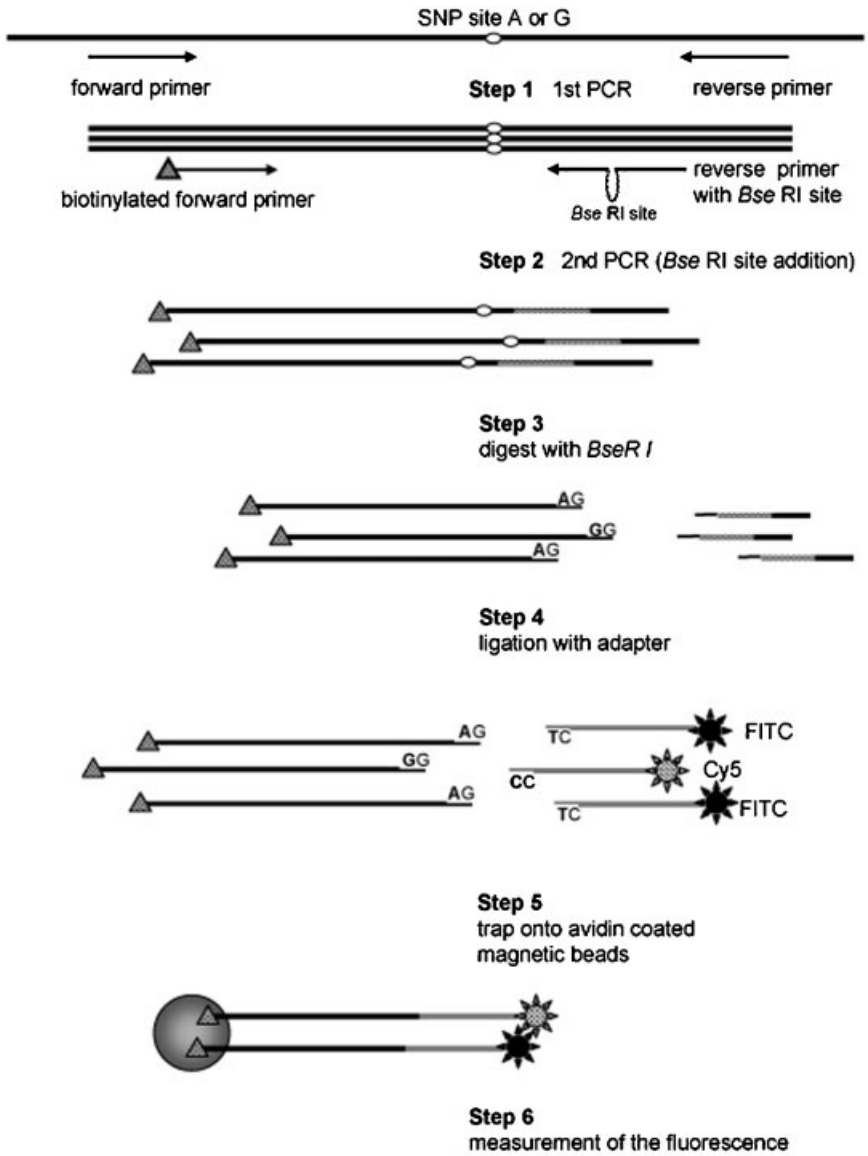

Figure 1. Schematic representation of the individual steps of SNPs typing by the adapter ligation method. In step 1, the DNA fragment containing polymorphic site to be determined is amplified by first PCR from genomic DNA. The products of the first PCR were amplified using the biotinylated primer and the primer with *Bse*RI recognition sequence in step 2. In step 3, the PCR products are digested with *Bse*RI restriction enzyme to expose the SNP site on two bases of single-stranded overhang. The nucleotides at SNP site are indicated with boldface. In step 4, fluorescently labeled adapter DNAs are ligated to the complementary cohesive ends generated in step 3. In step 5, the fragments are captured onto the streptavidin coated paramagnetic beads. In the last step (step 6), the fluorescence of the beads is measured with fluorescence micro plate reader.

further increase the reliability for calling SNP but is not necessary for most research purposes in general. Measurement of the ratios between the two fluorescence intensities in advance for each SNP allele is useful because they may slightly vary SNP by SNP.

Automated Single or Multiple SNP Typing Reaction

We have demonstrated that our method which utilizes adapter ligation is accurate enough without applying statistical processing to the results from multiple samples. The methods consisted of PCR amplification, restriction digestion and the ligation reaction with T4 ligase. Each reaction has been well-established as a basic tool in molecular biology. Combining these ''common'' reactions make the overall reaction highly reproducible and reliable. All SNPs used for this work were analyzed at the same condition without any requirements to optimize the reaction conditions specifically for each SNP locus. On the other hands, the method requires multiple enzymatic reactions, which is disadvantageous to other single reaction methods such as SSCP or TaqMan. In order to overcome the

DOI 10.1002/bit

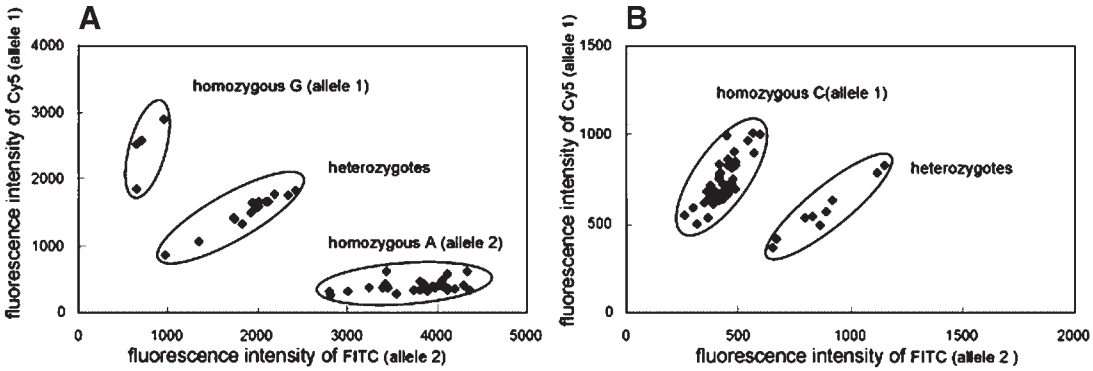

Figure 2. Scatter plot of the 50 samples for the polymorphic regions on the human ATase gene.Panels (A) and (B) show the result of exons 6 and 8, respectively. The horizontal and the vertical axes show the FITC and the Cy5 fluorescence intensities, respectively. The distinct clusters for each genotype are indicated by open ovals. There was no allele 2 homozygous for the SNP on exon 8 included in our samples.

drawback of our method, we have automated the reactions using MagSNiPer FD, the automated robot with the paramagnetic beads handling unit. We carried out the SNP typing reactions including restriction digestion, ligation and removal of excess fluorescently labeled adapters except PCR by this robot in a fully automated manner. The typing reaction for 95 individuals for thirteen SNP sites, which were selected from the database of Japanese Single Nucleotide Polymorphisms (JSNP) (Hirakawa et al., 2002), was carried out automatically. Various two base overhangs were generated from the SNPs, including palindromic, non-palindromic, high GC- and low GC-content sequences, which might affect the ligation efficiency. However, the genotypes of 95 individuals were correctly determined without any ambiguity by the single reaction condition for all the thirteen SNPs (Table II). The three average ratios from two homozygotes and a heterozygote for each SNP varied probably due to preference for one of the two alleles as a substrate in the PCR amplification, the restriction enzyme digestion and the ligation reactions. Thus, each locus showed a characteristic pattern of the three ratios for the three genotypes (Fig. 3, single SNP typing). Clear separation and reproducibility of the three ratios for each SNP allowed reliable discrimination for all the SNPs examined (Fig. 3, single SNP typing).

In order to improve the throughput of the adapter-mediated method, the typing reaction for two SNPs in a single tube was carried out. The duplex SNP typing reactions from 30 individuals were performed by the automated protocol. As shown in Figure 3, the two SNPs of these samples were determined without any ambiguity or discrepancy though the discrimination performance appeared lower than the single SNP typing mainly due to decrease of fluorescence intensities. Usage of Ligation-Convenience Kit instead of T4 DNA ligase improved the ligation efficiency when the duplex reaction was performed. Moreover, the robotics with Magtration®, MagSNiPer FD, allowed highly reproducible recovery of the paramagnetic beads and smaller dispersion of the fluorescent intensity as compared with the manual operation (data not shown).

Discussion

In this report, we have demonstrated that our method mediated by adapter-ligation has reliability and performance for SNP typing. The key feature of the present method is to utilize a sticky end ligation to attain highly reliable discrimination of a single base alteration. This allowed ligation between the sample and the reference DNAs with annealing length as short as two nucleotides. Therefore, the reaction condition for highly efficient ligation could be used without losing discrimination efficiency.

A DNA fragment containing a target SNP site was amplified by two steps PCR using the primer sets that was designed to amplify the SNP loci at first PCR and introduce *Bse*RI recognition site for the generation of two bases overhang including the SNP site at second PCR. The usage of first PCR reduced the amount of genomic DNA samples as template and brought good reproducibility of the PCR and SNP typing. In this work, all PCR for 15 SNPs yielded the amplified fragments with expected sizes for all individuals.

Other type IIS restriction enzymes, *Bce*AI and *Fok*I, were successfully applied to this method as well. Of the three type IIS restriction enzymes, *Bse*RI and *Bce*AI, the two-bases overhang producers, showed better discrimination than *Fok*I, the five-bases overhang producer. There are 16 different sequences generated by two bases, thus, 32 adapters, which consist of two sets (labeled with two different fluorescent dyes) of 16 adapters can be universally used for any SNPs. In fact, we have used fifteen labeled adapters for thirteen SNPs typing reactions (Table I). For example, TA-Cy5 adapter was used at the typing reaction for

Table II. Summary of the SNPs and the results determined by the automated robotics

SNP No	dbSNP ID[a]	Chr location	Allele 1	Allele 2	Number of allele homozygotes	Number of heterozygotes	Number of allele 2 homozygotes
					Average of fluorescence ratio	Average of fluorescence ratio	Average of fluorescence ratio
Single SNP typing							
SNP225	rs12637772	3	A	G	31	45	19
					8.01 ± 1.87	1.09 ± 0.17	0.09 ± 0.03
SNP158	rs1857883	3	C	T	14	61	20
					12.76 ± 3.83	2.45 ± 0.39	0.11 ± 0.05
A003	rs1681752	10	C	T	41	41	13
					16.88 ± 10.91	1.98 ± 0.23	0.08 ± 0.07
A004	rs2627197	10	T	A	30	46	19
					8.80 ± 3.50	1.59 ± 0.25	0.10 ± 0.25
ANP337	rs2118207	10	T	C	29	49	17
					7.78 ± 3.06	1.10 ± 0.15	0.05 ± 0.04
SNP331	rs2531685_	10	G	C	15	49	31
					6.22 ± 3.43	0.93 ± 0.15	0.04 ± 0.03
SNP332	rs740600_	10	A	G	16	48	31
					4.47 ± 1.36	0.60 ± 0.10	0.05 ± 0.03
SNP334	rs1419832	10	A	G	16	48	31
					17.12 ± 4.41	1.40 ± 0.10	0.04 ± 0.02
SNP336	rs1905544	10	A	T	17	48	30
					25.00 ± 3.41	1.80 ± 0.15	0.03 ± 0.02
SNP657	rs4929798	10	C	G	4	34	57
					15.04 ± 1.76	1.46 ± 0.13	0.09 ± 0.04
SNP909[b]	rs1438506	10	T	A	60	30	4
					8.20 ± 4.25	1.63 ± 0.23	0.12 ± 0.08
SNP531	rs870947	10	G	C	3	32	60
					7.12 ± 2.51	0.91 ± 0.14	0.07 ± 0.05
SNP678	rs10736889	10	A	G	28	46	21
					12.24 ± 3.26	1.19 ± 0.15	0.04 ± 0.02
Duplex typing							
SNP334		10	G	C	10	10	10
					9.04 ± 1.31	1.02 ± 0.13	0.07 ± 0.03
SNP336		10	A	G	10	10	10
					8.34 ± 1.18	2.65 ± 0.23	0.43 ± 0.05

[a]The ID number of NCBI dbSNP database.
[b]Ninety-four individual samples were used for SNP909.

SNP225, A003 and SNP334. Two SNPs can be simultaneously analyzed in condition that four possible overhangs produced from the two SNP sites differ from each other. The sequence of the two bases overhang may be changed by adjusting the position of the SNP site to the distal or the proximal end of the two bases overhang. Combination of *Bse*RI and *Bce*AI, which produce two bases but 3′- and 5′-protruding overhangs, respectively, may be a straight forward solution in case an overlap of the sequences produced from the two SNP sites would be inevitable.

The present method requires two steps enzymatic reactions and removal of excess fluorescently labeled adapters after the reaction. We have overcome these disadvantages by applying our automated robot equipped with the Magtration® unit. The robots based on the Magtration® technology have been widely used for DNA purification in both laboratory (Deggerdal and Larsen, 1997; Leren et al., 1993) and clinical use (Sawakami-Kobayashi et al., 2003). The MagSNiPer FD takes approximately 8 h for analysis of 96 samples by eight-time sequential reaction of 12 samples. Recently, we have developed a high-throughput robot, SX-96G which is equipped with the 96 channels Magtration® unit and the protocol for the purification of dye terminator sequencing products (Sawakami-Kobayashi et al., 2003). Considering that the Magtration® technology have been used for purifying DNA from blood, slight modification of the robot used in this work will automate the entire process of SNPs typing from blood. We are currently developing a bench-top robot, MagSNiPer FD II, which is equipped with the 96 cannels Magtration® unit, an automated PCR unit and an automated fluorescence detection unit to process from the first PCR to the fluorescent detection in an automatic manner. Fully automated SNPs typing technology would be of great importance for research and medical diagnostics as well

DOI 10.1002/bit

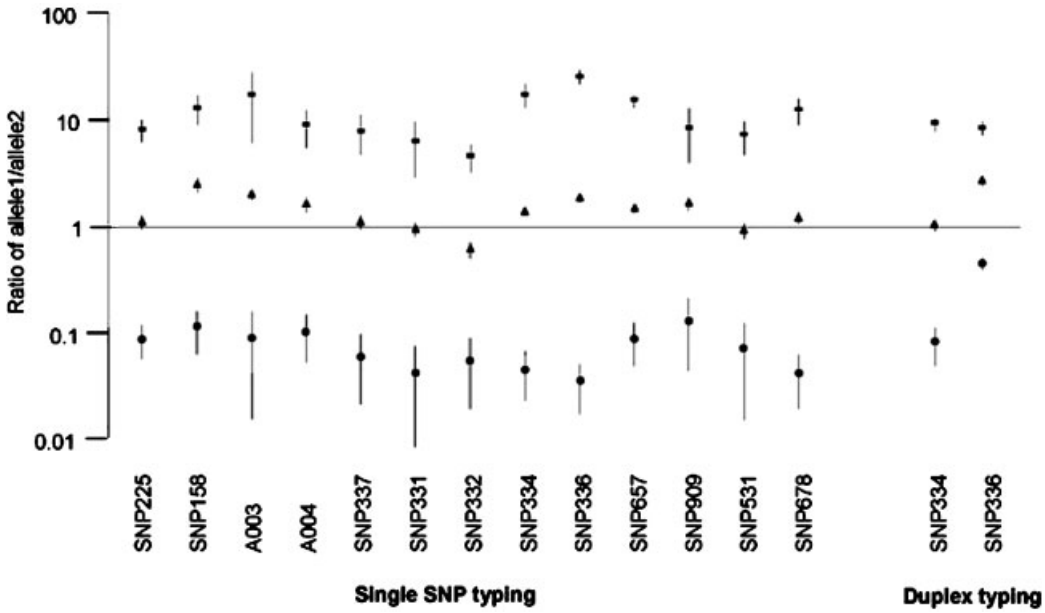

Figure 3. Average and distribution of the fluorescence intensity ratios by the automated reaction.The average and ±1SD of the samples from 95 individuals are plotted for the thirteen SNPs examined in the present work. Square, circle and triangle symbols correspond to the average ratios of two homozygous alleles and a heterozygote allele, respectively. SNP334 and SNP336 at the right side of the graph show the results of duplex SNPs typing. The reaction was performed by the automated robot as described in Materials and Methods.

as existing technologies including DNA microarray (Hacia et al., 1999), TaqMan and Invader methods, which have enabled large scale and high-throughput analysis of SNPs.

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DOI 10.1002/bit