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Analytical Biochemistry 341 (2005) 77–82
ANALYTICAL BIOCHEMISTRY
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MagSNiPer: A new single nucleotide polymorphism typing method based on single base extension, magnetic separation, and chemiluminescence

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Received 3 January 2005
Available online 25 March 2005

Abstract

We have developed a new method for typing single nucleotide polymorphisms (SNPs), MagSNiPer, based on single base extension, magnetic separation, and chemiluminescence. Single base nucleotide extension reaction is performed with a biotinylated primer whose 3′ terminus is contiguous to the SNP site with a tag-labeled ddNTP. Then the primers are captured by magnetic-coated beads with streptavidin, and unincorporated labeled ddNTP is removed by magnetic separation. The magnetic beads are incubated with anti-tag antibody conjugated with alkaline phosphatase. After the removal of excess conjugates by magnetic separation, SNP typing is performed by measuring chemiluminescence. The incorporation of labeled ddNTP is monitored by chemiluminescence induced by alkaline phosphatase. MagSNiPer is a simple and robust SNP typing method with a wide dynamic range and high sensitivity. Using MagSNiPer, we could perform SNP typing with as little as 10^{-17} mol of template DNA.

Keywords: Single nucleotide polymorphism; Magnetic separation; Chemiluminescence; Single base extension; Cytochrome P450

Single nucleotide polymorphisms (SNPs)[1] are the most abundant genetic variations in the human genome and appear on average once every 1.0–1.9 kb [1,2]. They are of medical and pharmacological interest in studies of disease susceptibility and drug response [3,4]. They are also regarded as ideal genetic markers for identifying genetic factors associated with common diseases due to their abundance and stability [5].

A number of techniques have been developed for typing SNPs, including restriction fragment-length polymorphism analysis, single-strand conformation polymorphism analysis [6], allele-specific oligonucleotide hybridization [7], allele-specific primer polymerase chain reaction (ASP–PCR) [8], oligonucleotide ligation assay [9], molecular beacon assay [10], TaqMan method [11], Invader assay [12], mass spectrometry [13], and pyrosequencing [14]. Using these technologies, a number of SNPs have been discovered. Recently, the International HapMap Project has started to determine common patterns of DNA sequence variation in the human genome by characterizing sequence variants, their sequence frequencies, and correlations between DNA samples from

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[1] *Abbreviations used:* SNP, single nucleotide polymorphism; ASP–PCR, allele-specific primer polymerase chain reaction; SBE, single base extension; B/W, binding and washing; S/N ratio, signal/noise ratio.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.03.015

populations with ancestries from parts of Africa, Asia, and Europe [15]. The project aims to genotype 600,000 SNPs spaced at approximately 5-kb intervals.

The availability of a large SNP database has enabled the diagnostic application of SNP typing, which in turn has emphasized the need for simple and robust methods of typing. To this end, we have developed a new SNP typing method, MagSNiPer, which is based on three commonly used methods: single base extension (SBE), magnetic separation, and chemiluminescence. SBE is an accurate and effective method of typing SNPs. Because of its high fidelity, it is used for various typing procedures [16–18]. To remove unincorporated ddNTPs or antibody conjugates, magnetic separation is adopted. Compared with centrifugation, magnetic separation enables a rapid and simple change of reaction buffers or reagents and facilitates automation. Because it is widely used for the purification of DNA from clinical samples, a fully automated SNP typing procedure would be realized by magnetic separation [19]. Chemiluminescence is widely used in immunoassays for clinical diagnosis due to easy handling and high sensitivity. The combination of SBE, magnetic separation, and chemiluminescence provides a new SNP typing method that is robust, highly sensitive, and suitable for automation.

Materials and methods

DNA sample preparation and SNP typing

Whole blood samples were obtained from 10 healthy adult volunteers with informed consent. Genomic DNA was prepared using a MagExtractor genomic DNA (Toyobo, Osaka, Japan). SNP types were determined by ASP–PCR using kits supplied by Toyobo (SNP Typing Kit cytochrome P450 2D6*2 and 2D6*10).

Oligonucleotide primers and reagents

Oligonucleotide primers for PCR amplification were 5′-TGTACCTCCTATCCACGTCA-3′ and 5′-CCTCG GCCCCTGCACTGTTT-3′ for CYP2D6*2 and 5′-CA GTCAACACAGCAGGTTCA-3′ and 5′-CCGAAAC CCAGGATCTGGGT-3′ for CYP2D6*10. SBE was performed using 5′ biotinylated primers: 5′-biotin-AAC GCTGGGCTGCACGCTAC-3′ for CYP2D6*10 and 5′-biotin-CAGGTCAGCCACCACTATGC-3′ for CYP 2D6*2. 5′-Biotin-AACGCTGGGCTGCACGCTAC- fluorescein-3′ was used for estimation of the sensitivity of MagSNiPer.

The PCR was performed using Ex *Taq* DNA polymerase (Takara Bio, Shiga, Japan). ThermoSequenase (Amersham Biosciences, Piscataway, NJ, USA) was used for SBE. Fluorescein-labeled ddNTP was a product of Perkin-Elmer (Wellesley, MA, USA), and ddNTPs were obtained from Amersham Biosciences. Fluorescein-labeled ddUTP was used for fluorescein-labeled ddTTP. Anti-fluorescein alkaline phosphatase conjugate and CDP–Star were obtained from Perkin-Elmer, and Tropix (Bedford, MA, USA), respectively.

PCR-amplified DNAs were purified using MagExtractor PCR (Toyobo) and were quantified using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA). The streptavidin-coated magnetic beads used were Dynabeads M-280 Streptavidin (Dynal, Oslo, Norway). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

PCR

PCR was performed using a thermal cycler (GeneAmp PCR System 9700, Applied Systems, Foster City, CA, USA). The reaction mixture (50 μl) contained 1 μl of human genome, 0.2 mM (each) dNTPs, 1× Ex *Taq* buffer, 1 μM of each primer, and 2.5 U of Ex *Taq* DNA polymerase. The reactions were run at 95 °C for 3 min, followed by 30 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. After the thermal cycling, the reaction mixtures were incubated at 72 °C for 10 min. PCR products were purified by MagExtractor PCR.

SBE, magnetic separation, and detection

SBE reaction was performed in a 10-μl reaction mixture containing DNA template, 1× sequencing buffer, 10 μM fluorescein-labeled ddNTP, 30 μM of the other three ddNTPs unlabeled, 1 μM biotinylated primer, and 1.6 U of ThermoSequenase. The reactions were carried out at 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

Streptavidin-coated magnetic beads (5 μl, Dynabeads M-280) were washed with 100 μl of 2× binding and washing (B/W) buffer (10 mM Tris–HCl, 1 mM EDTA, and 2.0 M NaCl, pH 7.5) and then resuspended in 80 μl of 1× B/W buffer. The SBE product (10 μl) was mixed with the streptavidin-coated magnetic beads at room temperature for 10 min. The beads were recovered and washed with 100 μl of 1× B/W buffer containing 0.1% Tween-20 twice by magnetic separation. After blocking the beads by incubation in 100 μl of TBSE-T (50 mM Tris–HCl, 100 mM NaCl, 2 mM EDTA, and 0.05% Tween-20) containing 5% skim milk for 10 min, anti-fluorescein alkaline phosphatase conjugate was added and the incubation was continued for 15 min at room temperature. The beads were washed with 100 μl of TBSE-T containing 5% skim milk three times and were applied for chemiluminescence detection after incubated in 100 μl of chemiluminescence substrate for alkaline phosphatase (CDP–Star) for 10 min.

SBE, magnetic separation by Magtration technology, and chemiluminescence detection were performed using

an automation system, MagSNiPer Station, which is equipped with a thermal cycler, an eight-channel tip dispenser for Magtration technology and a chemiluminescence detector (manuscript in preparation).

Results and discussion

MagSNiPer

We have developed a novel method of typing SNPs, MagSNiPer. Fig. 1 shows the typing procedure. After amplification of the genomic region containing the SNP, an SBE reaction with a specific biotinylated primer, whose 3′ end is contiguous to the SNP site, is performed on the PCR product in the presence of one tag-labeled ddNTP and the three other ddNTPs. The primers were captured on magnetic beads conjugated with streptavidin, and unincorporated ddNTPs were removed by magnetic separation. The magnetic beads were mixed with anti-tag alkaline phosphatase conjugates, and then excess conjugates were also removed by magnetic separation. Finally, the magnetic beads were incubated in chemiluminescence substrate for alkaline, and the chemiluminescence was measured.

Considering the sensitivity of chemiluminescence and the reliability of SBE, we can expect sensitive and reliable SNP typing using MagSNiPer. In addition, MagSNiPer employs magnetic separation to remove excess ddNTP or anti-tag alkaline phosphatase conjugates. Magnetic separation enables a rapid change of buffers or reagents, resulting in robust typing with a high signal/noise (S/N) ratio.

Typing of CYP2D6

To demonstrate MagSNiPer's typing capability, we performed SNP typing of the CYP2D6 gene, which encodes the cytochrome P450 debrisoquine 4-hydroxylase [20–22]. The gene is composed of nine exons and is located in the CYP2D6–8 cluster on chromosome 22 in association with the CYP2D7P and CYP2D8P pseudogenes [23,24]. The gene locus is highly polymorphic, with more than 70 known allelic variants. CYP2D6∗10, which involves a base substitution from C to T at position 100, has been reported to be relatively frequent in Japanese [25]. The phenotype of the homozygote for the CYP2D6∗10 allele is an intermediate metabolizer. CYP2D6∗2 involves two base substitutions (2850G to C and 4180G) and is generally distinguished by a base substitution at position 2850. It gives rise to only a slight decrease in metabolizing activity, and the phenotype of its homozygote is an extensive metabolizer.

DNA fragments containing SNP sites for CYP2D6∗2 and CYP2D6∗10 were amplified from genomic DNA of volunteers and purified. Then the fragments were applied for SNP typing by MagSNiPer. In this study, fluorescein-labeled ddNTP and anti-fluorescein alkaline

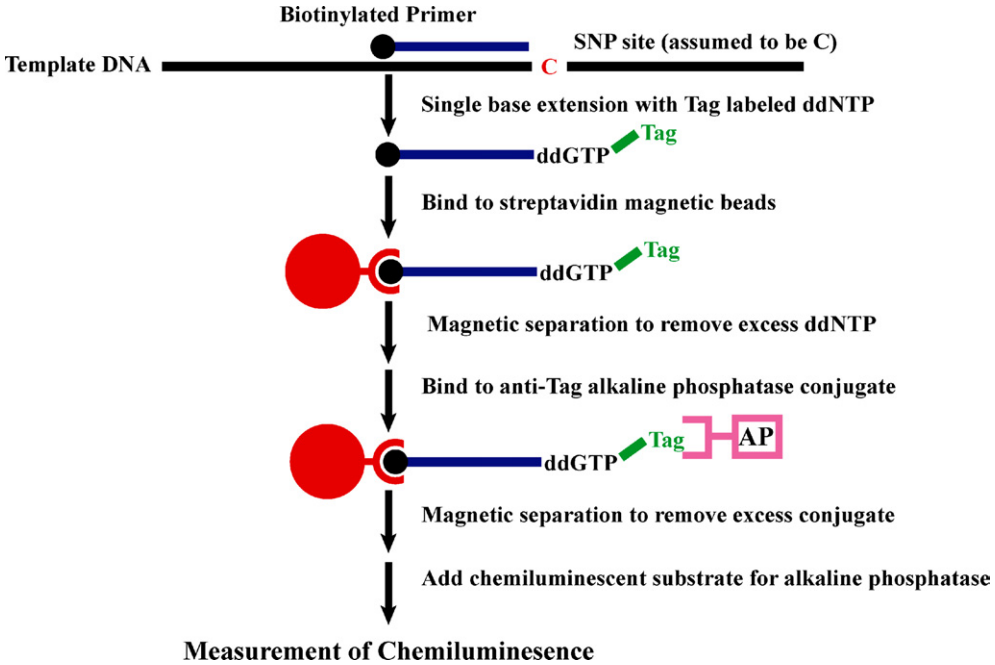

Fig. 1. Schematic diagram of SNP typing with MagSNiPer. SBEs are performed with biotinylated primers and tag-labeled ddNTP. The primers are captured by magnetic beads coated with streptavidin, and magnetic separation is performed to remove excess ddNTPs. The magnetic beads are then incubated with anti-tag alkaline phosphatase conjugate. To remove excess conjugate, magnetic separation is performed again, and the magnetic beads are incubated with a chemiluminescent substrate for alkaline phosphatase. Incorporated labeled ddNTPs are monitored by the measurement of chemiluminescence.

Table 1
SNP typing of CYP2D6 with MagSNiPer

Site	Genome	Chemiluminescence (a.u.)				Typing result	
		A	C	G	T	MagSNiPer	ASP–PCR
2D6∗10	1	79	729	72	829	C/T	C/T
	2	70	151	47	1120	T/T	T/T
	3	46	965	55	29	C/C	C/C
	4	53	526	47	905	C/T	C/T
	5	52	566	94	740	C/T	C/T
	6	52	1147	62	2306	C/T	C/T
	7	63	194	61	1815	T/T	T/T
	8	42	1994	64	24	C/C	C/C
	9	91	585	87	619	C/T	C/T
	10	103	1561	100	58	C/C	C/C
2D6∗2	1	1625	8644	1274	255	C/C	C/C
	2	1671	11,934	1465	324	C/C	C/C
	3	843	10,748	856	225	C/C	C/C
	4	1027	12,761	702	8165	C/T	C/T
	5	1004	10,286	937	154	C/C	C/C
	6	995	13,093	636	235	C/C	C/C
	7	1177	13,247	1137	414	C/C	C/C
	8	1103	7623	512	9998	C/T	C/T
	9	1349	11,796	904	8683	C/T	C/T
	10	1120	10,965	737	306	C/C	C/C

Note. The chemiluminescence obtained with the SBE product in the presence of each fluorescein-labeled ddNTP and the typing results are shown in the table. a.u., arbitrary units.

phosphatase conjugate were used. As shown in Table 1, each sample exhibits a clear typing result. In the results of 2D6∗10 genotyping, negative or background signals are very low. Their average value and standard deviation are calculated to be 72 and 37, respectively. The average of positive signals in 2D6∗10 genotyping is 1094, which is more than 10 times that of backgrounds. The data are very uniform among different template DNAs. On the contrary, the average value of positive signals in 2D6∗2 genotyping is 10,611, which is also more than 10 times that of negative signals, 851. The difference of the signal strength seems to reflect the amount of used DNA. In 2D6∗10 genotyping, 10 times diluted PCR products were applied for analysis. The high S/N ratio enables the unmistakable discrimination of SNP types.

Sensitivity and dynamic range of MagSNiPer

We have evaluated the sensitivity and dynamic range of MagSNiPer using an oligonucleotide primer labeled with biotin at the 5′ terminus and fluorescein at the 3′ terminus. The primer was captured by magnetic beads conjugated with streptavidin and then was used for detection. A linear relationship was observed between the chemiluminescence and the amount of labeled oligonucleotide from 10^{-18} to 10^{-14} mol or more (Fig. 2). The lower limit of detection is estimated to be approximately 10^{-19} mol.

In general, there exist 4500–8500 white blood cells in 1 μl of blood. This means that 10^{-20} mol DNA corresponds to the amount of genomic DNAs obtained from approximately 0.5 μl of blood. Therefore, if the sensitivity is higher than 10^{-19} mol, we will be able to perform SNP genotyping from only 5 μl of blood without PCR amplification.

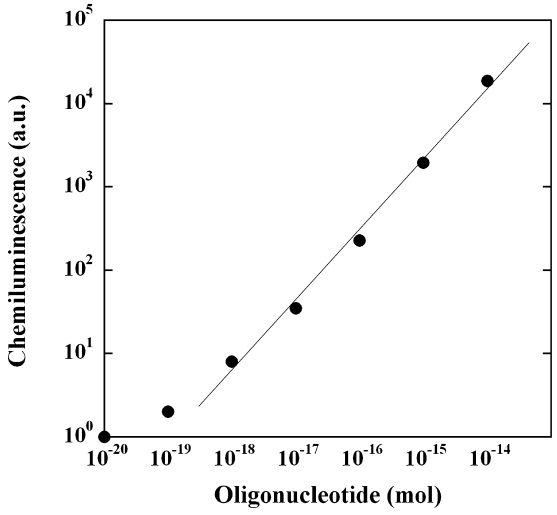

Fig. 2. Correlation between the amount of labeled oligonucleotide and chemiluminescence. The primer, 5′-bio-AACGCTGGGCTGCACGC TAC-fluorescein-3′, was used for detection by MagSNiPer. Chemiluminescence is plotted against the amount of primer used. a.u., arbitrary units.

Then we performed SNP typing using quantified PCR-amplified DNA. Fig. 3 shows the results obtained with 10^{-17} mol of target DNA. Even 10^{-17} mol of DNA gives clear typing results with a high S/N ratio. The S/N ratio was at least 3 (190/60 in Fig. 3A), which seems to be

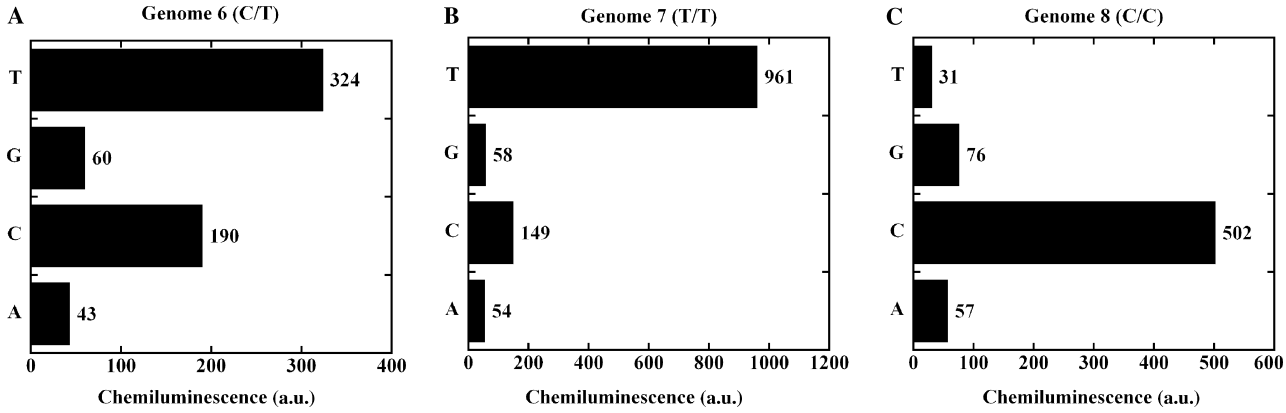

Fig. 3. SNP typing of CYP2D6∗10 using a small quantity of DNA. DNA fragments containing a CYP2D6∗10 SNP site were amplified from genomic DNA (A, 6; B, 7; C, 8) and were quantified with a PicoGreen dsDNA Quantitation Kit. A 10^{-17} mol concentration of PCR-amplified DNA fragment was used for SNP typing with MagSNiPer. a.u., arbitrary units.

enough to determine the genotype without statistical analysis. Compared with data shown in Table 1, the signals of negative samples are nearly the same, but the strength of positive signals is low. We had expected higher chemiluminescence because 30 cycles of primer extension were performed. But the chemiluminescence was nearly the same as that obtained with the same amount of labeled oligonucleotide (Fig. 2). Because it seems to be difficult to reduce the background chemiluminescence, we should optimize to conditions for primer extension to improve the sensitivity of MagSNiPer.

Previously, we developed a novel method for operating magnetic beads, Magtration technology, where the separation and resuspension of magnetic beads are performed within a disposable pipette tip [19]. Magnetic beads are captured on the inner wall of the disposable tip, and captured beads are resuspended in new buffer or are discarded with ease. This technology reduces cross-contamination between samples because all processes, including the suspension of magnetic beads, separation of magnetic beads, and resuspension of magnetic beads in new buffer, are performed within a disposable tip. Magtration technology has been widely used for the purification of genomic DNA for clinical applications [26] as well as for the purification of PCR-amplified DNA fragments. Moreover, this technology can be easily applied to automation because we need only add a mechanical magnetic separation unit to a dispenser. Thus, it enables fully automated typing from DNA purification to detection with ease.

Acknowledgments

The work reported here is part of the 21st Century Center of Excellence (COE) program of the "Future Nano-Materials" research and education project, which is financially supported by the Ministry of Education, Science, Sports, Culture, and Technology through Tokyo University of Agriculture and Technology.

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