Multiplex single nucleotide polymorphisms genotyping using solid-phase single base extension on magnetic nanoparticles

Hongna Liua, Song Li a,b, Lishang Liub, Lan Tian a, Nongyue He a,b,*

a State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China
b Hunan Key Laboratory of Green Packaging and Application of Biological Nanotechnology of Hunan Province, Hunan University of Technology, Zhuzhou 412008, China

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ABSTRACT

To fulfill the increasing need for large-scale genetic research, we have developed a new solid-phase single base extension (SBE) protocol on magnetic nanoparticles (MNPs) for multiplex SNP detection using adapter polymerase chain reaction (PCR) products as templates. Extension primers were covalently immobilized on the MNPs, and allele-specific extension took place along the stretch of target DNA for one-color ddNTP incorporation. The MNPs with fluorophores were spotted on a glass slide to fabricate a “bead array” to discriminate their genotypes. Eight SNP loci of three DNA samples were interrogated, and the experiment demonstrated that it is an efficient method for large-scale SNP genotyping.

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Single nucleotide polymorphisms (SNPs) 1 represent the abundant variation in the human genome. Genotyping large numbers of these SNPs in pathological samples should lead to insights into complex genetic traits, including many common human diseases [1]. Linkage and association analyses are generally used in these types of studies; thus, large-scale SNP typing has become necessary. However, multiplex SNPs must be genotyped by using limited amounts of DNA samples from both patients and normal subjects [2]. Up to now, a number of techniques have been reported in the literature for genotyping SNPs, but most of these methods are still difficult to analyze multiplex loci of different samples. To perform these large-scale genetic studies, efficient and highly parallel methods for genotyping SNPs are still required.

During recent years, use of nanoparticles has opened new dimensions in DNA detection. In particular, magnetic nanoparticles (MNPs) have emerged as a rapid and efficient separation tool in characterizing genetic variations because of their unique physical and chemical properties [3]. Previously, we developed some novel genotyping methods that were based on MNPs and dual-color hybridization, and we successfully demonstrated that SNPs of large amounts of samples could be efficiently and automatically genotyped using MNPs as DNA carriers. However, when using dual-color hybridization, the selectivity and quantification are dependent on the dissociation properties of target DNA hybridized with the allele-specific probes [4,5]. The difference in binding affinity between a perfectly matched probe and a one-base mismatched one is usually small; therefore, to achieve SNP discrimination, an optimized hybridization temperature and a stringent wash step are always needed.

Here we have developed a new approach that takes advantage of MNPs to fabricate “bead arrays” for multiplex detection of SNPs using solid-phase single base extension (SBE) as a platform. Currently, most of the multiplex SNP genotyping methods are still based on multiplex polymerase chain reaction (PCR) platforms. However, these multiplexing approaches are difficult to scale up because they are usually costly and laborious [6,7]. In this study, DNA template was prepared by adapter PCR, and it was demonstrated that adapter PCR-based solid-phase SBE is a flexible and reliable technology that can be adapted to multiplex SNP genotyping of DNA samples. It is hoped that this new method will have wide applications in clinical diagnostics.

A proof-of-concept scheme using solid-phase SBE followed by analysis was performed with MNPs to fabricate a bead array for SNP detection (see Fig. 1 in the Supplementary material), taking a T→C transition as an example. After amplification of DNA templates using adapter PCR, biotinylated extension primers were captured by streptavidin (SA)-coated MNPs and a solid-phase SBE reaction with the specific biotinylated primer was subsequently performed directly on the surface of MNPs in two separate tubes. One tube was used to detect the T allele signals (“W” tube) containing TAMRA–ddATP, and the other tube was used to detect the C allele signals (“M” tube) containing TAMRA–ddGTP. Finally, the genotype of each sample could be identified simultaneously by scanning the bead array printed with the DNA–MNP complexes. In principle, the MNPs from the W tube yielded strongly fluores-
cent signals, whereas the MNPs from the M tube yielded very low fluorescent signals, indicating that the TAMRA–ddATP could elongate with templates that showed homozygous wild-type samples. The homozygous mutant-type samples yielded a contrary result. For both kinds of TAMRA–ddNTP (dideoxynucleotide triphosphate) that could elongate with the templates, the heterozygote-type samples displayed strong fluorescences from both the W and M tubes.

Adapter PCR products were used as templates for SNP typing. Genomic DNA from three volunteers with informed consent were collected and then digested with the restriction endonuclease MseI. The cleaved ends of DNA were ligated to unphosphorylated adapters used as universal PCR primers. The adaptor sequences were made by hybridizing two oligonucleotides: H-24 (5'-AGGCAACTGTGCTATCCGAGGGAT) and H-12 (5'-TAATCCCTCGGA) (Sangon, Shanghai, China). The use of universal adaptors allows amplifying all target fragments in ligated DNA samples. Adaptor ligations and PCR reactions were performed as described previously [5]. The adaptor PCR products were stored at 4°C until use.

MNPs coated with colloid gold were prepared according to the reported method [8], and the average size of these MNPs was approximately 13 nm. The MNPs were functionalized with amino groups by reacting 20 mg MNPs with 20 mg/ml cysteamine solution in distilled water from which oxygen was removed for 24 h at room temperature. Then SA was covalently immobilized on the MNPs using glutaraldehyde as a linker between the amine group of cysteamine on the MNP surface and SA [4]. The SA-coated MNPs (SA–MNPs) were finally dispersed in PBS buffer with a concentration of 4 mg/ml and stored at 4°C.

In this research, we selected eight SNP loci from six genes: AGT (angiotensinogen), MTHFR (ethylenetetrahydrofolate reductase), MTR (5-methyltetrahydrofolate-homocysteine methyltransferase), COX-2 (cyclooxygenase-2), E-cadherin (epithelial cadherin), and IL-8 (interleukin 8). Eight biotinylated extension primers for the eight different polymorphic sites were designed for solid-phase SBE reactions (see Table 1 for sequences). Primer lengths were scaled up and down to find the equal annealing temperature for the extension step. MNP-bound primers were prepared using all 24-pmol biotinylated extension primers covalently immobilized onto 80 μg of SA–MNPs. Solid-phase SBE reactions, using either wild- or mutant-type TAMRA–ddNTPs, were performed simultaneously in 20 μl of reaction mixture (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 2.0 mM MgCl2, 60 μM of the other three ddNTPs unlabeled, 1.5 U of Taq DNA polymerase (TaKaRa, Japan), and 80 μg of MNP-bound extension primer) in two separate reactions. The reactions were carried out at 95°C for 5 min, followed by 35 cycles of primer extension at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. After the reaction, the DNA–MNP complexes were thoroughly washed with sterilized water (ddH2O) three times and then resuspended in 10 μl of sterilized ddH2O. To this end, MNPs from the W and M tubes were printed onto a cleaned glass slide to fabricate a bead array. After printing, the bead array was scanned with a 4100A microarray analysis system (Axon Instruments, Union City, CA, USA). The images acquired by the scanner were analyzed with GenePix Pro 6.0 software. For each fluorescent image, the average pixel intensity within each circle was determined and a local background using mean pixel intensity was computed for each spot. The net signal was determined by subtraction of this local background from the mean average intensity for each spot.

Here eight SNP loci from three human samples were analyzed. The fluorescent images obtained from the eight SNP loci and their relative fluorescent intensities are shown in Fig. 1. For example, in the polymorphism rs699, sample 1 (S1), yielding strongly fluorescent spots from the M tube, gave a fluorescence score of 11,586.5, while yielding very low fluorescent spots from the W tube, gave a...
fluorescence score of 235.25 near background, and the signal/noise ratio was 49.25. The results indicated that TAMRA-ddGTP elon-
gated with the template of sample 1, which showed homozygous mutant-type. Sample 3 (S3) is homozygous wild-type for which MNPs from the W tube gave a fluorescence score of 870.85, and those from the M tube gave a fluorescence score of 347.5, with a signal/noise ratio of 25.06. Sample 2 (S2) yielded strong fluorescent spots from both the W and M tubes, with fluorescence scores of 4657.75 and 4164.25, respectively, and a ratio of 1.12, indicating that this sample was heterozygote-type. So far as the other seven SNPs loci are concerned, the three allelic states for the loci were also very easily discriminated from Fig. 1. For all homozygous samples, we calculated the signal/noise ratios, which ranged from 21.87 to 76.01, and the fluorescent intensity ratios of heterozygote samples (W:M), which ranged from 0.82 to 1.22. The expected scores and good discrimination were obtained between the two alleles. The above results were validated by sequencing (data not shown).

In conclusion, we have successfully achieved multiplex SNP detection using MNPs as primer carriers, performing specific primers and one-color ddNTP incorporation of SBE reactions at an elevated temperature, avoiding the low discrimination of hybridization. Using this method, the signal/noise ratio for homozygous-type samples was more than 21, whereas the wild/mutant signal ratio for heterozygous samples was near 1.12, indicating that this sample was heterozygote-type. We also presented a new technology platform, the bead array, relying on detection of beads with fluorophores that can be easily controlled by using an external magnetic field. The acquisition of the reaction signals can be directly read out from the labels themselves, tremendously simplifying the detection process. On the other hand, all operations of genotyping can be easily controlled by using an external magnetic field and can be easily automated. Most important, when using adapter PCR products as DNA templates in solid-phase SBE, only a very small amount of DNA is required for multiplex SNP loci analysis and all of the templates can be amplified simultaneously in one adapter PCR, avoiding any complex multiplex PCR procedure [9]. Therefore, this multiplex SNP detection approach based on solid-phase SBE and bead array offers a low-cost, rapid, and flexible platform capable of high-throughput assays that are increasingly needed. This method is ideal for applications such as diagnostic detection of disease genes from clinical samples and screening of characteristic marker genes from many biological systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2008.11.035.

References


Table 1

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