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Multicolor-based discrimination of 21 short tandem repeats and amelogenin using four fluorescent universal primers

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ABSTRACT

The aim of this study was to develop a cost-effective genotyping method using high-quality DNA for human identification. A total of 21 short tandem repeats (STRs) and amelogenin were selected, and fluorescent fragments at 22 loci were simultaneously amplified in a single-tube reaction using locus-specific primers with 24-base universal tails and four fluorescent universal primers. Several nucleotide substitutions in universal tails and fluorescent universal primers enabled the detection of specific fluorescent fragments from the 22 loci. Multiplex polymerase chain reaction (PCR) produced intense FAM-, VIC-, NED-, and PET-labeled fragments ranging from 90 to 400 bp, and these fragments were discriminated using standard capillary electrophoretic analysis. The selected 22 loci were also analyzed using two commercial kits (the AmpFLSTR Identifiler Kit and the PowerPlex ESX 17 System), and results for two loci (D19S433 and D16S539) were discordant between these kits due to mutations at the primer binding sites. All genotypes from the 100 samples were determined using 2.5 ng of DNA by our method, and the expected alleles were completely recovered. Multiplex 22-locus genotyping using four fluorescent universal primers effectively reduces the costs to less than 20% of genotyping using commercial kits, and our method would be useful to detect silent alleles from commercial kit analysis.

Universal fluorescent polymerase chain reaction (PCR) is a cost-effective method for producing amplified fragments labeled by fluorescent dyes to enable genotyping of genetic variations [1]. In universal fluorescent PCR, fluorescent universal primer sequences are added to the 5′ termini of forward primers as universal tails. A two-step amplification procedure is conducted using combinations of forward primers with universal tails, reverse primers, and fluorescent universal primers. Intermediate products with universal tails are amplified in the first step, and these products are then fluorescently labeled using fluorescent universal primers (see Fig. 1). The amplification using two fluorescent universal primers can discriminate ≤40 alleles such as single nucleotide or insertion/deletion polymorphisms [2,3]. Although combinations of four universal primers have been also designed [4], the numbers of alleles that are detectable in simultaneous single-tube reactions have not been indicated.

Short tandem repeat (STR) genotyping is a powerful tool for forensic identification. During recent years, the number of STR targets has been expanded in both the Combined DNA Index System (CODIS) and the European Standard Set (ESS). Additional STRs beyond CODIS and ESS have also been analyzed in various populations [5–7], and these STRs can enhance discrimination of kinship relationships [8]. Twenty or more STR loci can be simultaneously analyzed by a single-tube amplification of fluorescent fragments using commercial kits and by discrimination of up to five fluorescent dyes of amplified fragments on subsequent electrophoresis. Intense signals from fluorescent fragments are expected using optimized concentrations of DNA (0.5–1.0 ng/reaction). However, STR analysis has also been performed in criminal investigations using postmortem degraded DNA [9] or low template DNA [10]. Moreover, identifying casework samples can be

Abbreviations used: PCR, polymerase chain reaction; STR, short tandem repeat; RFU, relative fluorescence units.

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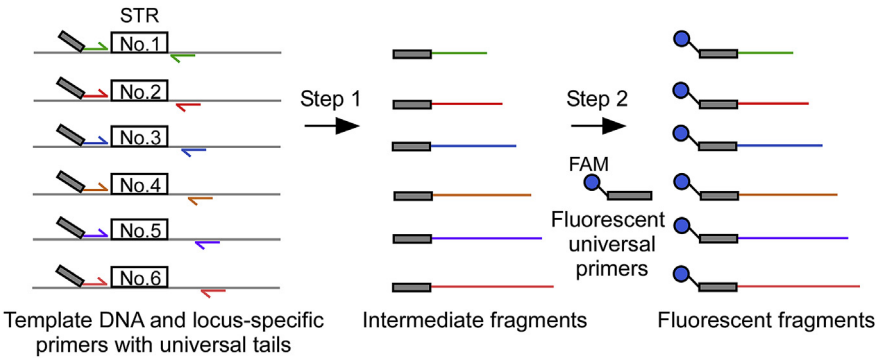

Fig.1. Fluorescent labeling of amplification products using fluorescent universal primers. Intermediate products with universal tails are amplified using locus-specific primers with universal tails. These products for up to 6 loci are labeled using single fluorescent universal primer. In this study, four fluorescent universal primers were designed, and fluorescent fragments at 22 loci were simultaneously amplified in a single-tube reaction using four fluorescent universal primers.

performed using reference samples of single-source high-quality DNA; at least 50 ng of reference sample DNA may be routinely collected from buccal cell samples. However, multi-locus fragments amplified using commercial kits are commonly labeled using forward or reverse primers conjugated with fluorescent dyes. Therefore, these methods can be expensive, depending on the number of STR loci targeted.

We have developed a cost-effective method for genotyping multiple STR loci using four fluorescent universal primers that have differences at three or four nucleotide bases. Fluorescent fragments from 22 loci were amplified in a single-tube reaction and discriminated by capillary electrophoretic analysis. The genotypes at 22 loci were determined from 100 samples by our method, and we compared these data with the profiles obtained from two commercial kits.

Materials and methods

Samples

Buccal cells were collected from 100 healthy Japanese individuals (81 male and 19 female), and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA concentrations were determined using a Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA). This study was approved by the ethics committee of Asahikawa Medical University.

Design of primers

We selected 21 of the autosomal STR loci targeted by the AmpFLSTR Identifiler Kit (Applied Biosystems) and PowerPlex ESX 17 System (Promega, Madison, WI, USA) and an amelogenin locus. Reference sequences were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Approximately 300–600 bases that included the repeat structures were targeted to design locus-specific primers using a web tool (Primer3). The product size ranges were limited to between 70 and 500 bases. Locus-specific primers for the 22 loci were divided into four groups (groups 1, 2, 3, and 4).

We designed combinations of four universal tails and fluorescent universal primers. A 24-base universal M13(-47) sequence was used as a standard sequence, and three sequences were designed with three or four nucleotide substitutions in the middle and at the 3′ terminus (Table 1), based on a previous report [11]. The four universal tails were added to the 5′ termini of the forward primers in each group. Four fluorescent universal primers were also designed using the universal tail sequences and labeled with the fluorescent dyes (FAM, VIC, NED, and PET).

Multiplex PCR conditions

Each reaction (total volume = 20 μl) contained 0.1 μM locus-specific primers, genomic DNA, fluorescent universal primers, and the reagent from the multiplex PCR kit (Qiagen) at 1 × concentration. Reaction conditions were as follows: 95 °C for 15 min; 27 or 28 cycles of 94 °C for 60 s, 62 °C for 60 s, 72 °C for 60 s; and a final extension at 72 °C for 60 min.

Sensitivity was evaluated for different concentrations (0.125–0.75 μM) of fluorescent universal primers and different amounts of template DNA (0.5–10 ng) from 20 selected individuals. Target sequences of the 22 loci were amplified using optimized concentrations of fluorescent universal primers (0.375 μM FAM-labeled primer, 0.25 μM VIC-labeled primer, 0.75 μM NED-labeled primer, and 0.375 μM PET-labeled primer) and 2.5 ng of DNA from 100 individuals.

Allelic ladders

We performed a singleplex PCR to generate fluorescent fragments of representative alleles at each locus using DNA from the Japanese individuals. Each reaction (total volume = 20 μl) contained 0.25 μM of each locus-specific primer, 4 ng of genomic DNA, and the reagent from the multiplex PCR kit (Qiagen) at 1 × concentration. Reaction conditions were as follows: 95 °C for 15 min; 32 cycles of 94 °C for 60 s, 60 °C for 60 s, 72 °C for 60 s; and a final extension at 72 °C for 60 min. The allelic ladder was a mixture of fragments from each locus, diluted to enable genotyping of DNA samples.

Detection of PCR products

PCR products were purified using a NucleoSpin Gel and PCR Clean-up Kit (Takara, Otsu, Japan). A 1-μl sample of purified PCR product was added to 10 μl of Hi-Di Formamide (Applied Biosystems) and 0.3 μl of GeneScan 600 LIZ Size Standard version 2.0 (Applied Biosystems). Detection of PCR products was performed on a 3500 Genetic Analyzer (Applied Biosystems) with a 36-cm capillary and performance-optimized polymer (POP4). Data were analyzed using GeneMapper software (Applied Biosystems). Peak heights are represented as relative fluorescence units (RFU), and a peak detection threshold of 100 was used. The bins and panels for the multiplex system were programmed for genotyping.