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The art of traditional native PAGE: The APLP 48-ID assay for human identification

Kazuo Umetsu [a,*], Isao Yuasa [b], Masaki Hashiyada [c], Noboru Adachi [d], Gotaro Watanabe [e], Toshihiro Haneda [a], Kentaro Yamazaki [a]

[a] Department of Forensic Medicine, Yamagata University School of Medicine, Yamagata 990-9585, Japan
[b] Division of Legal Medicine, Faculty of Medicine, Tottori University, Yonago 683-8503, Japan
[c] Department of Legal Medicine, Kansai Medical University, Hirakata 573-1010, Japan
[d] Department of Legal Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi 409-3898, Japan
[e] Forensic Science Laboratory, Yamagata Prefectural Police Headquarters, Yamagata 990-8577, Japan

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ABSTRACT

When full STR profiles cannot be obtained, further DNA analyses targeting single nucleotide polymorphisms (SNPs) may occasionally yield valuable information. Although the discrimination power of each SNP is relatively low, combined analysis of many SNPs can improve the personal identification ability to a level as high as that of commercial STR typing kits. In this study, we developed a new SNP typing method, named the amplified-product length polymorphism (APLP) 48-ID assay, for genotyping of 47 autosomal SNPs and two X and Y chromosomal markers for sex typing. Forty-seven SNPs were selected from all 22 autosomes, showing high diversity in European, Nigerian, Han Chinese, and Japanese population in the HapMap data. PCR primers were designed to generate amplicons 40–100 bp in length to increase the robustness of the PCR.

The APLP 48-ID assay consisted of four independent PCR reactions followed by electrophoretic run on four lanes in a polyacrylamide gel. Complete profiles were obtained when more than 1.2 ng of DNA was used. We applied this assay for genotyping of 236 Japanese individuals. The random matching probability was 3.3E-20, and the power of exclusion was greater than 0.9999999. This method is a rapid, robust, and cost-effective approach for human identification and paternity testing.

1. Introduction

Because of its highly polymorphic nature, short tandem repeat (STR) genotyping has been widely used as a powerful tool for human personal identification and paternity testing [1,2]. It is generally agreed that smaller amplicons are much more likely to be amplified in samples containing highly degraded DNA [3,4]. Because the product sizes of STR-based markers may be up to 400 bp, STR profiling sometimes fails when DNA is highly fragmented.

In general, single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms (INDELs) detected using PCR-based methods that generate amplicons shorter than the PCR products of STRs [5]. Although the discrimination power of each SNP is low, a combined analysis of many SNPs can increase the discrimination power to a level as high as that of STRs. Moreover, as an overwhelming number of SNPs have already been reported, selection of those that are suitable for personal identification is easier than for STRs and INDELs. For genotyping of SNPs, excellent methods such as SNaPshot single base extension system [6,7], Genplex SNP typing system [8], Digitag assay [9], and HID-Ion AmpliSeq Identity Panel [10] have already been established. However, these methods require expensive fluorescent dyes and detection equipment, and are time-consuming.

We previously developed a multiplex SNP typing method, termed the amplified-product length polymorphism (APLP). This method uses two allele-specific primers, one of which has a few non-complementary bases at the 5′ end, to detect SNPs as the difference in length of amplicons by subsequent PCR and electrophoresis. The APLP method has been successfully applied to ABO genotyping [11] and haplogrouping of modern and ancient mitochondrial DNA [12,13].

Here, we present a novel APLP system, named the APLP 48-ID assay, for human identification that analyze 47 unassociated SNPs selected carefully from all autosomes together with two XY sex

* Corresponding author.
E-mail address: kumetsu@med.id.yamagata-u.ac.jp (K. Umetsu).

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markers. To achieve simple and economical SNP genotyping, we employed traditional native PAGE [14,15] which does not require fluorescent primers and the expensive apparatus.

2. Materials and methods

2.1. DNA samples

Genomic DNA was extracted from blood or buccal cells from 236 healthy unrelated Japanese living in Yamagata prefecture (Northeast Japan) with informed consent. This study was approved by the Ethics Committee at Yamagata University School of Medicine. DNA was prepared from the samples according to the standard protocol. The quantity of DNA was determined using spectrophotometry.

2.2. SNP selection and primer design

Although an enormous number of SNPs are known, highly polymorphic SNPs, shared by many populations around the world, are relatively limited. To select SNPs for personal identification, we meticulously searched the HapMap database (http://www.hapmap.org). The criteria for selection of SNPs were as follows: (1) transversion to avoid misclassification of bases arising from deamination in highly degraded samples; (2) a minimum allele frequency of >0.25 in European, Nigerian, Han Chinese, and Japanese populations; (3) physical distance of >55 Mb between SNPs to avoid linkage; (4) design of primers with a similar Tm value (about 55–57 °C). The SNPs examined in the present study do not overlap with those examined in the previous studies [7,8]. Primers were manually designed to generate 40–100 bp of amplicons for analysis of highly degraded samples. Similarly, sex chromosomal markers were selected for sex typing. We divided primer candidates into four groups. All primer candidates in each group were checked for primer-dimer and hairpin structures using AutoDimer software (http://www.cstl.nist.gov/biotech/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm). Finally, we selected 47 SNPs on all autosomes and XY markers in the sex-determining region Y (SRY) and androgen receptor (AR) genes, and then we incorporated them into four separate 12-plex sets (A/B/C/D), based on the compatibility of the primers (Supplementary Table 1).

2.3. Multiplex APLP reaction and electrophoresis

Reaction for each set were performed in a 7.5-μl mixture containing 2–30 ng genomic DNA, 0.5 μM each primer (Supplementary Table 1), and 1× Platinum Multiplex PCR Master Mix (Life Technologies, Carlsbad, CA). Thermal cycling was performed in a GeneAmp PCR System 9700 (Life Technologies). A two-step PCR method was employed: 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 2 min, followed by a final extension step of 20 min at 60 °C.

Following multiplex PCR, native PAGE was performed according to the previously published protocols [12] with slight modifications. In brief, the separating gel (total acrylamide concentration = 10%, cross-linking rate = 5%, 65 mm long, 140 mm wide, 1 mm thick) were prepared containing 0.5% (v/v) EnhanceIT® polymer solution (10×) (Elchrom Scientific, Cham, Switzerland) and 375 mM Tris–HCl buffer (pH 8.9). The use of EnhanceIT® polymer solution improved the separation of the bands. The stacking gel (3.125%T, 5%C) consisted of 62.5 mM Tris–HCl buffer (pH 6.7). Three microliters of PCR products were loaded into the wells on a Wide-Mini BE-250 electrophoresis system (BIO CRAFT, Tokyo, Japan), and the gel was run for 60 min at a constant power supply of 5 W (room temperature) with running buffer (12.5 mM Tris, 96 mM glycine; pH8.3). After the migration, the DNA bands were stained with SYBR Green I (Life Technologies). All APLP analyses were performed at least twice to confirm reproducibility. Moreover, the results of SNP analysis by using our assay were confirmed by the direct sequencing of each SNP site in at least three individuals by using BigDye Termintor v.1.1 cycle sequencing kit (Life Technologies).

2.4. Sensitivity study

To determine the minimum amount of DNA required, we used a commercially available cell line male DNA 9948 (Promega Corporation, Madison, WI) containing 10 ng, 1 ng, 300 pg, and 50 pg of DNA. The duplicate tests were performed with the same DNA samples.

2.5. Analysis of degraded DNA samples

Artificially degraded DNA was used to assess the effectiveness of our APLP 48-ID assay in forensic and archaeological analyses. The artificially degraded DNA was prepared by incubating fresh blood DNA (10 μg) with DNase I. DNase-treated DNA and a standard 100 bp ladder marker were run on agarose gel followed by staining with ethidium bromide. The gel strip containing the DNA with various sizes of about 30–100, 100–200, 200–300, 300–400, 400–500, and 500–1000 bp was cut from the gel based on the location of the bands of a 100 bp ladder marker, followed by purification of DNA on a silica-based column. The quantity of recovered DNA was determined using spectrophotometry. Using this mock forensic DNA sample, the effectiveness of our method for degraded samples was compared with that of an STR typing method (*AmpFl*STR Identifiler Plus amplification kit and the ABI Prism 310 Genetic Analyzer, Life Technologies). The PCR and the STR analysis were performed as recommended by the manufacturer.

2.6. Statistical analysis

Forensic statistical parameters of 47 SNPs, including the mean match probability, combined power of discrimination, combined exclusion probability, and typical paternity index (trios) were calculated, based on the allele frequencies in each population group (Japanese and HapMap data). The statistical values were calculated with the Arlequin v3.5 software package [16] and PowerStats ver1.2 spreadsheet [17].

3. Results

Forty-seven SNPs fulfilling the criteria described in Section 2.2 were selected from the HapMap genome browser. Since no fluorescent dyes were used in this assay, it was impossible to perform 48-plex PCR amplifications for 47 SNPs and sex markers in one tube. The genotyping was done by four separate 12-plex PCR amplifications per sample followed by electrophoresis on four lanes. A drawback of multi-tube reactions is the need for excess DNA. All the results of this assay were confirmed in all experiments performed in the present study. The results of typing ($n = 3$) were 100% identical to those of direct sequencing. In automated fluorescent typing, a relative fluorescence unit cut-off value is a useful threshold for interpretation. On the other hand, genotyping in the present assay is simpler than that of the other methods, as the bands in question can be genotyped by comparing them with the corresponding bands in the adjacent lanes in a photograph. We analyzed 236 Japanese individuals using our 48-ID assay