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TECHNICAL NOTE

CRIMINALISTICS

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Rapid Direct PCR for ABO Blood Typing*

ABSTRACT: Many different molecular typing methods have been reported to complement routine serological ABO blood typing in forensics. However, these ABO genotyping methods are often time-consuming and call for an initial DNA isolation step that requires the use of expensive kits or reagents. We report here a rapid direct ABO genotyping method that eliminates the need for DNA extraction from fresh blood, hair, and body fluid stains before PCR. Using a fast PCR instrument and an optimized polymerase, the genotyping method—which employs a multiplex allele-specific primer set for the simultaneous detection of three single-nucleotide polymorphism (SNP) sites (nucleotides 261, 526, and 803)—identifies A, B, O01/O02, O03, and cis-AB01 alleles in around 70 min from sample collection to electropherogram. Not only will this ABO genotyping method be efficiently used in forensic practice for rapid screening of samples before full-blown multilocus short tandem repeat profiling, but it will also demonstrate an example of rapid direct genotyping of SNPs that offers the advantages of time- and cost-efficiency, convenience, and reduced contamination during DNA analysis.

KEYWORDS: forensic science, DNA typing, ABO genotype, rapid PCR, direct PCR, allele-specific primer

Since Yamamoto and colleagues (1) cloned ABO gene in 1990, many different molecular typing methods using DNA samples have been reported to complement routine serological ABO blood typing (2–9). ABO genotyping establishes the single-nucleotide polymorphism (SNP) information that determines the ABH antigen expression on the surface of the red blood cells (10). As 100 or more alleles exist for the four primary phenotypes (A, B, AB, and O) and the genotype does not always represent the phenotype, ABO genotyping can be considered to be prone to misinterpretation and to be no more effective than the hemagglutination technique. However, ABO genotyping of forensic specimens is of great value because it can be used as a rapid screen for samples before multilocus short tandem repeat (STR) profiling is performed. Exclusion at this screening level can save forensic laboratories much time and cost on DNA profiling. In addition, ABO genotyping can work efficiently even when serological test is not available, e.g., for identifying a decomposed body and blood group typing using body fluids of the nonsecretor. Therefore, if we could improve the PCR protocols of molecular genotyping methods that are often time-consuming and call for an initial DNA isolation step that requires the use of expensive kits or reagents, ABO genotyping will be used much more effectively with ease and robustness.

Here, we describe a rapid and robust ABO genotyping method directly using fresh blood, hair, and body fluid stains without prior DNA extraction. Using a fast PCR instrument and an optimized polymerase, the genotyping method—which employs a multiplex allele-specific primer set—can be performed in around 70 min from sample collection to allele designation.

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Materials and Methods

Samples

One hundred genomic DNA samples with known ABO sequences were obtained from a previous report (11). Fresh blood, hair, and buccal swab samples were obtained from 19 voluntary donors. All individuals gave their informed consent to participate, and the study protocol was approved by the institution's ethics committee. Blood and saliva stains were prepared by spotting the blood and by smearing buccal swabs onto a 2.5-cm-diameter Indicating FTA Classic Card (Whatman, Florham Park, NJ). 9947A standard DNA (Promega, Madison, WI), diluted to 1 ng, 500, 250, 125, 60, and 30 pg, was used to test the sensitivity of multiplex allele-specific PCR for ABO genotyping.

Design of Multiplex Allele-specific PCR Primers for ABO Genotyping

A multiplex allele-specific primer set was developed by first selecting three SNPs (nucleotides 261, 526, and 803) from exons 6 and 7 of the ABO locus to identify the A, B, O01/O02, O03, and cis-AB01 alleles (Fig. 1). Using the BatchPrimer3 program (12), two allele-specific primers and a shared fluorescence-labeled primer were designed for each SNP (Table 1). The 3′ end of an allele-specific primer was specific to one of the two alleles of a SNP (see bold), and an additional mismatch was included at the third or other position from the 3′ end (see underlined) to enhance the specificity in the allele-specific PCR. Common fluorescence-labeled primers did not have mismatches. One of the two allele-specific primers (i.e., ABO261G-F, ABO526G-F, and ABO803C-R) was

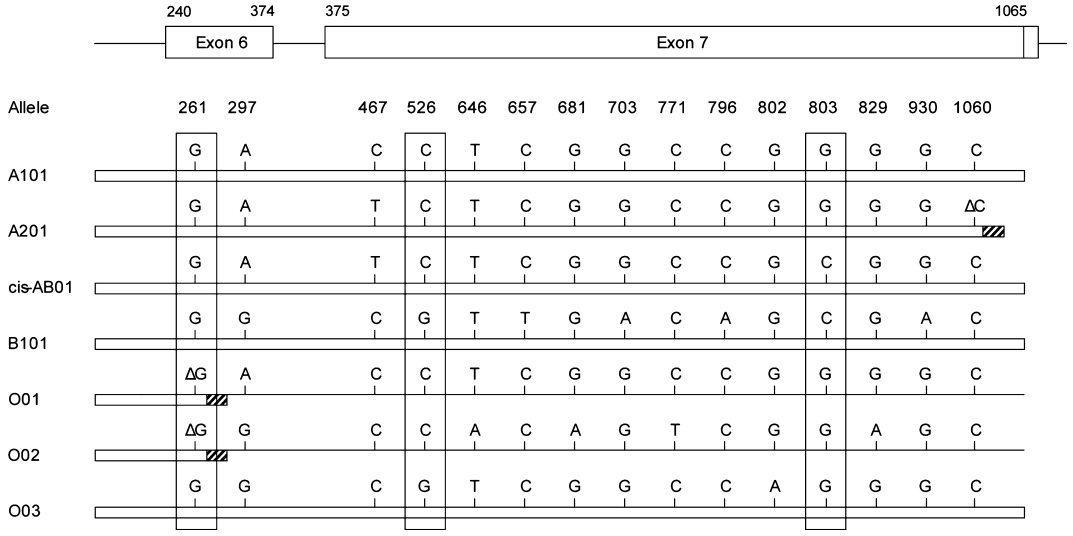

FIG. 1—*Representative alleles at the ABO locus. Nucleotide sequences at discriminative single-nucleotide polymorphism (SNP) sites are compared among various ABO alleles. Assessment of three SNPs at nucleotide 261, 526, and 803 revealed five alleles (A, B, O01/O02, O03, and cis-AB01). Nucleotide deletion is indicated by "Δ."*

TABLE 1—*A multiplex allele-specific primer set for rapid direct PCR for ABO blood typing.*

SNP		Primer	Sequence (5′-3′)*	PCR Size (bp)	Concentration (μM)
261	ΔG	ABO261d-F	AGGAAGGATGTCCTCGTGTT**A**C	98	0.37
	G	ABO261G-F	ttaAGGAAGGATGTCCTCGTTGT**G**	102	0.45
	–	ABO261-R	6FAM-GTTCTGGAGCCTGAACTGCT	–	0.82
526	C	ABO526C-F	AGCTGTCAGTGCTGGAGATG**C**	127	0.21
	G	ABO526G-F	ttatGCTGTCAGTGCTGGAGGAG**G**	130	0.19
	–	ABO526-R	6FAM-TCCACGCACACCAGGTAATC	–	0.40
803	G	ABO803G-R	CCGACCCCCCGAAGTAC**C**	154	0.27
	C	ABO803C-R	atatCCGACCCCCCGAAGATC**G**	158	0.30
	–	ABO803-F	6FAM-GAGATCCTGACTCCGCTGTT	–	0.57

*Two alleles of a single-nucleotide polymorphism (SNP) at the 3′ end of allele-specific primers are indicated in bold; additional mismatches included at the third or other position from the 3′ end to enhance the specificity in the allele-specific PCR are underlined; the tails inserted at the 5′ end of one of the two allele-specific primers to produce amplicons of different sizes are written in lower case.

modified to have a tail at its 5′ end, thereby allowing different alleles to produce amplicons of different sizes. Through screening PCR amplifications, the final set of primers was selected from several candidate primer pairs based on high amplification efficiency and specificity for SNPs. In addition, the absence of cross-reactivity of each primer pair for other species (e.g., chimpanzee, cat, and dog) was confirmed using in-silico PCR of the Human Genome Browser at UCSC (http://genome.ucsc.edu/cgi-bin/hgPcr) (13).

The performance of this multiplex allele-specific primer set was assessed by analyzing 100 genomic DNA samples with known ABO sequences. The sensitivity of the process was tested using 9947A standard DNA, which has a heterozygotic peak pattern at all of the three SNP sites, serially diluted from 1 ng to 30 pg and used for PCR.

Rapid Direct PCR for ABO Genotyping

Rapid direct PCR using fresh blood was performed with reaction mixtures consisting of 1 μL of whole blood, 2.5 U of Phire Hot Start DNA Polymerase (Finnzymes, Espoo, Finland), 4.0 μL of 5× Phire Reaction buffer (Finnzymes), each dNTP at 200 μM (Applied Biosystems, Foster City, CA), and each primer at the appropriate concentration in a total volume of 20 μL (Table 1). When using hairs, a hair root was directly placed in the PCR mixture. If a 1.2-mm-diameter punch of blood or saliva stain was used, a punch was washed twice with 20 μL of water at 50°C for 3 min and then PCR components were added directly to the rinsed punch after removing the water. Thermal cycling was performed in a Veriti 96-well thermal cycler (Applied Biosystems) using the following conditions: 98°C for 5 min for cell lysis and initial denaturation; 30 cycles at 98°C for 5 sec, 65°C for 10 sec, and 72°C for 10 sec; and 72°C for 1 min. All amplification reactions were accompanied by negative (no template DNA) and reagent blank controls for DNA extraction and PCR amplification. The obtained PCR products were then mixed with GeneScan-400HD (ROX) Size Standard (Applied Biosystems) and analyzed by capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and GeneScan 3.1 software (Applied Biosystems). The genotyping of PCR products at each locus was performed using Genotyper 2.5 software (Applied Biosystems) and an allelic ladder.

Results and Discussion

The multiplex allele-specific primer set utilized in the present study demonstrated sensitivity and accuracy in scoring three SNPs (nucleotides 261, 526, and 803) to identify five alleles (A, B, O01/O02, O03, and cis-AB01) (Fig. 2). It produced reliable results when the amount of DNA was as low as 60 pg, with no allelic drop-in or drop-out. The accuracy of the genotype discrimination was confirmed by concordance between ABO genotyping using this multiplex allele-specific primer set and the previous sequencing results of 100 genomic DNA samples (11, 23, 9, 16, 30, and 11 samples for

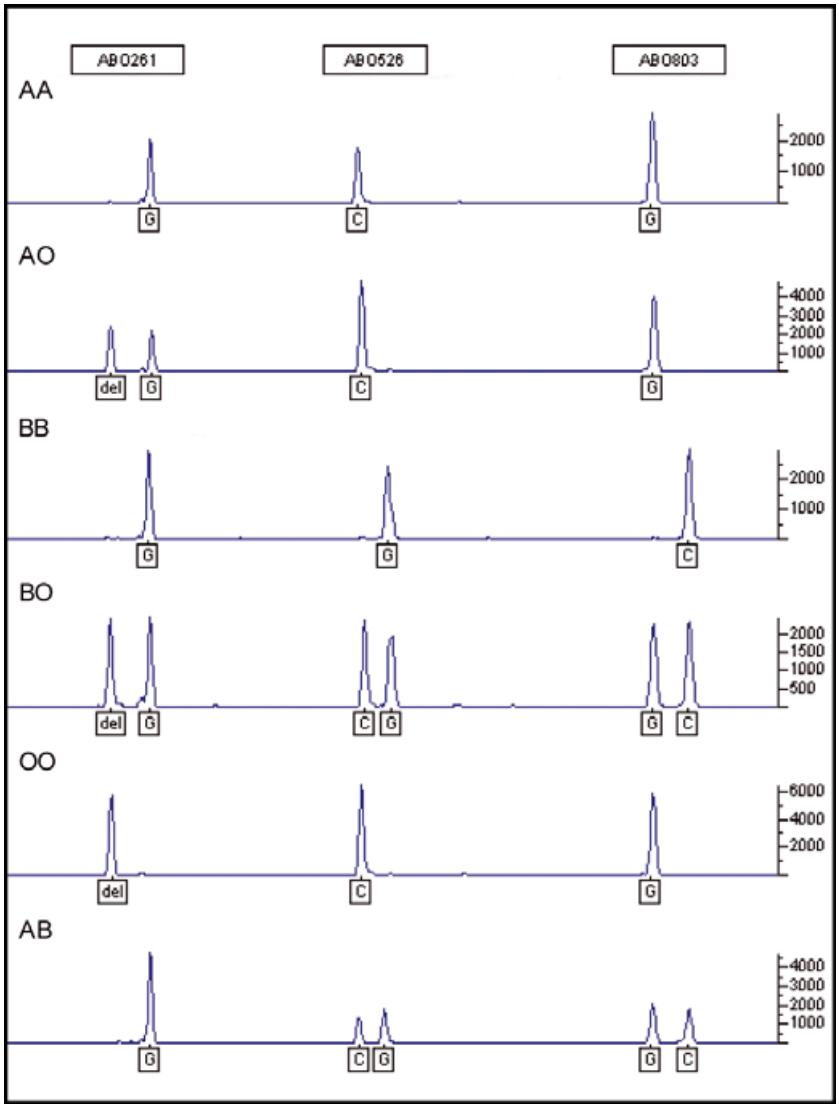

FIG. 2—*Electropherograms of six common ABO genotypes in a Korean population: AA, AO, BB, BO, OO, and AB. Using allele-specific primer pairs that have been modified at the 5′ end, the multiplex PCR set of this study facilitates ABO genotyping while allowing different alleles to produce amplicons of different sizes. Nucleotide deletion at 261 is indicated by ''del.''*

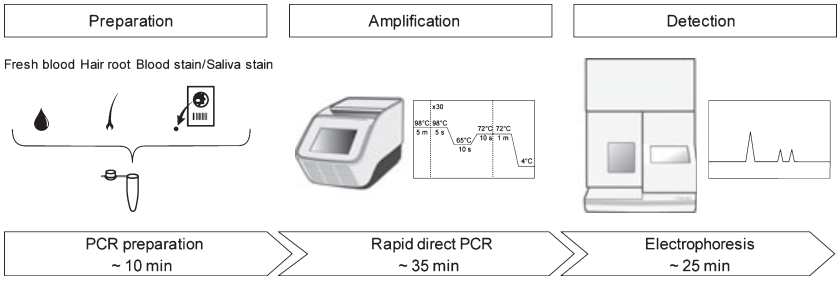

FIG. 3—*Diagram showing the rapid direct PCR protocol for ABO blood typing from sample collection to electropherogram.*

AA, AO, BB, BO, OO, and AB blood types, respectively) (11). Negative controls and reagent blanks did not show any PCR products.

Moreover, the ABO genotyping described herein, which utilizes a fast PCR instrument and an optimized polymerase, enables the direct characterization of blood, hair, and saliva samples without prior DNA extraction in around 70 min (Fig. 3). Just 1 μL of blood, one hair root, or a 1.2-mm-diameter punch of blood or saliva stain is sufficient for precise ABO genotyping, with good heterozygote peak height ratios and no locus drop-out occurring (Fig. 4). In addition, the absence of the need for prior DNA extraction reduces analysis time and removes the necessity of using expensive kits or reagents and the possibility of contamination. Moreover, this method will be acceptable for use in paternity investigations and human identifications. Especially, the rapid screen using this method could save forensic laboratories much time and cost on DNA profiling by enabling exclusions at this screening

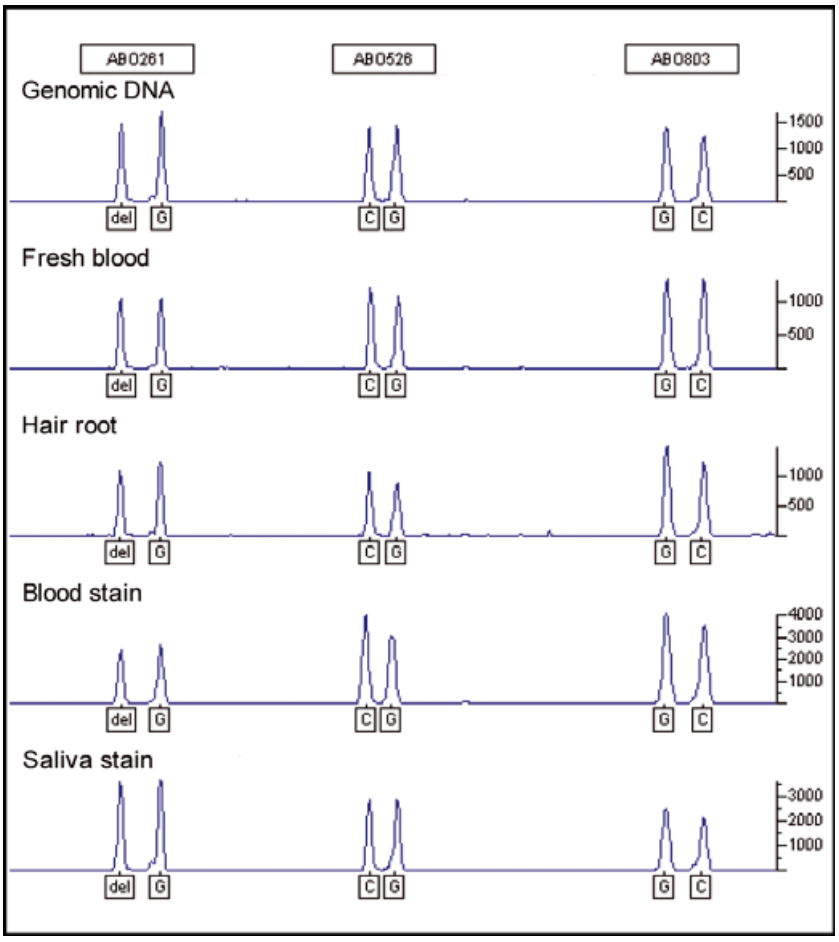

FIG. 4—*Representative electropherograms of rapid direct PCR for ABO genotyping using fresh blood, hair, and body fluid stains. This ABO genotyping protocol enables the direct characterization of blood, hair, and saliva samples using just 1 μL of blood, one hair root, or a 1.2-mm-diameter punch of blood or saliva stains, without prior DNA extraction, in around 70 min, with good locus-to-locus balance, good heterozygote peak height ratios, and no locus drop-out.*

level. When an inclusion is observed, the same statistics as in STR genotyping can be applied for paternity and human identification tests using known frequencies of A, B, O01/O02, O03, and cis-AB01 alleles in a certain population. In conclusion, this newly developed ABO genotyping method represents an example of rapid direct genotyping of SNPs that offers the advantages of time- and cost-efficiency, convenience, and reduced contamination during DNA typing analysis.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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