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ORIGINAL ARTICLE

Rapid and Reliable One-Step *ABO* Genotyping Using Direct Real-Time Allele-Specific PCR and Melting Curve Analysis Without DNA Preparation

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Abstract *ABO* genotyping is a molecular diagnostic technique important for transfusion and transplantation in medicine, and human identification in forensic science. Because *ABO* genotyping are labor intensive and time consuming, the genotyping cannot be firstly used to resolve the serological ABO discrepancy in blood bank. For rapid one-step *ABO* genotyping, we developed direct, real-time, allele-specific polymerase chain reaction (PCR), and melting curve analysis (DRAM assay) without DNA preparation. In DRAM assay, we used a special PCR buffer for direct PCR, a rapid RBC lysis buffer, white blood cells as template without DNA preparation, allele-specific primers for discriminating three *ABO* alleles (261G/del, 796C/A, and 803G/C), and melting curve analysis as a detection method. There was 100% concordance among the results of *ABO* genotyping by the DRAM assay, serologic typing, PCR–RFLP and PCR-direct sequencing of 96 venous blood samples. We were able to reduce the number of manual steps to three and the hands-on time to 12 min, compared to seven steps and approximately 40 min for conventional *ABO* genotyping using allele-specific PCR with purified DNA and agarose gel electrophoresis. We have established and validated the DRAM assay for rapid and reliable one-step *ABO* genotyping in a closed system. The DRAM assay with an appropriate number of allele-specific primers could help in resolving ABO discrepancies and should be valuable in clinical laboratory and blood bank.

Keywords *ABO* genotyping · ABO discrepancies · Direct PCR · Allele-specific PCR · Real-time PCR · Melting curve analysis

Introduction

ABO blood system has been routinely analyzed to ensure safe transfusion and transplantation procedures, and for individual identification in medicinal and forensic science applications, respectively [1]. Many DNA-based techniques for *ABO* genotyping have been reported, including polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR–RFLP) analysis [2], allele-specific PCR (ASP) [3], PCR-direct sequencing [4], denaturing gradient gel electrophoresis [5], single-strand conformation polymorphism (SSCP) [6, 7], multiplex single-base primer extension reaction (SNaP-shot) [8, 9], multiplex ligation-dependent probe amplification assay [10], pyrosequencing [11], real-time Taqman assay [12], real-time fluorescence resonance emission transfer-melting curve analysis [13], real-time quenching probes assay [14], real-time displacing probes assay [15], DNA chip [16], and more. However, these DNA-based techniques for *ABO* genotyping all involve a DNA preparation step to produce purified DNA. The DNA preparation is generally the rate-liming and most labor-intensive step in these methods. Therefore, *ABO* genotyping cannot be firstly used to resolve the serological ABO discrepancy in blood bank. Two research groups have reported attempts to use whole blood as template for direct *ABO* genotyping [17, 18]; however, the methods described have the disadvantage of

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requiring other techniques (i.e. agarose gel electrophoresis and capillary electrophoresis) in post-amplification steps.

We developed a direct, real-time, allele-specific polymerase chain reaction (PCR), and melting curve analysis (DRAM assay) for rapid and reliable one-step *ABO* genotyping without DNA preparation and other analytical device except a real-time PCR system.

Materials and Methods

Sample Collection and Serologic Typing

In 300 randomly selected K2EDTA-treated venous blood samples without any patient's information, we selected 96 venous blood samples of which 24 each had A, B, O, and AB phenotypes, according to the results of ABO serological typing. ABO serology was performed using anti-A (BioClone, monoclonal IgM, murine; Ortho-Clinical Diagnostics, Raritan, NJ) and anti-B (BioClone, monoclonal IgM, murine; Ortho-Clinical Diagnostics).

Preparation of Rapid Red Blood Cells (RBC) Lysis, Washing, and Suspension Buffers

Rapid RBC lysis buffer was 1 × phosphate buffer saline (PBS) pH 7.4 (Gibco, Grand Island, NY) containing 0.25% saponin from Quillaja bark (Sigma-Aldrich, St. Louis, MO) according to the modified protocol [19]. The composition of washing buffer was 0.125% saponin from Quillaja bark (Sigma-Aldrich) in 1 × PBS buffer (pH 7.4) (Gibco). We used 1 × PBS buffer (pH 7.4) (Gibco) as a suspension buffer.

Template Preparation for the DRAM Assay

200 µL venous blood and 800 µL rapid RBC lysis buffer were mixed in a 1.5 mL microcentrifuge tube. After inverting five times to mix, samples were incubated at room temperature for 3 min, then centrifuged at 10,000 rpm for 10 s. After discarding the supernatant, the white blood cells (WBC) pellet was suspended in 1 mL of washing buffer to remove the red blood cells (RBC) debris. After centrifugation at 10,000 rpm for 10 s and removal of the supernatant, the WBC pellet was resuspended in 200 µL of suspension buffer. The WBC suspension was used directly in the DRAM assay as template.

Primers and PCR Mixtures

The properties of all primers used for the DRAM assay are listed in Table 1. We selected three single nucleotide polymorphisms (SNPs) at nucleotide positions (nt) 261, 796, and 803 of the *ABO* gene for *ABO* genotyping. A DRAM set is made up of six individual DRAM reactions to detect 261G, 261A, 796C, 796A, 803G, and 803C alleles, respectively. Each reaction in a DRAM set was carried out in a final volume of 20 µL containing 10 µL of 2 × AnyDirect Mastermix (BioQuest, Seoul, Korea), 2 µL of each primer set at various concentrations (Table 1), 2 µL of WBC suspension, 1 µL of 20 × EvaGreen (Biotium, Hayward, CA). The *ABO* alleles, oligonucleotide primer positions relative to the consensus sequence, and properties (size and melting temperature) of the PCR products are presented in Table 1. The binding positions of the primers for the DRAM assay were in Fig. 1. Two external controls for the DRAM assay were a WBC suspension with BB genotype and a WBC suspension with OO genotype.

DRAM Reactions

The DRAM assay was run on a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. Thermal cycles were an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 40 s, and 72 °C for 40 s. Melting curves were acquired by measuring the fluorescence during a temperature transition from 72 to 95 °C, with the rate of 0.5 °C/s. We defined the reaction as positive when its − d(RFU)/dT melting peak was > 1000. We interpreted the results of the DRAM assay according to the criteria described in Table 2.

Accuracy of the DRAM Assay

To confirm the results of the DRAM assay, we performed PCR–RFLP and PCR-direct sequencing using purified DNA for 261 allele, and 796 and 803 alleles, respectively. Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). To perform PCR–RFLP for 261 allele, we used two primers (*ABO*261F; 5′-GGGTTTGTTCCTATCTCTTTG-3′ [1] and *ABO*261cmR), EmeraldAmp GT PCR Master Mix (Takara, Kyoto, Japan), and *Kpn*I restriction enzyme (New England Biolabs, Beverly, MA) according to the manufacturers' protocols. We performed PCR-direct sequencing using two PCR and sequencing primers (*ABO*803cmF and *ABO*796cmR), EmeraldAmp GT PCR Master Mix (Takara), and the ABI 3730XL Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol (Table 1).

Table 1 Primers for direct allele-specific PCR and their observed melting points (Tm) in melting curve analyses for *ABO* genotyping

Wells	Name	Sequence of primers (5′–3′)	Concentration (μM)	Amplicon length (bp)	Average melting point (°C)	Target	Major alleles specificity	References
A	261GspF	GCAGTAGGAAGGATGTCCTCGTGtTG	0.4	206	85.0–85.5	261G allele	A, B	[18]
	261cmR	AGACCTCAATGTCCACAGTCACTCG	0.5					[18]
B	261AspF	GCAGTAGGAAGGATGTCCTCGTGtTA	0.09	206	85.0–85.5	261A allele	O	[18]
	261cmR	AGACCTCAATGTCCACAGTCACTCG	0.91					[18]
C	796CspF	AGGACGAGGGCGATTTCTACTTCC	0.45	159	89.0–89.5	796C allele	A, O	This work
	796cmR	GCAGGTACTTGTTCAGGTGGCTCT	0.45					This work
D	796AspF	AGGACGAGGGCGATTTCTACTACA	0.25	159	89.0–89.5	796A allele	B	This work
	796cmR	GCAGGTACTTGTTCAGGTGGCTCT	0.25					This work
E	803GspR	CACCGACCCCCCGAAGAAGC	0.45	206	88.5–89.0	803G allele	A, O	This work
	803cmF	ACCTGGTGTGCGTGGACGTG	0.45					This work
F	803CspR	AACCGACCCCCCGAAGATCG	0.5	206	88.5–89.0	803A allele	B	This work
	803cmF	ACCTGGTGTGCGTGGACGTG	0.5					This work

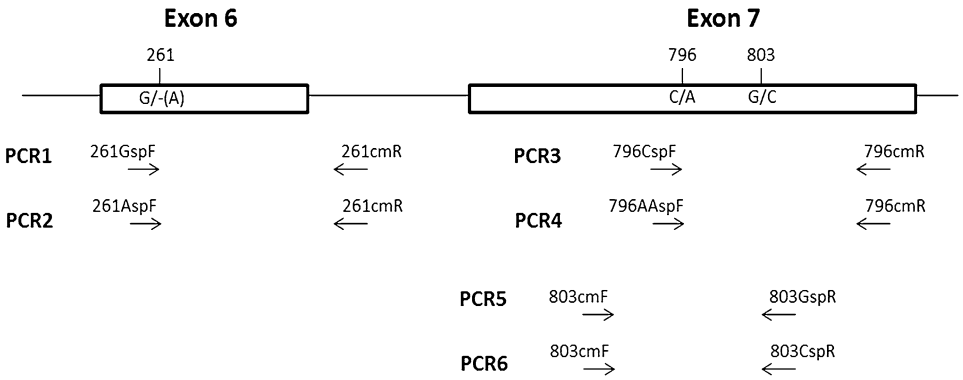

Fig. 1 Illustration of the primer positions for the six direct real-time allele-specific PCR reactions of direct *ABO* genotyping, consisting of four forward allele-specific primers and two reverse allele-specific primers, in relation to the *ABO* locus

Table 2 Interpretive criteria for determinations of the major A, B, O, and AB alleles from the results of the six DRAM reactions used in this study

PCR1 261G-specific	PCR2 261A-specific	PCR3 796C-specific	PCR4 796A-specific	PCR5 803G-specific	PCR6 803C-specific	Phenotype possible	Genotype
+	−	+	−	+	−	A	AA
+	+	+	−	+	−	A	AO
+	−	−	+	−	+	B	BB
+	+	+	+	+	+	B	BO
−	+	+	−	+	−	O	OO
+	−	+	+	+	+	AB	AB

Results

Melting Curve Analysis

Typical derivative melting curves from the DRAM assay for *ABO* genotyping are shown in Fig. 2. For each positive sample, a pattern representing the six primer pairs used in the assay was observed, with single peaks demonstrating melting points at 85.0–85.5 °C, 85.0–85.5 °C, 89.0–89.5 °C, 89.0–89.5 °C, 88.5–89.0 °C, and 88.5–89.0 °C for the 261G, 261A, 796C, 796A, 803G, and 803C allele-specific amplicons, respectively.

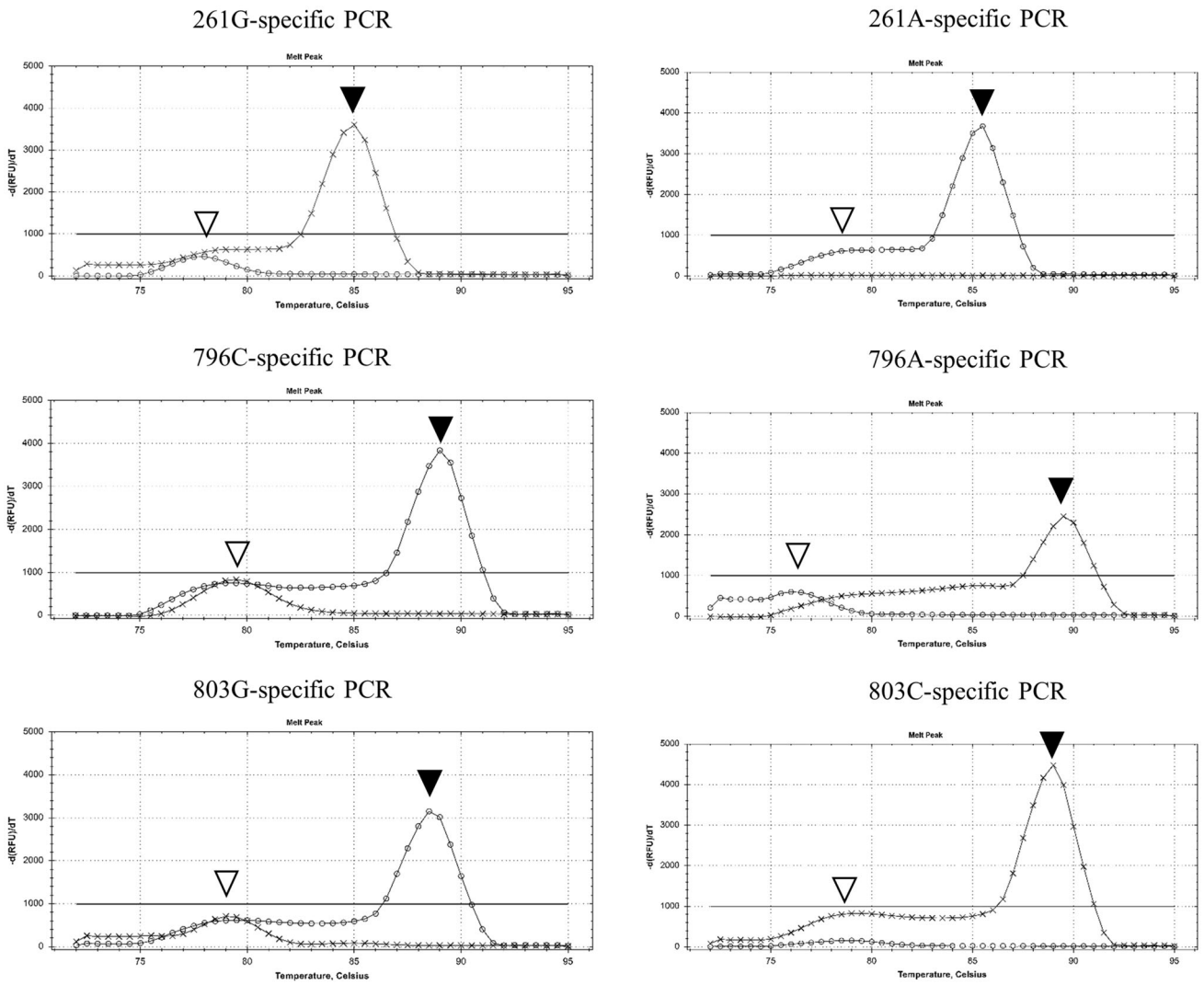

Fig. 2 Representative derivative melting curves of BB genotype (cross) and OO genotype (circle) in six melting analyses. Black triangles indicate the melting curves of target amplicons and white triangles indicate the melting curves of primer dimer

Running and Handling Times for the DRAM Assay

The durations of sample preparation, PCR reaction set up, amplification, melting curve analysis, interpretation, total running time, and hands-on time were 5 min, 5 min, 100 min, 19 min, 2 min, 131 min and only 12 min, respectively.

Accuracy

There was 100% concordance among the results of *ABO* genotyping by the DRAM assay, serologic typing, PCR–RFLP and PCR-direct sequencing (Fig. 3) of 96 venous blood samples (Table 3). Therefore, the accuracy of the DRAM assay was 100% in our study population.

Discussion

ABO genotyping is commonly used in cases of ABO blood group discrepancy, in forensic practices for individual identification, and in researches that reveals the relationship between *ABO* genotypes and cancer. For rapid *ABO* genotyping, we used a special PCR buffer for direct PCR, a rapid RBC lysis buffer, WBC as template without DNA preparation, allele-specific primers for discrimination of six alleles, and melting curve analysis as a detection method for rapid one-step *ABO* genotyping in a closed system. By summing up these techniques, the DRAM assay for *ABO* genotyping achieved a reduction in the number of manual steps and in hands-on time, compared to conventional allele-specific PCR using purified DNA and agarose gel electrophoresis.

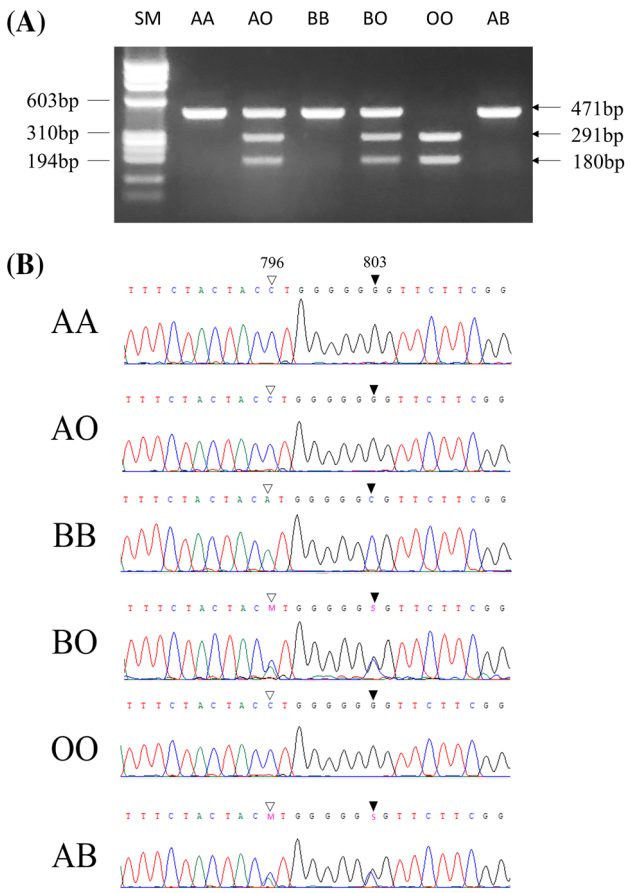

Fig. 3 Representative results of RFLP **a** for 261 alleles and sequencing **b** for 796 and 803 alleles in six major genotypes (AA, AO, BB, BO, OO, and AB). Lane SM, φX174/HindIII DNA size marker (TaKaRa, Siga, Japan)

Direct PCR is a method in which a sample is added directly into a PCR tube without being subjected to prior DNA preparation [20]. There are two major solutions for direct PCR: (1) inhibitor-resistant DNA polymerase and (2) various additives including pH optimization to relieve the inhibition and enhance amplification [21, 22]. To perform direct PCR in this study, we used AnyDirect Mastermix (BioQuest) including various additives in PCR buffer. Although Anydirect PCR buffer system (BioQuest) can amplify target DNA fragments directly from whole blood without the need for DNA preparation [23], we used WBC suspension as template in DRAM reactions due to a strong fluoroquenching effect of hemoglobin [24]. Unlike previous studies [17, 18] using whole blood, we could use a DNA-binding dye and a real-time PCR system for DRAM assay. For rapid RBC lysis, we used a saponin from Quillaja bark according to the modified protocol [19], which works in approximately 5 min compared with about 13 min for ammonium chloride solution [25].

The hands-on time and manual steps required for conventional molecular *ABO* genotyping mainly occur in the pre-amplification (i.e., DNA preparation) and post-amplification (i.e., restriction enzyme digestion, agarose gel electrophoresis, and/or sequencing) steps. Direct PCR can reduce the time of the pre-amplification step but cannot reduce the time of the post-amplification step. Because we used a DNA-binding fluorescent dye (EvaGreen; Biotium) in DRAM assay, there was no need to manipulate PCR products in post-amplification step and all the PCR reactions were performed in a closed system, which reduces assay time, labor, and the risk of contamination [26]. Also, the DRAM assay is economical because this assay used the DNA binding fluorescent dye and did not use Taqman probe. The unit price of eight DRAM reactions including two external controls (BB and OO) for one patient was approximately 11,370 Korean won (10.0 USD).

We selected three alleles (261, 796, and 803) to perform *ABO* genotyping. These alleles were also used for previous *ABO* genotyping works [11, 27]. The 261 allele was used to discriminate between O and non-O blood type. The combination of 796 and 803 alleles was used to discriminate between B and non-B blood type. The six allele-specific primer sets (Table 1) for three alleles used here can determine the six major *ABO* genotypes (AA, AO, BB, BO, BO, OO, and AB) as described in Table 2, and we accurately determined the genotypes of 96 blood samples with each of the six major *ABO* genotypes. Because the DRAM assay for *ABO* genotyping in this study used only six allele-

Table 3 Comparison of serologic results and genotypic results according restrictive fragment length polymorphism (RFLP), sequencing, and direct real-time allele-specific polymerase chain reaction and melting curve analysis (DRAM)

Serologic phenotypes (no)	Genotypes according to RFLP and sequencing (no)	Genotypes according to DRAM (no)	Concordance (%)
A(24)	AA(6)	AA(6)	100
	AO(18)	AO(18)	100
B(24)	BB(3)	BB(3)	100
	BO(21)	BO(21)	100
O(24)	OO(24)	OO(24)	100
AB(24)	AB(24)	AB(24)	100

specific primer sets, more allele-specific primers are needed to practically resolve ABO discrepancy. When allele-specific PCR with more allele-specific primers cannot discriminate *ABO* genotypes, PCR-direct sequencing should be performed to confirm the genotypes to distinguish more alleles [4].

Indeed, DRAM technique can be widely applied to many other genotyping for other blood group, newborn screening for congenital disorder, pharmacogenetics for personalized medicine, and so on [28, 29]. For example, a DRAM assay to test concurrently *ABO* genotypes and thalassemia trait genotypes can be performed in a case of ABO blood group discrepancy in thalassemia-endemic areas.

In conclusion, we have established and validated the DRAM assay for rapid and reliable one-step *ABO* genotyping in a closed system. The DRAM assay with an appropriate number of allele-specific primers could help in resolving ABO discrepancies and should be valuable in clinical laboratory and blood bank.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human blood samples were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent We obtained a waiver of informed consent from the research ethics committee on condition that we use discarded blood samples and remove all patient's information.

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