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PAPER

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Multicolor Real-Time PCR Genotyping of *ABO* System Using Displacing Probes

ABSTRACT: Rapid and informative ABO genotyping has become increasingly popular in forensic use. We developed a multiplex real-time polymerase chain reaction (PCR) approach to genotype ABO major groups and subgroups. Seven differently fluorophor-labeled displacing probes for O^1(261delG), A(261G), A(796C/803C), B(796A/803C), O^2 (802G>A), A^2 (1059delC), and A^2 (1009A>G) were combined in one or two PCRs to determine either *ABO* major groups or subgroups. The method correctly detected 13 reference DNA samples. A blind test of 237 samples resulted in complete agreement with their phenotypes, and 110 of these 237 samples as well as with PCR-SSP method. The whole analysis could be finished in less than 100 min at substantially low material cost and the template DNA ranging from 0.16 to 500 ng per reaction could be quantitatively detected. Despite the limited informativeness of ABO genotyping, the developed methods could find application in rapid and inexpensive screening of forensic settings.

KEYWORDS: forensic science, genotyping, ABO blood group, real-time polymerase chain reaction, displacing probes, single nucleotide polymorphisms

The ABO blood group is recognized as one of the most important markers in forensic testing. So far, a range of techniques have been reported for genotyping of major *ABO* alleles, such as polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) analysis (1), the mutagenically separated PCR (MS-PCR) (2), PCR with sequence-specific primers (PCR-SSP) (3,4), multiplexed allele-specific PCR (5,6), and single-strand conformation polymorphism (SSCP) (7). These methods are however disadvantaged by involving multiple post-amplification steps such as electrophoresis and/or enzyme cleavage and often thus suffer from the risk of carryover contamination. Recently, improved methods such as GeneScan fragment analysis (8,9), minisequencing analysis (10), and microarray-based genotyping (11,12) of *ABO* have also been reported. Unfortunately, post-PCR manipulations are still needed and the equipments introduced are not convenient to use on a routine basis.

We have developed multicolor real-time PCR genotyping method by taking advantage of the extremely high specificity of displacing probes (13). We hereby exploited this strategy to discriminate the major polymorphisms among the *ABO* alleles. We showed that using four differently labeled displacing probes, six genotypes of the four major ABO serotypes could be determined in a single PCR tube. Using seven differently labeled displacing probes, 15 genotypes of the $A^{1,2}BO^{1,2}$ could be determined in two PCR tubes. These reliable, rapid, simple, and cost-effective methods could serve as useful supplements to standard serotyping in forensic testing.

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Materials and Methods

Samples

Nine reference human genomic DNA samples of BO^2 (n = 2), O^1O^2 (n = 2), A^2O^1 (n = 2), A^2A^2 (n = 2), and O^2O^2 (n = 1) were provided by Dr. C. Gassner (Central Institute for Blood Transfusion and Immunological Department, General and University Clinics, Innsbruck, Austria). Two DNA samples with A206 allele and two DNA samples with A201 allele, which were identified by PCR-SSP and sequencing, were kindly provided by Dr. Qiong Yu (Blood Center of Shenzhen, China). Peripheral blood samples of 110 healthy blood donors whose phenotypes had been determined by serologic methods were collected from the Blood Center of Xiamen, and the phenotypes of these 110 samples included A (n = 43), B (n = 16), AB (n = 3), and O (n = 48). 127 DNA samples whose phenotypes had been determined by serologic methods were provided from Institute of Forensic Sciences, the Department of Public Security of Jiangxi Province, and the phenotypes of these 127 samples included A (n = 41), B (n = 32), AB (n = 3), and O (n = 51).

Genomic DNA of peripheral blood samples was extracted using a QIAamp DNA isolation kit (Qiagen, Hilden, Germany). For each extraction, 200 μL of whole blood was used and the concentration of the extracted DNA was determined on a spectrophotometer and diluted in Tris–HCl buffer (10 mM, pH 8.0) to a final concentration of 20 μg/mL.

Real-Time PCR Assay Design

Figure 1 illustrates the probe designs of 4-color real-time PCR genotyping. The O^1 allele has a single G deletion at nucleotide position 261, which distinguishes it from non-O^1 alleles. ROX-labeled probe O_{del} was designed to detect the G deletion and Cy5-labeled probe A_{del} was designed to be complementary to non-O^1 alleles. The *B* allele differs from *A* allele by substitutions

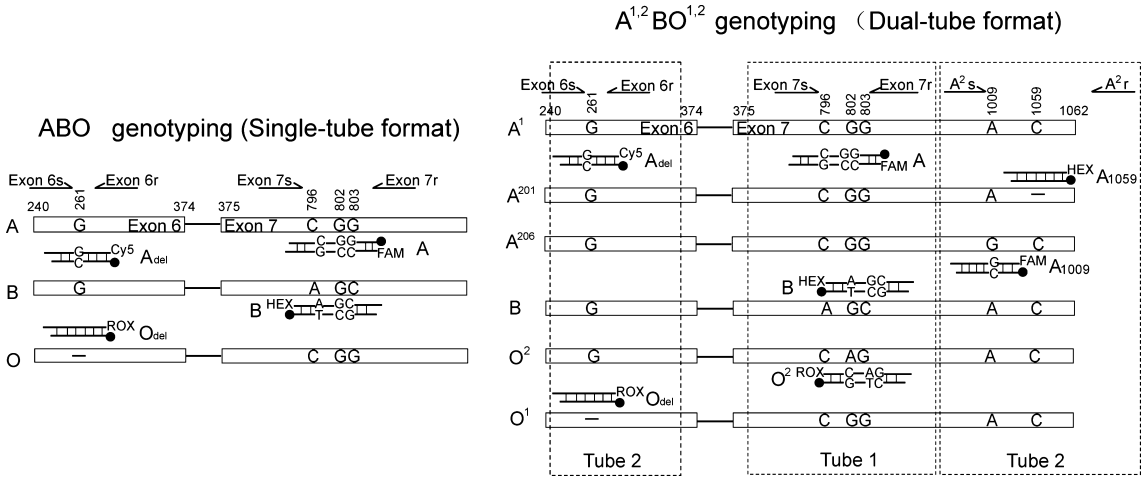

FIG. 1—*Schematic illustrations for probe design in 4-color real-time PCR genotyping. For genotyping of three major ABO alleles (left), four displacing probes (A, B, A_{del}, and O_{del}) are used in a single tube. For genotyping of major $A^{1,2}BO^{1,2}$ alleles (right), seven displacing probes (A, B, A_{del}, O_{del}, O^2, $A^2{}_{1009}$, and $A^2{}_{1059}$) are used in two separate PCR tubes: probe A, B, and O^2 in tube 1, probe A_{del}, O_{del}, $A^2{}_{1009}$, and $A^2{}_{1059}$ in tube 2. Key polymorphisms are indicated at codon 261, 796, 802, 803, 1009, and 1059, respectively. The labeling fluorophores (FAM, HEX, ROX, and Cy5) are shown on the 5′ end of the positive strand of the probes, while labeling quenchers (DABCYL) are shown as black dots on the 3′ end of the negative strand of the probes.*

at 796 (C>A) and 803 (G>C). HEX-labeled probe B was used to detect *B* allele and FAM-labeled probe A was used to detect *A* allele. As a whole, these four displacing probes, when included in a single PCR, could reveal a total of six genotypes corresponding to the four serotypes (Table 1). For further genotyping of the major O^2 and A^2 alleles, three additional probes were designed. ROX-labeled probe O^2 was used to detect 802 (G>A), HEX-labeled probe $A^2{}_{1059}$ was used to detect 1059 (C deletion), and FAM-labeled probe $A^2{}_{1009}$ was used to detect 1009 (A>G). A201 is the predominant A^2 allele in Caucasians (14), which is characterized by a single C deletion at codon 1059. In contrast, A206 is the predominant A^2 allele in Chinese (15), which is characterized by the substitution of 1009A>G. Accordingly, we designed one probe ($A^2{}_{1059}$) to detect a single C deletion at codon 1059 and another probe ($A^2{}_{1009}$) to detect the substitution of 1009A>G for different population detection. Altogether, seven displacing probes were included in two PCRs that could reveal a total of 15 genotypes (Table 1).

TABLE 2—*Primer sequences.*

Name	Sequence of Primers	Amplicon Length (bp)
Exon 6s	5′-ACGCCTCTCTCCATGTGCAGT-3′	79
Exon 6r	5′-AATGTGCCCTCCCAGACAATG-3′	
Exon 7s	5′-CCAGTCCCAGGCCTACATCC-3′	106
Exon 7r	5′-GTGGCAGGCCCTGGTGAG-3′	
A^2 s	5′-CGAGTACTTGTGGGACCAGCA-3′	129
A^2 r	5′-AGCCCTCCCAGAGCCCCT-3′	

Real-Time PCR Assay

All primers (Table 2) and probes (Table 3) were synthesized and PAGE-purified by Sangon Ltd (Shanghai, China) or Takara (Dalian, China). Displacing probes were prepared by mixing 5.0 nmol positive strand and 7.5 nmol negative strand to yield a

TABLE 1—*Genotyping interpretative criterion for the A^1, A^2, O^1, O^2, and B alleles.*

Phenotype	Genotype	A FAM	B HEX	A_{del} Cy5	O_{del} ROX	O^2 ROX	$A^2{}_{1059}$ HEX	$A^2{}_{1009}$ FAM
A^1	A^1A^1	+	−	+	−	−	−	−
A^1	A^1O^1	+	−	+	+	−	−	−
B	BB	−	+	+	−	−	−	−
B	BO^1	+	+	+	+	−	−	−
O	O^1O^1	+	−	−	+	−	−	−
A^1B	A^1B	+	+	+	−	−	−	−
A	A^1A^2	+	−	+	−	−	+	−
	A^2A^2	+	−	+	−	−	−	+
A^2	A^2O^1	+	−	+	+	−	+	−
		+	−	+	+	−	−	+
A^1	A^1O^2	+	−	+	−	+	−	−
A^2	A^2O^2	+	−	+	−	+	+	−
		+	−	+	−	+	−	+
B	BO^2	−	+	+	−	+	−	−
O	O^1O^2	+	−	+	+	+	−	−
O	O^2O^2	−	−	+	−	+	−	−
A^2B	A^2B	+	+	+	−	−	+	−
		+	+	+	−	−	−	+

The shadow area Indicates all possible genotyping results of the single-tube format.
+, positive; −, negative.

TABLE 3—*Probe sequences.*

Name	Sequence of Probes	Polymorphisms Detection Locus
A_{del}	5′-Cy5-TCCTCGTGGT***G***ACCCCTTGG-PO_4-3′ 3′-DABCYL-GGAGCACCA***C***TGGGGAAC-5′	Exon6-261Gdel
O_{del}	5′-ROX-TCCTCGTGG***TA***CCCCTTGGC-PO_4-3′ 3′-DABCYL-GGAGCACC***AT***GGGGAACC-5′	
A	5′-FAM-CCGAAGAAC***CC***CCCCA***G***GTA-PO_4-3′ 3′-DABCYL-GCTTCTTG***GG***GGGGT***C***CA-5′	Exon7-796C>A, 802G>A, 803G>C
B	5′-HEX-ACTAC***A***TGGGG***GC***GTTCTTCG-PO_4-3′ 3′-DABCYL-GATG***T***ACCCC***CG***CAAGAAG-5′	
O^2	5′-ROX-TAC***C***TGGGG***AG***GTTCTTCGG-PO_4-3′ 3′-DABCYL-ATG***G***ACCCC***TC***CAAGAAGC-5′	
$A^2{}_{1059}$	5′-HEX-TCCGGA***AC***CGTGAGCG-PO_4-3′ 3′-DABCYL-AGGCCT***TG***GCACTCG-5′	Exon7-1059Cdel
$A^2{}_{1009}$	5′-FAM-TCAGCTTCC***C***CAGGACGG-PO_4-3′ 3′-DABCYL-AGTCGAAGG***G***GTCCTGC-5′	Exon7-1009A>G

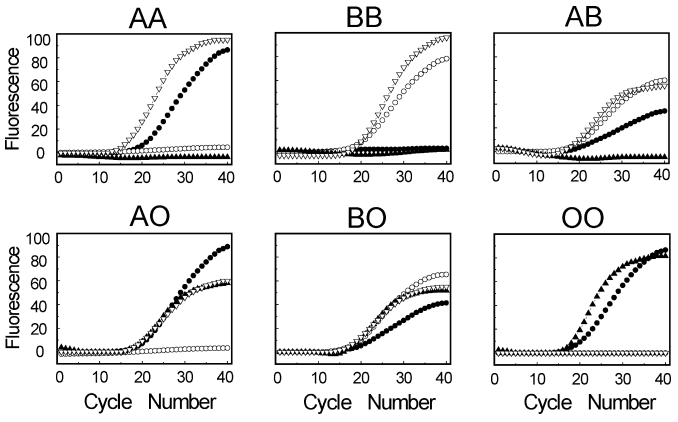

FIG. 2—*Typical genotyping results for three major ABO alleles using 4-color real-time PCR of the single-tube format. Four displacing probes, i.e., A, B, A_{del}, and O_{del}, were used in the single-tube format for detection of the three major ABO alleles. Each genotype is obtained through the corresponding fluorescence combination after PCR. FAM-labeled probe A: closed circles, HEX-labeled probe B: open circles, ROX-labeled probe O_{del}: closed triangles, Cy5-labeled probe A_{del}: open triangles.*

total volume of 50 μL of 10 mM Tris–EDTA buffer (pH 8.0). The concentration of positive strand represents the concentration of displacing probes throughout this work.

Each PCR tube contained 2.5 μL of 10 × PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl pH 8.8, and 0.1% wt/vol Tween 20], 2.5 μL of 25 mM $MgCl_2$, 0.3 μL of a mixture of 2.5 mM dNTPs, 0.2 μL each of forward primers and reverse primers with final concentration of 0.4 μM, 0.2 μL each of probes with final concentration of 0.1 μM and 1.0 U Taq DNA polymerase. After addition of 1 μL of 20 ng/μL genomic DNA template, the final reaction volume was brought to 25 μL by addition of distilled water.

Real-time PCR was run on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). Thermal cycling was started with denaturation at 96°C for 2 min, followed by 10 cycles of touchdown PCR (96°C for 15 sec, 68°C with 1°C/cycle decrease for 20 sec, then 72°C for 15 sec) and 40 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 15 sec. Fluorescence from all four channels (FAM, HEX, ROX, and Cy5) was recorded at the annealing step of 58°C during the later 40 cycles.

Genotyping by PCR-SSP and DNA Sequencing

PCR-SSP genotyping for $A^{1,\ 2}BO^{1,\ 2}$ was performed according to a method reported previously (3). Amplification products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized by an ultraviolet illuminator.

Sequencing of exon 6 was performed by using primer pair 5′-TTGGCTTATTTCAAAATGTATCAGTTCTTG-3′ (forward) and 5′-AAGACCCAGGTCCTCAGAGATCAC-3′ (reverse). Sequencing of exon 7 was performed by using primer pair 5′-TTGGCTTATTTCAAAATGTATCAGTTCTTG-3′ (forward) and 5′-AAGACCCAGGTCCTCAGAGATCAC-3′ (reverse). The PCR products were purified by a gel extraction kit (Promega, Madison, WI) and sequenced using a GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit on a Beckman CEQ 8800 DNA Analyzer (Beckman Coulter, Inc., Fullerton, CA).

Results

Firstly, a single-tube real-time PCR method for *ABO* genotyping was established using four differently fluorophore-labeled displacing probes. This method aimed to detect six genotypes of the four major serotypes. For each sample, the *ABO* genotype as well as the corresponding phenotype was determined based on the interpretative criterion (Table 1) in one round of PCR within 100 min. The method was proved by analyzing 237 blood samples with known serotypes. The genotypes of 237 samples included AO (n = 79), AA (n = 5), BO (n = 38), BB (n = 10), AB (n = 6), OO (n = 98), and one unidentifiable OO with an abnormal signal from HEX-labeled probe B. Typical results of six samples were shown in Fig. 2. For comparison, 110 of these 237 samples were also analyzed in a blind way using a PCR-SSP method. The two genotyping methods gave a complete concordant result.

To determine the sensitivity of each of the four probes for detection of three major *ABO* alleles in the single-tube format, genomic DNA of a BO^1 sample was quantified in the range of 1.28×10^{-3} to 1.0×10^2 ng/μL. Although the sensitivity and quantitative range varied with probes, all the four probes could detect at least 0.16 ng genomic DNA per reaction and the quantitative range was from 0.16 to 500 ng genomic DNA per reaction (Fig. 3).

In order to identify the major O^2 and A^2 alleles, we also established a dual-tube real-time PCR protocol with additional three probes. In this protocol, a total of seven displacing probes were used to detect 15 genotypes (Table 1). This protocol was again proved using 13 reference DNA samples and followed by a blind test with 237 human genomic samples. The genotypes of 237 samples included A^1O^1 (n = 79), A^1A^1 (n = 5), BO^1 (n = 38), BB (n = 10), A^1B (n = 6), O^1O^1 (n = 98), and one unidentifiable O^1O^1 with an abnormal signal from HEX-labeled probe B. Using dual-tube protocol, on the 36-well real-time PCR machine used in this

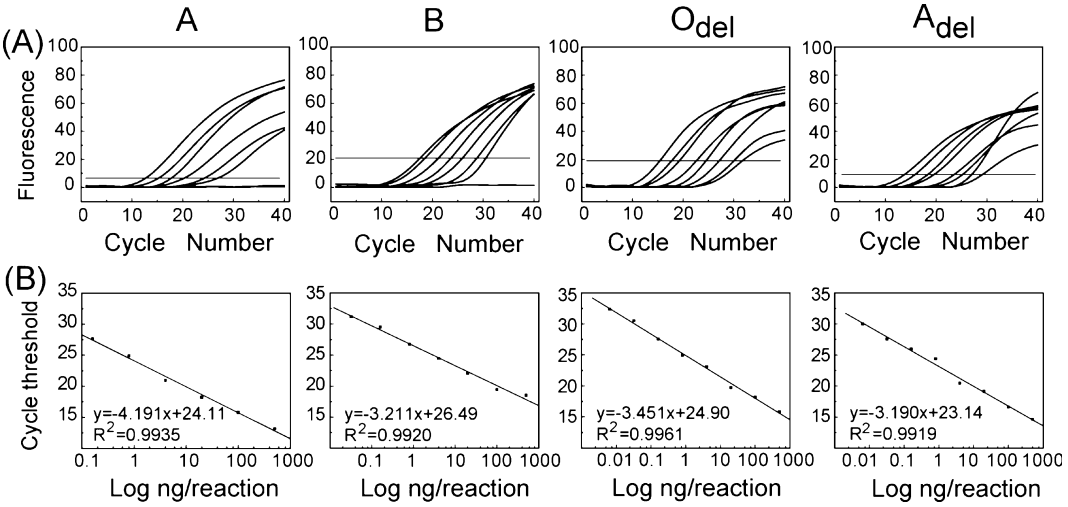

FIG. 3—*Quantification study of real-time PCR for the three major ABO alleles in the single-tube format. (A) Real-time PCR using genomic DNA of a BO^1 sample that diluted serially in 5-folds in the range of 6.4×10^{-3} to 500 ng/reaction. Probe A could detect as low as 0.16 ng/reaction, probe B could detect 3.2×10^{-2} ng/reaction, both probe O_{del} and A_{del} could detect 6.4×10^{-3} ng/reaction. (B) The linear relationship between the number of threshold cycle values and the logarithm of concentration of genomic DNA.*

study, up to 18 samples could be analyzed in one round PCR within 100 min. The results were once again concordant with PCR-SSP method. Typical results of $A^{1,\ 2}BO^{1,\ 2}$ of five samples are shown in Fig. 4.

We then compared the genotyping results of the 237 samples with their corresponding phenotypes determined previously. The result showed that all the genotypes obtained from either single-tube or dual-tube protocol agreed with their corresponding phenotypes but with one exception. The inconsistent sample of O serotype displayed a typical O^1O^1 homozygous pattern but with an unusual positive B signal in both real-time PCR and PCR-SSP. This sample was subjected to sequence analysis on both exon 6 and 7, and the result showed that it was 261delG homozygous but heterozygous at 703(G >A), 796(C >A), and 803(G >C), respectively, demonstrating that it was indeed an O^1O^{1v}-*B* genotype (16,17). As previously reported, the unexpected positive B could be caused by the O^1-B hybrid allele resulting from an earlier crossover event (17,18).

Finally, we estimated the overall cost for genotyping of one sample using our real-time PCR protocols. Except the DNA extraction cost, the overall cost for ABO system genotyping was estimated to be \$0.5 and \$0.8 per sample for single- and dual-tube PCR, respectively.

Discussion

We described hereby a single-tube, displacing probes-based real-time PCR protocol to genotype the major ABO groups, and a dual-tube real-time PCR protocol to genotype the major O^2 and A^2 alleles. The flexibility together with its simplicity and accuracy should advance the acceptance of real-time PCR genotyping in ABO blood group system. Obviously, real-time PCR detection format eliminates all postmanipulations, and the detection results could be directly converted to genotypes. Thus, it has great potential for automation in *ABO* genotyping.

ABO genotyping is increasingly used due to its distinct advantages over serotyping in many respects (6,19). Nevertheless, technical difficulties inherent in current approaches greatly hindered their wide acceptance. So far, PCR-SSP is a widely recognized genotyping method for ABO system (3). However, the tedious post-PCR manipulations involved in PCR-SSP make it low throughput and amenable to carryover contamination. As observed in this study, although PCR-SSP gave equivalent genotyping results with real-time PCR, it took 1 week to detect the 237 samples. The difficulties increased significantly regarding the labors involved in gel electrophoresis, bands visualization, and manual readout. Another widely used method, PCR-RFLP, which relies on restriction digestion and subsequent PAGE electrophoresis analysis, suffers the risk of incomplete digestion of the PCR products and the difficulty of visualization of short DNA fragments (20).

By taking advantages of the high specificity of the displacing probes, 4-color real-time PCR genotyping could be easily accomplished with increased overall throughput and decreased average cost. Unlike other probes used in real-time PCR, displacing probes have a synergistic specificity-enhancing effect that allows single-nucleotide mismatch detectable within a wide temperature range, a characteristic that is indispensable for genotyping of various mutations (21). The extremely high specificity of displacing probes has been proved particularly useful in multicolor genotyping (13,22), which is otherwise a challenging task for most other types of probes for real-time PCR detection. Although methods developed in this study were all of 4-color in one tube, it can be expected that, with the advent of 6-color real-time thermocycler, a 6-color $A^{1,2}BO^{1,2}$ genotyping method could be readily established by including six displacing probes in a single tube. Also, the throughput could be further improved if a 96- or 384-well platform with fast thermocycling is used. In these ways, even lower cost per sample could be achieved.

The high sensitivity of our real-time PCR methods is also valuable in practical use. Because only a small amount of DNA is enough for genotyping, many alternative materials other than blood could be used for DNA extraction. For example, a buccal swab or natal cells from amniocentesis may be suitable for genotyping. Another advantage of our method is the use of short amplicon (no longer than 129 bp), which allows partially degraded DNA to be used for genotyping. These advantages altogether made our method particularly useful in forensic applications, where old bloodstains, sperm, hair, etc., could be DNA sources (23).

Our methods aimed to screen common alleles only and thus hybrids as well as rare O alleles were not included. Nevertheless, it

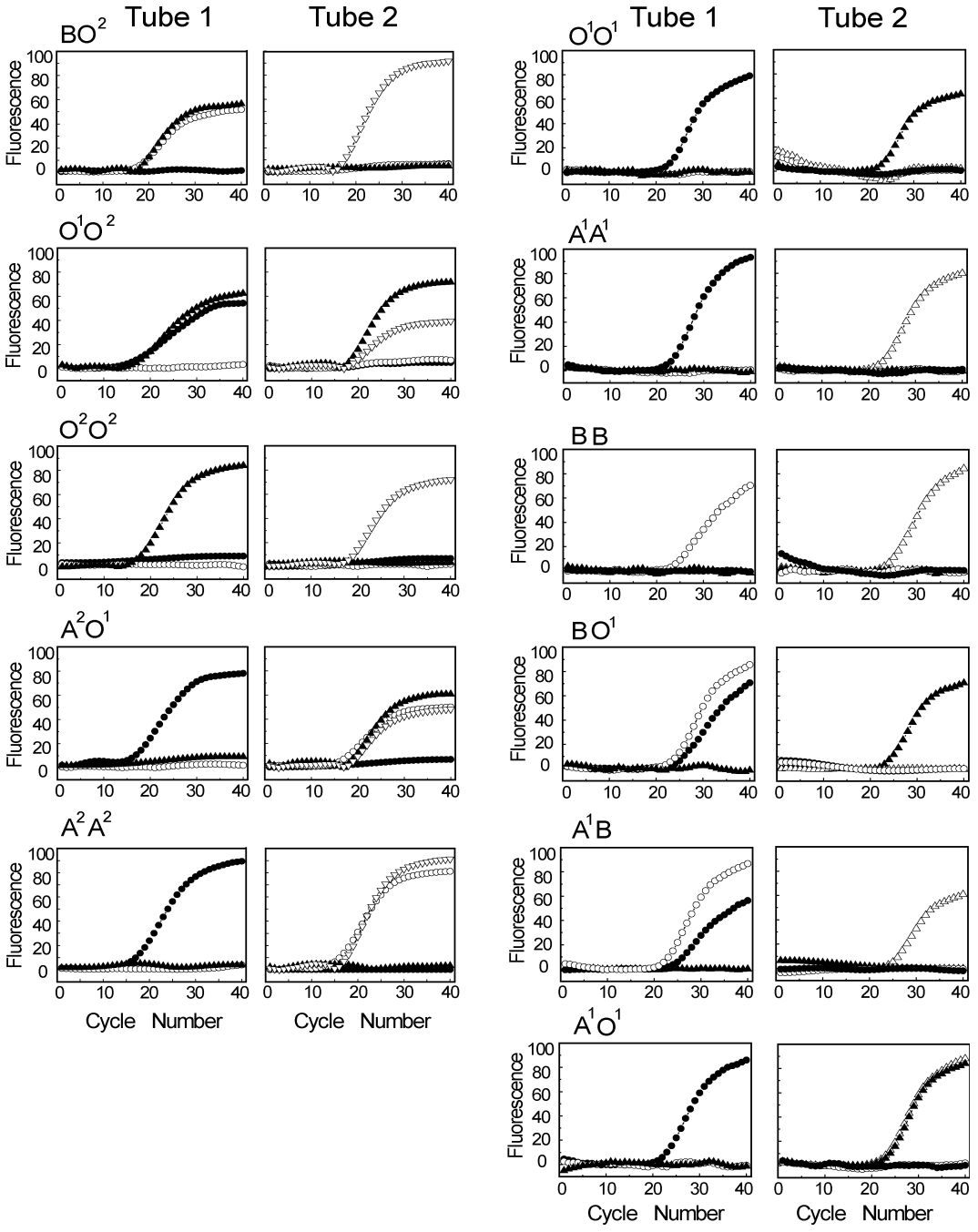

FIG. 4—*Typical genotyping results for major $A^{1,2}BO^{1,2}$ alleles using 4-color real-time PCR of the dual-tube format. Seven displacing probes, i.e., A, B, A_{del}, O_{del}, O^2, A^2_{1009}, and A^2_{1059}, were used in the dual-tube format for detection of the 15 genotypes with probe A, B, and O^2 in tube 1 and probe A_{del}, O_{del}, A^2_{1009}, and A^2_{1059} in tube 2. Each genotype is obtained through the corresponding fluorescence combination of the two tubes after PCR. In tube 1, probe-A-FAM: closed circles, probe-B-HEX: open circles, probe-O^2-ROX: closed triangles; in tube 2, probe-A^2_{1009}-FAM: closed circles, probe-A^2_{1059}-HEX: open circles, probe-O_{del}-ROX: closed triangles, probe-A_{del}-Cy5: open triangles.*

could be used to screen major *ABO* genotypes and is thus helpful in forensic testing. It should be noted that because the phenotype-genotype correlation remains to be established in some rare *ABO* alleles as observed here in the case of O^1O^{1v}-*B* genotype, considerations must be taken when interpreting molecular typing results from such rare hybrid alleles (20,24,25). It has been suggested that *ABO* genotyping strategies should consider all variations distributed across the entire coding region in order to achieve safe phenotype prediction (6,26), and this work is currently being undertaken using real-time PCR strategy described here.

In conclusion, we established and validated 4-color real-time PCR methods using displacing probes for rapid, accurate, and cost-effective *ABO* system genotyping. These methods could serve as a useful complement to classic serological ABO typing and should be helpful in forensic applications.

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