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Technical Note

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Minisequencing-Based Genotyping of Duffy and ABO Blood Groups for Forensic Purposes

ABSTRACT: Duffy and ABO blood group genetic polymorphisms were studied by minisequencing analysis of single-nucleotide polymorphisms (SNPs) at nucleotide positions—33, 125, 265, and 298 of the Duffy gene and at nucleotide positions—261, 297, 467, 646, and 703 of the ABO gene. In an Italian population sample, we found four alleles and seven genotypes for the Duffy and six alleles and 16 genotypes for the ABO systems. The lower limit for reproducible results was 200 pg DNA, with a range of up to 10 ng and an optimum at 1 ng. All of the 16 analyzed inclusive paternity tests were also consistent with parentage and two out of four inconsistencies with parentage cases were excluded by one or more SNPs. Although Duffy and ABO SNP typing show lower informativeness than most current forensic tests, their robustness, the limited population distribution of *FY*Fy* type, and the sensitivity of the minisequencing technology suggest that these markers can be useful in selected forensic applications.

KEYWORDS: forensic science, minisequencing, single nucleotide polymorphisms, duffy blood group, ABO blood group, forensic application

Since the discovery of the molecular genetic basis of the Duffy and ABO blood group systems, several DNA-based assays, including PCR-RFLP, alleles-specific PCR, sequences-specific PCR as single or multiplex assays, and real-time PCR, have been developed to identify defined single-nucleotide polymorphisms (SNPs) to complement routine serological typing (1–3).

The Duffy blood group system is polymorphic in most populations with two distinct antigens Fy^a and Fy^b, encoded respectively by *FY*A* and *FY*B* at the *FY* locus on chromosome 1. They differ in one SNP from G to A at nucleotide (nt) position 125 of the major cDNA transcript (125 G > A). The Fy(a–b–) serotype, resulting from the allele *FY*Fy*, is the predominant serotype among populations of black people and it is rare among non-black populations (4). It is associated with a substitution of T with C at the GATA box motif of the *FY*B* promoter (− 33 T > C). This SNP disrupts the binding site for the GATA-1 erythroid transcription factor, resulting in a silent *FY*B* in the erythroid lineage but not in other tissues (5). Concomitant SNPs at nt 265 from C to T (265 C > T) and at nt 298 from G to A (298 G > A) are associated with the phenotype known as Fy^x. These SNPs cause a weakening of Fy^b antigen expression to the extent that only some anti-Fy^b reagents detect the antigen by haemagglutination. The SNP at nt 298 from G to A (FY^*B^{298}) alone is expression neutral (6).

The ABO blood group locus that has more than 100 alleles (7) was previously analyzed by a minisequencing approach for five SNPs (nt 261, 297, 467, 646, and 703) and 16 different genotypes were found (8).

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Here, we describe a new, rapid, robust, and inexpensive assay to simultaneously genotype the four Duffy-based SNPs (nt − 33, 125, 265, and 298) using a minisequencing multiplex reaction that results in a complete Duffy genotype. We additionally report the sensitivity of Duffy and ABO minisequencing assays and their performance on different biological stains in paternity testing cases, and data from an Italian population sample from Bologna (North Italy).

Materials and Methods

DNA from buccal swabs and peripheral blood obtained from 60 unrelated volunteers from Bologna (North Italy) and seven Masai from Kenya tested as controls were extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Duffy serotyping of samples was performed by hemagglutination methods with commercially available anti-Fy^a and anti-Fy^b (BioRad and Diagast, Marne La Coquette, France). Cotton cloth soaked in 0.5 μL saliva or semen and aged bloodstains ranging from 4 to 13 years old with known phenotype were extracted by the QIAamp DNA micro-kit (Qiagen). For the Duffy assay, the PCR primer pairs and extension primers were selected using the publicly available Web-based primer selection software Primer3 (http://www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) and are listed in Table 1. Duffy DNA fragments of 164 and 282 bp, including the selected SNPs, were amplified in a duplex PCR reaction in a volume of 25 μL containing 1 × PCR Gold buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM of each primer and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) at 94°C for 5 min followed by 34 cycles of 1 min at 95°C, 1 min at 58°C, 1 min at 72°C, and a final extension for 7 min at 72°C. The products were analyzed by electrophoresis using 2% agarose gels.

TABLE 1—*Primer sequences and concentrations for multiplex amplification and detection of Duffy single-nucleotide polymorphisms.*

Primers	Primer Sequences	Final Concentration (μM)	
Duffy 1 F	TGA TTC CTT CCC AGA TGG AG	20 mer	0.2
Duffy 1 R	GCA GAG CTG CGA GTG CTA C	19 mer	0.2
Duffy 2 F	GTG GGG TAA GGC TTC CTG AT	20 mer	0.3
Duffy 2 R	TCA CCC TGT GCA GAC AGT TC	20 mer	0.3
Duffy 125 R	GCA GCT GCT TCC AGG TTG GCA	21 mer	0.35
Duffy -33 F	**GGA CT**C TCA TTA GTC CTT GGC TCT TA	26 mer	0.3
Duffy 265 F	TGT CCT CTT CAT GCT TTT CAG ACC TCT CTT	30 mer	0.25
Duffy 298 F	**GAC GAC TGA CTG ACT** TGC CCT GGC TGG CCT GTC CTG	36 mer	0.25

Nonspecific primer tails are given in bold.

All PCR products were purified using a treatment with ExoSapIT (USB Corporation, Cleveland, OH). Multiplex primer extension reaction were carried out in a volume of 10 μL with 2.5 μL of ABI Prism SNaPshot multiplex kit mix (Applied Biosystems) using 1 μL of purified product. Following the manufacturer's recommendations, postextension treatment was performed by SAP (Amersham Bioscience, Piscataway, NJ). Each extension primer was tested first by a uniplex reaction under the same conditions. Electrophoresis of minisequencing products was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). K562 standard DNA and serial dilutions ranging from 100 ng to 20 pg were submitted to the analysis. ABO serotyping and genotyping were performed as described previously (8). ABO and Duffy assays were applied in 20 paternity cases. Of these, 16 were inclusive based on analysis performed using AmpF*l*STR Identifiler (Applied Biosystems). Statistical parameters were calculated using PowerStatsV12 (http://www.promega.com) and PopGen32 (http://www.ualberta.ca/ ~ fyeh/).

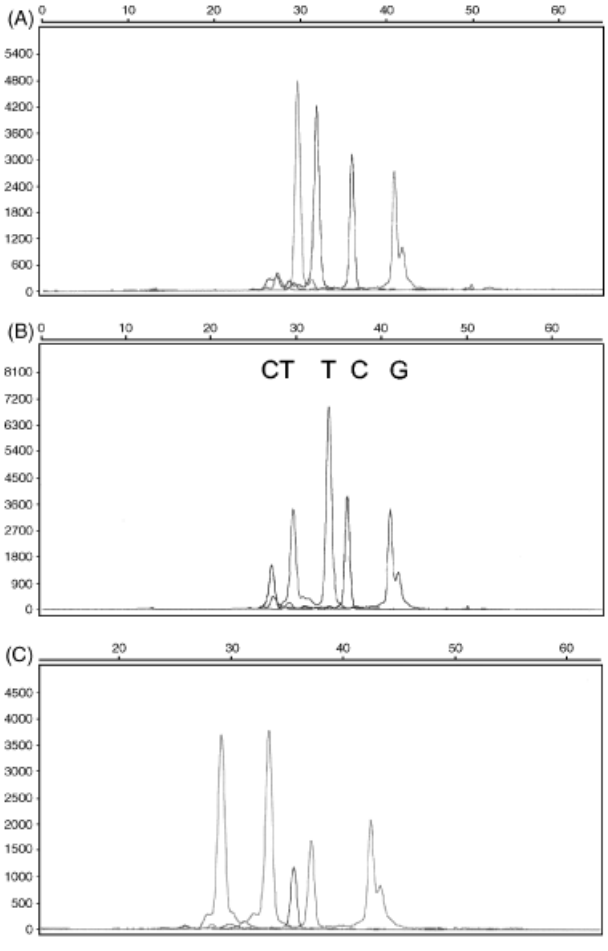

FIG. 1—*Representative electropherograms showing minisequencing of three Duffy genotypes: (A) FY^*Fy/FY^*Fy; (B) FY^*A/FY^*B; (C) FY^*X/FY^*B^{298}. From left to right: nucleotide polymorphisms at positions 125, −33, 265, and 298 of Duffy gene (detailed analysis indicated the SNP 298 has an artefactual peak one base bigger than the true peak).*

Results and Discussion

Duffy PCR products of 164 and 282 bp were amplified successfully in a duplex PCR reaction with the new set of primers. As a first step toward a multiplex assay, each SNP site was tested under singleplex minisequencing reactions to confirm the correct size and base assignment. Electrophoretic patterns from minisequencing (Fig. 1) were interpreted with a table of reference (Table 2) to assign the Duffy genotype. Table 2 shows the base substitution at each SNP site as expected from previous studies (9) (blood group antigen gene mutation database, www.bioc.aecom.yu.edu/bgmut/index.htm). Initial SNaPshot results were always concordant with serotypes. Observed allele frequencies, genotypes, and statistical analysis at Duffy and ABO loci are shown in Table 3. In the limited sample of 60, we found four alleles and seven genotypes for the Duffy and six alleles and 16 genotypes for the ABO systems. Gene frequencies at both loci agreed with those reported in European populations (10). The results confirm the high frequency of FY^*B, the relatively lower frequency of FY^*A, and the absence of FY^*Fy in the Italian population. The expression of neutral FY^*B^{298} occurs at a frequency of 8.3%, which is lower than that reported for Sweden and white donors from the New York area (6). As expected, all seven control black Masai donors were monomorphic for the FY^*Fy genotype.

For the ABO locus, the *O2* allele was present at a relative frequency of 0.8%, confirming its infrequency in the European population. The frequency of the *O1V* allele varies greatly between different ethnic groups, and that found in our population is similar to the frequency in the German population (11).

TABLE 2—*Base substitution at single-nucleotide polymorphism sites in the Duffy gene for each Duffy allele.*

	Nucleotide Position			
Allele	125*	33	265	298
FY^*A	C	T	C	G
FY^*B	T	T	C	G
FY^*B^{298}	T	T	C	A
$FY^*X^{(265+298)}$	T	T	T	A
FY^*Fy	T	C	C	G

*The base substitution in the antisense strand is shown.

TABLE 3A—*Forensic efficiency.*

	Duffy	ABO	Duffy+ABO
Forensic matching probability	0.294	0.106	0.044
Expressed as 1 in . . .	3.4	9.4	22.7
Power of discrimination	0.706	0.894	0.956
PIC	0.48	0.7	
Paternity power of exclusion	0.188	0.333	
Typical paternity index	1	1.36	
Allele frequencies			
Homozygotes	50%	36.7%	
Heterozygotes	50%	63.3%	
Total alleles	120	120	
P	0.094	0.798	
LR test for Hardy–Weinberg equilibrium			

TABLE 4—*Sensitivity of the Duffy minisequencing assay using K562 standard.*

DNA (ng)	− 33	125	265	298
0.02	NR	NR	NR	NR
0.05	NR	NR	NR	NR
0.1	168	115	NR	NR
0.2	435	353	131	112
0.5	832	725	454	405
1.0	2155	1555	854	779
5.0	5885	5198	3325	3128
10.0	6725	6165	4378	4123

Each dilution was typed for five replicates.
The values shown are the average peak heights of each single-nucleotide polymorphisms in RFUs.
NR (no results) indicates no alleles were observed.

The lower limit for reproducible results was 200 pg DNA, with a range of up to 10 ng and an optimum at 1 ng (Table 4). With higher amounts of template DNA, there were nonspecific products or some pull-up peaks becuase the dye channel saturation. Old bloodstains and experimentally prepared stains were correctly typed for both markers.

The success of molecular methods used in forensic laboratories must not depend on the quality of the nucleic acid purified from specimens, because, often, forensic samples showed marked DNA degradation and/or limited quantity. The minisequencing application on blood-group genotyping shows high accuracy and robustness, avoiding both the use of alternative time-consuming classical strategies and the need for high quantities of DNA template.

For forensic analysis, it is well known that Duffy group serotyping is very difficult in old stains (12), even though there has been successful determination of Fy^a and Fy^b in small amounts of relatively fresh bloodstained material using an absorption-elution technique (13), and a weak reaction of eluate in a 4-year-old bloodstain (14) has been reported. The minisequencing approach may provide useful information about old and degraded DNA specimens in a variety of scenarios in which Duffy and ABO typings were previously recorded from the reference samples.

By Duffy and ABO minisequencing assays, all the 16 analyzed inclusive paternity tests were also consistent and two out of four exclusion cases were genetically inconsistent with parentage by one or more SNPs.

We consider that this method is suitable as an adjunct paternity test when STR mutations cause inconsistencies that raise doubt about the diagnosis. As previously demonstrated, additional typing of SNPs loci seems preferable to an STR typing system, as the mutation rate of SNPs has been estimated to be lower than that of STRs (15).

This multiplex approach for the Duffy gene genotyping, investigating four SNPs, all involved in serotype expression and useful in the diagnosis of normal, weak, and null alleles, is substantially different from that of Inagaki et al. (16), who examined only one SNP for Duffy, other blood type genes, and several autosomal SNPs to obtain a highly discriminating assay.

In addition, the FY^*Fy genotype is considered to be a useful anthropologic marker because of its almost exclusive occurrence among the black population (4). In fact, this genotype is exceedingly rare in white populations and the racial variations in the distribution of the Duffy alleles provide one with few known examples of selective advantage resistance to vivax malaria conferred by a blood group genotype (4–6). It was found that *FY* was the most powerful locus to distinguish African Americans from European Americans, and in 96% of the cases in which an unknown stain donor is African American, this locus alone will determine probable ancestry (17). Thus, FY typing is very useful in particular forensic casework to supplement the criminal investigation. In conclusion, although Duffy and ABO SNP typings shows lower informativeness than most current forensic tests, their robustness, the limited population distribution of FY^*Fy type, and the sensitivity of the minisequencing technology suggest that these markers can be useful in selected forensic applications.

TABLE 3B—*Allele frequencies in a population sample from Bologna.*

	Allele	Frequency (%)
DUFFY	*FyA*	35
	FyB	55.8
	FyB^{298}	8.3
	$FyX^{265+298}$	0.8
ABO	*A1*	20
	A2	8.3
	B	11.7
	O1	40.8
	O1V	18.3
	O2	0.8

ABO: 16 different genotypes ranging from 1.6% to 20%.
DUFFY: 7 different genotypes ranging from 1.6% to 40%.
COMBINED: 32 different genotypes ranging from 1.6% to 8.33%.

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