A New Method for Analyzing the Duffy Blood Group Genotype by TaqMan Minor Groove Binding Probes

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Background: Duffy blood group genotyping is useful to ensure transfusion safety and determine the association of Duffy blood group polymorphism with diseases, and therefore has its clinical significance. In order to improve the existing methods for genotyping of Duffy blood group, which normally require post-PCR manipulation, a new method was developed by using 5'-nuclease assay (NA) with TaqMan minor groove binding (MGB) probes. **Methods:** Primers and TaqMan-MGB probes were designed and synthesized to genotype *FY*A* and *FY*B* alleles at Duffy blood group locus on a real-time PCR platform. A total of 120 samples were genotyped by using the new 5'-NA and conventional polymerase chain reaction with allele-specific

primers (PCR-ASP). The results obtained by the two methods were compared.

Results: There was a complete concordance of results for all samples genotyped by 5'-NA and PCR-ASP. The retesting results of 5'-NA were consistent with those of the initial testing. The detection limit of 5'-NA was determined as 100 pg per reaction. The *FY*A* and *FY*B* allelic frequencies were 93.3% and 6.7% respectively in the Chinese Han population in Dalian. **Conclusions:** The 5'-NA for genotyping of Duffy blood group is simple, rapid, reliable, reproducible, sensitive, and high-throughput and is superior to PCR-ASP used in routine genotyping. J. Clin. Lab. Anal. 29:203–207, 2015. ©

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Key words: hematology; clinical laboratory techniques; real-time polymerase chain reaction; molecular probe assays; genetic testing

INTRODUCTION

Duffy blood group is a clinically significant blood group system. The antigens of Duffy blood group system are located on the surface of the red blood cells and include *Fy^a*, *Fy^b*, *Fy3*, *Fy4*, *Fy5*, and *Fy6* (1). Among these antigens, *Fy^a* and *Fy^b* are the most important ones in transfusion medicine. Using both antibodies against either *Fy^a* or *Fy^b*, four phenotypes have been defined: *Fy* (a+b-), *Fy* (a+b+), *Fy* (a-b+), and *Fy* (a-b-) (2). The *Fy* (a-b-) phenotype is dominant among black populations and rare among nonblacks (3). The Duffy blood group locus is located on the long arm of chromosome 1 (1.q22-1.q23) and consists of three major alleles: *FY*A* (encoding the *Fy^a*), *FY*B* (encoding the *Fy^b*), and *FY*B^{ES}* (4, 5). The *FY*A* and *FY*B* alleles differ by a single nucleotide polymorphism (SNP) at nucleotide 125 (G125A, rs12075) resulting in the amino acid change Gly42Asp (6). *FY*B^{ES}* whose

homozygote corresponds to the *Fy* (a-b-) phenotype is caused by a SNP from T to C at position-33 in the GATA box motif of the *FY*B* promoter. This SNP disrupts the binding site for the GATA-1 erythroid transcription factor, abolishes erythroid expression of the *FY*B* in the erythroid lineage but not in other tissues (7, 8). *FY*B^{ES}* is also common in black people, but is rare in many other populations (9).

Clinically, both antibodies against *Fy^a* or *Fy^b* may result in hemolytic disease of the newborn and hemolytic

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Received 29 October 2013; Accepted 3 March 2014

DOI 10.1002/jcla.21751

Published online in Wiley Online Library (wileyonlinelibrary.com).

transfusion reactions (1, 10). Moreover, several recent studies have shown that *FY*A* and *FY*B* polymorphism is associated with serum concentration of monocyte chemoattractant protein-1 (MCP-1) and affects susceptibility to *Plasmodium vivax* malaria (11, 12). Therefore, the genotyping of *Fy^a* and *Fy^b* antigens is useful to ensure transfusion safety and determine the association of *FY*A* and *FY*B* polymorphism with diseases.

The discovery of the molecular basis of Duffy blood group system has promoted the development of several molecular methods. So far, several methods have been described to genotype *Fy^a* and *Fy^b* antigens, such as polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) (13), polymerase chain reaction with allele-specific primers (PCR-ASP) (14), melting curves analysis (15, 16), multiplex SNaPshot (17), and high-resolution melting (HRM) analysis (18). Among them, both PCR-ASP and PCR-RFLP are most widely used.

Recently, 5'-NA with TaqMan minor groove binding (MGB) probes has been widely used for SNP genotyping (19). Because this assay is simple, rapid, sensitive, and accurate, it may be useful for genotyping of blood group. In this study, we developed a new 5'-NA with TaqMan-MGB probes to genotype *FY*A* and *FY*B* alleles at Duffy blood group locus on the high-throughput 96-well platform of the ABI Prism® 7300 Sequence Detection System. We additionally reported gene frequencies of *FY*A* and *FY*B* in the indigenous Chinese Han population.

MATERIALS AND METHODS

Blood Samples

A total of 120 EDTA-anticoagulated whole blood samples were collected from randomly selected unrelated blood donors of Chinese Han nationality in Dalian Blood Center. All the blood donors have given informed consent. Genomic DNA was isolated from whole blood samples using commercially available DNA isolation kit on MagCore® Automated Nucleic Acid Extractor (RBCBio-science, Taipei, Taiwan) according to the manufacturer's instructions.

PCR-ASP

Conventional PCR-ASP was performed to genotype *FY*A*, *FY*B*, and *FY*B^{ES}* alleles according to a method reported by Olsson et al. (14). PCR products were electrophoresed on 2% agarose gel containing 0.5 µg/mL ethidium bromide and visualized under UV transillumination.

5'-NA with TaqMan-MGB Probes

Sequences for the Duffy blood group genes were obtained from the GenBank (NG_011626). For *FY*A* and *FY*B* alleles, sequence-specific forward, reverse primers, and two allele-specific TaqMan-MGB probes were designed by using Primer Express Software 2.0 (Applied Biosystems, Foster City, CA, USA) (Table 1). The probes were labeled with the reporter dye FAM or VIC at 5' ends, corresponding to the sequence of the allele *FY*A* or the allele *FY*B*, respectively. The 3' ends were labeled with a MGB and a nonfluorescent quencher (MGB-NFQ). The primers and probes were synthesized by Applied Biosystems.

The PCR mixtures included 2 µL of purified genomic DNA, 12.5 µL of 2X TaqMan Universal PCR Master Mix II (Applied Biosystems, Foster City, CA, USA), 1.125 µL of each primer (20 µM), 0.25 µL of each primer (20 µM) and 9.125 µL of distilled water in a final reaction volume of 25 µL. The 5'-NA was performed on the ABI Prism 7300 sequence detection system (Applied Biosystems) under the following conditions: 1 pre-PCR heat step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. After the PCR was completed, results were analyzed on the ABI Prism® 7300 sequence detection system with the SDS software v1.4 (Applied Biosystems), using the allele discrimination function to detect the end-point fluorescent intensity of FAM and VIC in each well. The genotyping results were sorted into three distinct groups, corresponding to the three genotypes, homozygous *FY*A*/*FY*A*, *FY*B*/*FY*B* and heterozygous *FY*A*/*FY*B*.

Statistical Analysis

Comparisons of the *FY*A* and *FY*B* allelic frequencies between Dalian and other Chinese Han populations were assessed by the χ^2 test. A two-tailed *P*-value of less than 0.05 (*P* < 0.05) was considered statistically significant difference.

RESULTS

One hundred and twenty blood samples were genotyped using the 5'-NA with TaqMan-MGB probes. Of them, 105 samples were genotyped as *FY*A*/*FY*A*, 14 samples were genotyped as *FY*A*/*FY*B*, and one were genotyped as *FY*B*/*FY*B*. Representative results are shown in Figure 1.

To test the reproducibility of the 5'-NA, five *FY*A*/*FY*A*, five *FY*A* /*FY*B*, and one *FY*B*/*FY*B* samples were reexamined. The retesting results were consistent with those of the initial testing.

TABLE 1. Primer and TaqMan-MGB Probes Sequences Used in 5'-NA

Primers or probes	Sequence (5'-3')	T _m (°C)	Length (mer)	Final concentration (μM)
Forward primer	TGTGAATGATTCTTCCAGATG	59.8	23	0.9
Reverse primer	CACTGGTGAGGATGAAGAAGGG	59.6	22	0.9
Probe for <i>FY*A</i>	FAM-AGACTATG GT GCCAAC—MGB	65.0	16	0.2
Probe for <i>FY*B</i>	VIC-AGACTATG AT GCCAAC—MGB	66.0	17	0.2

The polymorphic sites are bolded and underlined.

In order to determine the detection limit of 5'-NA for genotyping of Duffy blood group, the quantified DNA obtained from homozygous *FY*A/FY*A*, *FY*B/FY*B*, or heterozygous *FY*A/FY*B* was diluted with distilled water to a final concentration ranging from 12.5 pg to 1 pg/μL. Results showed that at least 50 pg of genomic DNA for homozygous *FY*A/FY*A* and *FY*B/FY*B*, and 100 pg of genomic DNA for heterozygous *FY*A/FY*B* could be detected. Thus, the detection limit of 5'-NA for genotyping of Duffy blood group was determined as 100 pg per reaction.

The samples tested by the 5'-NA were also genotyped by conventional PCR-ASP. There was a 100% concordance between the two assays (Table 2). As expected, no *FY*B^{ES}* allele was observed in the 120 tested samples by PCR-ASP.

The *FY*A* and *FY*B* allelic frequencies in the Chinese population from different geographic regions are listed in Table 3. The allelic frequencies in the Chinese Han population in Dalian are similar to those reported in the other areas of China (20–22).

DISCUSSION

Conventional PCR-ASP and PCR-RFLP are widely used for Duffy blood group genotyping. However, there are several disadvantages in these methods. For example, post-PCR manipulation such as agarose gel electrophoresis and visual interpretation are required, which are time-consuming and laborious, and also increase the

Fig. 1. Genotyping of *FY*A* and *FY*B* alleles at Duffy blood group locus using 5'-nuclease assay with TaqMan minor groove-binding probes. (A) Representative amplification curves of homozygous *FY*A/FY*A*. Homozygous *FY*A/FY*A* are reflected by an increase in the fluorescent intensity of dye FAM but not VIC. (B) Representative amplification curves of homozygous *FY*A/FY*B*. Heterozygous *FY*A/FY*B* are reflected by both increase in the fluorescent intensity dye FAM and VIC. (C) Representative amplification curves of homozygous *FY*B/FY*B*. Homozygous *FY*B/FY*B* are reflected by an increase in the fluorescent intensity of dye VIC but not FAM. (D) End-point fluorescent signals from several samples. Homozygous *FY*A/FY*A* showed an increased fluorescence along the Y-axis, homozygous *FY*B/FY*B* along the X-axis, whereas heterozygous *FY*A/FY*B* showed an increase in fluorescence intensity along both the X-axis and Y-axis.

TABLE 2. Comparison of the 5'-NA and the PCR-ASP for Genotyping of Duffy Blood Group

Methods	Genotypes				Internal control primers	Post-PCR manipulation	Testing time ^a	Risks of cross-contamination	Data capture and analysis
	<i>FY[*]A/FY[*]A</i>	<i>FY[*]A/FY[*]B</i>	<i>FY[*]B/FY[*]B</i>	<i>FY[*]B/FY[*]A</i>					
5'-NA	105	14	1	No	No	About 2 h	96 tests/96-well plate	Low	Automated
PCR-ASP	105	14	1	Yes	Yes	About 3 h	48 tests/96-well plate	High	Manual

^aTesting time does not include time for DNA extraction.

possibility of cross-contamination. Moreover, data capture and analysis for genotyping is not readily automated.

Besides PCR-ASP and PCR-RFLP, a multiplex SNaPshot method for genotyping of Duffy blood group has been reported (17). This method can simultaneously genotype both Duffy and Dombrock blood groups. However, the multiplex SNaPshot method also needs post-PCR manipulation. Moreover, because the initial purchase of the genetic analyzer is rather costly, it is normally not available for routine genotyping of Duffy blood group.

To overcome the disadvantages of the methods mentioned above, we have developed a new method of 5'-NA with TaqMan-MGB probes for genotyping of Duffy blood group. By using this method, *FY^{*}A* and *FY^{*}B* alleles in genomic DNA samples can be readily discriminated according to the fluorescence intensity of the reporter dye FAM and VIC, specific to the allele *FY^{*}A* and *FY^{*}B* in a single-closed tube. Because no post-PCR manipulation is needed for 5'-NA, testing time is saved and potential cross-contamination is minimized. The comparison between 5'-NA with TaqMan-MGB probes and PCR-ASP demonstrates that the 5'-NA with TaqMan-MGB probes can be readily applied to Duffy blood group genotyping with a complete concordance with PCR-ASP. In addition, the new method is a simple, rapid, reliable, reproducible, and high-throughput assay in comparison to the conventional assays. Furthermore, data capture and result interpretation can be automated that will largely decrease genotyping errors. In this study, the detection limit of this method was examined using three samples and a good detection limit was obtained, which suggests that this method can be applied to the genotyping of Duffy blood group from limited amounts of DNA samples. Maeda et al. (23) reported a 5'-NA with TaqMan-MGB probes for genotyping of ABO blood group in forensic casework and the detection limit of the method was similar to our method. It suggests that our method may be useful for forensic casework.

Recently, the Light Cycler™, SYBR Green I fluorescent dye-based melting curves analysis and HRM analysis methods for Duffy blood group genotyping have been reported (15, 16, 18). Although these methods do not need post-PCR manipulation such as agarose gel electrophoresis, the melting curves analysis is disadvantaged by the low throughput. In the HRM analysis method, the special equipment for measuring HRM is now rarely used in medical laboratory.

It is worth noting that the 5'-NA with TaqMan-MGB probes was used for genotyping of G125A but not GATA-1 sites, thus, the *FY^{*}B^{ES}* from *FY^{*}B* alleles cannot be discriminated by using our method. Nevertheless, *FY^{*}B^{ES}* is rare in nonblack populations (9). In this study, no *FY^{*}B^{ES}* allele was detected by using PCR-ASP in the 120 Chinese Han donors. Therefore, our method is suitable for

TABLE 3. Gene Frequencies of Duffy Blood Group in the Chinese Populations from Different Geographic Regions

Alleles	Geographic region			
	Dalian ^a (N = 120)	Chengdu (20) (N = 198)	Jiangsu (21) (N = 146)	Zhejiang (22) (N = 102)
FY*A	93.3%	94.7%	93.8%	94.1%
FY*B	6.7%	5.3%	6.2%	5.9%

^aThis study.

Dalian vs. Chengdu, $P = 0.476$; Dalian vs. Jiangsu, $P = 0.814$; Dalian vs. Zhejiang, $P = 0.735$.

Duffy blood group genotyping in Chinese and other non-black populations. For black populations, the combination of 5'-NA with TaqMan-MGB probes and PCR-ASP is needed.

In conclusion, we have developed a new method for genotyping of Duffy blood group using the 5'-NA with TaqMan-MGB probes, which is simple, rapid, reliable, reproducible, sensitive, and high-throughput. This method is superior to conventional PCR-ASP used in routine genotyping of Duffy blood group. In future, we will develop a 5'-NA with TaqMan-MGB probes for the genotyping of GATA-1 site at Duffy blood group locus.

ACKNOWLEDGMENTS

We would like to thank Dr. Y. Hu for help in critically reviewing the manuscript.

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