REPORT

Validation of a blood group genotyping method based on high-resolution melting curve analysis

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The detection of polymorphism is the basis of blood group genotyping and phenotype prediction. Genotyping may be useful to determine blood groups when serologic results are unclear. The development and application of different methods for blood group genotyping may be needed as a substitute for blood group typing. The purpose of this study is to establish an approach for blood group genotyping based on a melting curve analysis of real-time polymerase chain reaction (PCR). Using DNA extracted from whole blood, we developed and validated a DNA typing method for detecting *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02,* and *GYPB*03/GYPB*04* alleles using a melting curve analysis. All assays were confirmed with a commercial reagent containing sequence-specific primers (PCR-SSP), and a cohort of the samples was confirmed with sequencing. Results for all blood groups were within the range of specificity and assay variability. Genotypes of 300 blood donors were fully consistent with PCR-SSP data. The obtained genotype distribution is in complete concordance with existing data for the Chinese population. There are several advantages for this approach of blood group genotyping: lower contamination rates with PCR products in the laboratory, ease of performance, automation potential, and rapid cycling time. ***Immunohematology* 2014;30:161–165.**

Key Words: blood group genotyping, real-time PCR, melting curve analysis

Currently, typing for blood group antigens is performed by hemagglutination. As the gold standard for blood group phenotyping, hemagglutination assays may be inaccurate in some cases, such as for multiply transfused patients and patients with autoantibodies.[1] In addition, the necessary reagents are costly and often limited in availability. Molecular typing techniques are emerging as a supplement to standard serology.[2] Genotyping is often focused on a specific single-nucleotide polymorphism (SNP) of a blood group gene; as a result, the assay may not detect additional rare polymorphisms that may be responsible for the formation of a different antigen.[3] In addition, the method of polymerase chain reaction (PCR) amplification with sequence-specific primers (PCR-SSP) for blood group genotyping may lead to contamination from the laboratory during the process of opening the reaction tube for electrophoresis, especially when screening a large number of samples. The purpose of this study was to explore a new method of blood group genotyping as a substitute for blood group typing. We developed a DNA method for genotyping *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02*, and *GYPB*03/GYPB*04* alleles using a melting curve analysis. Assay results were within the range of specificity and assay variability.

Materials and Methods

Samples and Primers

A total of 300 EDTA blood samples were obtained from volunteer blood donors at Chengdu Blood Center, Sichuan, China. Genomic DNA was extracted using the commercially available TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) from 500-µL blood samples and prepared for subsequent PCR amplification. The special primers for *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02,* and *GYPB*03/GYPB*04* were designed with Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA). The forward primers for the alternative pairs of each blood group gene are the same, whereas the reverse primers are different by only one or two bases at the 3′ terminal because of the specific SNP of the blood group genes. Human β-actin primer served as the internal control for *DO*01/DO*02, DI*01/DI*02, and LU*01/LU*02,* and human growth hormone (HGH) primer was the internal control for *GYPB*03/GYPB*04*. Sequences of primers are shown in Table 1.

Melting Curve Analysis

PCR was carried out in a 10-µL reaction volume consisting of 10 ng purified genomic DNA, 10 pmol of each of the primers, and 1 U Taq DNA polymerase (Takara, Dalian, China). PCR was performed with 1 cycle at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 62°C for 15 seconds, and 72°C for 15 seconds. After the amplification phase, a melting curve analysis for *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02,* and *GYPB*03/GYPB*04* was performed. Each allele was amplified separately; for example, *DO*01* was interrogated in one tube, and *DO*02* in another tube.

Table 1. Sequences of primers

Primer*	Sequence	Position†	Size (bp)	Product Temperature (°C)
Di-F	TGGGGTGGTAGAGGGTGC	21,746 to 21,763	161	88.2
Dia-R	GGGCAGGGCCAGGGAGGCTA	21,901 to 21,882		
Dib-R	GGGCAGGGCCAGGGAGGCAG	21,901 to 21,882		
Do-F	GTTTGGGAACCAGACACTATT	7839 to 7859	155	82.3
Doa-R	GACCTCAACTGCAACCAGCT	7994 to 7977		
Dob-R	GACCTCAACTGCAACCAGCC	7994 to 7977		
Lu-F	GGGACCAGGGAGACCCATAA	7954 to 7973	173	86.7
Lua-R	CATCTCAGCCGAGGCTAGTT	8127 to 8110		
Lub-R	CATCTCAGCCGAGGCTAGTC	8127 to 8110		
Ss-F	CCCGCAGAACAGTTTGAT	2070 to 2087	112	77.5
SS-R	ACGATGGACAAGTTGTCCCA	2181 to 2162		
ss-R	ACGATGGACAAGTTGTCCCG	2181 to 2162		
ß-actin-F	GATGAGATTGGCATGGCTTT	1207 to 1226	125	77.1
ß-actin-R	CACCTTCACCGTTCCAGTTT	1331 to 1312		
HGH-F	GCCTTCCCAACCATTCCCTT	893 to 913	427	88.0
HGH-R	TCACGGATTTCTGTTGTGTTTC	1319 to 1298		

* F and R indicate forward and reverse primers, respectively.

† The last base of the downstream primers is the single-nucleotide polymorphism. Numbering of *DO, DI, LU,* and *GYPB* (Ss) is according to the sequenced BAC clone, GenBank acc. no.: NG_007477.1, BC099629.3, NG_007480.1, and HQ_402219, respectively; ß-actin and human growth hormone (HGH) reference sequences, GenBank acc. no.: XM-007447079 and M13438, respectively.

Validation Process

The assays were validated in terms of specificity with another commercial reagent (Human MNS, Duffy, Kell, Dombrock, Diego, Kidd and Lutheran blood type genotyping kit, PCR-SSP, Tianjin Super Biotechnology Developing Co. Ltd., China). PCR assays were performed to ascertain the specificity with 1 cycle at 96°C for 2 minutes followed by 5 cycles of 96°C for 20 seconds and 68°C for 60 seconds; 10 cycles of 96°C for 20 seconds, 65°C for 45 seconds, and 72°C for 30 seconds; and 15 cycles of 96°C for 20 seconds, 62°C for 45 seconds, 72°C for 30 seconds, and 72°C for 2 minutes. The final PCR products were separated by 2 percent agarose gels that were stained with ethidium bromide.

In addition, the validity of the melting curve analysis was also verified by sequencing. Five samples of Dombrock phenotype (DO) that included 2 *DO*01/DO*02,* 1 *DO*01/DO*01,* and 2 *DO*02/DO*02* defined by our laboratory-developed method were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI 3730 analyzer (Applied Biosystems, Inc., Foster City, CA).

Results

All assays performed best with a DNA input of 10 ng, which showed the highest and best-defined peaks. For all reactions, there was no measurable influence of different users or amplification instruments, underscoring the reproducibility of this approach. In all cases, the genotypes were easily identified by the differences of melting curves. The genotype results for the Do(a–b+) phenotype are shown in Figure 1A [Do(a–)] and 1B [Do(b+)], and results for Di(a–b+) are shown in Figure 1C [Di(a–)] and 1D [Di(b+)], as well as Lu(a–b+) and Ss(S–s+) in Figure 2. To determine the specificity of the approach based on the melting curve analysis, we compared the *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02,* and *GYPB*03/GYPB*04* blood groups with the determined genotype. It revealed a 100 percent genotype correlation with the different genotypes (Table 2). Genotypes of 300 blood donors were fully consistent with the data obtained by PCR-SSP. The sequencing results further supported the validity of this typing method (Fig. 3). The obtained genotype distribution is in complete concordance with existing data for the China population (Table 3).[4,5]

Discussion

There are many ways to genotype for blood groups. Some papers described a genotyping method by high-resolution melting curve analysis to predict red cell antigen expression.[6–8] Genotyping can also be performed by allele-specific fluorescence. Some closed-tube fluorescent methods for blood group genotyping do not require probes, but most methods are based on PCR and use fluorescent oligonucleotide probes. Chen et al.[8] reported that the Ael and Bel blood types were rapidly detected by real-time PCR and melting curve analysis. In their methods, the real-time PCR was performed in the Lightcycler thermal cycler and the allele-specific melting behavior of fluorophore-labeled hybridization probe was used to detect Ael- and Bel-specific genotyping. However, labeled probes are costly when compared with real-time PCR with SYBR Green I. Homogeneous, closed-tube methods for blood group genotyping that do not require a separation step are attractive for their simplicity and containment of amplified products. Genotyping of SNPs by high-resolution melting curve analysis in products has been reported.[9] We developed a blood group

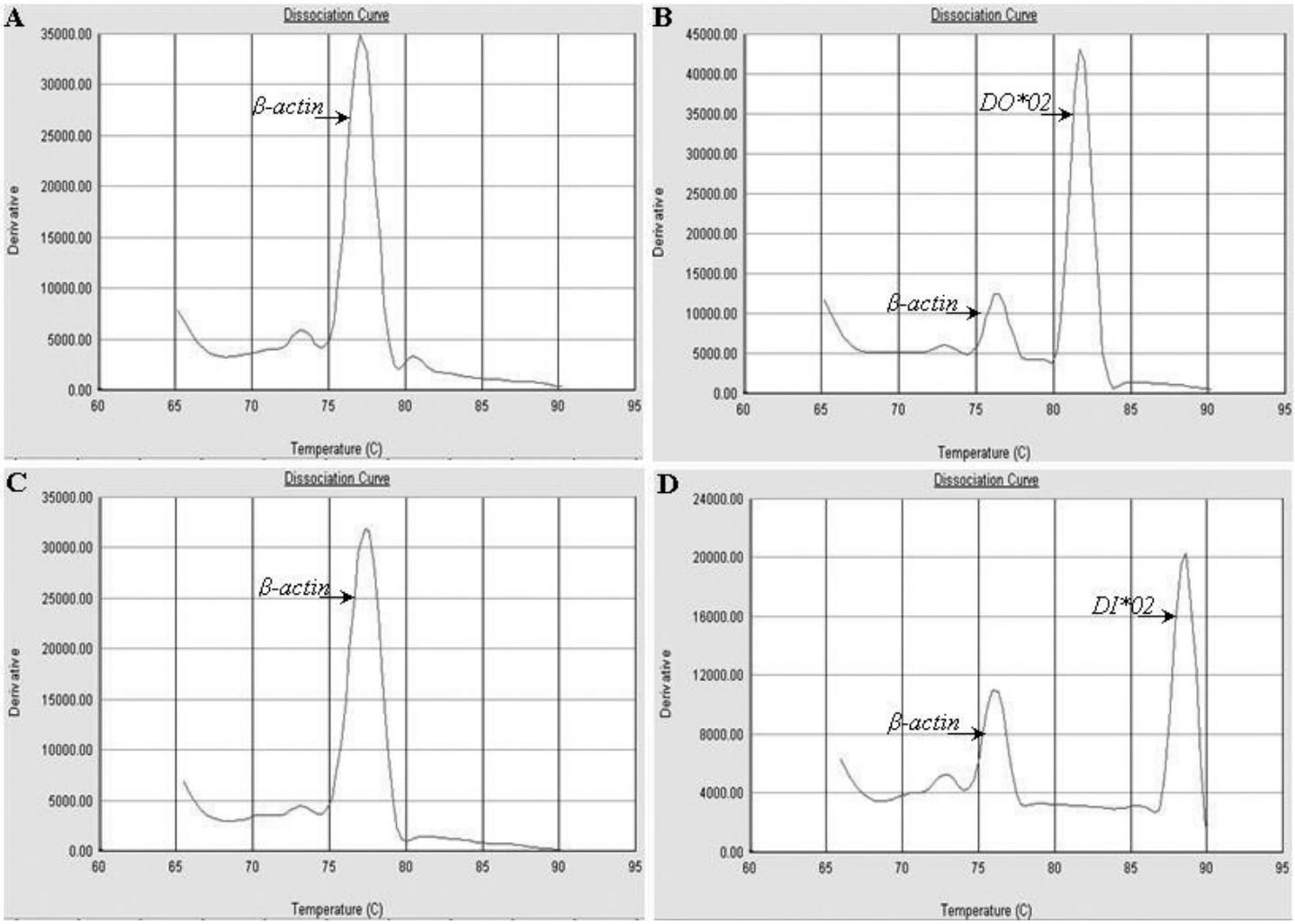

Fig. 1. Melting curve analysis of *DO*01/DO*02* and *DI*01/DI*02.* The genotype results of Do(a–b+) phenotype are shown in panels **A** and **B. A:** Do(a–), no reaction for *DO*01,* only the ß-actin peak identified. **B:** Do(b+), both the ß-actin and *DO*02* peaks are visible. The genotype results of Di(a–b+) phenotype are shown in panels **C** and **D. C:** Di(a–), no reaction for *DI*01,* only the ß-actin peak identified. **D:** Di(b+), both the ß-actin and *DI*02* peaks are visible.

genotyping method based on allelic discrimination by melting curve analysis that is suitable for the different requirements in an immunohematologic setting.[10] As the costs for reagents remain the same, differences exist in consumables. The cost of this laboratory-developed high-resolution melting curve assay is approximately one-third the cost of the commercial SSP-PCR genotyping kit in our laboratory. From an economical point of view, the melting curve analysis offers a platform for rare blood group molecular screening from a large number of samples. The detection of blood groups in a closed-tube reaction by unique melting behavior of the primer resulting from sequence variations saves on reagent costs.

Novaretti et al.[6] described a real-time PCR and melting curve analysis method for Diego blood group genotyping. We also tried to perform the testing using one tube for both alleles of *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02,* and *GYPB*03/GYPB*04,* but we were unable to identify both alleles because the product's size and melting temperature are high owing to the similarity of sequences between the two alleles.

Ansart-Pirenne et al.[7] described *FY*02.M* real-time PCR with melting curve analysis associated with a complete one-step real-time *FY* genotyping. Forty-seven samples were studied by real-time PCR, based on fluorescence resonance energy transfer (FRET) technology. Obviously, although the method of real-time PCR and FRET-PCR is useful for the correct typing of blood donors, it is less suitable for use in the screening of rare blood groups because of the technical skills and precision instruments required.

We were unable to validate the assay for the *LU* c.230A SNP. For future work, we will consider obtaining commercially

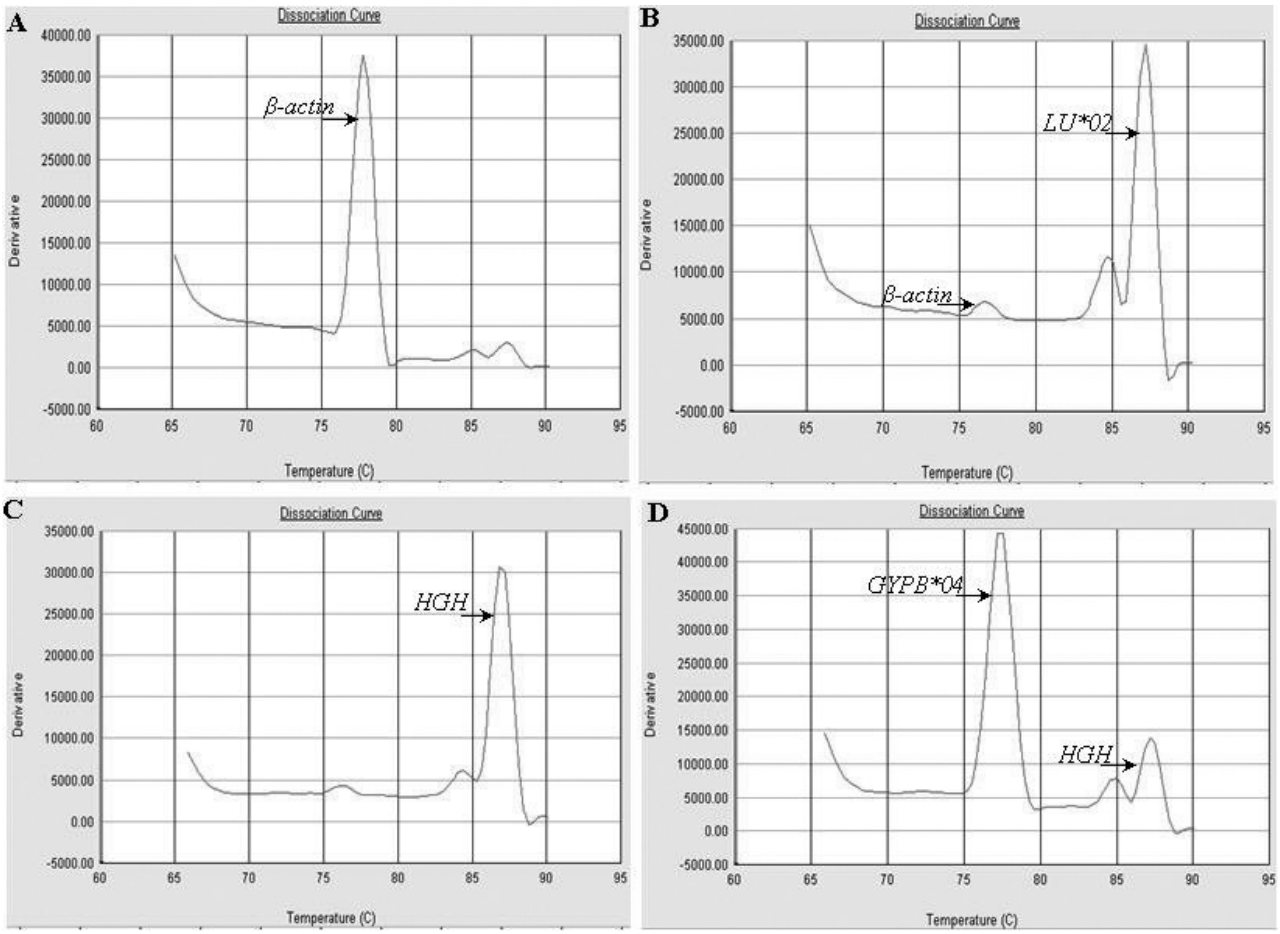

Fig. 2. Melting curve analysis of *LU*01/LU*02* and *GYPB*03/GYPB*04.* The genotype results of the Lu(a–b+) phenotype are shown in panels **A** and **B. A:** Lu(a–), no reaction for *LU*01,* only the ß-actin peak identified. **B:** Lu(b+), both the ß-actin and *LU*02* peaks are visible. The genotype results of *GYPB*03/GYPB*04* (S–s+) are shown in panels **C** and **D. C:** S–, no reaction for *GYPB*03,* only the human growth hormone (HGH) peak identified. **D:** s+, both the HGH and GYPB*04 peaks are visible.

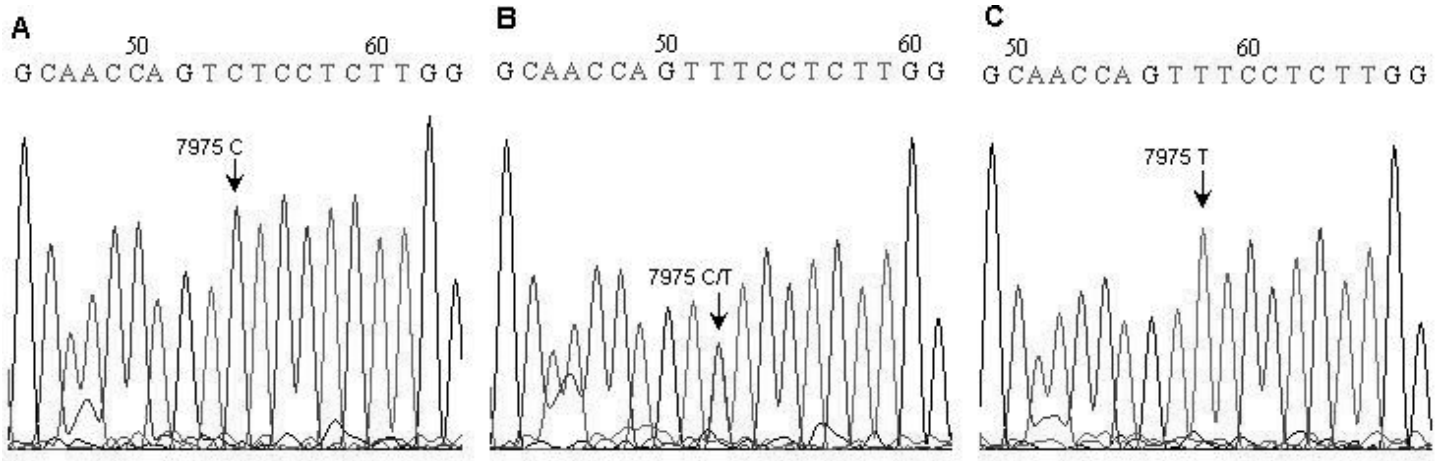

Fig. 3. Sequencing results (reverse) for the Dombrock blood group. The Dombrock genotype of donors A, B, and C, tested by the new method, is *DO*01/DO*01, DO*01/DO*02,* and *DO*02/DO*02,* respectively. The arrow indicates nucleotide position at 7975(NG_007477.1), and the genotypes of donors A, B, and C have the base C, C/T, and T at 7975 of the *DO* gene, respectively. Dombrock genotypes of these three donors were consistent with the data of sequencing.

Table 2. Genotypes detected by melting curve analysis and PCR-SSP

Phenotype	Genotype	Melting curve analysis (cases)	PCR-SSP (cases)
Do(a+b+)	*DO*01/DO*02*	26	26
Do(a+b–)	*DO*01/DO*01*	1	1
Do(a–b+)	*DO*02/DO*02*	273	273
Di(a+b+)	*DI*01/DI*02*	18	18
Di(a+b–)	*DI*01/DI*01*	0	0
Di(a–b+)	*DI*02/DI*02*	292	292
Lu(a+b+)	*LU*01/LU*02*	0	0
Lu(a+b–)	*LU*01/LU*01*	0	0
Lu(a–b+)	*LU*02/LU*02*	300	300
Ss(S+s+)	*GYPB*03/GYPB*04*	9	9
Ss(S+s–)	*GYPB*03/GYPB*03*	1	1
Ss(S–s+)	*GYPB*04/GYPB*04*	290	290

PCR = polymerase chain reaction; SSP = sequence-specific primer.

available DNA (such as from Coriell Cell Repositories) from individuals of other ethnic groups to include in genotyping assay validations. The laboratory-developed high-resolution melting curve method is a medium throughput assay.

The advantages of this real-time PCR method are its rapid PCR performance and the fact that the whole amplification and detection process is performed in a closed system, therefore minimizing the contamination risk. The software-operated calculation of melting points enhances the certainty of the results. Methods applied in screening for rare blood groups must show a high degree of robustness. The local genotype and allele distribution for all examined blood groups mirror the known occurrence in a Chinese population as described before.[4,5] In summary, the combination of real-time PCR and melting curve analysis provides a useful tool for blood group determination. The genotyping process can be finished within 90 minutes after DNA purification. We suggest that this particular molecular methodology may be successfully adopted as a method for screening a large number of samples for rare blood groups, and we foresee the further extension of real-time PCR assays for routine genotyping.

Acknowledgments

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Table 3. *DO*01/DO*02, DI*01/DI*02, LU*01/LU*02,* and *GYPB*03/GYPB*04* distribution among China and Chengdu populations

	Frequency (*n*)							
Area	*DO*01*	*DO*02*	*DI*01*	*DI*02*	*LU*01*	*LU*02*	*GYPB*03*	*GYPB*04*
China	0.1027 (292)	0.8973 (292)	0.0566 (299)	0.9990 (299)	0 (114)	1.0000 (114)	0.0225 (111)	0.9950 (111)
Chengdu†	0.0900 (300)	0.9100 (300)	0.0600 (300)	0.9400 (300)	0 (300)	1.0000 (300)	0.0333 (300)	0.9666 (300)

†These data were detected by the melting curve method.