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Rapid detection of GYPA, LDLR, HBGG, D7S8 and GC alleles by real-time fluorescence PCR

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Abstract

We developed the allele-specific TaqMan polymerase chain reaction (AS-TaqMan PCR) and SYBR Green PCR assays for detecting glycophorin A (GYPA), low-density lipoprotein receptor (LDLR), hemoglobin G (HBGG), D7S8 and group-specific component (GC) alleles. We improved the specificity of detecting a nucleotide substitution by introducing the additional mismatches at position 2 (3 in GYPA). The differences between threshold cycle (Ct) values of different genotypes on each of the loci were statistically significantly different. All the genotypes agreed with the results using the AmpliType PM+DQA1 PCR Amplification and Typing kit. The AS-TaqMan PCR and SYBR Green PCR assays are simple, rapid, and accurate, as well as suitable for high-throughput applications in a forensic investigation.

Keywords: Allele-specific TaqMan PCR assay; SYBR Green PCR assay; AmpliType PM kit; TaqMan probe; Additional mismatch

1. Background

Recently, the TaqMan polymerase chain reaction (TaqMan PCR) assay has been used for detection of known sequences and quantitation of gene expression. The allele-specific TaqMan polymerase chain reaction (AS-TaqMan PCR) assay combines allele-specific primers and hybridization of the TaqMan probe to the target DNA. The TaqMan probe (Applied Biosystems, USA) contains a reporter dye at the 5′ end and a quencher dye at the 3′ end of the probe. During PCR, AmpliTaq Gold DNA polymerase (Applied Biosystems) cleaves the TaqMan probe by its 5′ nuclease activity. As a result of separation of the

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reporter dye and the quencher dye by cleavage of the probe, the amount of fluorescence of the reporter increases, and an accumulation of PCR products is detected. Genotypes are detected according to the different threshold cycles (Ct) of the each allele-specific primer, respectively.

We developed the AS-TaqMan PCR assay for detecting glycophorin A (GYPA), low-density lipoprotein receptor (LDLR), hemoglobin G (HBGG), D7S8 and group-specific component (GC) alleles.

2. Materials and methods

2.1. DNA samples

Buccal swabs were obtained from 24 unrelated healthy Japanese volunteers. DNA was extracted using Chelex-100 (BioRad, USA), proteinase K and phenol/chloroform.

2.2. DNA typing

GYPA, LDLR, HBGG, D7S8 and GC alleles were identified using AmpliType PM+DQA1 PCR Amplification and Typing kit (Applied Biosystems) according to the manufacture's recommendations.

2.3. TaqMan probes and primers

The TaqMan probes (Applied Biosystems) contain 6-carboxyl-fluorescein (FAM) at the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) with a phosphate molecule at the 3′ end. Allele-specific PCR usually relies on the presence of a 3′-end mismatch (at position 1) between a PCR primer and a target sequence. We improved the specificity by introducing additional mismatches at position 2 (3 in GYPA), which further destabilizes the allele-specific PCR primers. Allele-specific primers were designed with an additional mismatch at position 2 (3 in GYPA) from the 3′ end of each primer. The selected allele-specific primers with an additional mismatch, common primers and TaqMan probes used in this study are shown in Table 1.

2.4. AS-TaqMan PCR

The AS-TaqMan PCR assay for detecting polymorphisms of GYPA, LDLR, HBGG, and D7S8 was performed according to the methods described previously by Mizugaki et al. [1]. In GC, two nucleotide substitutions at np35706th and np35717th were detected, and AS-TaqMan PCR assay was not carried out, only real-time fluorescence PCR based on SYBR Green was carried out. TaqMan PCR was carried out in a 30 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) containing 0.4 μM forward primers (two types) and common reverse primers, 0.1 μM TaqMan probes and 30 ng of genomic DNA. Thermal cycling was performed in MicroAmp Optical 96-well reaction plate (Applied Biosystems) with MicroAmp optical caps (Applied Biosystems) using the

Table 1
Allele-specific primers, common primers and TaqMan probes

GYPA (TG673.672GA)	
A	TTGAAGTGTGCATTGCCACAC
B	TTGAAGTGTGCATTGCCAA(C)CT
Common primer	TGTGAGGGAATTTGTCTTTTGCA
TaqMan probe	GATTAAGAACGAGGTGACTGAGCGGACCA
LDLR (C114T)	
A	TGGGTGAGGTTGTGGAAGAT(G)G
B	TGGGTGAGGTTGTGGAAGAT(G)A
Common primer	CACAGGTTCCGATGTCAACTTG
TaqMan probe	CCTCTGGGGACAGTAGGTTTTCAGCCA
HBGG (T805G)	
A	GGCCTCCAGATAACTACACACA(C)A
B	GGCCTCCAGATAACTACACACG(C)C
Common primer	GGGAAAGCAAAATCTCAGGCT
TaqMan probe	CAAGCCTATGTTAACTTCCCTCAAAGCCTG
D7S8 (C/T)	
A	GACCTTATTGCTCCCCTTTCG(C)C
B	GACCTTATTGCTCCCCTTTCT(C)T
Common primer	AAAGCATACAAAAATGGCTGCC
TaqMan probe	AGCTCTGAGAAGGCAGCCATTTTTGTATGC
GC (T35706G)	
AorB	CGACTAAAAGCAAAATTGCCTGT(A)T
C	CGACTAAAAGCAAAATTGCCTGG(A)G
Common primer	AAAGTCTGAGCGCTTGTTAACCA
GC (A35717C)	
A	GCCTGAGGCCACACCCG(A)A
BorC	GCCTGAGGCCACACCCT(A)C
Common primer	ACAGCAGTTGGAGGCAAAGTCT

The original nucleotide sites at position −2 are shown in parenthesis. The mismatched nucleotide sites at position −2 are underlined.

PRISM 7700 sequence detection system (Applied Biosystems). This instrument monitors increase in fluorescence of the reporter dye during the reaction. Amplification and detection were carried out with the following conditions: 50 °C for 2 min, 95 °C for 5 min and 40 cycles each of 95 °C for 10 s and 60 °C for 35 s. Samples were detected at any given cycle when the emission intensity of the reporter dye (FAM in this study) exceeded the threshold defined by the instrument's software, Sequence Detector Ver. 1.7. The threshold cycle (Ct) is the cycle at which a significant increase in the magnitude of the signal is detected first. The Ct values correlated with relative copy numbers [2].

2.5. Real-time fluorescence PCR based on SYBR Green

We have also applied the real-time fluorescence PCR based on SYBR Green, which is a double-stranded DNA-selective fluorescent dye, for genotyping of 5 loci. PCR was carried out in a 30 μl of SYBR Green PCR Master Mix (Applied Biosystems) containing 0.4 μM forward primers (two types) and common reverse primers, 20 ng of genomic DNA and no

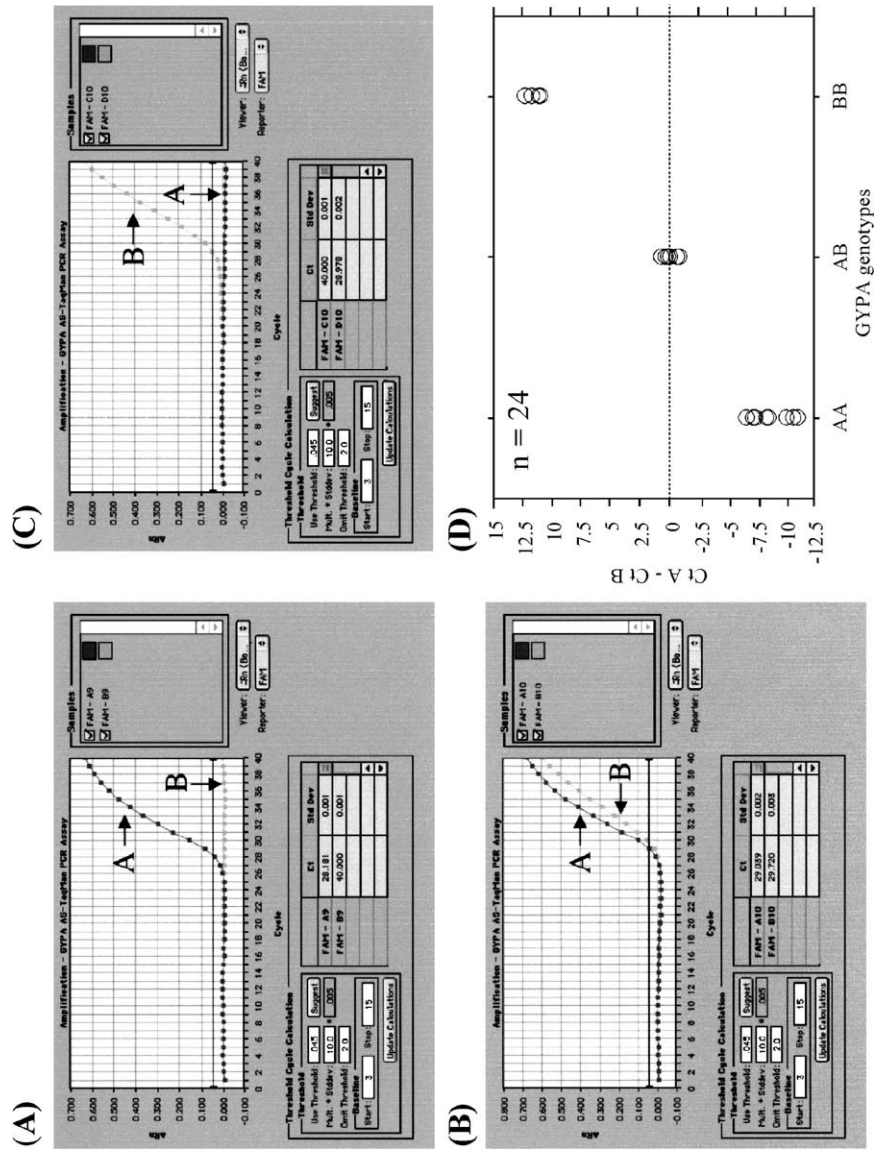

Fig. 1. GYPA detection using AS-Taqman PCR assay.

TaqMan probes. Amplification and detection were carried out in the same condition with AS-TaqMan PCR assay.

2.6. Statistical analysis

The differences of Ct values between different genotypes on each of the loci were analyzed statistically using the Mann–Whitney and the Kruskal–Wallis tests.

3. Results

The results of AS-TaqMan PCR assay about GYPA genotypes AA, AB and BB are shown in Fig. 1(A), (B) and (C), respectively. Differences of Ct values between different genotypes obtained using A-type and B-type AS-primers in the assay were statistically significantly different ($p < 0.001$) [Fig. 1(D)].

At the other four loci, LDLR, HBGG, D7S8 and GC (np35706th and np35717th), the differences between the Ct values were statistically differed significantly ($p < 0.001$) (Table 2). All the genotypes agreed with the results using AmpliType PM + DQA1 PCR Amplification and Typing Kit.

Using AS-TaqMan PCR assay, it is not necessary to use the reverse dot hybridization technique for detecting 5 loci which are detected by AmpliType PM + DQA1 PCR Amplification and Typing Kit.

We improved the specificity of detecting a nucleotide substitution by introducing the additional mismatches at position 2 (3 in GYPA). Additional mismatches destabilized the allele-specific PCR primers further (Table 1).

In SYBR Green PCR assay, genotypes are separated according to the different threshold cycles of the each allele-specific primer, without the TaqMan probe. All the genotypes by SYBR Green PCR assay is corresponding to the results of AS-TaqMan PCR

Table 2
The relation between Ct and genotypes

	Means of Ct (range, min–max) $n = 24$		
	AA	AB	BB
GYPA	−8.650 (−11.01 ~ −6.62)	−0.061 (−0.787 ~ 0.681)	11.609 (10.985 ~ 12.305)
LDLR	ND	−0.628 (−0.972 ~ −0.364)	6.319 (4.997 ~ 7.563)
HBGG	−3.297 (−3.399 ~ −3.195)	0.169 (−0.958 ~ 0.771)	6.784 (5.460 ~ 8.538)
D7S8	−5.906 (−8.616 ~ −4.260)	0.614 (0.027 ~ 0.961)	12.731 (11.788 ~ 13.674)
	AA, AB, BB	AC, BC	CC
GC 35706	−8.392 (−11.330 ~ −5.409)	0.293 (−0.520 ~ 0.987)	7.155 (6.038 ~ 8.271)
	AA	AC, BC	BB, BC, CC
GC 35717	−6.269 (−7.713 ~ −5.030)	0.050 (−0.312 ~ 0.627)	7.292 (5.858 ~ 11.493)

ND; not detected.

assay. This assay is more cost-effective than AS-TaqMan PCR assay because it does not need the TaqMan probe.

4. Conclusions

We developed the AS-TaqMan PCR and SYBR Green PCR assays for detecting GYPA, LDLR, HBGG, D7S8 and GC alleles. The differences of Ct values between different genotypes on each of the loci were statistically significantly different.

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