Acta Med. Okayama, 2005
Vol. 59, No. 5, pp. 179-194
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Acta Medica Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

A New HLA-DRB1 Genotyping Method Using Single Nucleotide Polymorphism (SNP) Analysis with Multiplex Primer Extension Reactions and Its Application to Mixed Samples

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We have improved on conventional methods for HLA-DRB1 genotyping and devised a new method that is simple, cost-effective, and adequately applicable to routine forensic practice. This method consists of group-specific polymerase chain reaction (PCR) of the exon 2 region of the HLA-DRB1 gene and simultaneous detection of single nucleotide polymorphisms (SNPs) at multiple sites using multiplex primer extension reactions. With this method, we successfully detected HLA-DRB1 genotypes from the following materials: the peripheral blood of 142 donors, 6 aged saliva stains of known DRB1 genotype stored for 5-10 years at room temperature, 10 aged bloodstains of unknown DRB1 genotype stored for 29 years at room temperature, and minimal bloodstains and saliva stains from 3 donors of known DRB1 genotypes. Furthermore, we were able to type DRB1 alleles of the minor component in mixed samples at a proportion of 1/1,000 or 1/10,000. In a criminal case, DRB1 alleles detected from mixed bloodstains on a sword found at the scene enabled us to explain the case. This method is expected to be useful for forensic medicine.

Key words: HLA-DRB1 genotyping, group specific primer, single nucleotide polymorphism, multiplex primer extension reactions, application to mixed samples

Among the human leukocyte antigen (HLA) genes on chromosome 6, the class II DRB1 gene is known to be highly polymorphic. More than 200 alleles, including some rare ones, can be found in databases [1]. In the fields of transplantation and transfusion, a number of reports on HLA-DRB1 genotyping methods have been published [2-8].

In forensic medicine, DRB1 genotyping using forensic samples, such as blood, bloodstains, and saliva stains, provides a useful means of personal identification and paternal tests [9-13]. The polymerase chain reaction (PCR) methods conventionally used for DRB1 genotyping include PCR-restriction fragment length polymorphism (RFLP), PCR sequence-specific primers (SSP), and PCR sequence-specific oligonucleotide probes (SSOP). These methods are seldom used at present for routine forensic tests because they require more complex manipulations and are more costly and time-consuming than microsatellite (STR) polymorphism typing, which has often been used for forensic medicine in recent years.

Received February 16, 2005; accepted March 11, 2005.
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Toward the goal of establishing a forensic testing method that is simpler and more cost-effective than conventional DRB1-typing methods and that is applicable to routine forensic practice, we have recently attempted DRB1 genotyping by single nucleotide polymorphism (SNP) analysis, making use of multiplex primer extension reactions. With this method, DRB1 genotyping is performed in 2 steps. The first step is allele group typing by PCR with group-specific primers [8]. The second step is DRB1 allele typing by the simultaneous detection of SNPs at multiple sites in the HLA-DRB1 gene using multiplex primer extension reactions.

In addition, mixed samples — those that include materials from more than one individual, such as mixed bloodstains and semen mixed with vaginal fluid — are sometimes investigated in routine forensic practice. If STR polymorphism analysis using conventional PCR is applied to mixed samples, competition may occur between the major and minor components of the samples during annealing. Therefore, it is difficult to detect the minor component of a mixed sample using such a method. However, our new method makes it possible to detect the minor component in a mixed sample by amplifying only the allele originating from that component. In this study, we first conducted a basic experiment using mixed samples to examine how this method could be applied to forensic medicine. We then applied the method to mixed samples collected during actual forensic cases, and attempted to type the HLA-DRB1 genes originating from the samples' major and minor components.

Materials and Methods

Materials. DNA samples were extracted from the peripheral blood lymphocytes of 142 individuals who had given informed consent (114 Japanese, 24 Germans, 2 Turks, 1 Peruvian, and 1 Paraguayan). Minimal bloodstains and saliva stains were prepared from 3 volunteers know to have the DRB1 genotype. Blood and saliva were sampled, and each sample (0.5 μl) was applied to a cotton cloth. The aged stains included 6 saliva stains with known DRB1 genotypes that had been stored for 5-10 years at room temperature, as well as 10 bloodstains with unknown DRB1 genotypes that had been stored for 29 years at room temperature. The numbers of mixed DNA solutions were 3 pairs and DNA solutions from 2 different individuals known to have the DRB1 genotype were combined at various ratios (from 1:1 to 1:10,000). Mixed bloodstains with varying mixture ratios were prepared for 8 pairs. Peripheral blood samples were collected from 6 volunteers known to have the DRB1 genotype, and mixed blood samples were prepared from pairs of individuals at mixture ratios ranging from 1:1 to 1:10,000. To produce mixed-sample bloodstains, each sample of mixed blood (200 μl) was applied to the surface of a wooden plate, and this coat was air-dried for 24 h at room temperature.

In a criminal case, bloodstains at 3 locations on a sword were collected using a bleached cloth for subsequent DNA extraction. The blood of the 2 cadavers at the scene of the crime was also collected during autopsies in order to compare the alleles of the sword bloodstains with the alleles of each cadaver.

To extract DNA from each sample, a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used. The DNA concentration was measured using GeneQuant II (Amersham Biosciences, Piscataway, NJ, USA).

Designing the primers for allele group-specific PCR. As shown in Table 1, individual alleles of the DRB1 gene can be divided into 10 groups according to their serological specificity. In our method, in accordance with the method adopted at the 11 th International Histocompatibility Workshop, these alleles are divided into 7 groups [14]. The DRB1 exon 2 region was amplified by PCR using a primer specific to each allele group. For the mixed samples, which were expected to have multiple alleles belonging to the DR3 group, a primer specifically amplifying alleles of the DR8 and DR12 groups was also employed [13]. Fig. 1 shows the

Table 1 Serological groups and alleles of HLA-DRB1 gene

Groups	Alleles
DR1	0101-0104
DR2	1501-1504
	1601-1605
DR3	0301-0302
DR4	0401-0421
DR5	1101-1103
(DR11, DR12)	1201-1202
DR6	1301-1307
(DR13, DR14)	1401-1412
DR7	0701-0704
DR8	0801-0804
DR9	09012
DR10	10011-10012

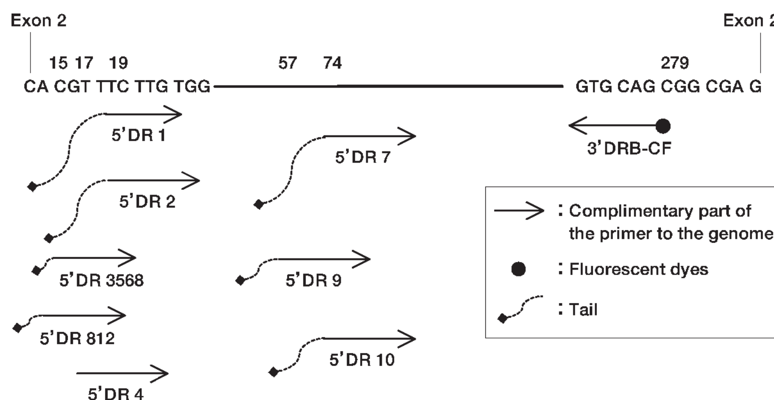


Fig. 1 Exon 2 region of HLA-DRB1 gene and the position of the group-specific primers. The sequence of about 20 terminal bases of the forward primers was designed to be complementary to the genome, while TGA and variable-length poly-T tails were attached to the 5'-terminal. A reverse primer was common to each amplification group.

Table 2 Primers for PCR amplification

Corresponding allele group	Name	Sequence (5'-3')	Product length with a tail (bp)*	Actual length of amplified region (bp)
Forward primer				
DR1	5'DR1	TGA-36T-TTCTTGTGGGAGCTTAAGTT	301	262
DR2	5'DR2	TGA-25T-TTCCTGTGGCAGCCTAAGAGG	290	262
DR3, 5, 6, 8	5'DR3568	TGA-8T-ACGTTTCTTGGAGTACTCTACG	277	266
DR4	5'DR4	GTTTCTTGGAGCAGGTTAAAC	264	264
DR7	5'DR7	TGA-47T-AGTTCCTGGAAAGACTCTTCT	257	207
DR9	5'DR9	TGA-17T-GGACGGAGCGGGTGCGGTATC	242	222
DR10	5'DR10	TGA-21T-GGTTGCTGGAAAGACGCGTCC	231	207
DR8, 12	5'DR812	TGA-4T-CACGTTTCTTGGAGTACTCTACGGG	274	267
Reveres primer				
Common	3'DRB-CF	Cy5-FITC-dT-CCGCTGCACTGTGAAGCTCT		

*The product length with a tail means the whole length of the amplification product obtained by adding the length of the actually amplified DNA region to the length of the tail attached to forward primers.

exon 2 region of the DRB1 gene and the positions of group-specific primers. Table 2 shows the sequences of the PCR primers. Thus, the lengths of the amplification products of each allele group were changed in order to allow simultaneous detection of amplification products during capillary electrophoresis. A common reverse primer was used for each amplification group, and its 5'-terminal was labeled with Cy5 and FITC.

Positive control for amplification. To form a positive control for amplification, we prepared PCR primers for exon 2 of the human immunoglobulin heavy constant gamma 3 (IGHG3) gene on chromosome 14 (Table 3). To obtain 7 different amplification products of IGHG3 with different lengths corresponding to the 7 allele groups of the DRB1 gene, the 5'-terminal of a common reverse primer was attached to a TGA tail and labeled with Cy5.

Allele group-specific PCR. Using the primers shown in Tables 2 and 3, we carried out group-specific PCR for each allele group. The amplification was performed in a 10 μl reaction mixture containing 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.2 mM of each dNTP, 3.0 mM $MgCl_2$, 0.8 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 4.0 μg bovine serum albumin (Takara Bio, Otsu, Shiga, Japan), 0.25 mM of each primer for DRB1 and IGHG3

Table 3 Positive control primers

Name	Sequence (5'-3')	Product length with a tail (bp)*	Corresponding allele group
Forward primer			
IGHG3-F1	TGA-21T-GTGCTGTTGTACTGGTCCTC	194	DR1
IGHG3-F2	TGA-17T-GTGCTGTTGTACTGGTCCTC	190	DR2
IGHG3-F3568	TGA-13T-GTGCTGTTGTACTGGTCCTC	186	DR3, 5, 6, 8
IGHG3-F4	TGA-9T-GTGCTGTTGTACTGGTCCTC	182	DR4
IGHG3-F7	TGA-5T-GTGCTGTTGTACTGGTCCTC	178	DR7
IGHG3-F9	TGA-1T-GTGCTGTTGTACTGGTCCTC	174	DR9
IGHG3-F10	GTGCTGTTGTACTGGTCCTC	170	DR10, DR8, 12
Reveres primer			
IGHG3-R	Cy5-TGACCCCCCAAAACCCAAGGATACC		Common

*The product length with a tail means the whole length of the amplification product obtained by adding the length of the actually amplified DNA region to the length of the tail attached to forward primers.

amplification (positive control primers), and template genomic DNA. For amplification of the DR4 and DR9 groups, the $MgCl_2$ concentration was set at 2.0 mM. As a rule, 10 ng of template DNA was contained in 10 μl of the PCR reaction mixture. For mixed samples, the amount of total template DNA contained in 10 μl of PCR reaction mixture was set at 100 ng. PCR was performed for pre-denaturation at 95 °C for 11 min and at 96 °C for 2 min, and 40 amplification cycles, each consisting of denaturation at 96 °C for 50 sec, annealing at 60 °C for 50 sec, and extension at 72 °C for 50 sec, were conducted using a thermal cycler PHC-3 (Techne, Cambridge, UK).

Analysis of group-specific PCR products. A loading cocktail was prepared by mixing 3.5 μl of fluorescent ladder (CXR) 60-400 bp (Promega, Madison, WI, USA) and 1 ml of Hi-Di™ Formamide (Applied Biosystems) before capillary electrophoresis. One μl of each allele group-specific PCR product was combined in a single tube. One μl of this mixture was combined with 10 μl of the loading cocktail. When the amount of template DNA was very small, 1 μl of each PCR product was combined with 10 μl of the loading cocktail. The mixture was heated at 95 °C for 3 min and then ice-cooled before capillary electrophoresis. Electrophoresis was performed for 24 min at a voltage of 15 kV and a temperature of 60 °C using an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with a 47 cm capillary and performance-optimized polymer 4 (POP4, Applied Biosystems). GeneScan Analysis Software (version 3.2.1; Applied Biosystems) was employed for analysis of the electrophoresis data. Allele groups were typed on the basis of DNA size markers and homemade ladders. The ladders for allele group typing were prepared in 2 steps: (1) group-specific PCR using DNA known to be of the DRB1 genotype, and (2) mixing the PCR products for 7 alleles belonging to each allele group.

For the allele group for which amplification of the DRB1 gene had been confirmed, 5 μl of the PCR reaction mixture was added to 10 units of exonuclease (USB, Cleveland, OH, USA) and 1 unit of shrimp alkaline phosphatase (SAP, USB). The mixture was incubated at 37 °C for 30 min and then at 80 °C for 15 min. In this manner, non-reactive primers and dNTP were decomposed, yielding a template for the subsequent multiplex primer extension reactions.

Designing SNP typing primers. To detect SNPs by means of multiplex single-base extension reactions, alleles were divided into 3 groups (A, B, and C) and primers for typing SNPs were prepared for each group. As shown in Table 4, 7 SNP-typing primers were prepared for group A alleles belonging to DR1, 2, 7, 9, and 10. group B was subdivided into B-1 and B-2, and 5 SNP-typing primers were prepared for each of the B-1 and B-2 groups (10 primers in total), because DR3, 5, 6, and 8 were composed of many alleles. In group B-1, alleles were subjected mainly to low-resolution typing, whereas in group B-2 they were subjected to high-resolution typing. In group C, 6 SNP-typing primers were prepared to type the DR4 alleles. These SNP-typing primers were designed so that the 3'-terminal of SNP-typing primers would be located immediately before each SNP detection site. Furthermore, to allow simultaneous detection of multiple products of single-base

Table 4 SNP-typing primers for DRB1 allele typing

Groups for allele typing		Name	Length (mer)	SNP site	Direction	Sequence (5'-3')
Group A (DR1, 2, 7, 9, 10)		P1	24	159	Forward	GGAGTACCGGGCGGTGACGGAGCT
		P2	31	199	Forward	TGATGCCGAGTACTGGAACAGCCAGAAGGAC
		P3	34	82	Forward	10T-TGGACGGAGCGGGTGCGGTTTCTG
		P4	41	216	Reverse	11T-GTTGTGTCTTCAGTAGGTGTCCACCGCGGC
		P5	48	234	Reverse	27T-AACCCCTTAGTTGTGTCTGCA
		P6	56	257	Forward	36T-CAGACAGAACTACGGGGTTG
		P7	64	119	Reverse	31T-GTCACCGCCCGGTACTCCCCCACGTCGCTGTCG
Group B (DR3, 5, 6, 8)	B-1	P8	26	46	Reverse	CACCCGCTCCGTCCCATTGAAGAAAT
		P9	27	84	Forward	TGGGACGGAGCGGGTGCGGTTCCTGGA
		P10	33	178	Reverse	2T-TCTTCCAGGAGGTCCTTCTGGCTGTTCCAGT
		P11	41	221	Reverse	11T-CCGTAGTTGTGTCTGCAGTAGGTGTCCACC
		P12	51	234	Roverse	21T-GCTCTCACCAACCCCGTAGTTGTGTCTGCA
	B-2	P13	24	48	Reverse	CACCCGCTCCGTCCCATTGAAGAA
		P2	31	199	Forward	TGATGCCGAGTACTGGAACAGCCAGAAGGAC
		P14	37	94	Reverse	8T-GTCGAAGCGCACGTACTCCTCTTGGTTAT
		P15	46	169	Forward	17T-ACCGGGCGGTGACGGAGCTGGGGCGGCCT
		P16	58	258	Reverse	31T-AACTCGCCGCTGCACTGTGAAGCTCTC
Group C (DR4)		P17	27	221	Reverse	TAGTTGTGTCTGCAGTACGTGTCCACC
		P18	35	212	Reverse	5T-TGTCTGCAGTAGGTGTCCACCCCGGCCCGC
		P19	39	169	Forward	9T-TACCGGGCGGTGACGGAGCTGGGGCGGCCT
		P20	47	110	Forward	18T-GGACAGATGCTTCTATCACCAAGAGGAGT
		P21	53	174	Reverse	23T-CAGGAGGTCCTTCTGGCTGTTCCAGTACTC
		P6	56	257	Forward	36T-CAGACAGAACTACGGGGTTG

Group B was subdivided into Group B-1 and Group B-2 because of many alleles belonging to DR3, 5, 6 and 8.

extension reactions during capillary electrophoresis, poly-T was added to the 5'-terminal and the lengths of SNP-typing primers were varied within each SNP-typing group.

Multiplex single-base extension reactions. Using the SNP-typing primers, a multiplex single-base extension reaction was performed for each amplification-confirmed group by using a SNaPshot Multiplex Kit (Applied Biosystems) (Table 4).

Each single-base extension reaction was performed using a 10 μl reaction mixture containing 5 μl of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), SNP-typing primers (each 0.1–3.0 pmol), and 1 μl of PCR products. The reaction mixture was combined with group-specific PCR amplification primers and positive control primers. Furthermore, $(NH_4)_2SO_4$ (Katayama Chemical Industries, Osaka, Japan) was added to the reaction mixture in a final concentration of 20 mM to suppress nonspecific reactions caused by factors such as the high-level structure of SNP-typing primers and the formation of primer dimers [15]. Single-base extension reactions were induced for 25 cycles with a PHC-3 thermal cycler, with each cycle consisting of denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec, and extension at 60 °C for 30 sec.

Analysis of products from multiplex single-base extension reactions. The product from each extension reaction (5 μl) was combined with 1 unit of SAP. The mixture was incubated at 37 °C for 30 min and then at 80 °C for 15 min to digest the non-reactive ddNTP. One μl of the product was combined with 10 μl of Hi-Di™ formamide. The mixture was heated at 95 °C for 3 min and then ice-cooled, yielding a sample for capillary electrophoresis. Electrophoresis was performed for 15 min at a voltage of 15 kV and a temperature of 60 °C, using an ABI Prism 310 Genetic Analyzer with a 47 cm capillary and POP4. GeneScan Analysis Software (version 3.2.1) was used for analysis of the electrophoresis data to type the base substitution at each SNP site. DRB1 alleles were typed by comparing the

combinations of substitute bases detected by capillary electrophoresis with those expected from the database (Table 5) [1].

Confirming HLA-DRB1 genotypes. Group-specific PCR products from samples were subjected to 8% polyacrylamide gel electrophoresis. The target bands were eluted, yielding template DNA for sequencing. Cycle sequencing was performed using a BigDye Terminator Kit (Applied Biosystems), with the sequence complementary to the forward primer genome serving as the sense primer and the reverse primer without fluorescent dye serving as the antisense primer. The protocol attached to the kit was used for analysis of the sequences. Sequences were determined using an ABI Prism 310 Genetic Analyzer and the Sequence Analysis Software (version 3.0, Applied Biosystems). DRB1 genotypes were determined using the modified PCR-RFLP method reported by Ota [8] and Inoue [12].

ABO blood typing and multiplex STR typing. In the criminal case, routine ABO blood typing and an STR polymorphism test using a PowerPlex 16 system (Promega, Madison, WI, USA) were performed.

Results

Typing of DNA extracted from peripheral blood lymphocytes. When the DNA of 143 individuals was examined from peripheral blood lymphocytes and the products from group-specific PCR were analyzed by capillary electrophoresis, 7 peaks of amplified fragments of the IGHG3 gene (positive control for each of the group-specific reaction systems) were noted in the low molecular weight range. In the high molecular weight range, 1 or 2 peaks of amplified fragments of the DRB1 gene were noted in each sample. The allele group of the DRB1 gene was determined by comparing these amplified fragments of the gene with the ladders for allele group typing. If the alleles from the same individual belonged to 2 allele groups, 2 peaks of amplified fragments of the DRB1 gene were detected. In cases where both alleles of an individual belonged to the same allele group or in homozygous cases, 1 peak of amplified fragments of the DRB1 gene was detected. The combinations of the detected bases were compared with Table 5 to determine the types of DRB1 alleles. Fig. 2 shows an example of typing for 1 individual with a DRB1 genotype common among Japanese. Fig. 2A shows the electropherogram after group-specific PCR. This sample was interpreted as possessing DR4 and DR3 alleles. Fig. 2B shows the results of group B and group C SNP-typing for this sample. This combination of bases allowed us to judge that the DR3 group allele for this sample was DRB1*1405. In the typing of group C, which includes DR4, the allele was judged to be DRB1*0410, judging from the combination of detected bases. Fig. 3 shows the typing results of the DRB1 allele and 'heterozygous' cases, where both of the alleles of an individual belong to the same allele group. The results of 66 DRB1 typing with this method coincided with the results of DRB1 typing carried out by sequencing or PCR-RFLP.

Sensitivity. The amount of DNA available for forensic analysis in actual cases is often small. We therefore evaluated the proposed method's sensitivity (Table 6). In all allele groups, peaks of PCR products were detected and typing was possible when the template DNA was available in amounts greater than 5 pg. Fig. 4 presents a portion of the results, showing the electropherogram for group-specific PCR products for the DR9 allele group (DRB1*09012).

Minimal stains and aged stains. Because the amounts of extracted DNA are expected to be small for minimal samples and aged samples, we examined the utility of this method in such cases. This method enabled the correct typing for all 9 minimal bloodstains and all 9 minimal saliva stains of known DRB1 genotype; these samples had been left at room temperature for 1 to 7 days after they were prepared (data not shown). Six of the aged saliva stains stored for 5–10 years and all 10 of the aged bloodstains stored for 29 years were correctly typed by this method (Tables 7 and 8).

Mixed DNA solutions. We first conducted a basic experiment using mixed DNA solutions. Fig. 5 shows the electropherogram for group-specific PCR products from mixed DNA samples of individual A (DR2 allele group: DRB1*1501; DR4 allele group: DRB1*0405) and individual B (DR4 allele group: DRB1*0403; DR9 allele group: DRB1*09012). At mixture ratios (individual A to individual B) ranging from 1:1 to 1:1,000, the amplification products of each allele group corresponding to the allele groups possessed by each individual (DR9, DR4, and DR2) were observed. When the proportion of the minor component was further decreased, to a 1:10,000 mixture ratio, no peak of the DR2 allele group (possessed only by individual A and serving as the minor component) was noted.

Table 5 Combinations of HLA-DRB1 alleles and bases detected by each SNP-typing primer in this method

Allele		SNP-typing primers									
Group A		P1	P2	P3	P4	P5	P6	P7			
DR1	DRB1*0101	G	C	G	*C	*G	G	*A			
DR2	DRB1*1501	G	A	G	*C	*G	T	*A			
	DRB1*1502	G	A	G	*C	*G	G	*A			
	DRB1*16011	G	T	G	*G	*G	G	*A			
	DRB1*16012	G	T	G	*C	*G	G	*A			
	DRB1*16021	G	C	G	*G	*G	G	*A			
	DRB1*16022	G	C	G	*C	*G	G	*A			
DH7	DRB1*0701	A	A	—	—	*C	G	*A			
	DRB1*0704	A	A	—	*C	*G	G	*A			
DR9	DRB1*09012	G	T	C	*C	*C	G	*A			
DR10	DRB1*10011	G	C	—	*A	*G	G	*T			
	DRB1*10012	G	C	—	*G	*G	G	*T			
Group B		B-1					B-2				
		P8	P9	P10	P11	P12	P13	P2	P14	P15	P16
DR3568	DRB1*03011	*G	C	*A	*C	*G	*A	C	*G	G	*C
	DRB1*03021	*G	G	*A	*C	*G	*A	C	*G	G	*A
	DRB1*0801	*A	C	*A	*A	*G	*A	T	*A	A	*A
	DRB1*08021	*A	C	*A	*A	*G	*A	T	*A	G	*A
	DRB1*08032	*A	C	*A	*A	*G	*A	A	*A	A	*A
	DRB1*08041	*A	C	*A	*A	*G	*A	T	*A	G	*C
	†DRB1*11011	*G	C	*A	*G	*G	*A	T	*A	G	*A
	DRB1*1102	*G	C	*A	*G	*G	*A	A	*A	G	*C
	DRB1*1103	*G	C	*A	*G	*G	*A	T	*A	G	*C
	DRB1*1201	*A	G	(*A)	*G	*A	*A	A	*G	G	*C
	DRB1*12021	*A	G	(*A)	*G	*A	*A	T	*G	G	*C
	DRB1*12022	*A	G	(*A)	*G	*G	*A	T	*G	G	*C
	DRB1*13011	*G	C	*A	*G	*G	*A	A	*G	G	*C
	DRB1*13021	*G	C	*A	*G	*G	*A	A	*G	G	*A
	DRB1*1304	*G	C	*A	*G	*G	*A	A	*A	A	*C
	†DRB1*13071	*G	C	*A	*G	*G	*A	T	*A	G	*A
	DRB1*14011	*G	C	*G	*T	*A	*A	C	*G	G	*C
	DRB1*1402	*G	G	*A	*G	*G	*A	C	*G	G	*A
	DRB1*1403	*G	G	*A	*A	*G	*A	C	*G	G	*A
	DRB1*1405	(*G)	C	*A	*T	*A	*T	C	*G	G	*C
	DRB1*1406	*G	G	*A	*G	*G	*A	C	*G	G	*C
	DRB1*1407	*G	C	*G	*T	*A	*A	C	*G	G	*A
	DRB1*1412	*G	G	*A	*A	*G	*A	C	*G	G	*C
Group C		P17	P18	P19	P20	P21	P6				
DR4	DRB1*0401	*G	*T	G	A	*G	G				
	DRB1*0402	*G	*T	G	A	*G	T				
	DRB1*04031	*T	*C	G	A	*G	T				
	DRB1*04032	*T	*C	G	A	*A	T				
	DRB1*0404	*G	*C	G	A	*G	T				
	DRB1*0405	*G	*C	A	A	*G	G				
	DRB1*0406	*T	*C	G	C	*G	T				
	DRB1*0407	*T	*C	G	A	*G	G				
	DRB1*0408	*G	*C	G	A	*G	G				
	DRB1*0409	*G	*T	A	A	*G	G				
	DRB1*0410	*G	*C	A	A	*G	T				

The bases marked with asterisk indecate bases on the antisense chain of template DNA. The bases in parentheses are those for which the signals of extension reaction products are impossible to detect or very weak because of the presence of mutation in the template DNA near the 3'-terminal of the SNP-typing primers for SNP sites detection. Hyphens indicate that the SNP sites detected by the SNP-typing primers was not included in the region of group-specific PCR, resulting in the absence of single-base extension reactions and the lack of signal of such reactions.
†The SNP-typing primers of this method can't distinguish HLA-DRB1* 11011 from *13071.

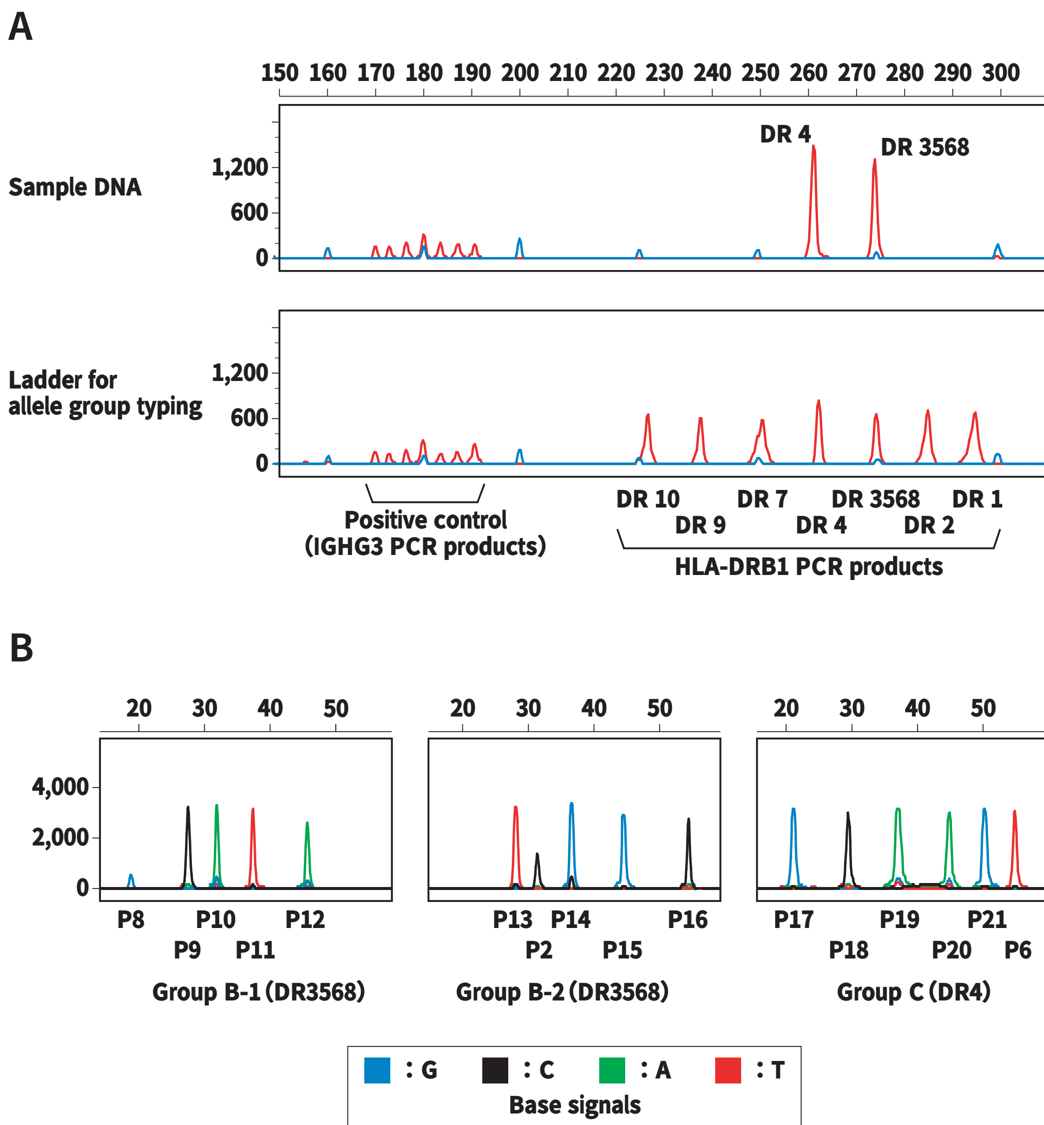


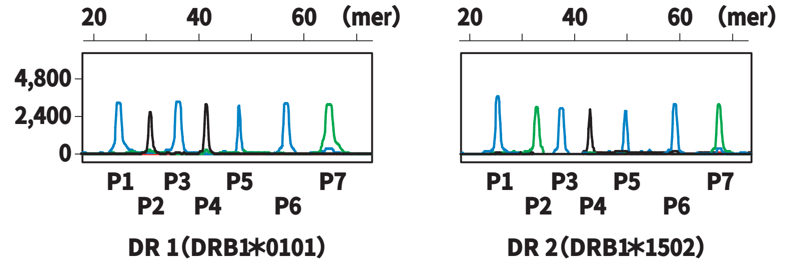
Fig. 2 An example of typing for an individual with a DRB1 genotype common among Japanese. **A**: Electropherogram showing the results of group-specific PCR. The lower part shows the ladders used for determining DRB1 allele groups. Seven red peaks of amplified fragments of positive controls were noted in the low molecular weight range (160–200 bp). Seven red peaks of amplified fragments for each allele group (DR1 through DR10) were noted in the high molecular weight range (220–300 bp). The blue peaks represent DNA size markers. The sample DNA showed 7 red peaks of the positive control in the low molecular weight range. In addition, 2 red peaks at about 240 and 275 bp, indicating amplification of the DRB1 gene, were noted in the high molecular weight range. **B**: Electropherogram showing the results of SNP typing after multiplex single-base extension reactions. In the typing of group B (B-1 and B-2), the P8 to P16 and P2 primers detected the following bases, respectively: G, C, A, T, A, T, G, G, C, and C. In group C typing, the combination of bases was G, C, A, A, G, and T.

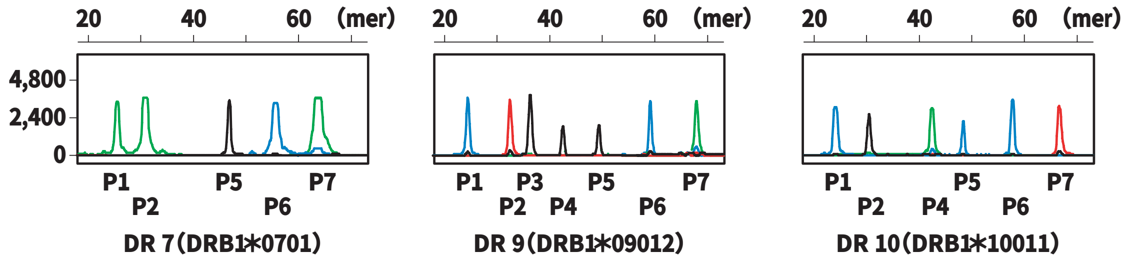
Table 6 Sensitivity of group-specific PCR

Allele group	Number of samples	Template DNA			
		1 ng	10 pg	5 pg	1 pg
DR1	3	+	+	+	—
DR2	3	+	+	+	—
DR3, 5, 6, 8	3	+	+	+	—
DR4	3	+	+	+	—
DR7	1	+	+	+	—
DR9	3	+	+	+	—
DR10	3	+	+	+	—

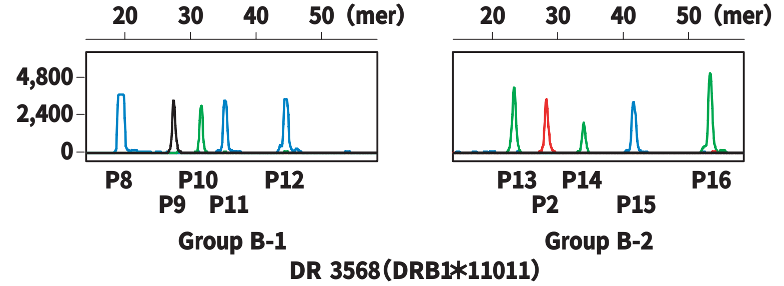
+, Detected; —, Not detected.

Group A
(DR 1, DR 2, DR 7,
DR 9, DR 10)

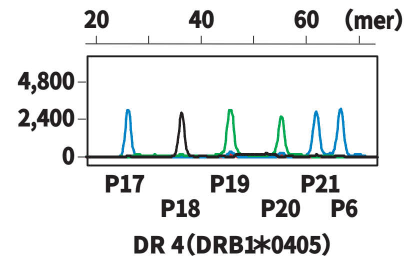




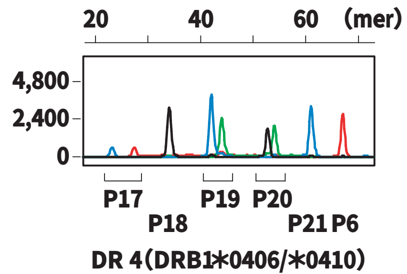
Group B
(DR 3568)



Group C
(DR 4)



(DR 4 ; heterozygous)



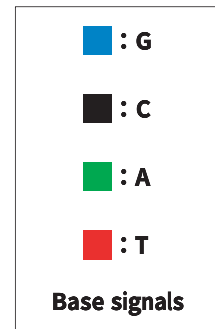


Fig. 3 Electropherogram showing the results of DRB1 genotyping for alleles common in Japanese. The figure also contains the result of typing for a 'heterozygous' case (DR4).

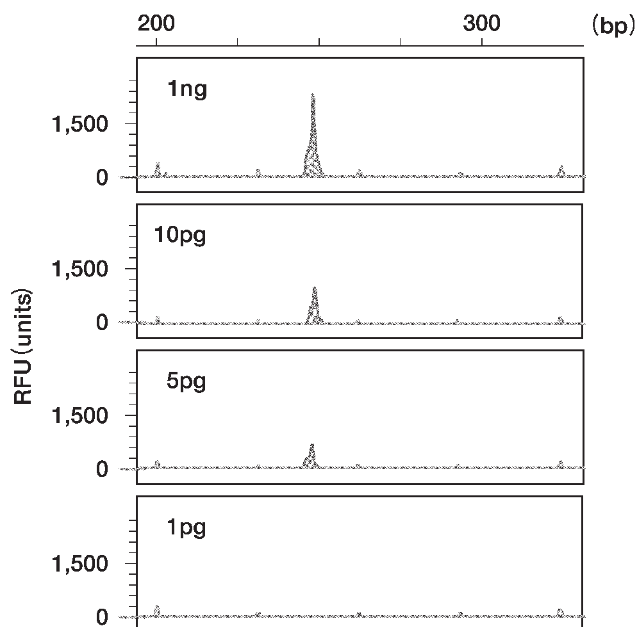


Fig. 4 Sensitivity of group-specific PCR. Electropherogram of group-specific PCR products for the DR9 allele group are shown. Template DNA (1 ng to 1 pg) was examined.

Table 7 HLA-DRB1 typing from aged saliva stains

Aged saliva stain	Allele groups detected	Alleles detected	Known DRB1 genotypes
1	DR3 DR9	DRB1*1302 DRB1*09012	*1302/*09012
2	DR3 DR9	DRB1*1302 DRB1*09012	*1302/*09012
3	DR1 DR3	DRB1*0101 DRB1*1406	*0101/*1406
4	DR3 DR9	DRB1*08032 DRB1*09012	*08032/*09012
5	DR1 DR9	DRB1*0101 DRB1*09012	*0101/*09012
6	DR2 DR3	DRB1*1502 DRB1*1401	*1502/*1401

Fig. 6 shows the electropherogram for the results of multiplex single-base extension reactions. When individuals A and B shared an allele group (DR4 group), the minor component was detected only at the 1:1 and 1:10 mixture ratios. However, when the proportion of the minor component was less than 1:10, only the major component was detected (Fig. 6A). When individuals A and B had no shared allele group (DR2 and DR9 allele groups), the allele DRB1*1501, originating from individual A, was detected when the proportion of the minor component was as low as 1:1,000 (Fig. 6B). The allele DRB1*09012, originating from individual B (possessed only by individual B and serving as the minor component), was typed at all of the ratios (Fig. 6C). For the 2 other mixed DNA solutions, the bases originating from the minor component at a 1:1,000 ratio were detected when the minor component shared either no allele group or only 1 allele group of the major component.

Mixed bloodstains. Table 9 shows the results of typing for the allele groups found only in the minor component when mixed bloodstains were used. When the proportion of the minor component was 1:1,000 or higher, PCR products originating from the minor component were detected in almost all cases. When the propor-

Table 8 HLA-DRB1 typing from aged bloodstains

Aged bloodstain	Allele groups detected	Alleles determined
1	DR3 DR10	*1406 *10011
2	DR2 DR4	*1502 *04031
3	†DR9	*09012
4	‡DR4	*0405
5	DR3 DR4	*1405 *04011
6	DR1 DR4	*0101 *0406
7	DR1 DR2	*0101 *1502
8	DR1 DR9	*0101 *09012
9	DR2 DR9	*1502 *09012
10	DR3 DR4	*08021 *0406

†: homozygous (*09012/*09012 or *09012/—)
‡: homozygous (*0405/*0405 or *0405/—)

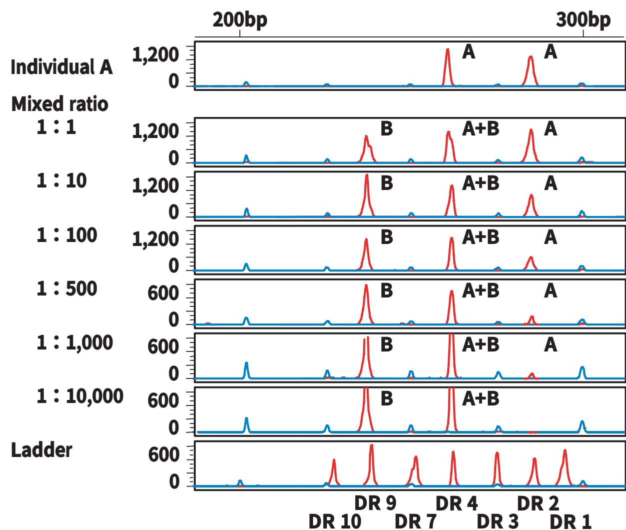


Fig. 5 Electropherogram showing the results of group-specific PCR of mixed DNA solutions. DNA solutions from individual A (DR2: DRB1*1501; DR4: DRB1*0405) and individual B (DR4: DRB1*0403; DR9: DRB1*09012) were mixed at ratios of 1:1 to 1:1,000, and then subjected to group-specific PCR. At the mixture ratios 1:1 to 1:10, the peak height of the DR2 allele group (possessed only by individual A and serving as the minor component) was comparable to that of the DR9 allele group (possessed only by individual B and serving as the major component) and that of the DR4 allele group. At mixture ratios of 1:100 and 1:1,000, the peak height of the DR2 allele group was lower than that of the DR9 or DR4 allele group.

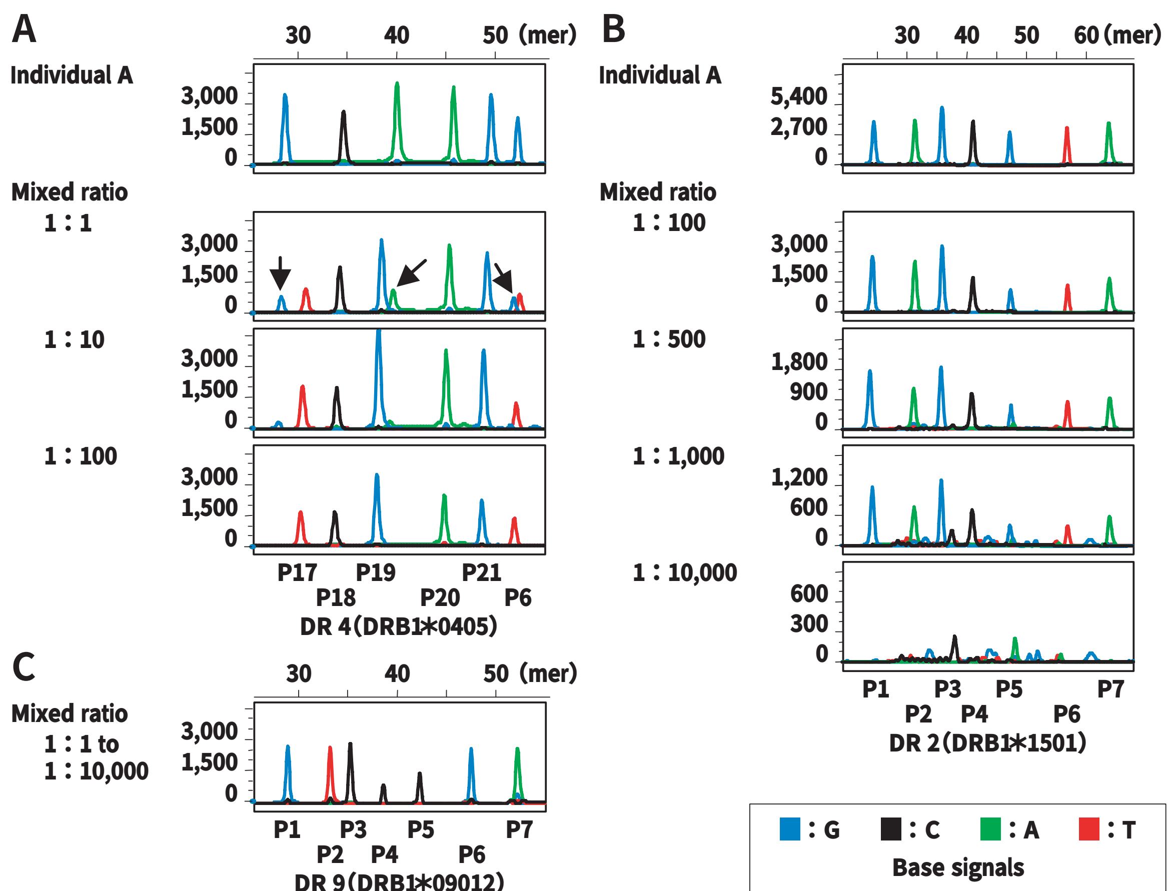


Fig. 6 Electropherogram showing the results of multiplex single-base extension reactions from group-specific PCR products of mixed DNA solutions. **A**, Individuals A and B shared an allele group (DR4 allele group); **B**, Individuals A and B had no shared allele group (DR2 allele group); **C**, Individuals A and B had no shared allele group (DR9 allele group).

Table 9 Results of HLA-DRB1 typing from mixed bloodstains

Mixed bloodstain	Allele groups of minor component	Mixed ratio (amount of minor component expected)					
		1:1,000 (100 pg)			1:10,000 (10 pg)		
		Number detected	/	number examined	Number detected	/	number examined
1	DR3	3	/	3	2	/	3
2	DR4	3	/	3	3	/	3
3	DR9	3	/	3	3	/	3
4	DR1	1	/	3	0	/	3
	DR3	3	/	3	0		3
5	DR2	3	/	3	0	/	3
	DR4	3	/	3	3	/	3
6	DR2	3	/	3	2	/	3
	DR9	3	/	3	3	/	3
7	DR2	3	/	3	3	/	3
	DR9	3	/	3	3	/	3
8	DR4	3	/	3	2	/	3
Total		34	/	36	24	/	36

tion was 1:10,000, PCR products from the minor component were detected in more than half the cases. In subsequent multiplex single-base extension reactions, SNPs were detected from which amplified fragments were observed, and allele typing was possible.

Criminal case: Examination of bloodstains on a sword. A male and a female were found dead indoors, facing each other. Blood, probably originating from both persons, had pooled around the bodies, between which lay a bloodstained sword. To determine whether or not this weapon was responsible for both deaths, the bloodstains on it were examined for comparison with the blood of the 2 bodies.

In ABO blood typing, the male was judged as type B and the female as type A. All 3 of the bloodstains found on the sword were rated as type B. In STR polymorphism analysis of the bloodstains, typing was possible for the component originating from the male but not for that originating from the female. However, when DRB1 genotyping was performed by this method, 3 alleles were detected on each of the 3 bloodstains, and it was possible to type the alleles of each DRB1 gene originating from the male and the female. The bloodstains were rated as DRB1*0405, DRB1*08032, and DRB1*1302; the male was rated as a homozygous type (DRB1*0405) and the female as DRB1*08032 and DRB1*1302 (both alleles belonging to the DR3 group).

Fig. 7 and 8 show some of the results of DRB1 typing in this case. In the electropherogram of group-specific PCR products (Fig. 7), the bloodstains showed amplification of the DR4 and DR3 allele groups; the female showed amplification of the DR3 allele group and the male showed that of the DR4 allele group. In the subsequent SNP typing by multiplex single-base extension reactions (Fig. 8), the bloodstains were judged to have alleles DRB1*08032 (DR3), DRB1*13021 (DR3), and DRB1*0405 (DR4); the female possessed alleles DRB1*08032 (DR3) and DRB1*13021 (DR3) whereas the male possessed allele DRB1*0405 (DR4). Thus, considering the DRB1 genotypes of the comparative samples from these 2 cadavers, the DRB1 alleles detected in the bloodstains on the sword were judged to be identical to the alleles expected for a mixture of DNA from this male-female couple. The findings about the 3 bloodstains on the sword did not contradict the view that they were produced by a mixture of blood from these 2 cadavers. We therefore concluded that the sword was the weapon responsible for the death of both individuals.

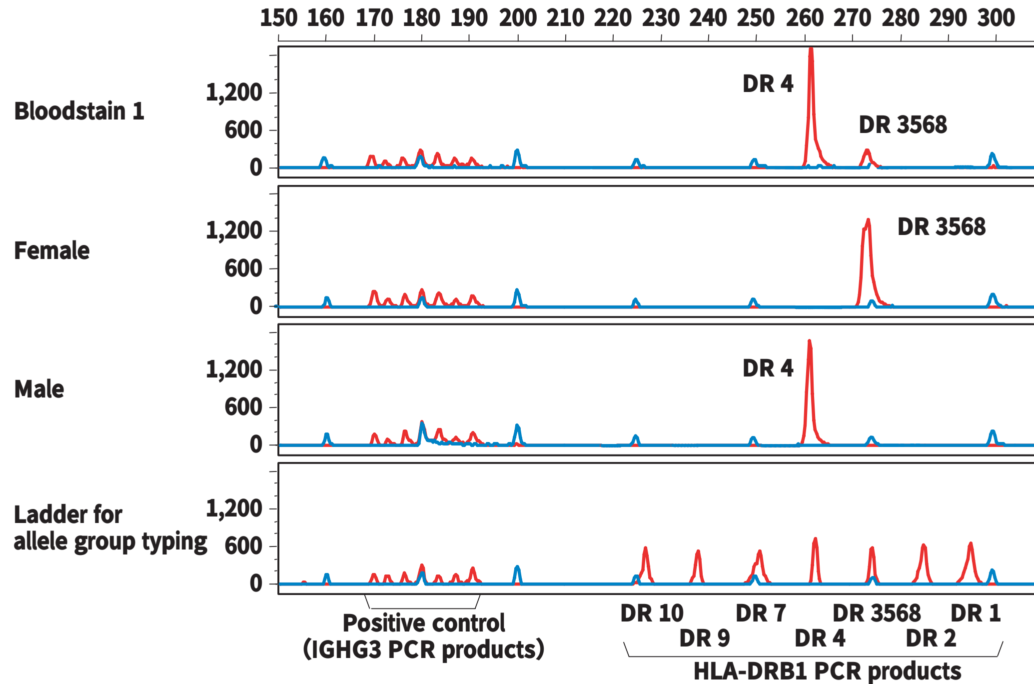


Fig. 7 Electropherogram showing the results of group-specific PCR in a criminal case. The bloodstain contained DR4 and DR3 allele groups, and the female and male possessed the DR3 and DR4 allele groups, respectively.

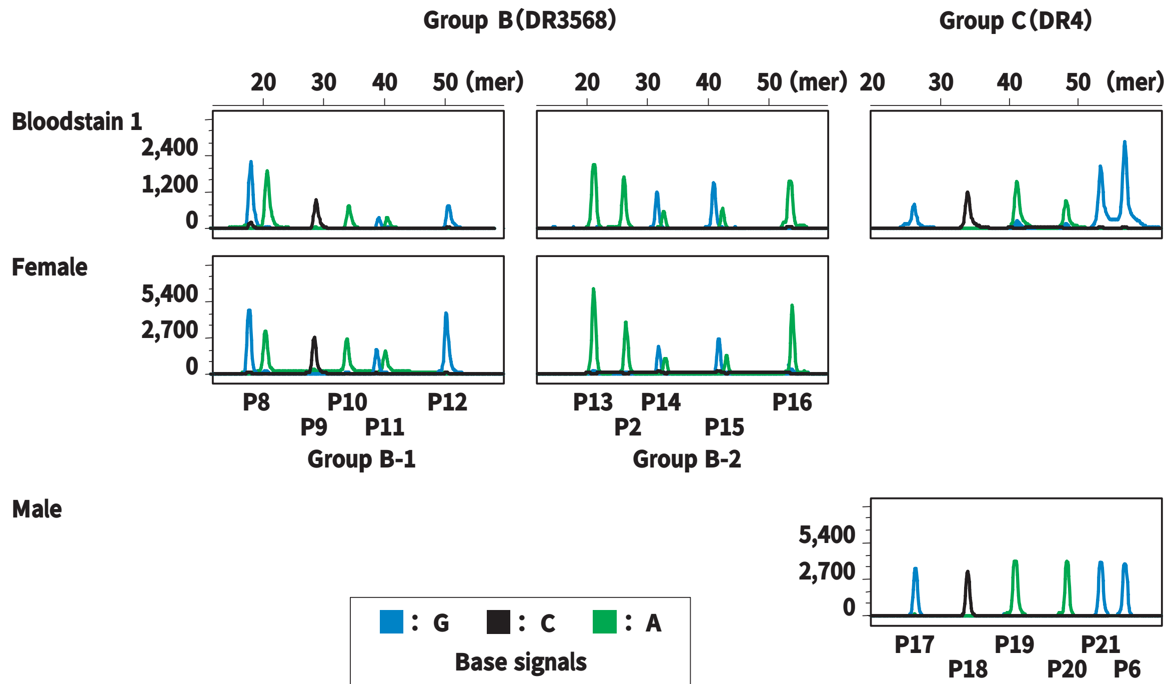


Fig. 8 Electropherogram showing the results of SNP typing after multiplex single-base extension reactions in a criminal case. The bloodstain was determined to have alleles DRB1*08032 (DR3), DRB1*13021 (DR3), and DRB1*0405 (DR4), while the female was typed as DRB1*08032 (DR3)/*13021 (DR3) and the male as DRB1*0405 (DR4)/*0405 (DR4) or DRB1*0405 (DR4)/-.

Discussion

As illustrated above, the DRB1 alleles typed by this method coincided with the results of allele typing by sequencing or PCR-RFLP. With this method, typing was also possible for 'heterozygous' cases, where both alleles possessed by an individual belonged to the same allele group.

This method is compared with the following conventional genotyping methods of the DRB1 gene. Sequencing requires large amounts of template DNA and complex manipulations, and is not suitable for minimal samples and/or degraded samples in forensic practice. The PCR-RFLP method requires complex manipulation, and it is impossible to detect multiple recognition sites simultaneously. Further, PCR-RFLP is subject to mistyping because of incomplete digestion by restriction enzymes [16]; in particular, this method is likely to mistype contaminated forensic samples. The PCR-SSP method, meanwhile, is simple. If it were performed in a multiplexed format, it would enable the simultaneous detection of multiple SNP sites. However, exact PCR conditions are necessary for accurate typing. Moreover, the PCR-SSP method tends to give rise to false-positive bands and false-negative results, especially for minute, degraded, or mixed samples. PCR-SSOP, on the other hand, involves time-consuming and troublesome procedures of hybridization using numbers of oligonucleotide probes. Although this method allows the detailed determination of DRB1 alleles, it requires strict hybridization conditions for accurate typing and causes false-positive and/or unclear-positive signals for minute, degraded, or mixed samples, possibly leading to incorrect results.

The new method we evaluated in this study requires only 1 round of PCR for each allele group and the following multiplex primer extension reactions. Multiple SNP sites can be detected simultaneously in a single tube and then analyzed with an automated capillary electrophoresis device. Thus, this new method takes less time and money than conventional methods, and allows detailed DRB1 genotyping more conveniently. Since this method involves allele group-specific PCR and subsequent multiplex single-base extension reactions, accurate allele typing was possible even when very small amounts of DNA (about 5 or 10 pg) were used as the template. These amounts of DNA correspond to the amounts of genomic DNA contained in 1 or 2 human cells [17]. The sensitivity of this method was thus comparable to or higher than that of DRB1 genotyping with nested PCR reported by Ota [11], Inoue [12], and Allen [10, 13]. With this method, the DRB1 genotype can be easily determined even from aged or minimal samples, as long as the amount of extracted DNA is not smaller than the detectable limit. It has thus been shown that this method is useful in personal identification from biological samples for forensic purposes.

When DNA is to be extracted from a mixed sample, which is often encountered during routine forensic practice, it is usually difficult to separately extract the DNA of each individual and to determine the genotype of the minor component. To examine the applicability of this method to mixed samples, we also attempted to type the DRB1 gene alleles of the major and minor components of mixed samples prepared by combining multiple samples of extracted DNA or blood at varying mixture ratios. If it was determined that the alleles belonged to both individuals in a mixed sample, the alleles belonging to different allele groups do not compete for the same primer during group-specific PCR. This is probably why it was possible, using this method based on group-specific PCR, to amplify the template DNA originating from the minor component whose proportion in a given mixed sample was 1:1,000 or 1:10,000. Also, during multiplex single-base extension reactions, there was no influence from the amplification products of the major component. This is probably why it was possible to detect the substitute bases from the minor component. In this attempt, typing was successful for alleles originating from the minor component. In addition, the calculated amount of DNA from the minor component was only 100 pg in a group-specific DNA from the minor component in a group-specific PCR reaction mixture at a 1:1,000 ratio. Inoue [12], Allen [13], and Gyllensten *et al.* [18], though Gyllensten performed HLA-DQA gene typing, also performed PCR using group-specific primers for mixed samples. The lowest proportion of the minor component in a mixed sample in which the minor component was genotyped was 1:1,000 in the study by Inoue *et al.* [12], 1:20,000 in the study by Allen [13], and 1:25,000 in the study by Gyllensten [18]. If the alleles belonged to both individuals in a mixed sample, the alleles belonging to the same allele group compete for the same primer during group-specific PCR. For this reason, when the proportion of DNA from the minor component in a given mixed sample was less than 1:100, the signals of bases originating from the minor component disappeared and only the bases

originating from the major component were detected following multiplex single-base extension reactions. This detection limit of the minor component was almost equal to that of the PCR-based method for minisatellite or microsatellite regions which were utilized for detection [17, 19].

In criminal cases such as the one discussed here, mixed blood samples from 2 individuals are most frequently encountered. The proposed method seems applicable not only to such mixed samples but also to all biological samples composed of a mixture of DNA originating from several individuals. In cases of samples composed of a mixture of semen and other bodily fluids, the method reported by Yoshida *et al.* [20] allows separate extraction of DNA from each component. However, even this conventional method will inevitably lead to simultaneous extraction of DNA originating from the suspects when a sample is composed of a mixture of semen, such as in the case of gang rape, or when it is composed of a mixture of DNA from the victim's epidermic cells and DNA from the suspect's oral epithelial cells (*e.g.*, if the suspect left saliva on the victim's skin). In such cases, DRB1 genotyping with our method may be useful.

It was recently reported that fetal DNA was found in maternal blood [21]. This suggests that fetal DNA could be sampled with less stress on the mother and with higher safety than through the conventional amniocentesis, and that this method may be useful for paternity testing during gestation.

The possibility of using this method to type the minor component's alleles in a mixed sample was calculated on the basis of the reported frequency of each allele group among Japanese (H. Maeda [22]). The results indicated that typing would be possible in about 95% (19 of 20) of cases by the possible combinations of allele groups of each individual.

Thus, the proposed method is promising not only as a means to determine the genotype of HLA-DRB1 using DNA samples from individuals for personal identification or to determine genotype for paternity testing, but also as a means to genotype samples composed of a mixture of biological materials from several individuals, such as in the case of mixed stains. This method is expected to be useful in forensic medicine.

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