

A new 39-plex analysis method for SNPs including 15 blood group loci

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Abstract

A novel 39-plex typing system for single nucleotide polymorphisms (SNPs) has been developed. This multiplex approach has the advantage of being able to type 38 autosomal SNPs and one sex-discriminating base exchange site on the X and Y chromosomes rapidly and simultaneously. The SNP loci on the autosomes, which we examined, contain 15 loci distributed on blood type genes: three on RhCE, two each on Km and Gc, and one each on Duffy, AcP1, Tf, MN, GPT, EsD, PI, and Kidd genes.

Thirty-seven genomic DNA fragments containing a total of 38 SNPs and one sex-discriminating site were amplified in one multiplex PCR reaction. Following the reaction, single nucleotide primer extension reaction was performed by dividing these SNP loci into five groups. The SNP type of each of the 39 loci was determined at one time by capillary electrophoresis using the newly designed multi-injection method.

The combined PD (power of discrimination) of this typing system was $(1-1.1) \times 10^{-14}$, and the MEC (mean exclusion chance) was 0.9990. We applied this system to forensic cases, including 16 paternity testing cases (13 non-exclusion and three exclusion cases) and one personal identification case. For the paternity testing cases, the highest Essen Möller's *W*-value was 0.9999995. The pM (matching probability) of the personal identification case was 2.22×10^{-17} . These data showed that this system was an excellent tool for use in forensic cases of paternity testing and personal identification.

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1. Introduction

Presently, short tandem repeats (STRs) contribute to many forensic cases because of their large number of alleles [1]. In addition, multiplex amplification methods for STR markers have been developed [2] and exploited for forensic purposes.

On the other hand, single nucleotide polymorphisms (SNPs), the majority of which are biallelic markers, are the most abundant form of DNA polymorphisms observed in

the human genome [3]. The discrimination power of each SNP is inferior to that of a STR, but a combination of many SNPs could realize a high discriminating power.

The number of reported SNPs is also on the increase, and many techniques have been developed for detection of SNPs using the DHPLC [4], Invader assay [5], DNA microarray [6], and so on. In this study, we employed a multiplex assay of single nucleotide primer extension reactions for typing the SNP loci because the multiplex assay [7] has the advantage of not only convenience but also using only a small amount of DNA samples, and a single nucleotide primer extension reaction is a simple and cost-effective method. In addition, we have devised a multi-injection

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method with which to analyze 39 loci simultaneously by capillary electrophoresis.

This 39-plex system contains 15 loci distributed on blood type genes. These blood types are often utilized for forensic samples, and their allele frequencies have been well investigated by serological analysis or genotyping. Furthermore, this system includes a single nucleotide difference between the amelogenin locus on the X chromosome and an amelogenin-like sequence on the Y chromosome, used to determine sex.

We utilized this high throughput SNP typing method for forensic cases of paternity testing and personal identification, and confirmed its usefulness in forensic practice.

2. Materials and methods

2.1. DNA samples

To determine the allele frequencies of 38 SNPs, 102 unrelated DNA samples were collected from Japanese subjects living in Okayama Prefecture, western Japan.

Paternity testing cases: DNA samples were extracted from peripheral blood of 16 trios. Among the 16 trios, 13 were inclusions cases and the remaining three were exclusions.

A personal identification case: DNA samples were extracted from the muscle of a decomposed female body that had been in the sea for about 3 weeks and from a sample of dried blood on blood type test paper that had come from an elderly woman who was missing and suggested to be the person concerned. The dried blood sample, a comparative sample, had been taken in a hospital 5.5 years before the elderly woman went missing.

2.2. Selection of SNP loci

We selected 39 loci (Table 1); 15 loci are in the regions of the blood type genes RhCE [8], Duffy [9], Km [10], ACPI [11], Tf [12], Gc [13], MN [14,15], GPT [16], EsD [17], PI [18], and Kidd [19]. In the other 23 loci, we took advantage of the JSNP database [20]: <http://snp.ims.u-tokyo.ac.jp/>. The SNPs selected were at least 50 mega-bases apart. The remaining locus is not a SNP but a single nucleotide that differs between the amelogenin gene on the X chromosome and an amelogenin-like sequence on the Y chromosome.

2.3. Amplification of DNA fragments

PCR primers are shown in Table 1. The primer pairs suitable for the multiplex amplification were designed under the following criteria: (1) amplified length of DNA fragments are short (71–232 bp), (2) the sequences of primers, especially those 3' ends, avoid the formation of primer dimers or second structures, (3) the 3' end base of the primers is guanine or cytosine, (4) all primers have GC contents of 41–61%, and (5) all primers show the similar T_m values. Table 1 lists the T_m values for each primer calculated

from the melting temperature formula [21]. Any software was not used to design PCR primers. All of the primers except that for *RHCE* were checked to bind a unique region using BLAST search [22] (<http://blast.genome.ad.jp/>). The primer pair for the *RHCE* gene coincidentally amplified the *RHCE* and *RHD* loci, due to the high sequence homology between these loci [8]. Primers sequences of the *MN* locus were partially changed from those in [15].

Thirty-seven-plex PCR reaction was performed in a volume of 20 µl containing 1 ng template DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 4.5 mM MgCl₂, 0.2 mM each dNTP, 0.25–35 pmol of 74 primers (see Table 1 for concentrations), 4 µg BSA, and 1 U AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA). The reaction consisted of one cycle at 95 °C for 11 min and 96 °C for 2 min; 10 cycles at 94 °C for 1 min, 60 °C for 1 min, and 70 °C for 2 min; followed by 30 cycles at 90 °C for 1 min, 60 °C for 1 min, and 70 °C for 2 min; and a final extension at 70 °C for 30 min.

2.4. Pretreatment of the PCR amplification products

After multiplex PCR, reaction products were digested with exonuclease I (EXO I) to dissolve unincorporated primers.

Next, to avoid an undesirable fluorescent ddATP addition to the 3' end of the amplified products in the next single nucleotide primer extension reaction, we performed beforehand a dNTP addition reaction in a volume of 10 µl containing 9 µl amplified products, 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 0.25 U AmpliTaq DNA polymerase (Applied Biosystems). The reaction was performed at 65 °C for 15 min, and the product was digested with shrimp alkaline phosphatase (SAP) to dissolve unincorporated dNTPs.

2.5. Single nucleotide primer extension reaction

Single nucleotide primer extension reactions were carried out by dividing the 39 loci into five groups (groups A–E) (Table 2), using an ABI PRISM® SNaPshot™ ddNTP Multiplex Kit (Applied Biosystems) containing fluorescently labeled ddNTPs and DNA polymerase. The single nucleotide primer extension reaction was performed in a volume of 5 µl containing 1 µl of PCR product, 1.25–20 pmol of typing primers (see Table 2 for concentrations), 15 mM (NH₄)₂SO₄, and 2.5 µl of ABI PRISM® SNaPshot™ ddNTP Multiplex Kit. The reaction cycle consisted of 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. The reaction product was then digested with SAP to inactivate unincorporated fluorescence-labeled ddNTPs.

2.6. Design of typing primers

Typing primers were designed to anneal to the target DNA next to the 3' end of the SNPs. Each primer

Table 1
Thirty-nine locus used in this study and primer sequences for multiplex PCR

Locus number	SNP loci	Product size (bp)	Locus	Allele	Serological type	Direction	Primer sequence (5' 3')	Tm	Primer concentration (pmol)
1	Amelogenin	85	X, Y	C/T	C = Xchr T = Ychr	F R	CATGGGTGGATGGCTGC CCACTGGGATGTGGTGATG	57.9 58.2	5.00 5.00
2	RhC 48	109	1p34.3 36.1	C/G	C T = CC C/G C/T = Cc	F R	TCTAAGTACTCGCGGTCTGTCTC CTAAGGAAGCGTCATAGTGGG	58.5 58.5	2.00 2.00
3	RhC 307			C/T	G C = cc	F R	TGTGCAGTGGGCAATCCTG AAGTGATCCAGCCACCATCC	58.2 58.3	0.25 0.25
4	RhE	211		C/G	C = E G = e	F R	GAGTGTGATTCTGGCCAACC GCTCACCATGCTGATCTTCT	58.3 58.5	1.00 1.00
5	Duffy	141	1q21 22	A/G	G = Fy ^a A = Fy ^b	F R	GAGAACTCAAAGTCAGCTGGAC TGCAGAGTCATCCAGCAGG	58.5 58.2	0.70 0.70
6	Km 153	186	2p12	C/T	T C = Km*1 C C = Km*1, 2	F R	AACTTCTATCCAGAGAGGCC CTGATGGGTGACTTCGCAG	58.5 58.2	2.00 2.00
7	Km 191			C/G	C G = Km*3				
8	ACP1	232	2p25.3	A/G	G = P ^a A = P ^b	F R	ATGTTTCAGAAGACCCTAGCA TGTTGGGTCTTCATATTAGCCC	54.6 56.7	2.25 2.25
9	IMS JST108553	162	2q23.2	A/G		F R	GGAAGACTAAGAAGACTGCGG TCATTTCTGTAAGCATTTCCCCC	58.5 56.9	0.65 0.65
10	IMS JST105631	150	2q37.1	A/G		F R	AGCAGATGCCAAACACCACG ATAGGTGAGGGTGGCCTG	58.3 58.1	13.50 13.50
11	Tf	121	3q21	C/T	C = TfC ₁ T = TfC ₂	F R	CCATGGGCTAAGAATCTGAATG TGACCACAGCGTGATTCGG	56.7 58.2	1.40 1.40
12	IMS JST013730	110	3q28	A/C		F R	GGTCTGTAGTGAAAGGTCATAG AAGGGCACAGGAGTGCTG	56.5 58.1	4.50 4.50
13	IMS JST073428	99	3p14.2	A/C		F R	ATGAAGCCCAATTACTCCACG CTCTTCTCGGCAGATTTACAGG	56.5 58.5	1.50 1.50
14	IMS JST066012	134	3p25.3	C/T		F R	GGTCTTGGGAACAGAAGCC GGTCGAGAAAGTGTTCTATTCC	58.3 56.7	9.00 9.00
15	IMS JST035151	187	4p16.3	C/T		F R	CCAAGGTACTTAATGGGCAGC CTCAAACCTCTGGACTCAAACG	58.5 58.6	13.50 13.50
16	Gc 416	176	4q11 13	G/T	T C = Gc ^{1F} G C = Gc ^{1S}	F R	GTTTTTCAGACTGGCAGAGCG CTGCCATGTTAAGTGGAGGG	58.5 58.3	4.50 4.50
17	Gc 420			A/C	T A = Gc ²				
18	MN	202	4q28.2 31.1	C/T	C = M T = N	F R	CCTTTCTCAACTTCTATGTTATACAGC TGACAGGTCCCTTAAAATGGGTTA	57.5 57.1	12.50 12.50
19	IMS JST013519	149	5p15.3	A/G		F R	TGTGGGAGCCAAGCTGAC ATGTCCAAGTGACAGGCATC	58.1 56.3	0.50 0.50
20	IMS JST059517	173	5q13.1	A/T		F R	GAGTTCCTGAAAGACAGGGAC GGAATGGCTCACACAGAACATG	58.5 58.6	35.00 35.00
21	IMS JST013752	136	5q35.2	C/T		F R	CCCTGGCCAGGTTCAAAG GCAGTTTGCTCAACCACTTCC	58.1 58.5	10.00 10.00
22	IMS JST047902	208	6p22.3	A/T		F R	ACCTAGGTCTCTGCATTGTAAC CCACACTTTGCATCTGTATCATAC	56.7 57.1	10.00 10.00
23	IMS JST157403	193	6q14.1	C/T		F R	ACGAAAGCACAAATTCAAAGCAGG GGAAAGCCTGGTTCATCAGTG	57.1 58.5	3.25 3.25
24	IMS JST051887	183	6q26	A/G		F R	GAGAAGTCTTCATGCTCACAG AAGCCTTTTGCCTAGCATCAAC	56.5 56.7	1.00 1.00
25	IMS JST028975	96	7p22	C/G		F R	TCTCAAGTCCACGCAGAGC GCCGTCAAGTCCCTTCATG	58.2 58.1	5.00 5.00
26	IMS JST011881	128	7q11	C/T		F R	GGTGAGTCAAGGAAACACTC TTGAGCTTTTGCCCTGGGTG	58.5 58.3	3.00 3.00
27	IMS JST160788	114	7q36.3	C/T		F R	CAAGCCTGGAAGAGCCTG CTGTAAACCGAGAACGTGCA	58.2 56.3	0.50 0.50
28	IMS JST047432	99	8p23.3	C/G		F R	AGGACACCCGCGTTCCAG TGTCGGAGGAACAGACAGC	60.4 58.2	0.75 0.75
29	IMS JST005748	123	8q13.3	A/T		F R	GGCATTGCTAATTGTGATTGAGC CTGCCATATTCCTTGTGATAGTG	56.9 57.1	2.50 2.50
30	GPT	85	8q24.3	A/C	C = Gpt ¹ A = Gpt ²	F R	GTAGAGTCATGGCCTCGAG ATGCCGTCCAGCGTCAG	58.2 57.9	10.00 10.00
31	IMS JST031054	93	9p22	A/G		F R	AGATTGACGTTGAGGTGCAG GTGTGGAGACATCTGAGAC	58.5 56.1	2.00 2.00
32	IMS JST074073	71	10p13	C/T		F R	ATGCAGCAGGAGTGGCAG CCATCCTGGACACGTCTAG	58.1 58.3	0.50 0.50

Table 1 (Continued)

Locus number	SNP loci	Product size (bp)	Locus	Allele	Serological type	Direction	Primer sequence (5' 3')	Tm	Primer concentration (pmol)
33	IMS JST175673	102	12p33.33	A/T		F	CAGCCTCTCAGAACAAATGCTG	58.5	1.50
						R	CAGGCTCTGGTTTGAC	58.2	1.50
34	IMS JST032847	146	12q24	A/G		F	CCACTTATCAGTAGCAGGCAG	58.5	5.00
						R	ACCTCTGACCTCTGCCTC	58.1	5.00
35	EsD	195	13q14.11	A/G	G = EsD ¹ A = EsD ²	F	ATTTGCAACCCCTGTA CTGTC	56.7	3.00
						R	AATTGTCTCAGCTCTAGAGCC	56.7	3.00
36	IMS JST030395	183	13q34	C/T		F	CTTCTAGCATTTTCTCTGGCAG	56.7	25.00
						R	TGCCCCCAGAACTTCCTG	58.2	25.00
37	IMS JST030323	174	14q11.2	C/T		F	GGTACATTGGATCGCCCTTC	58.3	18.00
						R	CTGGCAAAGCCCAAGTAGAC	58.3	18.00
38	PI	160	14q32.1	A/G	G = M1 or M3 A = M2	F	CAAGGCTCACACTCACGATG	58.3	30.00
						R	GCTGAGGAACACGCCATTG	58.2	30.00
39	Kidd	88	18q12 21	A/G	G = Jk ^a A = Jk ^b	F	TCCTGTCTTAACAGGACTCAG	56.5	5.00
						R	AATGCAGGCCAGAGAGCTG	58.2	5.00

sequence complementary to the target sequence was limited to 30 mers from the 3' end. The poly(dT) were added to the 5' end of typing primers longer than 30 mers to reduce the risk of forming primer dimers or second structure. Moreover, *T* exhibits higher mobility as mentioned below and makes easier to adjust the length of primer desired. Exceptionally, in the Kidd locus, the template specific part of the typing primer was limited to 25 mers from the 3' end, and the poly(dT) was added to the part longer than 25 mers of the primer to avoid making primer dimer with the IMS-JST 032847 primer (Table 2).

Typing primers are ought to be designed so that the primer extension products to show the electrophoresis peak at similar intervals to each locus. The electrophoretic mobility of the primer extension products is thought to correlate basically with the nucleotide composition and the molecular weight of the typing primer, and the fluorescence-labeled dideoxy nucleotide incorporated at the 3' end of primer.

In the present study, we actually have adjusted the length of typing primers by assuming empirically the mobility of primer extension products as follows: at first, the mobility of each nucleotide was calculated based on the molecular weight and electrical charge. That is, A (adenine) was regarded to show 0.9 base of the mobility, G was 1.3, C was 0.7 and T was 1.1. Second, the sum of the mobility of each nucleotide was calculated to estimate the mobility of the typing primer. This empirical estimation showed a fairly good performance for the primers of 20–50 mers. When the length of primer was approximate to 20 mers, this estimate gave lower values than the actual mobility.

As the 5' end base of the amelogenin typing primer, C was employed, which was not complementary to the reported target sequences (C on X chromosome, T on Y chromosome), to avoid the large difference between the targets in annealing efficiency.

2.7. Detection and analysis of SNPs

Following dilution with the Hi Di formamide (Applied Biosystems) and heat denaturation, the products of the single nucleotide primer extension reaction were electrophoresed simultaneously by the newly designed multi-injection method [23] with an ABI PRISMTM 310 Genetic Analyzer (Applied Biosystems) using the POP4 polymer and the 310 capillary, 47 cm × 50 μm (Applied Biosystems).

In this multi-injection method, syringe pump time and collection time of control module, GS POP4 (1 ml) E5, were changed and renamed as shown in Table 3: GS POP4 (1 ml) multi-1 was for group A, GS POP4 (1 ml) multi-2 was for groups B and C, GS POP4 (1 ml) multi-3 was for group D, and GS POP4 (1 ml) multi-4 was for group E. The collection time was set to 1 or 2 min for groups A–D, and 20 min for group E. The syringe pump time was changed from 150 to 1 s for groups B–E. Thus, the polymer was not exchanged before the injections of groups B–E. The 1 s of syringe pump times, which keep the appropriate distance between the syringe drive and the plunger head of the syringe, were favorable to initiate electrophoresis of the next series multi-injection. Other electrophoresis conditions were: voltage 13 kV, temperature 60 °C. In Table 3, module parameters of the multi-injection method were shown. After five samples were taken up to in the tray of an ABI PRISMTM 310 Genetic Analyzer, the products were injected in turn at 1 or 2 min intervals. Just after group E was injected, groups A–E components were all in one capillary with adequate intervals, electrophoresed as one sample for 20 min, and recorded as a group E data file. Following electrophoresis, the data file of group E was analyzed by GeneScan[®] Analysis 3.1.2 software (Applied Biosystems).

2.8. Statistical analysis

Divergence from Hardy Weinberg expectations (HWE) was determined by χ^2 -test. Linkage disequilibrium across

Table 2
Primer sequences used for multiplexed single nucleotide primer extension reactions

Group	Locus number	SNP loci	Direction	Primer sequence (5' 3')	Primer concentration (pmol)
A	1	Amelogenin	R	CCTGCAGGGTGTGAGTC	5.00
	2	RhC-48	R	AGAGCTGCTCCAGTGTAGGGC	7.50
	3	RhC-307	F	CTGCTGGAGCGCTTCTGAGCAGTTCCT	5.00
	4	RhE	F	TTTTTTTGTGGATGTTCTGCGCAAGTGTCAACTCT	10.00
	5	Duffy	F	TTTTTTTTTTTTTTTTTTTTTGTGATCTCTCCAGATGGAGACTATG	3.00
	6	Km-153	F	TTTTTTTTTTTTTTTTTTTTTTAGGCCAAAGTACAGTGGAGGTGGATAACG	6.25
	7	Km-191	F	TTTTTTTTTTTTTTTTTTTTTTTCTGACAAAGCAGACTACAGAAACACAAA	1.25
	8	ACP1	F	TTTTTTTTTTTTTTTTTTTTTTTACAGAGATTGTAATAGAAAAGTAATC	3.75
	9	IMS-JST108553	F	TTTTTTTTTTTTTTTTTTTTTTTCTCTCTTTAGATCTGGTTGTAGGTTTAT	2.50
	10	IMS-JST105631	F	TTTTTTTTTTTTTTTTTTTTTTTACCAGCACAGGACAACCTGCAGGGACAGAG	5.00
B	11	Tf	F	CTTGATGGTACCAGGAAA	5.00
	12	IMS-JST013730	F	CGCCAGGGAGATGATAGTGATGGCA	10.00
	13	IMS-JST073428	F	TTTATGGTTCCTGCTGGGTATTTTCAGAGGA	2.00
	14	IMS-JST066012	F	TTTTTTTTTTTACAGGATTTTATGAAAGCAATGAGCTAG	5.00
	15	IMS-JST035151	F	TTTTTTTTTTTTTTTTTCTGTAATCCAGCACTCTGGGAGGTGAAGA	2.00
	16	Ge-416	F	TTTTTTTTTTTTTTTTTTTGGCAGAGGACTAAAGCAAAATTCCTGA	3.75
	17	Ge-420	R	TTTTTTTTTTTTTTTTTTTTTTTGTAGCGCTTGTTAACAGCTTTGGCAGTTCC	2.50
	18	MN	F	TTTTTTTTTTTTTTTTTTTTTTTGTATACAGCAATGTGAGCATATCAGCAT	5.00
C	19	IMS-JST013519	F	AGTATCGCTGGTTATCC	1.25
	20	IMS-JST059517	F	CAATACTGGCAAAATGTAAITGCCCAITAA	15.00
	21	IMS-JST013752	R	TTTTTTTTTACAGGAAGATCAAGGTTGACAAATCTGG	12.50
	22	IMS-JST047902	F	TTTTTTTTTTTTTTTAAATGACCTCTATCTAAAGTATTTCTTTT	3.75
	23	IMS-JST157403	F	TTTTTTTTTTTTTTTTTTTCTTAAATCTTACCATCAGGAITTTA	3.75
	24	IMS-JST051887	F	TTTTTTTTTTTTTTTTTTTTTTCTCTCTCCAGCCAGTACTACTTTGAAGC	10.00
	25	IMS-JST028975	F	TTTTTTTTTTTTTTTTTTTTTTTTTTTGTAGCATAGATTGGGGTGGAGAGTGGCTTTC	5.00
	26	IMS-JST011881	F	TTTTTTTTTTTTTTTTTTTTTTTTTTTCTGCCCAATCAGGAATGAGGTCAAACTCC	2.50
	27	IMS-JST160788	F	TTTTTTTTTTTTTTTTTTTTTTTTTTTCTACTGCCTCTAGACTGGCTCTCAGTTC	2.50
D	28	IMS-JST047432	F	AGGCTGGTTCGGAAAGGGCCTCTGTGG	1.25
	29	IMS-JST005748	F	TTTTTGTATGAGCTAAAAATATGAATAACTGAT	8.75
	30	GPT	F	TTTTTTTTTTTACACAGGTGACCGAGCGCAGCGGGTGAGG	5.00
	31	IMS-JST031054	R	TTTTTTTTTTTTTTTTTGGGTACAGCAATACACTCTCTCTCTCTCC	5.00
	32	IMS-JST074073	R	TTTTTTTTTTTTTTTTTTTGTAGCCTCACCCCTCGCACCTGGAGGCAC	5.00
E	33	IMS-JST175673	F	AGGCATGCAGGATCTGTGGTTC	2.00
	34	IMS-JST032847	R	TTTTTGGGTAATTTGGGGATTAACGGAGACCT	2.00
	35	ESD	F	TTTTTTTTTTTTTCTGCTCCCTGGGCAAAAAGCCTTTAGTG	5.00
	36	IMS-JST030395	F	TTTTTTTTTTTTTTTTTTTTTTTATAGCAAAAACAGACCTTAGCCCA	15.00
	37	IMS-JST030323	F	TTTTTTTTTTTTTTTTTTTTTTTGGGTGATCAACGATGCTGATGTGAGGGAT	2.50
	38	PI	F	TTTTTTTTTTTTTTTTTTTTTTTACATCCATGAACCCCTTCCAGGAACCTCTCC	20.00
	39	Kidd	F	TTTTTTTTTTTTTTTTTTTTTTTTTTTACTAGTCTTTCAGGCCCAATTGAG	10.00

Table 3
The module parameters of the multi-injection method

Injection number	Tube and sample name	Modules	Injection (s)	Injection (kV)	Run (kV)	Run (°C)	Run time (min)
1	A1-group A	GS POP4 (1 ml) multi-1	5	13	13	60	2
2	A3-group B	GS POP4 (1 ml) multi-2	5	13	13	60	2
3	A5-group C	GS POP4 (1 ml) multi-2	5	13	13	60	2
4	A7-group D	GS POP4 (1 ml) multi-3	5	13	13	60	1
5	A9-group E	GS POP4 (1 ml) multi-4	5	13	13	60	20

loci was tested using a computer program, GENEPOP ON THE WEB, based on a Markov chain method (<http://wbio-med.curtin.edu.au/genepop/>).

The power of discrimination (PD), the mean exclusion chance (MEC), the paternity index (PI), Essen Möller's *W*-value, and the probability of match (pM) were calculated according to Jones [24] and Hummel [25].

For each of the RhCE, Km, and Gc loci, the genotypes were determined by two or three sites, and RhCE, Km, and Gc were considered as one locus through the statistical analysis.

3. Results and discussion

3.1. Thirty-seven-plex amplification

First, 37 pairs of primers were tested by dividing them into groups A–E (Table 2). After confirming amplification with polyacrylamide gel electrophoresis and the single nucleotide primer extension reaction, the amplification primers of these five groups were mixed together.

Successful completion of the 37-plex PCR was verified by analyzing the data of single nucleotide primer extension reaction (Figs. 1 and 2). The major reasons the 37-plex PCR is successful are as follows: the short fragment sizes of the target amplicons, specificity of primer sequences confirmed by BLAST search, and appropriate primer concentration (Tables 1 and 2). The concentration of the primers used had to be varied to obtain each peak clearly and at a similar height in the following single nucleotide primer extension reaction. When the peak height of a certain locus was considerably higher than that of the other loci, amplification primers of that locus were reduced. Conversely, primers were added when the peak of one locus was lower than those of the others. With regard to the concentration of the amplification primers, most of those used less than 10 pmol. There were exceptions: in the loci IMS-JST105631, IMS-JST035151, MN, IMS-JST059517, IMS-JST030395, IMS-JST030323, and PI, a primer concentration of 13.5–35 pmol was required. Since the amplification inefficiency of these loci had no obvious correlation with product size, character of annealing would largely affect the efficiency of amplification.

However, no amplification primers over 35 pmol were added. Because the amplification primers were initially dissolved in TE at a dilution of 50 nmol/ml, the total volume of primer solution was limited. Therefore, to obtain optimal peak heights, the reaction conditions were adjusted by increasing the typing primers in the single nucleotide primer extension reaction, as mentioned below.

3.2. Pretreatment with extra A addition

Pretreatment with extra A addition was a simple and effective process to eliminate the noise of unexpected fluorescent fragments. Without this pretreatment, a fluorescent ddATP contained in the ABI PRISM® SNaPshot™ ddNTP Multiplex Kit was added to the 3' end of the amplified products by the extra A addition activity of the Taq polymerase. In the electrophoretic analysis step, this would cause the “noise” of peaks of fluorescent A nucleotides to overlap the true peaks. Therefore, to avoid a fluorescent ddATP additive effect unlabeled dNTP was added before the single nucleotide primer extension reaction. Fig. 3 shows an effect of pretreatment with extra A addition. The electropherogram in the top panel represents a primer extension reaction product of group E was not pretreated by the extra A addition. In the bottom panel was with the A addition. Many noises (Fig. 3, painted green), probably caused by the multiplex PCR products or primer dimers, were reduced or eliminated after the extra A addition. By this treatment, we could type the SNPs correctly, without the noise of unexpected fluorescent peaks.

3.3. Detection and analysis of SNPs

By the multi-injection method employed in the electrophoretic analysis, the single nucleotide primer extension reaction products of the five groups were successfully analyzed in one electrophoretic run. Compared with the total time of electrophoresis using ordinary separate injections, the multi-injection method was more than twice as efficient. Using this typing system, all 39 loci tested could be typed simultaneously and clearly (Figs. 1 and 2).

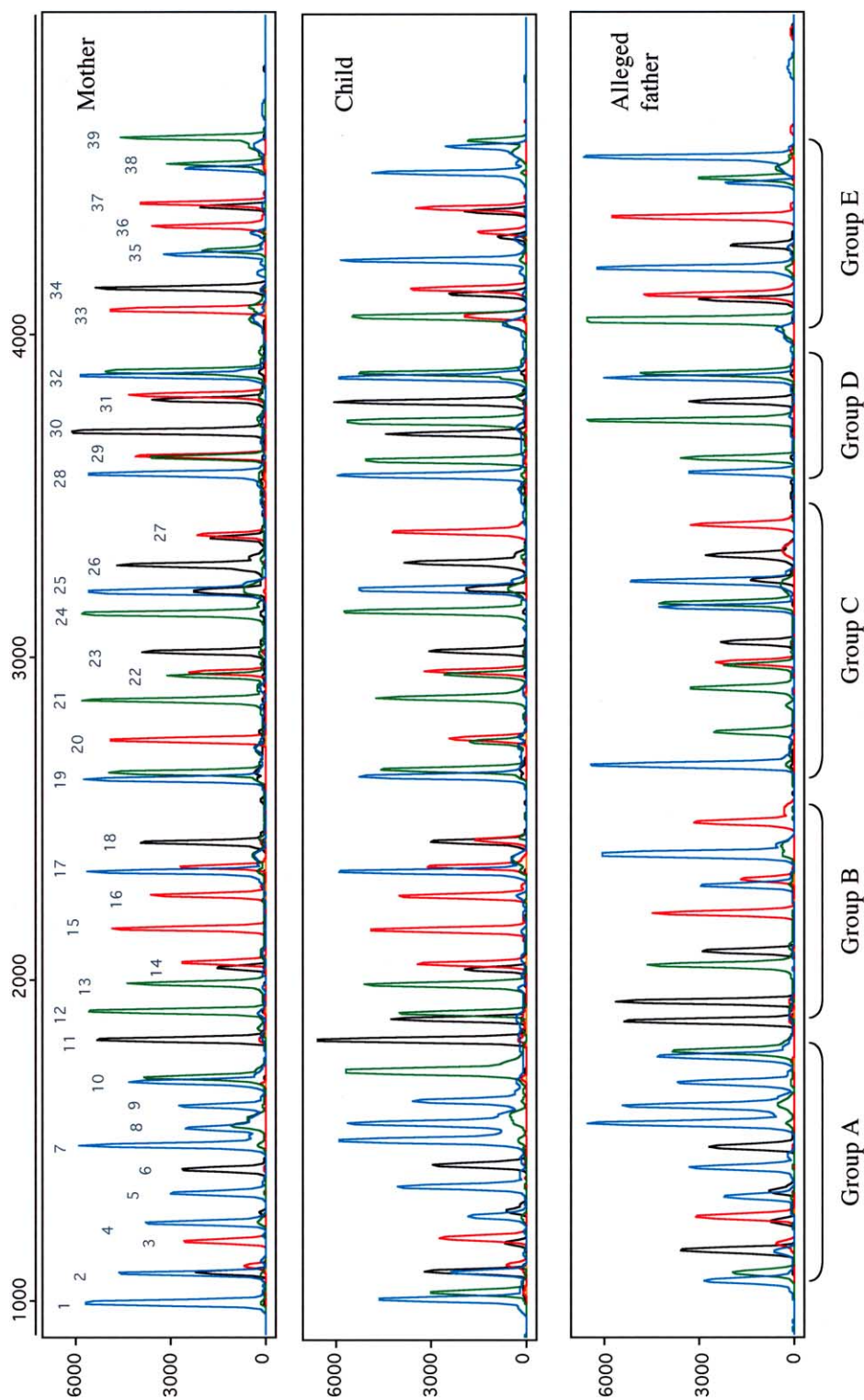


Fig. 1. Electropherograms of an inclusive paternity testing case, mother (top), child (middle), and alleged father (bottom). The number above each electrophoretic peak indicates the locus number as shown in Tables 1 and 2.

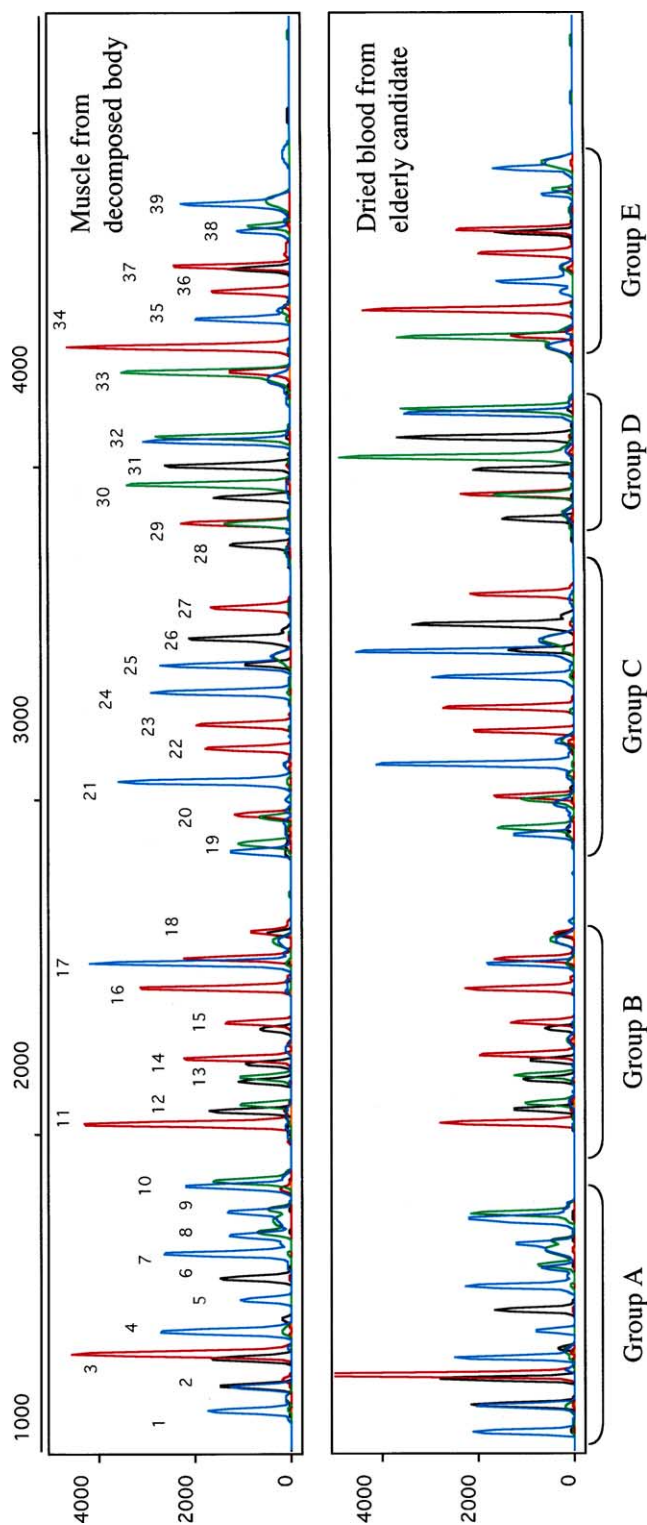


Fig. 2. Electropherograms of a personal identification case, based on a sample from muscle of the decomposed body of the victim (top) and dried blood on blood type test paper of the elderly candidate (bottom). The number above each electrophoretic peak indicates the locus number as shown in Tables 1 and 2.

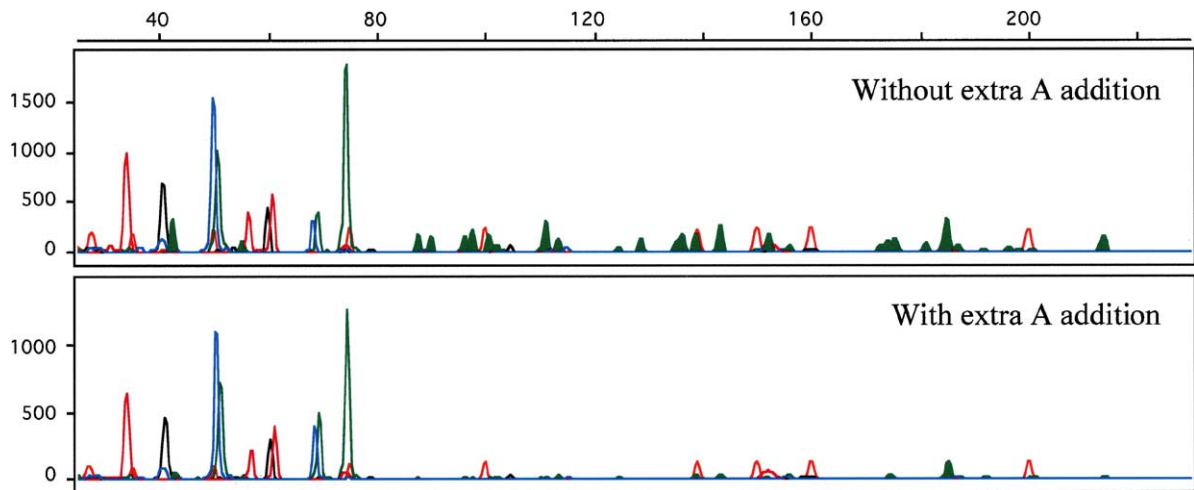


Fig. 3. Comparison of electropherograms from a primer extension reaction product of group E with treated (bottom) and untreated (top) with extra A addition. As internal size standard, GeneScan™-500LIZ™ (Applied Biosystems) was used.

In a homozygous locus, each locus appeared as a single peak, though in a heterozygous locus, two distinct nucleotide peaks appeared closely together because fluorescence-labeled dideoxy nucleotides incorporated at the 3' end of primer differently influenced the mobility of the extension products. The two peaks of heterozygous locus were electrophoresed reproducibly in the same order. Thus, it was possible to type confidently the nucleotides in each SNP locus.

The detected nucleotides are shown in Tables 7 and 8.

3.4. Statistical analysis

No deviations from Hardy Weinberg expectations were observed in any locus ($P > 0.05$) (Table 4).

Linkage analysis suggested no evidence of linkage disequilibrium among loci on the same chromosome, though the P -values of 16 pairs of loci on different chromosomes were less than 0.05 (Table 5).

The combined PD was estimated to be $(1-1.1) \times 10^{-14}$, and the cumulative MEC was 0.9990. This means the performance of the present system is adequate for forensic application.

3.5. Forensic cases

In a paternity testing, 13 cases that were inclusive based on the typing results of 15 STR loci using the PowerPlex® 16 kit (Promega) were also inclusive by the present SNP typing results (Table 6). Using this SNP typing method, the three exclusion cases were excluded by six to nine loci.

The highest value of Essen Möller's W in these inclusion cases, 0.9999995, was obtained from case 9.6.9 (Fig. 2 and

Table 4

Allele frequencies, P -values for HWE and MEC in a sample of 102 unrelated Japanese subjects

SNP locus	Allele	Frequency	P -value	MEC
RhCE	Ce	0.623	0.260	0.235
	cE	0.324		
	ce	0.054		
Duffy	Fy ^a	0.902	1.000	0.081
	Fy ^a	0.098		
Km	Km1.2	0.265	0.201	0.157
	Km3	0.735		
AcP1	P ^a	0.181	0.094	0.126
	P ^b	0.819		
IMS-JST108553	A	0.471	0.081	0.187
	G	0.529		
IMS-JST105631	A	0.549	1.000	0.186
	G	0.451		
Tf	TfC ₁	0.784	1.000	0.141
	TfC ₂	0.216		
IMS-JST013730	A	0.431	1.000	0.185
	C	0.569		
IMS-JST073428	A	0.637	0.203	0.178
	C	0.363		
IMS-JST066012	C	0.544	1.000	0.187
	T	0.456		
IMS-JST035151	C	0.520	0.689	0.187
	T	0.480		
Gc	Gc ^{1F}	0.451	0.681	0.355
	Gc ^{1S}	0.255		
	Gc ²	0.294		

Table 4 (Continued)

SNP locus	Allele	Frequency	P-value	MEC
MN	M	0.564	0.841	0.185
	N	0.436		
IMS-JST013519	A	0.466	0.693	0.187
	G	0.534		
IMS-JST059517	A	0.436	0.229	0.185
	T	0.564		
IMS-JST013752	C	0.569	0.110	0.185
	T	0.431		
IMS-JST047902	A	0.461	0.555	0.187
	T	0.539		
IMS-JST157403	C	0.564	0.687	0.185
	T	0.436		
IMS-JST051887	A	0.534	1.000	0.187
	G	0.466		
IMS-JST028975	C	0.480	1.000	0.187
	G	0.520		
IMS-JST011881	C	0.466	0.116	0.187
	T	0.534		
IMS-JST160788	C	0.427	0.542	0.185
	T	0.574		
IMS-JST047432	C	0.363	0.138	0.178
	G	0.637		
IMS-JST005748	A	0.446	0.551	0.186
	T	0.554		
GPT	Gpt ¹	0.608	0.399	0.182
	Gpt ²	0.392		
IMS-JST031054	A	0.544	0.233	0.187
	G	0.456		
IMS-JST074073	C	0.480	0.231	0.187
	T	0.520		
IMS-JST175673	A	0.451	0.430	0.186
	T	0.549		
IMS-JST032847	A	0.510	0.429	0.187
	G	0.490		
EsD	EsD ¹	0.642	0.398	0.177
	EsD ²	0.358		
IMS-JST030395	C	0.456	0.557	0.187
	T	0.544		
IMS-JST030323	C	0.456	1.000	0.187
	T	0.544		
PI	M1, M3	0.701	0.813	0.166
	M2	0.299		
Kidd	Jka	0.412	0.838	0.184
	Jkb	0.588		

Table 5

P-values for linkage disequilibrium test between two loci for 35 loci in 102 unrelated Japanese subjects

Loci	P-value
RhCE/IMS-JST105631	0.0347
RhCE/IMS-JST013730	0.0224
RhCE/IMS-JST157403	0.0047
RhCE/IMS-JST074073	0.0276
RhCE/IMS-JST032847	0.0286
Km/IMS-JST031054	0.0021
ACP1/IMS-JST013752	0.0017
IMS-JST108553/IMS-JST059517	0.0333
IMS-JST108553/GPT	0.0367
IMS-JST1055631/IMS-JST051887	0.0205
Tf/Gc	0.0082
IMS-JST073428/IMS-JST028975	0.0469
IMS-JST073428/IMS-JST011881	0.0109
IMS-JST035151/PI	0.0184
MN/IMS-JST047902	0.0368
IMS-JST013752/Kidd	0.0105
IMS-JST157403/IMS-JST030323	0.0391
IMS-JST011881/IMS-JST032847	0.0143
IMS-JST160788/GPT	0.0415
EsD/IMS-JST032847	0.0323
EsD/Kidd	0.0326

Table 7). The W-value calculated based on the typing results of the PowerPlex[®] 16 system in the same case was 0.99999992, that is, remarkably different from the present estimate. No mutation was noted in the present paternity testing case.

Since the mutation rate of SNPs has been estimated to be lower than that of STRs [26,27], an SNP typing system seems a preferable alternative to an STR typing system, particularly in paternity testing, in which a hereditary discrepancy caused by mutation in STR locus can occasionally

Table 6

The paternity index (PI) and Essen-Möller's W-value in 13 paternity testing cases

Case	W-value	PI value
8.9.30	0.9999912	113827
9.6.9	0.9999995	2196365
9.9.8-2	0.9997901	4763
11.6.2	0.9999867	74985
11.6.3	0.9999815	54039
12.3.27	0.9999693	32615
12.6.26B	0.9998555	6921
12.7.6	0.9997503	4004
12.8.3	0.9999596	24743
13.2.13	0.9999275	13792
14.3.24	0.9999690	32285
14.8.5	0.9998906	9143
15.1.28	0.9999980	492652

Table 7

Detected nucleotide of an inclusive paternity testing case

Locus number	SNP loci	Mother	Child	Alleged father	Y/X	W
1	Amelogenin	XX	XY	XY	—	—
2–4	RhCE	CeCe	CecE	CecE	0.647	0.607
5	Duffy	Fy ^a /Fy ^a	Fy ^a /Fy ^a	Fy ^a /Fy ^a	0.902	0.526
6, 7	Km	Km [*] 3/Km [*] 3	Km [*] 3/Km [*] 3	Km [*] 3/Km [*] 3	0.735	0.576
8	AcP1	P ^a /P ^b	P ^a /P ^a	P ^a /P ^a	0.181	0.846
9	IMS-JST108553	G/G	G/G	G/G	0.529	0.654
10	IMS-JST105631	A/G	A/A	A/G	1.098	0.477
11	Tf	TfC ₁ /TfC ₁	TfC ₁ /TfC ₁	TfC ₁ /TfC ₁	0.784	0.560
12	IMS-JST013730	A/A	A/C	C/C	0.569	0.638
13	IMS-JST073428	A/A	A/A	A/A	0.637	0.611
14	IMS-JST066012	C/T	C/T	C/C	1.000	0.500
15	IMS-JST035151	T/T	T/T	T/T	0.480	0.675
16, 17	Gc	Gc ^{1F} /Gc ²	Gc ^{1F} /Gc ²	Gc ^{1F} /Gc ^{1S}	1.412	0.415
18	MN	M/M	M/N	N/N	0.436	0.696
19	IMS-JST013519	A/G	A/G	G/G	1.000	0.500
20	IMS-JST059517	T/T	A/T	A/A	0.436	0.696
21	IMS-JST013752	T/T	T/T	T/T	0.431	0.699
22	IMS-JST047902	A/T	A/T	A/T	1.000	0.500
23	IMS-JST157403	C/C	C/C	C/C	0.564	0.640
24	IMS-JST051887	A/A	A/A	A/A	1.069	0.483
25	IMS-JST028975	C/G	C/G	C/G	1.000	0.500
26	IMS-JST011881	C/C	C/C	C/C	0.466	0.682
27	IMS-JST160788	C/T	T/T	T/T	0.574	0.636
28	IMS-JST047432	G/G	G/G	G/G	0.637	0.611
29	IMS-JST005748	A/T	A/A	A/A	0.446	0.692
30	GPT	Gpt ¹ /Gpt ¹	Gpt ¹ /Gpt ²	Gpt ² /Gpt ²	0.392	0.718
31	IMS-JST031054	A/G	G/G	G/G	0.456	0.687
32	IMS-JST074073	C/T	C/T	C/T	1.000	0.500
33	IMS-JST175673	T/T	A/T	A/A	0.451	0.689
34	IMS-JST032847	G/G	A/G	A/G	1.020	0.495
35	EsD	EsD ¹ /EsD ²	EsD ¹ /EsD ¹	EsD ¹ /EsD ¹	0.642	0.609
36	IMS-JST030395	T/T	C/T	C/C	0.456	0.687
37	IMS-JST030323	C/T	C/T	T/T	1.000	0.500
38	PI	M1, M3/M2	M1, M3/M1, M3	M1, M3/M2	1.402	0.416
39	Kidd	Jk ^b /Jk ^b	Jk ^a /Jk ^b	Jk ^a /Jk ^a	0.412	0.708

Electropherograms of this case is shown Fig. 1; W = 0.9999995.

complicate the diagnosis. Therefore, the number of paternity testing cases excluded by only one locus is considered to be smaller by the present SNP typing system than by the STR multiplex typing system.

In the personal identification case, all 38 SNPs and a single nucleotide difference in the amelogenin gene could be typed clearly by the SNP typing method (Fig. 2 and Table 8). SNP types of every locus matched between the tissue from the victim and the dried blood on the blood type test paper of the elderly candidate. Additionally, the peaks of amelogenin showed that both samples were from females. The matching probability calculated was 2.22×10^{-17} , and we could conclude that the candidate was the victim. It was suggested that this system would be useful for application with small amounts of DNA or degraded DNA.

Table 8

Detected nucleotide of a personal identification case

Locus number	SNP loci	Type of kidney and test paper	Probability
1	Amelogenin	CC	0.500
2–4	RhCE	Ce/ce	0.067
5	Duffy	Fy ^a /Fy ^a	0.814
6, 7	Km	Km [*] 3/Km [*] 3	0.541
8	ACP1	P ^a /P ^b	0.297
9	IMS-JST108553	AG	0.498
10	IMS-JST105631	AG	0.495
11	Tf	TfC ₂ /TfC ₂	0.047
12	IMS-JST013730	AC	0.491
13	IMS-JST073428	AC	0.462
14	IMS-JST066012	CT	0.496

Table 8 (Continued)

Locus number	SNP loci	Type of kidney and test paper	Probability
15	IMS-JST035151	CT	0.499
16, 17	Gc	Gc ^{1F} /Gc ²	0.265
18	MN	MN	0.492
19	IMS-JST013519	AG	0.498
20	IMS-JST059517	AT	0.492
21	IMS-JST013752	CC	0.323
22	IMS-JST047902	TT	0.291
23	IMS-JST157403	TT	0.190
24	IMS-JST051887	GG	0.217
25	IMS-JST028975	CG	0.499
26	IMS-JST011881	CC	0.217
27	IMS-JST160788	TT	0.329
28	IMS-JST047432	CC	0.132
29	IMS-JST005748	AT	0.494
30	GPT	Gpt ¹ /Gpt ²	0.477
31	IMS-JST031054	GG	0.296
32	IMS-JST074073	CT	0.499
33	IMS-JST175673	AT	0.495
34	IMS-JST032847	AA	0.260
35	EsD	EsD ¹ /EsD ¹	0.412
36	IMS-JST030395	TT	0.208
37	IMS-JST030323	CT	0.496
38	PI	M1, M3/M2	0.419
39	Kidd	Jk ^a /Jk ^a	0.170

Electropherograms of this case is shown Fig. 3; pM = 2.22 × 10⁻¹⁷.

4. Conclusion

In conclusion, the novel 39-plex SNPs analysis system we developed was shown to be useful and highly discriminating, and could detect SNPs types even from miniscule and degraded specimens. This method would be reliable for forensic practices.

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