J Forensic Sci, Mar. 2003, Vol. 48, No. 2
Paper ID JFS2002259_482
Available online at: www.astm.org

TECHNICAL NOTE

Natsuko Mizuno,[1] *D.V.M.; Kazumasa Sekiguchi,*[1] *M.Sc.; Hajime Sato,*[1] *D.V.M., Ph.D.; and Kentaro Kasai,*[1] *Ph.D.*

Variant Alleles on the Penta E Locus in the PowerPlex® 16 Kit*

ABSTRACT: Penta E in the PowerPlex® 16 kit is a pentanucleotide tandem repeat marker located on Chromosome 15, containing an AAAGA repeat motif. Variant alleles (18.4 and 19.4) were found in the Japanese population. A sequence analysis revealed that both the variant alleles had a partial repeat motif of AAAA, resulting in one-base-shorter alleles compared to known alleles. Despite the relatively large amplicon sizes (379 to 474 bp) of Penta E, an accurate allele assignment can be reliably made by capillary electrophoresis. However, alleles differing in size by only one base (e.g., 18.4 and 19) were not separated and appeared as a single broad peak. The Genotyper® software assigned one of the component alleles to this peak. Therefore, such broad peaks require careful interpretation so as to not overlook the other component allele contained by the peak. As an index to recognize a peak containing two alleles, the ratio of peak area to peak height was found to be useful.

KEYWORDS: forensic science, DNA typing, pentanucleotide tandem repeat marker, capillary electrophoresis, Penta E, short tandem repeat, PowerPlex 16

Short tandem repeat (STR) markers are polymorphic DNA loci that consist of a repeated nucleotide sequence (1). The simultaneous analysis of multiple STR loci by capillary electrophoresis has become a common practice for purposes of human identification in forensic applications (2). Penta E is a pentanucleotide tandem repeat marker included in a commercially available kit that employs multiplex PCR using fluorescently labeled primers. It has been reported that Penta E is a highly polymorphic marker among various populations, including the Japanese (3–10). Two Penta E variant alleles differing from known Alleles 19 and 20 in size by one base, respectively, were found in the Japanese population. However, at present, no sequence data for the Penta E variant allele are available. The objective of this study was to present the sequence data of the variant alleles and to assess the resolution of alleles differing in size by one base using capillary electrophoresis.

Materials and Methods

Samples

DNA was isolated from blood samples obtained from 132 unrelated Japanese subjects by MagNA Pure LC (Roche Diagnostic, Mannheim, Germany), the extraction procedure of which is based on the adsorption of the DNA to magnetic glass particles (11).

[1] Biology Section, Department of First Forensic Science, National Research Institute of Police Science, 6-3-1 Kashiwanoha Kashiwa, Chiba, 277-0082, Japan.

* This work was presented in poster at the 13th International Symposium on Human Identification (October 2002).

Received 27 July 2002; and in revised form 15 Oct. 2002; accepted 16 Nov. 2002; published 12 Feb. 2003.

Amplification and Typing

The sixteen DNA loci, including Penta E, were simultaneously amplified using the PowerPlex® 16 kit (3,4,12) (Promega, Madison, WI) following the manufacturer's recommendations. The fluorescent-tagged amplified products were analyzed using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster, CA) according to the manufacturer's recommended protocol (i.e., using a 47 cm capillary and a POP-4™ polymer (Applied Biosystems), 3 s injection time, 15.0 kV both in injection and run voltage and run at 60 ºC) (13). Allele designations were determined by comparison of the sample fragments with those of allelic ladders supplied with the kit, utilizing the GeneScan® analysis software (Ver. 3.1), Genotyper® software (Ver. 2.5) (Applied Biosystems) and the PowerTyper™ 16 Macro (Promega) that contains the manufacturer's recommended criteria.

Sequence Analysis

Monoplex PCR was employed to amplify the Penta E locus alone. All reactions were performed in a total volume of 50 μL containing 0.4 μ*M* each of unlabeled Penta E primers (12), 50 ng of genomic DNA, 200 μ*M* each dNTP, 1.5 m*M* $MgCl_2$, 5U AmpliTaq Gold® DNA polymerase, and 1 X GeneAmp® PCR Gold buffer (Applied Biosystems). In the case of heterozygous samples, amplified fragments corresponding to an allele of interest were isolated and collected with the Wave system (Transgenomics, Omaha, NE), which utilizes DHPLC (denaturing high-performance liquid chromatography) in conjunction with a DNA separation cartridge (14). Since the DNA separation cartridge is not compatible with BSA contained by GoldST*R Buffer (Promega) in the kit, BSA-

free GeneAmp® PCR Gold buffer was used for the PCR reactions. The collected DNA fragments were reamplified and sequenced on an ABI Prism 310 Genetic Analyzer using the ABI Prism Big Dye terminators Version 2 cycle sequencing kit (Applied Biosystems).

Resolution of Alleles Differing in Size by One Base

To access the resolution of alleles differing in size by one base by capillary electrophoresis, PCR product mixture samples from two individuals were utilized. First, PCR was performed each using a DNA sample extracted from one individual possessing Allele 18.4, one individual possessing Allele 19, three individuals possessing Allele 19.4, and one individual possessing Allele 20. All donors were heterozygous. There was no overlapping Penta E allele between samples from two donors to be mixed. The six PCR product samples were analyzed separately to collect data on peak heights of heterozygote alleles and a ratio between heterozygote alleles. Based on this information, two PCR product samples were then mixed so as to make the estimated heights of peaks corresponding to a variant allele and the corresponding wild one in the following ratios, 1:8, 1:4, 1:2. 1:1, 2:1, 4:1, and 8:1. This was done with each PCR product sample containing a variant allele. Thus, in each ratio, four combinations of mixed samples were prepared. The mixtures were analyzed by capillary electrophoresis following the manufacturer's protocol.

Results and Discussion

Penta E is a pentanucleotide tandem repeat marker having an AAAGA repeat motif. It has been reported that Penta E is a highly polymorphic marker among various populations (3–10). Out of 132 Japanese samples, two variant alleles were detected, fragments of which were reproducibly sized as approximately one-base-shorter compared to Alleles 19 and 20 in the allelic ladder marker, respectively, by capillary electrophoresis. The genotypes carrying the variant alleles were 17/19 variant, 11/20 variant, 12/20 variant, and 16/20 variant. Each variant allele was isolated and collected from each sample. By sequence analysis, the variant alleles were found to have a partial repeat motif of AAAA (Fig. 1). Following ISFG (International Society of Forensic Genetics) recommendations (15), the variant alleles were designated as 18.4 and 19.4, respectively (Table 1).

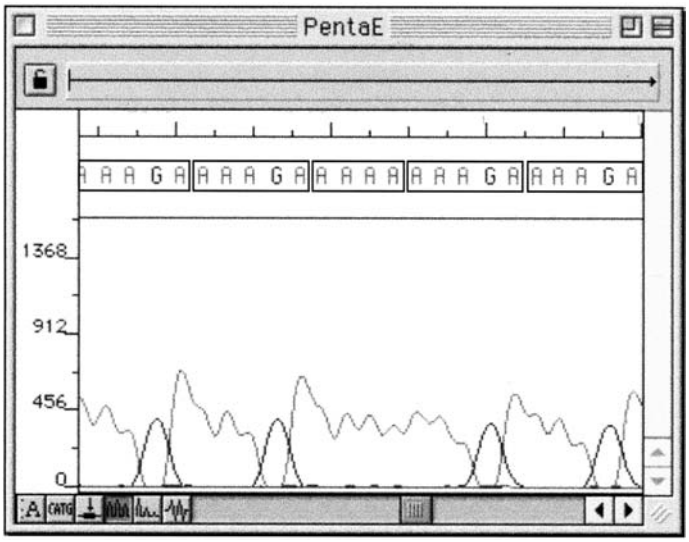

FIG. 1—*Partial repeat motif "AAAA" observed in a Penta E variant allele. Sequence of a Penta E variant allele is shown. Repeat motifs are enclosed by open boxes.*

Since Alleles 18.4 and 19.4 were found, the windows to type Alleles 19 and 20 should be narrowed from ±0.75 to ±0.50, similar to Alleles 9.3 and 10, which differ in size by only one base on the TH01 locus (1) in the same kit. Using DNA samples that possess Allele 19 or 20, the size differences between an allele amplified from a sample and the corresponding allele in the allelic ladder were examined (Table 2). The results were similar to the size precision data reported by Krenke et al. obtained from comparing the sizing of alleles from 13 ladder marker injections (12). The high precision in sizing the alleles ensured that narrowing the windows to ±0.50 would have no effect on allele assignment.

To examine the resolution of alleles by capillary electrophoresis, the PCR product mixture containing alleles that differ in size by one base was analyzed (Fig. 2). Alleles 18.4/19 (448/449 bases) and 19.4/20 (453/454 bases) failed to separate and appeared as one broad peak. The Genotyper software assigned one of the component alleles to the peaks. Therefore, when encountering such broad peaks, careful interpretation is recommended so as to not overlook the other component of the peak. As a potential index for recognizing a peak that contains two alleles, the ratios of peak area to peak height were calculated using PCR product samples from a single source DNA and PCR product mixtures from two donors (Table 3). The significant differences between peaks corresponding to a single component and one that contains two components indicated that the ratio constitutes a useful index for recognizing a peak containing two components.

A cutoff value for a peak containing a single component was determined by taking the mean value of a single component peak (9.70) plus five times the standard deviation (0.33) of a single component peak. Based on this, the resulting cutoff value for a single

TABLE 2—*Size differences between an allele amplified from a sample and the corresponding allele in the allelic ladder.*

Allele	*n**	Mean	S.D.
19	9	0.063	0.061
20	10	0.040	0.083

* The number of *n* data obtained from PCR product samples amplified from *n* different individuals was used.

TABLE 1—*Fragment length and repeat composition of the variant Penta E alleles.*

Allele Designation	Fragment Length (bp)	Repeat Region	Number of Sequenced Alleles*
18.4	448	(AAAGA)6(AAAA)1(AAAGA)12	1
19	449	(AAAGA)19	1
19.4	453	(AAAGA)6(AAAA)1(AAAGA)13	3
20	454	(AAAGA)20	1

* Each variant allele observed in the Japanese population was sequenced. Alleles 19 and 20 were also sequenced as controls.

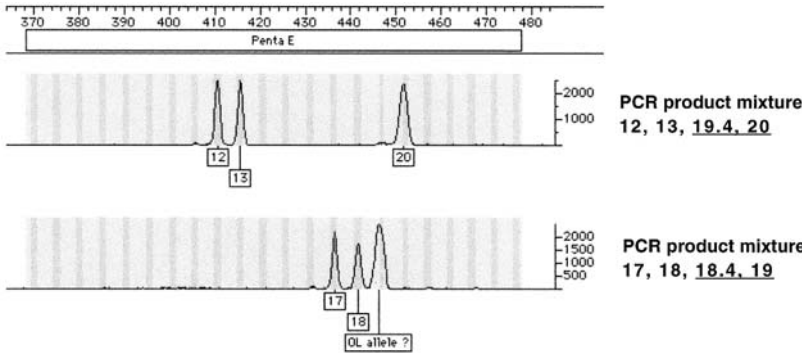

FIG. 2—*Broad peaks composed of two alleles that differ in size by one base. The windows to type Alleles 19 and 20 were narrowed from ±0.75 to ±0.50. Allele 18.4 and 19.4 are typed as an OL (off-ladder) allele by the Genotyper software. PCR product samples from two donors were mixed so as to make the estimated heights of peaks corresponding to the alleles differing in size by one base in a ratio of 1:1. By capillary electrophoresis, however, those alleles (underlined) were not separated and appeared as a single broad peak Genotyper software assigned only one of the component alleles to this peak.*

TABLE 3—*An index for recognizing peaks that contain alleles differing in size by one base.*

			Peak Area Divided by Peak Height			
Peak Component	*n*	Mixture Ratio	Minimum	Maximum	Mean	S.D.
A single allele	234*	…	8.96	10.74	9.70	0.33
A single allele?†	15†	…	9.24	10.22	9.76	0.26
Two alleles	4‡	8:1	10.73	11.60	11.33	0.40
18.4/19, 19.4/20	4‡	4:1	11.73	13.20	12.35	0.63
	4‡	2:1	13.92	14.70	14.14	0.37
	4‡	1:1	14.55	15.77	15.04	0.54
	4‡	1:2	13.37	14.76	13.88	0.64
	4‡	1:4	11.55	13.41	12.63	0.78
	4‡	1:8	10.72	11.91	11.33	0.56

* Out of 132 Japanese samples, 117 individuals were heterozygous. Therefore, 234 peaks obtained from those 117 heterozygous individuals were obviously single component peaks.

† The remaining 15 individuals appeared homozygous. However, since two alleles differing in size by one base cannot be separated, the peak might contain two alleles. Therefore, those peaks were not pooled with the obvious single component peaks.

‡ Two PCR product samples were mixed so as to make the estimated heights of peaks corresponding to the alleles differing in size by one base in the various ratios. They were the estimated peak heights, since those alleles were not separated by capillary electrophoresis. In each ratio, four PCR product mixtures were prepared. Each mixture contained a variant allele amplified from a different individual.

component peak was determined to be 11.35 with a 99.99994% certainty that no false positive for two alleles in a peak that actually contains only one allele will occur. Using this cutoff value, it was possible to recognize a peak as containing two components when the minor component was at 20% of the total DNA. Further, based on this observation and the cutoff value, 15 individuals who appeared homozygous were positively identified as homozygous.

In conclusion, the variant Penta E alleles have a partial repeat motif of AAAA, resulting in one-base-shorter alleles compared to known Penta E alleles. An accurate allele assignment, including the variant alleles, can be readily achieved by capillary electrophoresis. However, due to limitations in resolution, a careful examination of the electropherogram is necessary when the possibility exists that a sample contains alleles differing in size by one base. As an index to recognize a peak containing two alleles, the ratio of peak area to peak height was found to be useful. In general, peak shape is not significantly affected by peak height; however, it is affected by both peak and signal quality. The values for the ratios of peak area to peak height tend to become larger in a poor-quality electropherogram compared with one of good quality. Therefore, when we examine electropherograms, it is necessary to be sure that peak broadening caused by unusually poor resolution did not occur during the runs (this can be efficiently checked by taking the ratio of area to height using internal lane standard peaks coelectrophoresed with PCR products), signals are not saturated, and an elevated baseline is not present both in raw data and data after analysis by the GeneScan Analysis software. Ultimately these precautions simply mean that in order to use the index, electropherograms of sufficient quality for use in the field of forensic science are needed.

References

1. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 1991;49:746–56.
2. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Brown AL, Budowle B. Validation of STR typing by capillary electrophoresis. J Forensic Sci 2001;46:661–76.
3. Budowle B, Masibay A, Anderson SJ, Barna C, Biega L, Brenneke S, et al. STR primer concordance study. Forensic Sci. Int 2001;124:47–54.
4. Budowle B, Sprecher CJ. Concordance study on population database samples using the PowerPlex 16 kit and AmpFℓSTR Profiler Plus kit and AmpFℓSTR COfiler kit. J Forensic Sci 2001;46:637–41.
5. Fernandes AT, Brehm A, Alves C, Gusmao L, Amorim A. Genetic profile of the Madeira Archipelago population using the new PowerPlex16 System kit. Forensic Sci Int 2002;125:281–3.
6. Gangitano DA, Garofalo MG, Juvenal GJ, Budowle B, Lorente JA, Padula RA. STR data for the PowerPlex 16 loci in Buenos Aires population (Argentina). J Forensic Sci 2002;47:418–20.

7. Garofano L, Pizzamiglio M, Donato F, Biondi F, Rossetti M, Budowle B. Italian population data on two new short tandem repeat loci: D2S1338 and Penta E. Forensic Sci Int 1999;105:131–6.
8. Hashiyada M. Short tandem repeat analysis in Japanese population. Electrophoresis 2000;21:347–50.
9. Robino C, Gino S, Torre C. Allele frequencies for the PowerPlex 16 STR loci in an Albanian population sample from northern Italy. J Forensic Sci 2001;46:998–9.
10. Steinlechner M, Grubwieser P, Scheithauer R, Parson W. STR loci Penta D and Penta E: Austrian Caucasian population data. Int J Legal Med 2002;116:174–5.
11. Kessler HH, Muhlbauer G, Stelzl E, Daghofer E, Santner BI, Marth E. Fully automated nucleic acid extraction: MagNA Pure LC. Clin Chem 2001;47:1124–6.
12. Krenke BE, Tereba A, Anderson SJ, Buel E, Culhane S, Finis CJ, et al. Validation of a 16-locus fluorescent multiplex system. J Forensic Sci 2002;47:773–85.
13. Promega Corporation. GenePrint® PowerPlex™ 16 System Technical Manual, Part#TMD012 (4/00). Madison WI, 2000, www.promega.com/tbs/TMD012/TMd012.html.
14. Devaney JM, Girard JE, Marino MA. DNA microsatellite analysis using ion-pair reversed-phase high-performance liquid chromatography. Anal Chem 2000;72:858–64.
15. Olaisen B, Bar W, Brinkmann B, Budowle B, Carracedo A, Gill P, et al. DNA recommendations 1997 of the International Society for Forensic Genetics. Vox Sang 1998;74:61–3

Additional information and reprint requests:
Natsuko Mizuno, D.V.M.
Senior Scientist
Fourth Biology Section
Department of First Forensic Science
National Research Institute of Police Science
6-3-1 Kashiwanoha Kashiwa, Chiba, 277-0882
Japan
E-mail: mizuno@nrips.go.jp