CASE REPORT

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Male DNA Typing from 25-Year-Old Vaginal Swabs Using Y Chromosomal STR Polymorphisms in a Retrial Request Case

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ABSTRACT: We report here the application of Y chromosomal DNA analysis in a retrial request case, raised officially by Sapporo High Court, Japan, of a condemned criminal whose capital punishment has been suspended. DNA was extracted from mixed seminal/vaginal secretion stains collected 25 years ago from two raped and murdered victims, and Y chromosome STR loci (DYS19, 390, 393, YCAII) were amplified and sequenced to clarify the DNA type of the rapist. Alkaline proteinase and sodium hydroxide were used before phenol/chloroform extraction to achieve high quality DNA from very old samples. In addition, amplified fragments of DYS19, DYS390, and DYS393 were sequenced using an automated DNA sequencer. Four Y STR DNA types detected from vaginal swabs were found identical to those of the accused criminal and confirmed that the two rape and murder cases had been committed by the same person. Sapporo High Court accepted the results and rejected the retrial request in February 1998.

KEYWORDS: forensic science, DNA typing, Y chromosome, vaginal swab, short tandem repeats, DYS19, DYS390, DYS393, YCAII, old evidence

Mixed seminal/vaginal secretion stains are one of the most important evidential materials for criminal investigation in sexual assault cases. Recently, Y STR analysis has become one method of choice for analysis of vaginal swabs from sexual assault cases having mixed female and male fraction (1).

To identify DNA types of a rapist from old vaginal swabs, it is necessary to overcome the problem of effectively isolating seminal DNA from mixed stains. The conventional method for extracting seminal DNA from mixed seminal/vaginal secretion stains involves two-step differential extraction (2,3), but it is not always successful in the case of extremely old stains in which cells have been lysed. Furthermore, if we detected mixed DNA types of rapist and victim because of imperfect DNA separation, we do not have any sure way to know the DNA type of the rapist. Therefore, we applied a new method using alkaline proteinase to extract high quality genomic DNA without separating seminal DNA from such stains. To detect male DNA type selectively from mixed male/female DNA, we performed Y STR DNA typing (4–6).

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Case History

From 1972 to 1973, two successive rape/murders and another rape occurred in Hokkaido, the northern part of Japan. Two years later, one suspect was arrested after police investigation. As the suspect confessed to the crimes during the trial, he was sentenced to life imprisonment. However, the suspect later insisted on his innocence, and the defense side appealed for a second trial. He was again found guilty and sentenced to death. The defense then appealed to the Supreme Court, but the capital punishment was still not overturned. After the judgment of the Supreme Court, the defense demanded the opportunity of a retrial on the grounds that judgment based on a confession was unsatisfactory. Fortunately, vaginal swabs of two victims, stored for 25 years, were discovered recently, and the defense asked the Sapporo High Court for DNA typing using these evidentiary materials. DNA typing was carried out at the Department of Legal Medicine, Osaka University Medical School.

Materials and Methods

Vaginal swabs of approximately 5 cm^2 which had been collected from two raped and murdered victims (coded as AK and ET) during autopsy were used for DNA typing of the rapist. They were preserved in dried condition at room temperature for 25 years since collection. Both victims were 19 years old at the time of death, and one was putrefied (AK) when found.

Instead of proteinase-K, 10 mM NaOH and alkaline proteinase were used for tissue destruction and digestion. As DNA is stable as single strands at high pH, it can be extracted more effectively by the simultaneous use of NaOH and alkaline proteinase (7).

Vaginal swabs were placed in 10 mL of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5% SDS, 1 mM EDTA, 10 mM NaOH) containing 1 mg/mL alkaline proteinase (Promega, Madison) and incubated at 50°C for 6 h adjusting the pH to 8.5 with 0.1M Tris-HCl, pH 7.6. Then, DNA was extracted using the phenol/chloroform procedure. After dialysis against 10 mM Tris-HCl (pH 8.0)

and 5 mM EDTA, extracted DNA was precipitated with ethanol, and concentrated with Centricon 30 (Amicon), then dried and stored at −20°C at a DNA concentration of 10 ng/μL after diluting with an appropriate amount of 20 mM Tris-HCl (pH 7.6). Concentration of extracted DNA was measured using a DNA Quant 200 fluorometer (Hoefer Phamacia Biotech, San Francisco, CA).

X-Y homologous amelogenin PCR (8) was carried out according to the published protocol to confirm whether male DNA was present in the samples. Male DNA was detected and so further detection of Y chromosomal fragments could be carried out and confirmed that a seminal fraction was present in the vaginal swabs of two rape victims.

The Y STR system can detect male DNA type selectively from very old mixed samples of semen and vaginal epithelium without separation during DNA extraction. DYS19, DYS390, DYS393, and YCAII PCR were performed using modified primer sequence compared to the published Genome Data Base (GDB) sequences (9,10).

Amplification Conditions

The reverse primers were labeled with different fluorescent dyes as recommended by the manufacturer of automated DNA sequencer (PE Applied Biosystems, Foster City, CA). Published primer sequences were used for PCR (9) reaction mixtures of 25 μL containing 2 μL of template (20 ng DNA), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X100, 1.5 mM $MgCl_2$, 0.2 mM d-NTPs, 25 pmol each primer, and 1U *Taq* polymerase (Promega, Madison). Forward primer sequences and modified reverse primer sequences of DYS19 and DYS393, and cycling conditions were as follows:

DYS19: Forward 5′-CTACTGAGTTTCTGTTATAGT-3′
Reverse 5′-GTAATACTTCGGGCCATGG-3′

Denaturation at 94°C for 2 min, followed by 30 cycles of 94°C × 1 min, 56°C × 1 min, 72°C × 1 min with a final extension at 72°C × 5 min in Perkin Elmer PJ2000 thermal cycler (Perkin-Elmer, Norwalk, CT).

DYS393: Forward 5′-GTGGTCTTCTACTTGTGTCAATAC-3′
Reverse 5′-TCAAGTCCAAAAAATGAGGTATG-3′

Cycling conditions were as described by Kayser et al. (9).

Aliquots of 1 μL of the amplified products were mixed with 2 μL of 1% dextrane blue/formamide loading dye, loaded on 6% polyacrylamide gels and run in 1X TBE (90 mM Tris, 90 mM Boric acid, 1 mM EDTA) including 8 M Urea and electrophoresed at 2500 V, 45 mA, 30 W in an automated DNA sequencer (PE Applied Biosystems, Foster City, CA: Model 373A). Sequenced allelic ladder was loaded in the lane flanking the samples to determine DNA type.

After detection of four kinds of Y STR types, direct sequencing of PCR products from both forward and reverse directions was performed. Dye terminator cycle sequencing: forward/reverse primer 871

3.2 pmol, template DNA-50 ng (PCR product), premix from Dye Terminator Cycle sequencing FS Ready Reaction Kit, including A,C,G,T-DyeDeoxy, dGTP, dATP, dCTP, dTTP, Tris-HCl (pH9.0), Buffer, Amplitaq DNA Polymerase FS (PE Applied Biosystems, Foster City, CA)-9.2 μL. Dye terminator cycle sequencing reaction (PE GeneAmp PCR System 2400 thermal cycler; PE Applied Biosystems, Norwalk, CT: 96°C 10 sec, 50°C 5 sec, 60°C 5 min for 25 cycles. Products were precipitated with ethanol and sequenced with a Model 377 DNA sequencer (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

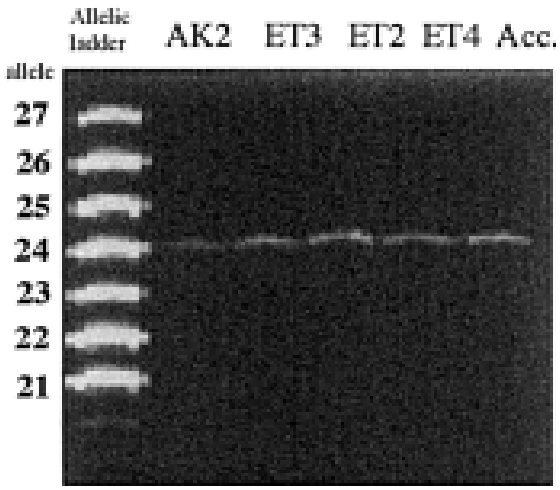

FIG. 2—*DNA typing of DYS390 locus by ABI PRISM® Model 373A. AK2: Male DNA from 25-year-old vaginal swab (victim AK; DYS390*24), ET3; ET2; ET4: Male DNA from 25-year-old vaginal swab (victim ET; DYS390*24), Acc: Accused blood DNA:DYS390*24.*

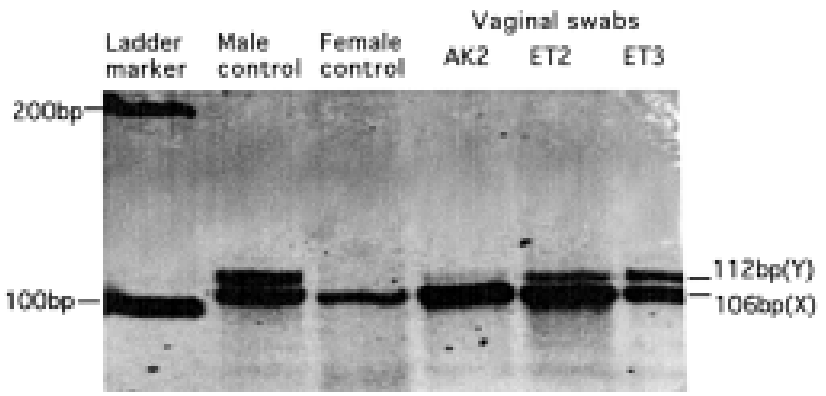

FIG. 1—*Amplification of XY homologous amelogenin from vaginal swab samples. PCR products run in 4% metaphor gel (FMC). AK2: DNA from vaginal swab (victim AK), ET2; ET3: DNA from vaginal swab (victim ET), and Y specific bands from male (112 bp) were visible in AK2, ET2, and ET3.*

Results

A considerable amount of DNA (from approximately 0.5 μg to 45 μg from 5 cm^2 gauze) was extracted from very old mixed stains samples of two victims using alkaline proteinase digestion. Male DNA was detected (Fig. 1) and we were able to proceed to Y STR DNA typing.

Types from four Y STR loci, DYS19, DYS390, DYS393, and YCAII, were detected from the vaginal swabs of two victims and their DNA types were found to be the same, namely: DYS19*13, DYS390*24, DYS393*14, YCAII*3/3. These results were reported to the court before collection from accused blood. Allelic designation was according to the Y chromosome STR information (9).

DNA Typing of the Accused

Ten mL of blood was collected from the accused detained in prison as witnessed by Sapporo prison officials. DNA was extracted from this blood sample by conventional phenol/chloroform extraction, and Y STR DNA typing was performed. The results were as follows: DYS19*13, DYS390*24, DYS393*14, YCAII* 3/3, identical to those of vaginal swabs. Figure 2 shows the gel image of one of Y STR locus, DYS390.

Direct sequencing of DYS19, DYS390, and DYS393 fragments was also carried out and confirmed that there was no contradiction between DNA type and nucleotide sequence (Figs. 3, 4). Repeated sequences were as follows: DYS19*13 = $(gata)^3(GGTA)(gata)^{10}$,

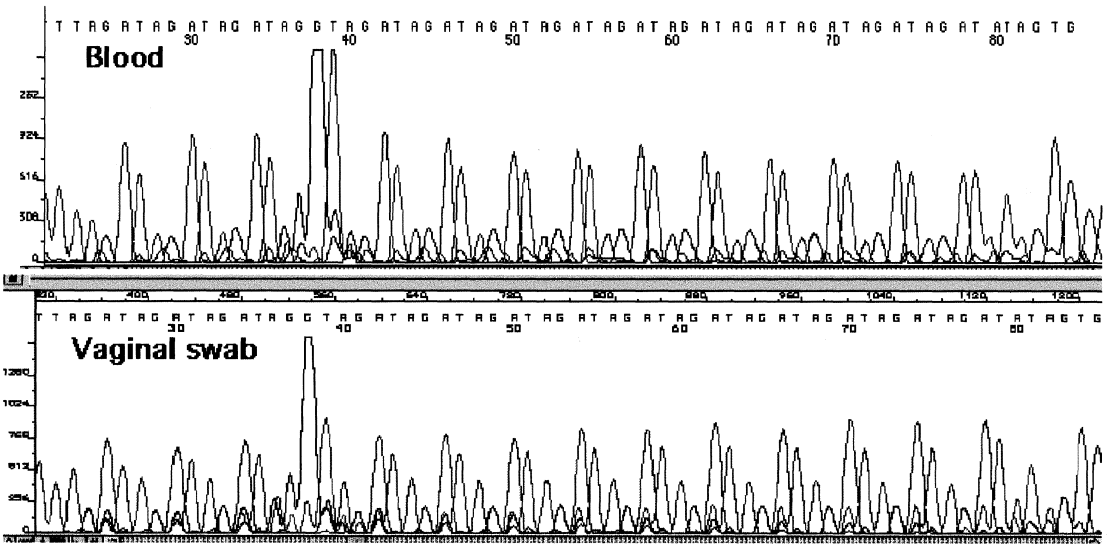

FIG. 3—*Direct sequencing of DYS19. Upper panel: sequencing of accused blood DNA, Lower panel: sequencing of vaginal swab (ET3). Both sequences were completely identical:DYS19*13 = $(gata)^3$ (GGTA)$(gata)^{10}$.*

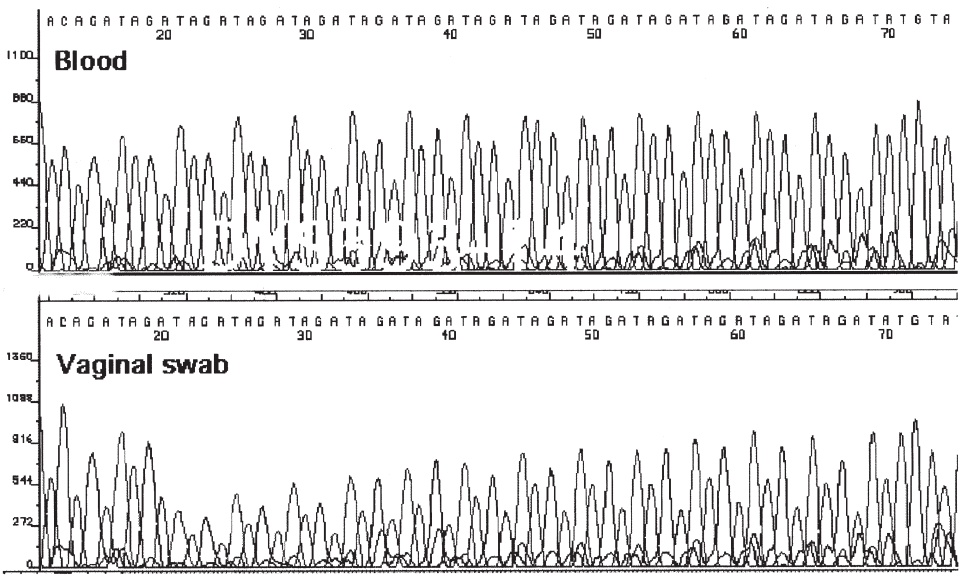

FIG. 4—*Direct sequencing of DYS393. Upper panel: sequencing of accused blood DNA, Lower panel: sequencing of vaginal swab (ET3). Both sequence were completely identical: DYS393*14 = $(gata)^{14}$.*

DYS390*24 = $(ctgt)^8(ctgt)^{16}$, DYS393*14 = $(gata)^{14}$. On August 31st, 1997, the results were summarized in an appraisal and reported to the Sapporo High Court.

Discussion

In this case, the physical evidence of the crime presented to the court was inconclusive. Therefore, when the accused retracted his confession, the court had to reinvestigate the physical evidence once again. The results of the ABO blood typing was one of the original pieces of evidence, but it was not very strong and also inconclusive.

Vaginal swabs from two raped and murdered victims which had been stored for 25 years were available. However, they were very old and it was thought that DNA extraction for subsequent male DNA typing might be difficult. To overcome the possible problems, alkaline proteinase extraction (7,11) and Y STR typing (1) is the method of choice. Recently, Mechthild Prinz and co-workers (12) reported that a mixture ratio with high female background as high as female:male of 2000:1 does not interfere with Y STR analysis. Our experiments showed similar results, in that 10 pg of male DNA mixed with 10 ng of female DNA in stains could be amplified and typed using fluorescent labeled primers and detection in an automated DNA sequencer. Interpretation of the results is also easier than with autosomal systems because Y STR systems always display a single type of the male without showing that of female, and loss of DNA during the differential lysis procedure can be avoided.

Although there are many proteinases, Proteinase-K is routinely used because of its nonspecific protein digestion activity. A pretreatment method involving cell lysis by heating at 95°C in 0.04 M NaOH was reported (13), and a new DNA extraction method using alkaline proteinase (14) with 10 mM NaOH for extraction from seminal/vaginal secretion stains was applied (15). To compare the effectiveness of alkaline proteinase extraction to conventional proteinase-K extraction, blood DNA was used as a control. The results demonstrated that this method was twofold more effective than conventional proteinase-K extraction (Fig. 5).

Although 14 evaluated Y STR loci are known to data (9,10), we selected a panel of 4 loci for extremely old samples based on the results of previous experiments using vaginal swabs stored for 32 years in our laboratory (11,15). The panel of systems selected combines high informativity (gene diversity for DYS390, DYS19, YCAII > 0.65) with short amplicons (108 bp and 227 bps for the shortest and longest allele of the four systems, respectively). The primers and PCR conditions used were basically the same as those described at the forensic Y user workshop (Berlin 1996) but modified reverse primers of DYS19 and DYS393 were used for more sensitive detection.

The use of DNA testing has been doubted by lawyers in Japan because recent DNA judgments have been overturned. Consequently, some restrictions were placed on the present investigation. First, we were required to submit the results of DNA types detected from these old vaginal swabs before examining the blood DNA type of the accused. Secondly, the record of detailed procedure and raw data of experiments were made available for the law court as exhibits.

Although compliance with the second request was simple, we were hesitant about the first because extracted DNA were not so large enough to repeated use. To determine DNA types of vaginal swabs correctly, allelic designation and reliable allelic ladder maker have to be established previously. This problem was resolved as the Y user workshop has distributed latest information and sequenced allelic ladders were produced and kindly distributed by P. de Knijff, Leiden University. Even without allelic ladder markers, it is possible to determine DNA type by calculation using

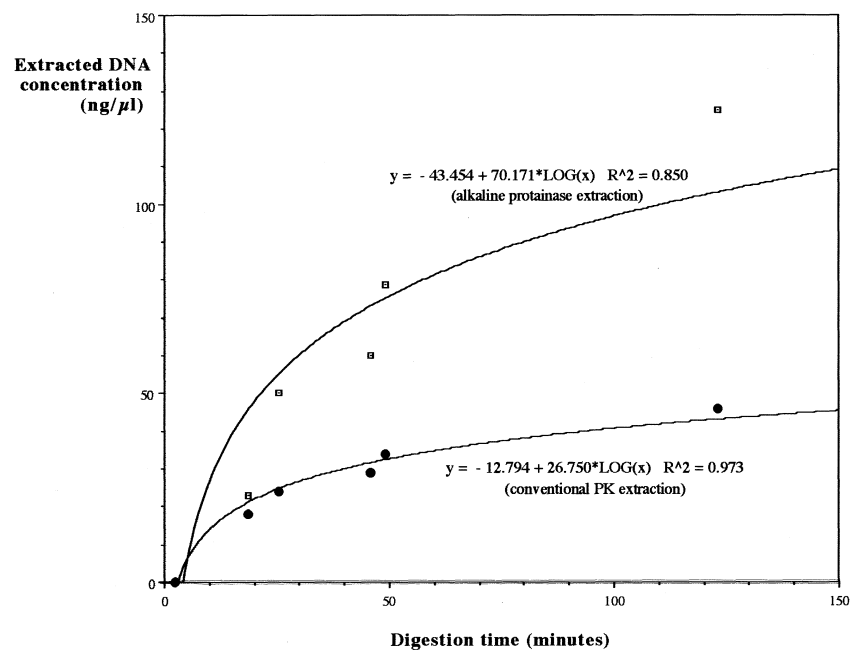

FIG. 5—*Increasing concentration of extracted DNA by alkali-proteinase from whole blood. 10 μL each of whole blood was digested with 10 μg each of alkali-proteinase (Promaga) with 10 mM NaOH and conventional proteinase-K in 100 μL TE buffer. Digestion was stopped at 2.5 min, 18.5 min, 25.5 min, 46 min, 49 min, and 123 min, and DNA was extracted using 10% chelex (Biorad) resin. DNA concentration was measured by a DNA Quant 200 fluorometer (Hoefer Phamacia Biotech). Square dot: alkali-proteinase extraction, Round dot: proteinase-K extraction. Regression curves were fitted as logarithmic.*

fragment analysis software using internal size standard. However, we found a discrepancy of one or two base pairs between the true and calculated sizes because small errors occur through software calculation or preparing gel for electrophoresis. In addition, mistyping of any one locus may result in false evidence of innocence. As forensic DNA testing requires precise results, allelic markers are an absolute requirement for DNA typing for use in the law court. In addition, direct sequencing of Y STR locus products showed complete identity between those of vaginal swabs and the accused, and confirmed DNA typing. Since all four loci are linked on the Y chromosome a conclusive statistical analysis of the match probability cannot be given without haplotype analysis (9,10). However, if the published single locus frequencies for Japanese (9) is used, the rate of an accidental match has been calculated to be 1/2500 for the detected 4-locus Y STR types.

We also examined X chromosome STR systems, HUMHPRTB and HUMARA (16) and found three fragments of each X chromosomal locus, two from the victim and one from the rapist. Each was the same as those of the accused (data not shown). These results demonstrated that DNA obtained from old vaginal swabs could be used for DNA typing.

We have demonstrated the utility and reliability of Y STR systems through practical application, and settled a confusion unsolved for as long as 25 years. Sapporo High Court accepted this appraisal, and announced in February 1998 that it became extremely difficult for the retrial request to be recognized. In addition, Mr. Kentaro Kasai of the National Research Institute of Police Science, an appraiser recommended by the prosecution, who compared conventional DNA types of vaginal swabs from one of two victims (ET) with the accused blood simultaneously, and found no remarkable inconsistencies between them.

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References

1. Roewer L, Epplen JT. Rapid and sensitive typing of forensic stains by PCR amplification of polymorphic simple repeat sequences in case work. Forensic Sci Int 1992;55:163–71.
2. Gyllensten U, Josefsson A, Schemschat K, Saldeen T, Petterson U. DNA typing of forensic material with mixed genotypes using allele-specific enzymatic amplification (Polymerase chain reaction). Forensic Sci Int 1992;52:149–60.
3. Yoshida K, Sekiguchi K, Mizuno N, Kasai K, Sakai I, Sato H, et al. The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with sperm. Forensic Sci Int 1995;72:25–33.
4. Arnemann J, Jakubiczka S, Schmidtke J, Schäfer R, Epplen JT. Clustered GATA repeats (Bkm sequences) on the human Y chromosome. Hum genet 1986;73:301–3.
5. Roewer L, Arnemann J, Spurr NK, Grzeschik KH, Epplen JT. Simple repeats sequences on the human Y chromosome are equally polymorphic as their autosomal counterparts. Hum Genet 1992;89:389–94.
6. Santos FR, Pena SDJ, Epplen JT. Genetic and population study of a Y-linked tetranucleotide repeats DNA polymorphism with a simple non-isotopic technique. Hum Genet 1993;90:655–6.
7. Honda K, Nakatome M, Tun Z, Wakasugi C. Detection of male specific DNA in vaginal swab to certify sexual offenses. In: Wakasugi C, editor. Advances in Legal Medicine. Proceedings of the 3rd International Symposium of Advances in Legal Medicine (ISALM); 1996 Sept. 2–4; Osaka. 1997;316–7.
8. Mannucci A, Sullivan KM, Ivanov PL, Gill P. Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin. Int J Leg Med 1994;106:190–3.
9. Kayser M, Caglià A, Corach D, Fretwell N, Gehrig C, Graziosi G, et al. Evaluation of Y chromosomal STRs: A multicenter study. Int J Legal Med 1997;110:125–33.
10. de Knjjff P, Kayser M, Caglià A, Corach D, Fretwell N, Gehrig C, et al. Chromosome Y microsatellite: population genetics and evolutional aspects. Int J Legal Med 1997;110:134–40.
11. Honda K, Tun Z, Nakatome M, Shimada S, Sakurada M, Terada M, et al. Identification of rapist from old vaginal swabs using sex chromosomal STRs. In: Tatsuno Y, editor. Proceedings of the 6th Indo Pacific Congress on Legal Medicine and Forensic Sciences (INPALMS); 1998 July 26–30; Kobe, Japan,1999;755–7.
12. Prinz M, Boll K, Baum H, Schler B. Multiplexing of Y chromosome specific STRs and performance for mixed samples. Forensic Sci Int 1997;85(3):209–18.
13. Jiwa NM, van Gemert GW, Raap AK, van de Rijke FM, Mulder A, Lens PF, et al. Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation 1989;48:72–6.
14. Cleveland DW, Fisher SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 1977;252(3):1102–6.
15. Honda K, Nakatome M, Tun Z, Sakurada M, Misawa S. Universal method of hypersensitive nested PCR toward forensic DNA typing. In: Olaisen B, Brinkman B, Lincoln PJ, editors. Progress in Forensic Genetics 7. Proceedings of the 17th International ISFH Congress; 1997 Sept. 2–6, Oslo, Elsevier. 1998;28–30.
16. Edwards AL, 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.

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