CASE REPORT

Manfred N. Hochmeister,[1] M.D.; Bruce Budowle,[2] Ph.D.; Urs V. Borer;[1] Oskar Rudin;[1] Michael Bohnert, M.D.;[3] and Richard Dirnhofer,[1] M.D.

Confirmation of the Identity of Human Skeletal Remains Using Multiplex PCR Amplification and Typing Kits

REFERENCE: Hochmeister, M. N., Budowle, B., Borer, U. V., Rudin, O., Bohnert, M., and Dirnhofer, R., "**Confirmation of the Identity of Human Skeletal Remains Using Multiplex PCR Amplifcation and Typing Kits,**" *Journal of Forensic Sciences*, JFSCA, Vol. 40, No. 4, July 1995, pp. 701–705.

ABSTRACT: The identity of human skeletal remains found in a wooded area approximately one year after the person was reported missing was provisionally established by routine methods and circumstantial evidence. Multiplex PCR systems—the AmpliType® PM PCR Amplification and Typing Kit and the GenePrint™ STR Triplex Amplification and Typing Kit—were used to confirm the identification. DNA profiles from femur bone from the remains were compared with profiles derived from head hairs from a hairbrush recovered in the missing woman's apartment. In addition, a sex typing procedure using the X-Y homologous gene amelogenin was carried out. This is the first report of a case using commercially available multiplex PCR amplification and typing kits to confirm the identity of skeletal remains.

KEYWORDS: forensic science, human identification, deoxyribonucleic acid (DNA), polymerase chain reaction (PCR), genetic typing, HLA DQA1, LDLR, GYPA, HBGG, D7S8, GC, short tandem repeats (STR), TH01, TPOX, CSF1PO, amelogenin, sexing

Human skeletal remains were discovered in March 1994 in a wooded area near the city of Berne, Switzerland. A 56-year-old woman had been reported missing one year before the remains were discovered. The identity was provisionally established from X-rays, dental records and circumstantial evidence. DNA analysis was attempted to confirm the identification of the woman. Multiplex PCR product profiles from the femur bone DNA of the remains were compared with corresponding DNA profiles generated from head hairs from a hairbrush recovered by investigators from the woman's apartment.

Typing of genetic markers in human bone by DNA analysis was reported by Lee et al. in 1991 [*1*]. However, the detection of variable number of tandem repeat (VNTR) sequences by restriction fragment length (RFLP) analysis requires a minimum of 25 to 50 ng of relatively undegraded DNA and is usually not successful in decomposed human remains [*2*]. Genetic information from samples with degraded DNA or from samples which do not contain much DNA may be obtained by amplification of the DNA using the PCR. PCR-based tests, including the amplification of the HLA DQA1 locus, various VNTR loci [*2,3*], microsatellite typing [*4,5*], and mitochondrial DNA sequence analysis [*6,7*] were successfully applied to the analysis of DNA extracted from human skeletal remains and single hairs [*8,9*].

Currently, two multiplex PCR amplification and typing kits are commercially available that enable the simultaneous amplification of specific regions of several genetic loci. The AmpliType® PM PCR Amplification and Typing Kit (Perkin Elmer; Roche Molecular Systems, Inc., Branchburg, NJ) includes reagents that direct the simultaneous amplification of specific regions of six genetic loci: HLA DQA1 (previously refered to as HLA DQ alpha; PCR product size 242/239 base pairs) [*10*], Low Density Lipoprotein Receptor (LDLR; PCR product size 214 base pairs) [*11*], Glycophorin A (GYPA; PCR product size 190 bpbase pairs) [*12*], Hemoglobin G Gammaglobin (HBGG; PCR product size 172 base pairs) [*13*], D7S8 (PCR product size 151 base pairs) [*14*], and Group Specific Component (GC; PCR product size 138 base pairs) [*15*]. Typing these loci is performed by hybridization of the amplified PCR products to DNA oligoprobes immobilized on nylon membrane strips. The hybridized DNA is visualized upon enzymatic conversion of a colorless substrate to a blue colored precipitate.

The GenePrint™ STR Systems PCR Amplification Kit (Promega Corporation, Madison, WI, USA) [*16,17*] includes reagents that direct the simultaneous amplification of specific regions of three tetrameric STR loci: HUMTH01 (PCR product size 179–203 base pairs) [*18–20*], TPOX (PCR product size 232–248 base pairs) [*21*], and CSF1PO (PCR product size 295–327 base pairs) [*22*]. Typing these loci is performed by separation on denaturing polyacrylamide gels [*18,19,23*] and silver staining. Since the sizes of the three STR loci in this multiplex assay do not overlap, unequivocal typing of the three loci is possible. This report describes the analysis of DNA from skeletal human remains and reference head hairs using two multiplex PCR amplification and typing kits.

Received for publication 12 Oct. 1994; revised manuscript received 8 Dec. 1994; accepted for publication 8 Dec. 1994.

[1]Research Scientists, and Director, respectively, DNA Identity Laboratory, Institute of Legal Medicine, University of Berne, Switzerland.

[2]Research Chemist, Forensic Science Research and Training Center, Laboratory Division, Federal Bureau of Investigation Academy, Quantico, VA.

[3]Forensic Pathologist, Institute of Legal Medicine, University of Freiburg im Breisgau, Germany.

Copyright © 1995 by ASTM International

Materials and Methods

DNA Extraction from Bone

The DNA was extracted from two 5 g fragments of the femur bone using a procedure described previously [2] with some modifications. The bone was cleaned aggressively with sandpaper to remove the outer layer of foreign material, broken into small pieces, and then pulverized into a fine powder using liquid nitrogen. The bone powder was transferred into a sterile 50 mL polypropylene tube and decalcified in 40 mL of 0.5 M EDTA, at pH 8.0, on a rotator at 4°C for 24 h. After centrifugation at 2000 g for 15 min, the supernatant was discarded. The powder was washed with 40 mL of extraction buffer (0.5 M EDTA, 0.5% sarcosyl at pH 8.0) in order to remove excess EDTA. Then, the sample was centrifuged at 2000 g for 15 min, and the supernatant was discarded. The DNA was extracted by adding prewarmed (37°C) extraction buffer (0.5 M EDTA, 0.5% sarcosyl at pH 8.0) to a final volume of approximately 7 mL. 100 μL of proteinase K (20 mg/mL) were added and the tube was incubated at 37°C for 12 h. Subsequently, 100 μL Proteinase K (20 mg/mL) were added and incubation was continued at 37°C for an additional 12 hours. The solution was extracted two to three times with phenol/chloroform/isoamylalcohol (25:24:1). Then, one extraction with 20 mL water-saturated n-butanol was carried out to remove traces of phenol. The aqueous phase was concentrated using a Centricon™ 100 microconcentrator tube (Amicon Division, W.R. Grace & Co.—Conn., Danvers, MA) [24], which was subjected to several 30 min centrifugation steps at 1000 g. Finally, the retentate was washed three times with 2 mL of sterile water. The final sample volume was approximately 25 to 40 μL. Bone extraction was carried out independently in duplicate and a sample containing no bone served as a reagent negative control sample.

DNA Extraction from Single Hairs

Several head hairs, which had follicular sheath material attached were recovered from a hairbrush from the missing woman's apartment. DNA was isolated from one centimeter of the root portion from two hairs. Each hair was incubated overnight at 56°C in 400 μL stain extraction buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl, 39 mM DTT, 2% SDS) and 10 μL Proteinase K (20 mg/mL). On the following day an additional 10 μL Proteinase K (20 mg/mL) were added, and the samples were incubated for 2 more hours at 56°C. The solution was extracted with 500 μL phenol-chloroform-isoamylalcohol (25:24:1) and subsequently extracted in 1 mL water saturated n-butanol to remove traces of phenol. The aqueous phase was then transferred to a Centricon™ 100 microconcentrator tube containing 1 mL sterile water. The volume was brought up to a total of 2 mL with sterile water, the sample reservoir was sealed with parafilm, and the tubes were subjected to centrifugation at 1000 g for 30 min. Then 2 mL of sterile water were added to the sample reservoir, and the reservoir was sealed with new parafilm. Again the tubes were centrifuged at 1000 g for 30 min. The DNA was recovered by back centrifugation at 1000 g for 5 min. The final sample volume was approximately 25 to 40 μL. Isolation and analysis of the DNA was carried out independently from each of the two hairs from the hairbrush in order to be able to compare the results. A sample containing a head hair from a known donor and a sample containing no hair served as reagent positive and negative control samples, respectively.

Quantification of Human DNA

Ten percent of each retentate was used to determine the quantity of human DNA by slot blot analysis using the QuantiBlot™ Human DNA Quantitation Kit (Roche Molecular Systems, Inc., Branchburg, NJ), according to the manufacturer's protocol [25].

HLA-DQA1 and Polymarker Analysis

Two ng of DNA from each sample were amplified and typed according to the specifications of the AmpliType™ HLA-DQ alpha and the AmpliType® PM PCR Amplification and Typing Kits [26,27] except that each PCR contained 16 μg Bovine serum albumin (BSA, Sigma Cat. No. A 3350)/100 μL PCR rection mix.

Multiplex Short Tandem Repeat (STR) Analysis

Two ng of DNA from each sample were amplified and typed simultaneously at three polymorphic STR loci (TH01, TPOX and CSF1PO) using a GenePrint™ Triplex PCR Amplification and Typing Kit provided by the Promega Corporation (Madison, WI, USA) according to the specifications of the GenePrint™ STR Systems Technical Manual [17] and Huang et al. [28], except that each PCR contained 16 μg Bovine serum albumin (BSA, Sigma Cat. No. A 3350)/100 μL PCR rection mix. Briefly, the PCR was carried out in 50 μL reaction volumes in a Perkin Elmer 480 thermal cycler. Five μL of PCR product were mixed with 2.5 μL 3 × STR loading dye and 2.5 μL of this mix were loaded onto a denaturing polyacrylamide gel (4%T, 5%C, 31 cm long and 0.4 mm thick) containing 7 M urea and 0.5 × Tris-Borate-EDTA buffer [28]. Electrophoresis was carried out on an SA 32 Electrophoresis Apparatus (Gibco BRL, Gaithersburg, MD, USA). The conditions for electrophoresis were set at a constant power of 40 watts and electrophoresis was carried out at ambient temperature. Electrophoresis was stopped when the xylene cyanol dye migrated 6 cm from the anode (approximately 1 h 15 min).

Allele designations were determined by comparison of the sample fragments with those of the allelic ladders supplied in the kit. Allele designations were made according to recommendations of the DNA commission of the International Society of Forensic Haemogenetics [29].

Sex-Determination

Two ng of DNA from each sample were amplified at the X-Y homologous amelogenin gene, as described previously [30]. The amplification products were visualized on a 3% NuSieve/1% SeaKem agarose gel after ethidium bromide staining. Male and female control DNA was processed in the same manner.

Determination of the Frequency of the Derived Profile

Allele and genotype frequencies for the nine PCR-based genetic markers were determinated in a Swiss Caucasian population sample using the AmpliType™ HLA DQ alpha-, the AmpliType® PM-, and the GenePrint™ PCR Amplification Kits, as previously reported [31,32]. The frequency of the multiple locus DNA profile was estimated using the allele frequencies and the product rule under the assumption of independence [32].

Results and Discussion

From the femur bone samples an average of 12 ng of human DNA per gram of powdered bone and from the head hairs from

TABLE 1—*Typing results and genotype frequencies from the skeletal remains and the reference head hair samples.*

	DQA1	LDLR	GYPA	HBGG	D7S8	GC	TH01	TPOX	CSF1PO	Sex
Human Remains (Bone)	2,4	AB	AB	AA	AB	AB	6,8	8,11	10,11	female
Head Hairs from Hairbrush	2,4	AB	AB	AA	AB	AB	6,8	8,11	10,11	female
Genotype Frequency in Swiss Caucasians	0.095 1 in 10	0.45	0.57	0.24	0.51	0.13 1 in 2500	0.054	0.302 1 in 1.1 million	0.135	0.5

the hairbrush an average of 36 ng of human DNA were recovered. The bone DNA and the reference head hair DNA were successfully amplified and typed at the HLA DQA1, LDLR, GYPA, HBGG, D7S8 and GC loci (Fig. 1), at the STR loci TH01, TPOX and CSF1PO (Fig. 2) and at the X-Y homologous gene amelogenin (one 106 bp band consistent with a female type; data not shown). All control samples typed correctly.

The reference head hair samples showed the same typing results as the femur bone samples from the skeletal human remains (Table 1 and Figs. 1 and 2). Based on Swiss Caucasian population data and the assumption of independence [*32*] this particular DNA profile occurs in approximately 1 in 1.1 million women.

This report is an example of successful confirmation of the identity of skeletal remains by DNA analysis of bone using commercially available multiplex PCR amplification and typing kits. Additionally, a sex determination using the amplification of the X-Y homologous amelogenin gene [*30*] was carried out. Based on the physical examination of the skeleton remains the result supports that this method provides a reliable determination of the sex of skeletal human remains.

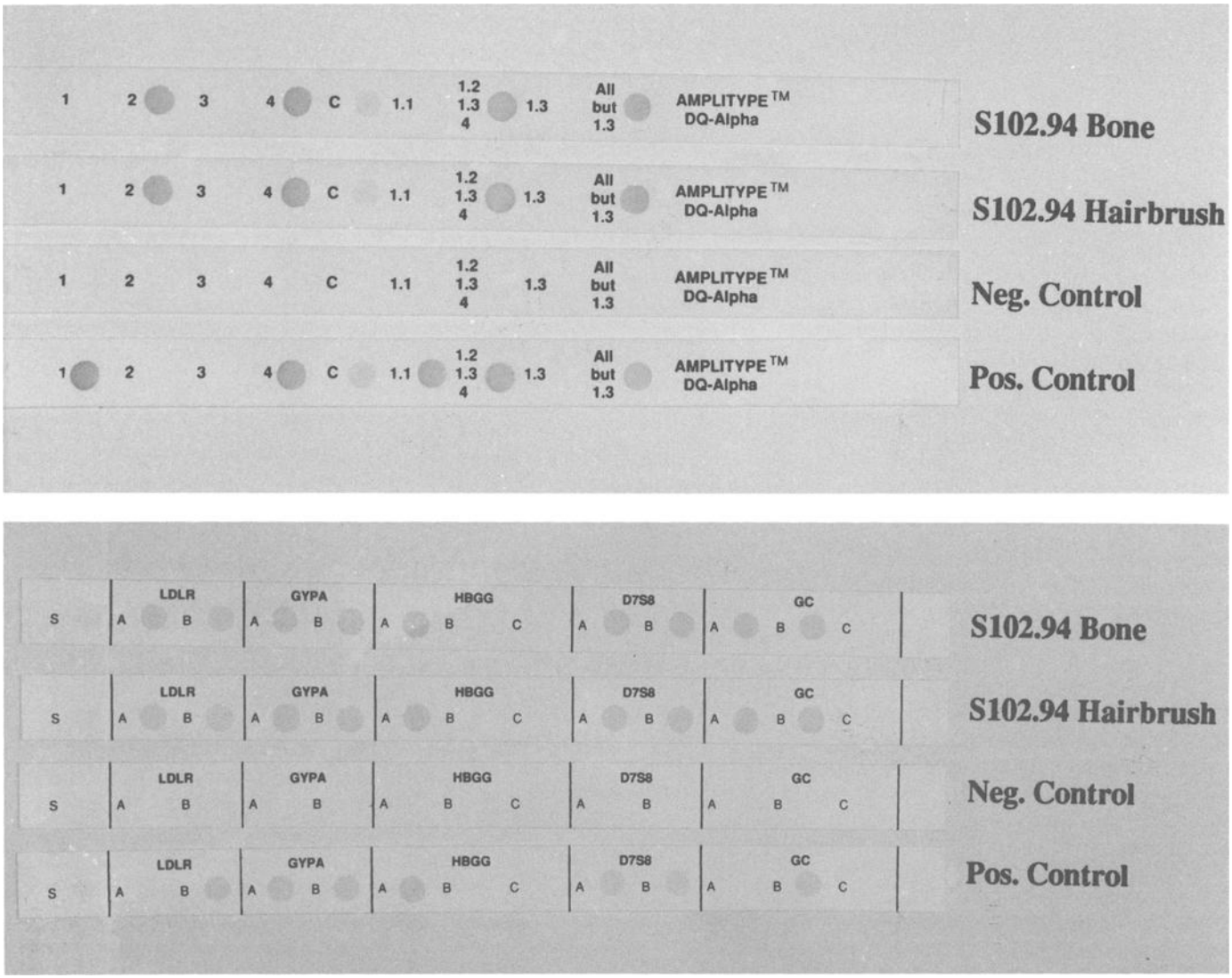

FIG. 1—*HLA DQA1 and Polymarker probe strips comparing DNA from bone from the skeletal human remains and a recovered head hair from a hairbrush. The DNA from the bone shows the same typing results as the DNA from the head hair (HLA DQA1: 2,4; LDLR: AB, GYPA: AB, HBGG: AA; D7S8: AB, and GC: AB).*

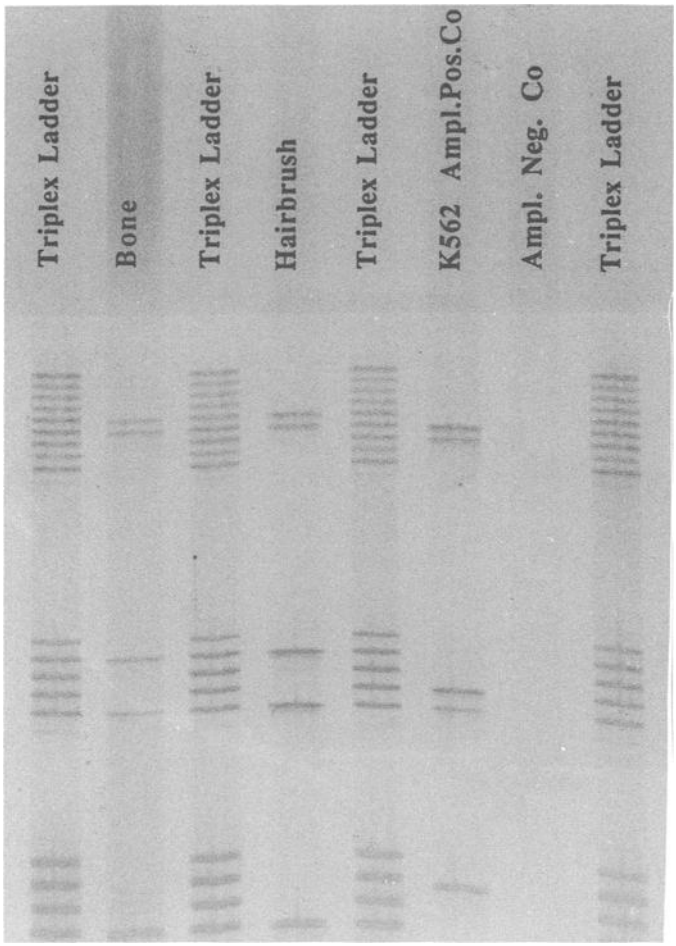

FIG. 2—*Silver-stained DNA profiles of the three simultaneous amplified STR loci TH01, TPOX, and CSF1PO. Thus, each lane represents genetic information for three loci. The DNA from the bone of the skeletal human remains shows the same typing results as the DNA from the recovered head hair from a hairbrush. The STR ladder alleles (# of repeats) from bottom to top are: TH01: 5,6,7,8,9,10,11; TPOX: 8,9,10,11,12; CSF1PO: 7,8,9,10,11,12,13,14,15. The typing results from the bone and head hair are: 6,8 for TH01; 8,11 for TPOX; and 10,11 for CSF1PO; and for the K562 allelic control DNA: 9.3,9.3 for TH01; 8,9 for TPOX; and 9,10 for CSF1PO.*

DNA analysis of human remains can yield reliable results; but caution is recommended regarding the interpretation of results. Hagelberg et al. [*3*] suggest that an apparent exclusion based solely on bone DNA analysis should be evaluated cautiously; it may be impossible to determine whether or not the exclusion is authentic or if it is the result of contaminating material. However, this concern might be more appropriate for ancient bone material analysis. Using our suggested typing approach, a laboratory introduced contamination will likely be apparent as extraneous dots on the PM strips and as additional bands on the Triplex PCR. When possible confirming the typing results with a second independent extraction and typing as well as the use of appropriate positive and negative control samples minimizes such a risk.

The analysis was carried out under the assumption that the hairs in the hair brush, recovered from the missing womans apartment, actually belonged to her. This assumption could be made due to special circumstances in this case, since this woman had no relatives, was very reserved, and was known to have lived alone. Furthermore, all head hairs found on the hairbrush were microscopically undistinguishable from each other, therefore suggesting a single donor. In this special case the identity of the DNA profiles from the bone and the hairs recovered from the hairbrush in the missing womans apartment could be used to confirm identity. Of course, since it is known that hairbrushes are often used by relatives or friends of the owner, unconfirmed hair samples can not be used to confirm an exclusion, since an erroneous interpretation could result.

In conclusion, these two multiplex PCR amplification and typing kits enable the amplification of specific regions of a total of nine genetic loci, thus providing a powerful tool for forensic identity testing. Furthermore, this PCR-based analysis is relatively simple and can be implemented into most application orientated laboratories.

Acknowledgment

We would like to thank Dr. James Schumm at Promega Corporation, Madison, WI, for kindly providing the GenePrint™ Triplex PCR Amplification and Typing Kit.

References

[*1*] Lee, H. C., Pagliaro, E. M., Berka, K. M., Folk, N. L., Anderson, D. T., Ruano, G., Keith, T. P., Phipps, P., Herrin, G. L., Jr., Garner, D. D., and Gaensslen, R. E., "Genetic Markers in Human Bone I: Deoxyribonucleic Acid (DNA) Analysis," *Journal of Forensic Sciences,* Vol. 36, 1991, pp. 320–330.

[*2*] Hochmeister, M. N., Budowle, B., Borer U. V., et al., "Typing of DNA Extracted from Compact Bones from Human Remains," *Journal of Forensic Sciences,* Vol. 36, No. 6, Nov. 1991, pp. 1649–1661.

[*3*] Sajantila, A., Ström, M., Budowle, B., Karhunen, P. J., and Peltonen, L., "The Polymerase Chain Reaction and Post Mortem Forensic Identity Testing: Application of Amplified D1S80 and HLA DQ Alpha Loci to the Identification of Fire Victims," *Forensic Science International,* Vol. 51, 1991, pp. 23–34.

[*4*] Hagelberg, E., Gray, I. C., and Jeffreys, A. J., "Identification of the Skeletal Remains of a Murder Victim by DNA Analysis," *Nature,* Vol. 352, August 1991, pp. 427–429.

[*5*] Jeffreys, A. J., Allen, M. J., Hagelberg, E., and Sonnberg, A., "Identification of the Skeletal Remains of Josef Mengele by DNA Analysis," *Forensic Science International,* Vol. 56, 1992, pp. 65–76.

[*6*] Holland, M. M., Fischer, D. L., Mitchel, L. G., Rodriguez, W. C., Canik, J. J., Merril, C. R., and Weedn, V. W., "Mitochondrial DNA Sequence Analysis of Human Skeletal Remains: Identification of Remains from the Vietnam War," *Journal of Forensic Sciences,* Vol. 38, No. 1, May 1993, pp. 542–553.

[*7*] Fisher, D. L., Holland, M. M., Mitchell, L., Sledzik, P. S., Webb Wilcox, A., Wadhams, M., and Weedn, V. W., "Extraction, Evaluation and Amplification of DNA from Decalcified and Undecalcified United States Civil War Bone," *Journal of Forensic Sciences,* Vol. 38, No. 1, January 1993, pp. 60–68.

[*8*] Higuchi, R., von Beroldingen, C. H., Sensabaugh, G. F., and Erlich, H. A., "DNA Typing from Single Hairs," *Nature,* Vol. 332, 1988, pp. 543–546.

[*9*] Uchihi, R., Tamaki, K., Kojima, T., Yamamoto, T., and Katsumata, Y., "Deoxyribonucleic Acid (DNA) Typing of Human Leukocyte Antigen (HLA)-DQA1 from Single Hairs in Japanese," *Journal of Forensic Sciences,* Vol. 37, 1992, pp. 853–859.

[*10*] Gyllenstein, U. B. and Erlich, H. A., "Generation of Single-Stranded DNA by the Polymerase Chain Reaction and its Application to Direct Sequencing of the HLA-DQ Alpha Locus," *Proceedings of the National Academy of Sciences USA,* Vol. 85, 1988, pp. 7652–7656.

[*11*] Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., and Russel, D. W., "The Human LDL Receptor: A Cysteine-Rich Protein with Multiple Alu Sequences in Its mRNA," *Cell,* Vol. 39, 1984, pp. 27–38.

[*12*] Siebert, P. D. and Fukuda, M., "Molecular Cloning of a Human Glycophorin B cDNA: Nucleotide Sequence and Genomic Relation-

ship to Glycophorin A," *Proceedings of the National Academy of Sciences USA*, Vol. 84, 1987, pp. 6735–6739.

[13] Slightom, J. L., Bechl, A. E., and Smithies, O., "Human Fetal g-Gammaglobin and Alpha-Globin Genes: Complete Nucleotide Sequences Suggest that DNA Can Be Exchanged Between These Duplicate Genes," *Cell*, Vol. 21, 1980, pp. 627–638.

[14] Horn, G. T., Richards, B., Merrill, J. J., and Klinger, K. W., "Characterization and Rapid Diagnostic Analysis of DNA Polymorphisms Closely Linked to the Cystic Fibrosis Locus," *Cell*, Vol. 21, 1980, pp. 627–638.

[15] Yang, F., Brune, J. L., Naylor, S. L., Apples, R. L., and Naberhaus, K. H., "Human Group-Specific Component (Gc) Is a Member of the Albumin Family," *Proceedings of the National Academy of Sciences USA*, Vol. 82, 1985, pp. 7994–7998.

[16] Puers, C., Lins, A. M., Sprecher, C. J., Brinkmann, B., and Schumm, J. W., 1994, Analysis of Polymorphic Short Tandem Repeat Loci Using Well-Characterized Allelic Ladders, In *Proceedings of the Fourth International Symposium on Human Identification 1993*, Promega Corporation, Madison, WI, USA

[17] Promega, Technical Manual, "GenePrint™ STR Systems," 1994, Promega Corporation, Madison, WI, USA.

[18] Edwards, A., Civitello, A., Hammond, H. A., and Caskey, C. T., "DNA Typing and Genetic Mapping with Trimeric and Tetrameric Tandem Repeats," *American Journal of Human Genetics*, Vol. 49, 1991, pp. 746–756.

[19] Edwards, A., Hammond, H., Jin, I., Caskey, C. T., and Chakraborty, R., "Genetic Variation at Five Trimeric and Tetrameric Repeat Loci in Four Human Population Groups," *Genomics*, Vol. 12, 1992, pp. 241–253.

[20] Polymeropoulos, M. H., Xiao, H., Rath, D. S., and Merril, C. R., "Tetranucleotide Repeat Polymorphism at the Human Tyrosine Hydrolase Gene (TH)," *Nucleic Acids Research*, Vol. 19, 1991, p. 3753.

[21] Anker, R., Steinbrueck, T., and Donnis-Keller, H., "Tetranucleotide Repeat Polymorphism at the Human Thyroid Peroxidase (hTPO) Locus," *Human Molecular Genetics*, Vol. 1, 1992, p. 137.

[22] Hammond, H. A., Jin, L., Zhong, Y., Caskey, C. T., and Chakraborty, R., "Evaluation of Thirteen STR Loci for Use in Personal Identification Applications," *American Journal of Human Genetics*, (in press).

[23] Puers, C., Hammond, H. A., Jin, L., Caskey, C. T., and Schumm, J. W., "Identification of Repeat Sequence Heterogeneity at the Polymorphic Short Tandem Repeat Locus HUMTH01 [AATG]n and Reassignment of Alleles in Population Analysis by Using a Locus-Specific Allelic Ladder," *American Journal of Human Genetics*, Vol. 53, 1993, pp. 953–958.

[24] Amicon, Centricon™ Microconcentrators User Guide, Amicon Division, W.R. Grace & Co.- Conn., Danvers, MA, 1989.

[25] Perkin Elmer, "Quantiblot™ Human DNA Quantification Kit User Guide," 1993, Roche Molecular Systems, Inc., Branchburg, NJ, USA.

[26] AmpliType™ HLA DQ alpha Forensic DNA Amplification and Typing Kit User Guide, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA, 1992.

[27] AmpliType™ PM PCR Amplification and Typing Kit User Guide, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA, 1994.

[28] Huang, N. E., Schumm, J., and Budowle, B., "Chinese Population Data on Three Tetrameric Short Tandem Repeat Loci—HUMTH01, TPOX, and CSF1PO-Devised Using Multiplex PCR and Manual Typing," *Forensic Science International*, (in press).

[29] Recommendations of the DNA Commission of the International Society of Forensic Haemogenetics 1994, *International Journal of Legal Medicine*, (in press).

[30] Sullivan, K. M., Mannucci, A., Kimpzon, C. P., and Gill, P., "A Rapid and Quantitative DNA Sex Test: Fluorescence-Based PCR Analysis of X-Y Homologous Gene Amelogenin," *BioTechniques*, Vol. 15, No. 4, 1993, pp. 636–641.

[31] Hochmeister, M. N., Budowle, B., Borer, U. V., and Dirnhofer, R., "Swiss Population Data on the Loci HLA-DQ alpha, LDLR, GYPA, HBGG, D7S8, GC, and D1S80," *Forensic Science International*, 1994, (in press).

[32] Hochmeister, M. N., Budowle, B., Borer U. V., and Dirnhofer, R., "Swiss Population Data on Three Tetrameric Short Tandem Repeat Loci—HUMTH01, TPOX, and CSF1PO—Derived using the GenePrint™ Triplex PCR Amplification and Typing Kit," *International Journal of Legal Medicine*, (accepted Dec. 1994).

Address requests for reprints or additional information to
Manfred N. Hochmeister, M.D.
Institute of Legal Medicine
Buehlstrasse 20, CH-3012 Berne, Switzerland
Fax: 41-31-631 38 33