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"Sexing" Deoxyribonucleic Acid (DNA) on DNA Fingerprint Gel: An Internal Control for DNA Fingerprint Evidence

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ABSTRACT: Deoxyribonucleic acid (DNA) isolated from male and female fresh blood samples was processed exactly as for routine DNA fingerprint analysis; that is, the DNA was digested with particular restriction endonucleases and fractionated by agarose gel electrophoresis. Ultraviolet (UV) visualization of ethidium-bromide (EtBr)-stained gels revealed a sex-specific banding pattern, which depended only on the restriction enzyme used. By means of this test, which is based on direct detection of particular sex-specific restriction fragments in human DNA digests, the authors succeeded in determining the sex of DNA obtained from biological specimens recovered as criminal evidence in rape cases. The data obtained demonstrate that direct sexing of DNA on DNA fingerprint gel appears to be useful as an intermediate control step in DNA fingerprinting analysis used for the purpose of assailant identification.

KEYWORDS: criminalistics, human identification, Deoxyribonucleic acid (DNA), sex determination, DNA fingerprints

The determination of the sex of biological materials recovered as criminal evidence is an important matter in forensic investigation. The routine tests for sex diagnosis are based on detection of sex-specific chromatin structures in interphase cell nuclei. However, these cytological methods have certain limitations. The limitations include the rather unspecified morphological criteria for estimation of sex-specific features at the chromatin level, the optional presence of true X and Y chromocenters in normal female and male cell nuclei, and the occasional presence of structures resembling chromatin that do not correlate with the sex of the individual.

The analysis of biological materials recovered as evidence at the molecular level considerably expands the means of discrimination. Several methods of "sexing" human deoxyribonucleic acid (DNA) were developed focusing on the detection of Y-specific chromosomal sequences [*1,2,3*]. Some of these methods were found to be of use in sexing DNA for forensic science purposes when incompletely degraded DNA was available [*4*]. Probes specific for repeat sequences derived from the heterochromatin region of the long

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arm of the Y chromosome [5] were used to develop the first screening tests for sex diagnosis through dot blot hybridization of unrestricted genomic DNA [6] or in situ hybridization [1]. Recently, a method has been presented in which the polymerase chain reaction (PCR) was used to detect the presence of sequences of this region [7]. In addition, the alphoid repetitive DNA sequences, which exhibit the properties of significant chromosome specificity, were offered as a probe for a sex diagnosis procedure for blotted DNA using PCR [3,4].

However, all these methods of sexing DNA, while having very high diagnostic power and sensitivity, apparently demand some special requirements, for example, specific primers or hybridization probes, or both. The authors of this paper propose a simple, rapid, and reliable test for sex diagnosis, based on direct detection of sex-specific restrictase fragments in total human DNA digests through ultraviolet (UV) visualization of ethidium-bromide (EtBr)-stained agarose gels. This method may be useful in screening of newborns and in research on the analysis of biological evidence. In particular, the DNA fingerprint technique [8–10] certainly needs such a test as an intermediate control step in cases of assailant identification from biological materials of mixed-sex origin. In this article, we demonstrate some evidence in proof of our proposition.

Materials and Methods

Isolation of DNA

High-molecular-weight DNA was isolated from 5 mL of peripheral blood by standard procedures [11] for samples sent to our laboratory for paternity determinations. Purified sperm DNA was prepared using a procedure described by Gill et al. [10]. Semen-contaminated vaginal swabs and mixed semen/vaginal fluid/blood stains recovered as criminal evidence were processed using the differential lysis methods described by Gill et al. [10] and Guisti et al. [12] with modifications described elsewhere [13,14]. The main steps were the following: Fabric containing dried body fluids and cotton vaginal swabs were cut into small pieces. The materials were then soaked with 10% aqueous ammonia with mild agitation for 36 h at 4°C. The solution was compressed through nylon mesh to prevent fabric contamination and then centrifuged for 10 min at 1500 × *g*, at 4°C, to pellet the suspended cells. The pellet was washed twice with TNE buffer [10m*M* tris(hydroxymethyl)aminomethane (Tris)/hydrochloric acid (HCl), 10m*M* sodium ethylenediaminetetraacetic acid (Na_2EDTA), and 100m*M* sodium chloride, at pH 7.6], resuspended in the same buffer containing 2% Sarkosyl (Serva), and centrifuged at 3000 × *g*, at 4°C. The reformed pellets were then suspended in TNE buffer containing 2% sodium dodecyl sulfate (SDS) and 50 μg of proteinase K per millilitre (Merck Co.) and incubated for 4 h at 65°C. The sperm nuclei were pelleted as described above and then lysed with the same SDS/proteinase K/TNE mixture in the presence of 3% β-mercaptoethanol at 37°C overnight. The sample solution was extracted three times with a phenol/chloroform/β-mercaptoethanol (49:49:1) mixture, then concentrated tenfold with *n*-butanol and dialyzed against TE buffer [10m*M* Tris-HCl and 1m*M* ethylenediaminetetraacetic acid (EDTA), at pH 7.5]. DNA was precipitated with 2.5 volumes of ethanol, after adding ammonium acetate up to a final concentration of 2.5*M*, washed with cold 75% ethanol, and dissolved in a small volume of TE buffer.

DNA Digestion and Electrophoresis of DNA Fragments

DNA samples (5 μg) were digested using a threefold excess of the appropriate restriction endonuclease—*Mva*I, *Alu*I, *Bsp*RI, *Hae*III, or *Eco*RII (Fermentas, U.S.S.R.)—at 37°C for 5 h and recovered by ethanol precipitation. The resulting DNA fragments were

electrophoretically separated on 0.8% horizontal agarose gels, 0.5 cm thick by 20 cm long, using TAE running buffer [40m*M* Tris-acetate and 2m*M* EDTA, at pH 7.6). Electrophoresis was carried out at 2 V/cm for 24 h. After electrophoresis, the gel was stained with ethidium bromide (EtBr) and photographed using an MP4 Polaroid Land Camera and a TS40 UV transilluminator (UV Products) as a light source.

DNA Fingerprinting Procedure

DNA fingerprint analysis using M13-phage DNA as a probe was carried out as previously described [*14,15*]. The procedure involved DNA transfer by blotting to a Schleicher and Shuell nitrocellulose membrane filter (BA85), Phosphorus-32 (^{32}P) labeling of the single-stranded M13 DNA to a specific activity of 10^9 cpm/μg of DNA was carried out using multiprime labeling kit (Amersham) hybridization and autoradiography. The hybridization solution contained 4× standard saline citrate (SSC), 0.1% SDS, and 10× Denhardt's solution. Incubation went on for 16 to 20 h at 59°C. After the hybridization, the filters were washed in 1× SSC and 0.1% SDS at 59°C, and exposed to X-ray film.

Results and Discussion

Determination of the Sex of Origin of DNA on DNA Fingerprint Gels

We examined 50 male and 50 female blood DNA samples—a total of 100 samples—using restriction endonucleases *Alu*I, *Mva*I, and *BspR*I, which, as we have recognized, are suitable and in most cases preferable for DNA fingerprinting of humans using M13-phage DNA as a probe [*14,15*]. These restrictases produce a wide spectrum of hybridizable bands, showing extensive variability, so the whole hybridization pattern in each case has a highly individualizing capacity.

In order to obtain clear restriction patterns composed of well-separated fragments, electrophoresis was carried out for at least 24 h at 2 V/cm using a large apparatus, so that the length of the gel corresponding to molecular sizes between 1.0 and 23 kilobases (kb) was about 15 cm. This is our standard procedure for obtaining the Southern-blot profile produced by the M13 minisatellite probe.

Each male and female DNA obtained from blood samples was digested with the corresponding enzyme and loaded on the three gels according to the enzyme used. An example of the results observed in this type of comparison is shown in Fig. 1 (here and further on we present negative photographs of the EtBr-stained agarose gels to maximize visual contrast of the picture).

The restriction patterns produced by the restrictases used are typical for DNA of high complexity, cleaved with restriction enzymes specific for four and relaxed five-nucleotide sequences. The density distribution along the lanes quantitatively reflects the mass distribution of the restriction fragments of different sizes. This is the normal distribution, and for nucleotide sequences of high complexity, it depends only on the restriction enzyme involved.

Usually, there is a smooth, continuous density distribution along the lane with a maximum of saturation at 1 to 4 kb. In this zone, one can see faint discrete bands ordered equally in male and female DNA digests. These bands, which obviously originated from some reiterated genomic elements, consist as tandem arrays of repeat sequences, such as the major satellites and satellite-like sequences [*16–18*]. This banding profile differs in different organisms, being species specific (data not shown), which thus makes it possible to discriminate DNA of different origins.

In this study, we emphasize the bands which exhibit the properties of sex specificity (these are indicated by arrows). These fragments are present only in male DNA digests,

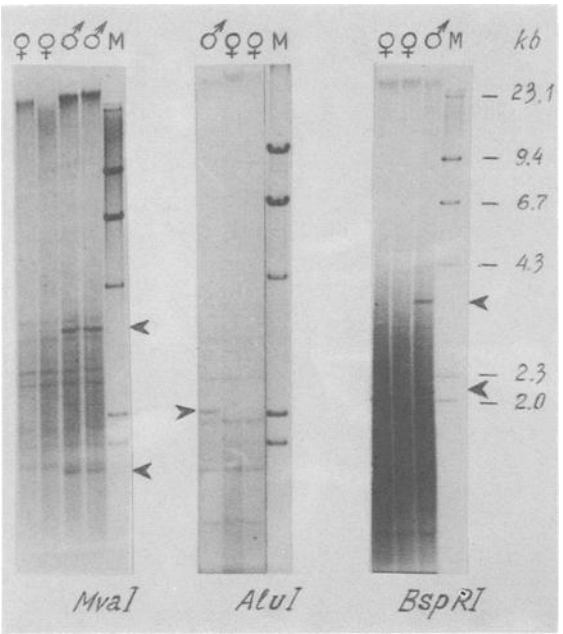

FIG. 1—*UV pattern of electrophoretically separated human blood DNA digests (negative photograph). Male and female DNA samples (designated by symbols) were digested with the appropriate restrictase and loaded onto 0.8% horizontal agarose gels. Each lane contained 5 μg of DNA. Arrowheads (in parentheses) indicate clear male-specific bands.* M *indicates* Hind*III-digested* λ *DNA used as a molecular size marker.*

being absent from female DNA, and thus appear to be the markers of sex heteromorphism of human DNA. Significantly, the sex-specific banding pattern is dependent on the restriction enzyme used.

There are two readily detectable male-specific bands in *Mva*I digests (1.7 and 3.5 kb) and in *Bsp*RI digests (2.1 and 3.5 kb), while *Alu*I reveals only one band of 2.3 kb.

No enzyme-dependent methylation differences were observed when comparing the digestion patterns of male blood DNA generated by the isoschizomeric restriction enzymes *Mva*I and *Eco*RII, which are respectively resistant and sensitive to 5-methylcytosine. In addition, the absence of tissue-dependent methylation differences in male-specific banding was demonstrated using the same pair of isoschizomeric restriction enzymes for sperm DNA and total blood DNA (Fig. 2), taking into account the higher level of methylation differences between the germ-line and somatic cells than between different somatic cells [*19*]. We should say here that we have not performed any special investigations concerning the nature of these male-specific fragments, but one can suppose that they have the same origin as the satellite-III-associated, 3.5-kb, Y-specific *Hae*III fragment previously described [*20*]. Therefore, these fragments appear to be unique to humans, being absent in similar digests of nonhuman DNA.

A Practical Case Report

We have demonstrated a very simple sex diagnosis test in which fresh blood and semen samples were used as a source of DNA for the restriction enzyme digests. Our subsequent task was to determine the potential range of applications of the method described for the examination of biological materials recovered as criminal evidence.

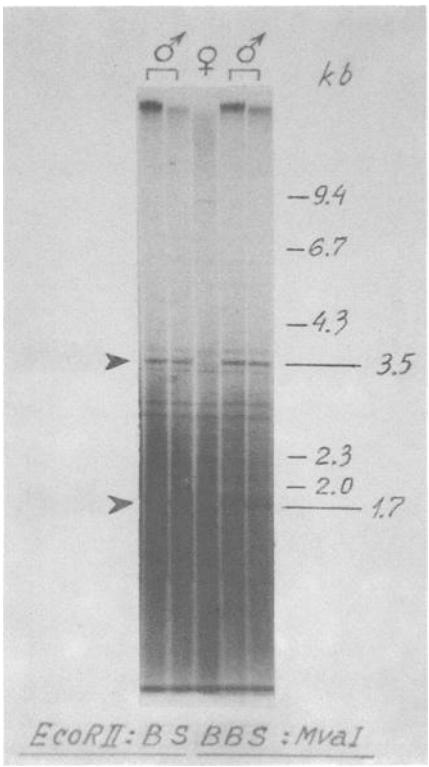

FIG. 2—*UV pattern of electrophoretically separated human blood* (B) *and semen* (S) *DNA digests (negative photograph). DNA samples obtained from blood* (B) *and semen* (S) *of a male individual were digested in parallel with isoschizomeric restrictases* MvaI *and* EcoR*II, which are respectively resistant and sensitive to 5-methylcytosin. Female blood DNA (designated by symbol) was digested with* MvaI. *Arrowheads indicate male-specific bands.*

The results presented in Fig. 3 illustrate DNA fingerprint analysis performed in our laboratory for assailant identification in three particular sexual assault cases. Summarizing these cases, we were requested to examine semen from (1) a vaginal cotton swab, (2) a stain deposited on the woman's cotton underwear, and (3) an agglutinated semen plug removed from the cervix, in addition to the blood of the suspected men. Because in all of these practical cases the semen was obviously contaminated with vaginal materials, we used a differential lysis procedure for obtaining DNA derived from only the sperm of the semen (see Materials and Methods).

Figure 3*a*, left panel, is a negative photograph of the EtBr-stained agarose gel before blotting. The three lanes are *Mva*I-digested DNA taken from the rape victim's vaginal swab (Lane 1), from the blood of the suspect (Lane 2), and from the victim's muscle (1 day postmortem) (Lane 3). One can see a typical faint sex-unspecific *Mva*I banding pattern in all the DNA samples, as well as distinct male-specific bands in Lanes 1 and 2. The right panel shows the DNA fingerprint pattern obtained from this gel. One can see that the total of more than 25 clear bands longer than 2.0 kb is completely identical to that of the blood from the suspected man. This indicates that the semen is the suspected man's.

Figure 3*b*, right panel, shows two DNA fingerprints generated by *Mva*I-digested DNA from a dried stain on the rape victim's undergarments (Lane 5) and from the blood of

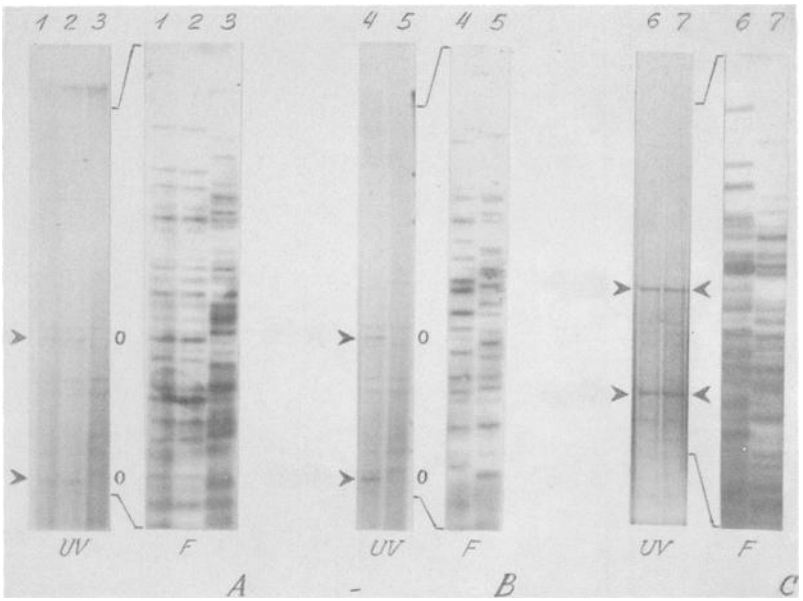

FIG. 3—*UV patterns of electrophoretically separated human DNA (negative photographs) and corresponding DNA fingerprints* (F) *generated by* ^{32}*P-labeled M13 DNA probe, providing evidence for expert evaluation in three particular cases (Cases A, B, and C) of forcible rape:* (a) *Case A:* Mva*I-digested DNA from the rape victim's vaginal swab (Lane 1), from the blood of the alleged rapist (Lane 2), and from the victim's muscle (Lane 3). The pattern obtained from the semen-contaminated swab is the same as that obtained from the blood of the suspected man. Arrowheads indicate male-specific bands in the DNA recovered from evidence.* (b) *Case B:* Mva*I-digested DNA from a dried stain on the rape victim's underwear (Lane 5) and from the blood of the suspected man (Lane 4). The two DNA fingerprint profiles do not match. Meanwhile, considering the absence of male-specific bands* (0), *the DNA recovered from the evidence proves to be female DNA. Therefore, in this particular case, the DNA fingerprinting test cannot provide any specific evidence to support the suspect's innocence.* (c) *Case C:* BspR*I-digested DNA from the agglutinated semen plug removed from the victim's cervix (Lane 7) and from the blood of the alleged rapist (Lane 6). The two DNA fingerprints do not match and male-specific banding in the DNA recovered from the evidence indicates its male origin, thus leading to a conclusion excluding the suspect from the crime.*

the alleged rapist (Lane 4). These profiles do not match and, ordinarily, this could mean the exclusion of a man charged with this criminal case. However, the UV visualization of the gel before blotting (left panel, negative photograph) has provided evidence that the DNA presented on Lane 5 appears to be female DNA, since both male-specific *Mva*I bands were absent from it. Considering the presence of typical nonspecific autosomal restriction fragments in this DNA digest, we could conclude that the absence of sex-specific bands was not caused by DNA degradation.

One should realize, that "female" most probably means the "overall" sex attribute of this particular DNA preparation, however. As we have recognized through our practice, in some cases, probably because of certain environmental conditions, vaginal cellular debris and blood cells, as well as sperm of the semen deposited on clothing left on the scene of crime, may become impervious to standard lysis procedures, thus making a two-step or differential lysis inefficient in removing female cells contaminating the sample. At the same time, it results in poor DNA extraction from recovered spermatozoa. Therefore, one could suppose that male DNA was present, but at levels too low to be seen on the gel. The point is, however, that a mass excess of female DNA in a thus mixed DNA preparation would inevitably mask the male fingerprint. Unfortunately, in this case

the woman's tissues were not available for examination, but we believe that Lane 5 does represents the DNA fingerprint of the victim.

The logical inference was that, in this particular case, the DNA fingerprinting test could not provide any specific evidence to support a suspect's guilt or innocence. It is noteworthy that later the suspected man was convicted on the strength of circumstantial and expert evidence. So, false exclusion by the DNA fingerprinting test might occur in a case of "blind" examination, that is, without sexing of the DNA obtained from a biological specimen retrieved from the scene of a crime.

An example of true exclusion is illustrated by Fig. 3*c*. Considering the presence of both male-specific *BspR*I fragments in DNA recovered from evidence (agglutinated semen removed from the cervix 12 h postcoitus) (Lane 6), one can be assured that the negative match observed between DNA fingerprints generated by this DNA and those by DNA in the suspect's blood specimen (Lane 7) argues in favor of the suspect's innocence.

Conclusions

These experiments demonstrate that DNA of a quality and quantity sufficient for determination of the sex of its origin by the method described can be recovered from at least some biological specimen retrieved from a victim of forcible rape. In general, DNA preparations acceptable for DNA fingerprint analysis are also suitable for direct sex diagnosis on DNA fingerprint gel. (We usually apply about 5 μg of DNA per lane.) Therefore, sexing DNA by means of restriction endonuclease digestion/agarose gel separation appears to be a simple intermediate control step in the DNA fingerprinting procedure used for purposes of identification. We hope that such a test will prove to be a useful addition to current sex determination techniques.

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References

[*1*] Lay, Y.-F. and Schonberg, S., "A Male-Specific DNA Probe Detects Heterochromatin Sequences in a Familial Yq-Chromosome," *American Journal of Human Genetics*, Vol. 36, 1984, pp. 1394–1396.

[*2*] Disteche, C., Luthy, I., Haslam, D. B., and Haar, D., "Prenatal Identification of a Deleted Y Chromosome by Cytogenetics and a Y-Specific Repetitive DNA Probe," *Human Genetics*, Vol. 67, 1984, pp. 222–224.

[*3*] Stalvey, J. R. D. and Erickson, R. P., "An Improved Method for Detecting Y Chromosomal DNA," *Human Genetics*, Vol. 76, 1987, pp. 240–243.

[*4*] Witt, M. and Erickson, P., "A Rapid Method for Detection of Y-Chromosomal DNA from Dried Blood Specimens by the Polymerase Chain Reaction," *Human Genetics*, Vol. 82, 1987, pp. 271–274.

[*5*] Cooke, H. J., "Repeated Sequences Specific to Human Males," *Nature*, Vol. 262, 1976, pp. 182–186.

[*6*] Lau, Y.-F., "Detection of Y-Specific Repeat Sequences in Normal and Variant Human Chromosomes Using In Situ Hybridization with Biotinylated Probes," *Cytogenetics and Cell Genetics*, Vol. 39, 1985, pp. 184–187.

[*7*] Kogan, S. C., Doherty, M., and Gitschier, J., "An Improved Method for Prenatal Diagnosis of Genetic Diseases by Analysis of Amplified DNA Sequences," *New England Journal of Medicine*, Vol. 317, 1987, pp. 985–990.

[*8*] Jeffreys, A. J., Wilson, V., and Thein, S. L., "Hypervariable 'Minisatellite' Regions in Human DNA," *Nature*, Vol. 314, 1985, pp. 67–73.

[9] Jeffreys, A. L., Wilson, V., and Thein, S. L., "Individual Specific 'Fingerprints' of Human DNA," *Nature*, Vol. 316, 1985, pp. 76–79.
[10] Gill, P., Jeffreys, A. J., and Werret, D. J., "Forensic Application of DNA Fingerprints," *Nature*, Vol. 318, 1985, pp. 577–579.
[11] Mathew, C. G. P., "The Isolation of High Molecular Weight Eukaryotic DNA," *Methods in Molecular Biology*, Vol. 2, J. M. Walker, Ed., Humana Press, Clifton, NJ, 1984, pp. 31–34.
[12] Guisti, A., Baird, M., Pasquale, S., Balazs, I., and Glassberg, J., "Application of Deoxyribonucleic Acid (DNA) Polymorphisms to the Analysis of DNA Recovered from Sperm," *Journal of Forensic Sciences*, Vol. 31, No. 2, April 1986, pp. 409–417.
[13] Boldescu, N. G., Gyske, L. I., Gurtovaya, S. V., and Ivanov, P. L., "Primary Evaluation of Biological Specimens Recovered as Criminal Evidence in Rape Cases in Terms of Their Accessibility for DNA Fingerprinting," *Sudebno-meditsinskaya Ekspertiza* (*Forensic Medical Examinations*), (in Russian), Vol. 1, 1991.
[14] Ivanov, P. L., Gurtovaya, S. V., Plaksin, V. O., Verbovaya, L. V., Ryskov, A. P., and Boldescu, N. G., " 'Genome Dactyloscopy' Using M13 DNA as a Probe: Expert Evaluation of Material Evidence and Personal Identification," *Sudebno-meditsinskaya Ekspertiza* (*Forensic Medical Examinations*), Vol. 4, 1989, pp. 39–42.
[15] Ryskov, A. P., Jincharadze, A. G., Prosnyak, M. I., Ivanov, P. L., and Limborska, S. A., "M13 Phage DNA as a Universal Marker for DNA Fingerprinting of Animals, Plants and Microorganisms," *F.E.B.S. Letters*, Vol. 233, No. 2, 1988, pp. 388–392.
[16] Singer, M. F., "Highly Repeated Sequences in Mammalian Genomes," *International Review of Cytology*, Vol. 76, 1982, pp. 67–112.
[17] Frommer, M., Posser, J., Tkachuk, D., Reisner, A. H., and Vincent, P. C., "Simple Repeated Sequences in Human Satellite DNA," *Nucleic Acids Research*, Vol. 10, 1982, pp. 547–563.
[18] Waye, J. S. and Willard, H. F., "Nucleotide Sequence Heterogeneity of Alpha Satellite Repetitive DNA: A Survey of Alphoid Sequences from Different Human Chromosomes," *Nucleic Acids Research*, Vol. 15, 1987, pp. 7549–7580.
[19] Berneman, Z. N., Raeymakers, P., Vanhoof, G., Van Bockstaele, D., Ramael, M., Peetermans, M. E., Vandenberghe, A., and Van Broeckhoven, C., "DNA Fingerprints Revealing Common and Divergent Human DNA Methylation Patterns," *F.E.B.S. Letters*, Vol. 255, 1989, pp. 226–230.
[20] Cooke, H. J., Fantes, J., and Green, D., "Structure and Evolution of Human Y Chromosome DNA," *Differentiation*, Vol. 23 (Supplement), 1983, pp. S48–S55.

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