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Restriction Fragment Length Polymorphism and Polymerase Chain Reaction-HLA DQα Analysis of Casework Urine Specimens

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ABSTRACT: DNA was isolated from casework urine samples previously submitted for toxicological analysis. The quality and quantity of DNA isolated was determined by spectrofluorometry and agarose yield gel electrophoresis. Hae III restricted samples were then resolved by analytical agarose gel electrophoresis, transferred to a membrane by Southern blotting and hybridized with a chemiluminescently-labelled (D2S44) probe. The DNA fragment banding patterns were indistinguishable from the DNA banding patterns of blood specimens collected from the same donor. Only 5 of 20 samples yielded banding patterns and the banding intensity relative to background was low. Genomic DNA was also obtained from casework samples by Chelex extraction, amplified by polymerase chain reaction (PCR) and then genotyped for human leucocyte antigen (HLA) DQα. Of 20 specimens, 13 (65%) were typed correctly producing identical results for urine and blood specimens obtained from the same donor. Aging studies of casework samples and normal samples (from a non-drug using population) were also conducted with PCR-HLA DQα analysis. Results of these studies indicate that amplification by PCR was more likely to produce positive results. Based on these findings, we conclude that PCR-initiated analysis is more suitable than RFLP analysis for individualization of urine samples.

KEYWORDS: toxicology, DNA, RFLP, polymerase chain reaction, HLA DQα, urine

The extraordinary level of illicit drug abuse in today's society has led to the general implementation of, and reliance upon, urine drug testing in the pursuit of a drug-free environment. As a result, toxicology laboratories may handle hundreds, if not thousands, of samples daily. The U.S. Armed Services conduct approximately 2.7 million urine drug tests yearly. Approximately 0.05% of these tests (1350) result in legal challenge and trial based on identification of the specimen donor [*1*]. Because the possibility of human error, such as sample mixup, will always exist, this study was undertaken to determine if a method of identity testing could be applied to urine samples routinely submitted for toxicological analysis. A suitable individualizing method would have to be reliable, sensitive enough to

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produce positive results on most urine specimens, economical and forensically informative, for example, produce either an exclusion or statistically significant inclusion if a match is found between the specimen and a suspect.

Urine generally contains erythrocytes, leucocytes and epithelial cells [2] as well as other components of forensic significance. Spermatozoa can also sometimes be found in urine specimens obtained from males and post-coital urine samples from females [3]. Soluble glycoprotein A, B, and H blood group antigens can generally be found in the urine of secretors [4,5] and Lewis blood group substances can be detected regardless of secretor status [5]. Proteins such as albumin, fibrin, proteases, myoglobin, and mucoproteins are also occasionally found in urine samples [6]. Normal urine also contains many metabolic waste products such as urea, endogenous molecules including antibiotics and vitamins and metabolic by-products. Unfortunately, the urinary concentration of these substances is extremely variable, depending on many factors including diet, physical activity, and health status and may therefore limit their forensic value.

Traditionally, individualization of urine samples has been attempted using well known serological methods. For example, ABO blood group determination from urine and/or urine stains of secretors is often accomplished by absorption-inhibition testing [4,5,7]. A two-dimensional absorption-inhibition procedure has also been applied to the individualization of normal urine samples [8].

In a study to determine the feasibility of conducting DNA fingerprinting on urine specimens, Roewer et al. [9] reported that at least 200 mL of urine was required to obtain useful results. In a later report, Brinkmann et al. described testing of urine samples with volumes of 10, 1 and 0.1 mL. Samples were also subjected to a temperature/aging study using restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AmpFLP) and short tandem repeat locus (STR) analyses [10]. RFLP analysis using a D2S44/Hinf I system was successful in 1 out of 3 samples that were 2 days old but failed to produce results in samples stored for a period of 2–5 weeks. The AmpFLP analyses were performed on both the COL2A1 locus as well as the 3′ end VNTR of the ApoB locus and were successful in producing results in 62% and 50% of 2 day old samples, respectively, but failed to provide results for samples stored for 5 weeks. Samples were stored at 4°C without preservation. STR analyses of SE33 and HUMTHO1 loci were positive in all samples in all cases. Nevertheless, the apparent instability of high molecular weight DNA in samples such as these suggests that one or more substances within urine may have an adverse effect on the integrity of native DNA [11]. There is some evidence for the presence of a PCR inhibitor in urine [1,12] which may explain the relatively small number of successful amplification results. Various serological and DNA methods of identification of urine specimen donors are described in a review article by Holland et al. [1].

Materials and Methods

Urine samples for this study were chosen at random from among samples submitted to NDA Laboratories, Farmingdale, NY for toxicological analysis. A matching blood sample was also obtained from each donor by venipuncture. Urine sample volume was variable ranging from 15 to 50 mL. Samples were refrigerated and stored for no longer than 5 days prior to analysis. Information regarding volume and storage period for each specimen was recorded. Urine specimens were characterized by the use of the Chemstrip 10 Urinalysis Strip (Boehringer Mannheim Diagnostics, Indianapolis, IN) which provides information regarding specific gravity, pH, presence of leucocytes, nitrites, protein, glucose, ketones, urobilinogen, bilirubin and hemoglobin content of urine.

RFLP Analysis—Casework Samples

Two groups of urine samples, 15 mL each, were collected and subjected to RFLP analysis within a one day period. Batch 1 consisted of 20 samples identified as 125u-144u (the

code-suffix "u" and "b" denote urine and blood specimens, respectively). Batch 2 consisted of 12 samples labeled as 145u–156u. After thoroughly mixing the samples, each was transferred to a centrifuge tube and centrifuged at 2000 × g for 5 min in a Beckman GS-6R refrigerated centrifuge. For each sample the supernatant was decanted and the pellet transferred to a 1.5 mL Sarstedt tube with a pasteur pipette. Restriction fragment length polymorphism analysis was performed according to the protocol developed by the FBI [*13*] and subsequently modified [*14,15*]. The samples were washed twice with 1X SSC (20X SSC = NaCl 173.3g + 88.2g $Na_3Citrate \cdot 2H_2O$, pH 7.5, in 1.0 liter of distilled water) and the washed material was then digested with Proteinase K. The digestion mixture consisted of 400 μL 0.2 M sodium acetate, 30 μL 10% sodium dodecyl sulfate (SDS), and 15 μL Proteinase K (25 mg/mL in distilled H_2O).

Following digestion at 56°C for 3 h, DNA was extracted using phenol/chloroform/isoamyl alcohol. The DNA was then resolubilized in 200 μL of TE^{-4} buffer (10 mM tris base, pH 7.5, 0.1 mM disodium EDTA) at 56°C overnight. 10 μL of sample were then electrophoresed on a 1% agarose yield gel to determine the quality and quantity of isolated DNA.

The total quantity of DNA isolated in each sample was then determined by spectrofluorometry using the Hoefer TKO 100 dedicated fluorometer and Hoechst 33258 dye [*16*]. Since bacterial contamination of urine is common [2], it was necessary to determine if the isolated DNA was of human origin. Therefore 0.5 μL of each sample was subjected to slot-blot analysis on the BRL Life Technologies Convertible Filtration Manifold System, using BioRad Zeta Probe Membrane, and the Lifecodes Quick-Light Nano Blot detection system. This system uses a human repeat probe (SLI-332) to specifically detect and quantitate human DNA [*17*]. Samples yielding sufficient high molecular weight DNA (as determined by yield gel analysis) were then restricted with Hae III as described in FBI Procedures (1989) [*13*]. The restriction reaction mixture was incubated at 37°C overnight. Samples were reprecipitated and suspended in 16 μL TE^{-4} buffer. A test gel was then run to assess the restriction process.

If restriction was complete, samples were then electrophoresed on a 1% agarose analytical gel overnight and transferred to a BioRad Zeta Probe membrane by Southern blotting. DNA fragments were covalently linked to the nylon membrane by baking for 1 h at 80°C in a vacuum oven. Blood samples were prepared according to the FBI's *Procedures for the Detection of Restriction Fragment Length Polymorphism in Human DNA* [*13–15*]. Membranes were then hybridized with the Lifecodes Quick-Light (chemiluminescent) YNH24 probe which is specific for the D2S44 locus. Chemiluminescent detection is sensitive and specific while its use eliminates the hazards and disposal problems associated with radioactively labelled probes [*18*]. Hybridization was performed in roller tubes in a hybridization oven (Hybaid Mini Oven MK2). The chemiluminescence substrate Lumi Phos 480 was applied to the membranes which were then sealed in development pouches. The hybridized membranes were then placed into an X-ray film cassette adjacent to a sheet of Kodak X-omat film. After development the resulting lumigrams were analyzed. Urine samples were compared with corresponding blood controls from the same donor.

Normal Urine Samples

In order to determine DNA stability over time in non-casework samples, specimens were collected randomly from normal (drug-free) volunteers, and processed as described above in order to obtain high molecular weight human genomic DNA. The quality and quantity of DNA that could be isolated from these types of samples were determined by agarose gel electrophoresis and spectrofluorometry. Samples were divided into two batches. The first group, identified as ON1–ON10, consisted of specimens, 15 mL each, and less than one day old. The second group of specimens, labeled N1–N10, were 50 mL each and were 5 days old (stored at 4°C).

PCR-HLA DQα Analysis

Several types of urine samples were analyzed by HLA DQα analysis of polymerase chain reaction amplified DNA. Ten fresh casework urine samples (submitted for toxicological analysis) were chosen. Half had a volume of 50 mL and the remainder had a volume of 15 mL. Ten casework samples that had been frozen in a −80°C freezer for 6 months were also analyzed. These 10 samples varied in volume ranging from 20 to 50 mL. Samples found to be positive for an illicit drug are generally stored frozen so that they may be retested at a later date if necessary. Seven fresh "normal" (drug-free) specimens, with volumes of 50 mL, were also randomly chosen and analyzed. To determine the minimal volume needed for successful PCR amplification and HLA DQα typing, 1, 5 and 10 mL aliquots were drawn from a fresh normal urine sample and studied. Matching blood samples were obtained from the urine donors for comparison purposes. Frozen specimens were allowed to equilibrate to room temperature and all samples were thoroughly mixed to ensure homogeneity before analysis. After being transferred to centrifuge tubes, the samples were centrifuged at 2000 × g for 5 min. The supernatant was decanted and the collected sediment transferred to 1.5 mL Sarstedt tubes with pasteur pipettes. DNA was isolated from urine and blood by Chelex extraction [*19*] and put through 32 cycles of temperature ramping consisting of denaturation at 94°C for 1 min, primer annealing at 60°C for 30 sec, and extension at 72° for 30 s. The Perkin-Elmer model 480 thermal cycler was programmed to link to the cycling segment a time delay of 7 min at 72°C. Amplified DNA was then hybridized to HLA DQ alpha probe strips as described in the Cetus (now Perkin-Elmer Corp.) Amplitype™ Manual [*20*].

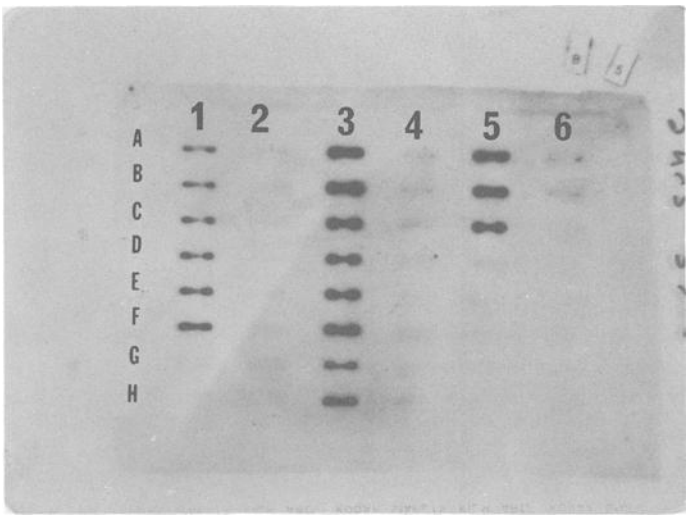
Results

RFLP Analysis—Casework Samples

Eighty-four percent (27) of 32 urine samples, labeled 125u–156u, yielded high molecular weight (HMW) DNA (Table 1). Slot blot analysis revealed that at least 50 ng of human DNA was present in each of the 0.5 μL aliquots of solubilized concentrate tested (Fig. 1). Of the 27 specimens yielding HMW DNA, 20 were selected with their corresponding blood samples for complete RFLP analysis using chemiluminescence detection. Approximately 1.5 μg of human DNA from each sample was analyzed. Examination of the resulting lumigrams showed that 5 of the 20 urine samples, 25%, produced banding patterns that were indistinguishable from the corresponding blood banding patterns (Fig. 2). Four of the 5 urine banding patterns were lighter than the corresponding bands obtained from blood and only 1 was darker. RFLP analysis of 5 of the 20 chosen urine samples produced inconclusive results for example, a clear banding pattern could not be discerned due to low signal to noise ratio. The remaining 10 urine samples produced no banding pattern at all.

TABLE 1—*Efficiency of DNA extractions on urine specimens.*

Sample #	Age	No. Samples	Volume	HMW DNA	% Yield HMW DNA
125–144u	1 day	20	10–15 mL	16	80%
145–156u	1 day	12	10–15 mL	11	92%
ON1–ON10	≤1 day	10	15 mL	8	80%
N1–N10	5 day	10	50 mL	3	30%

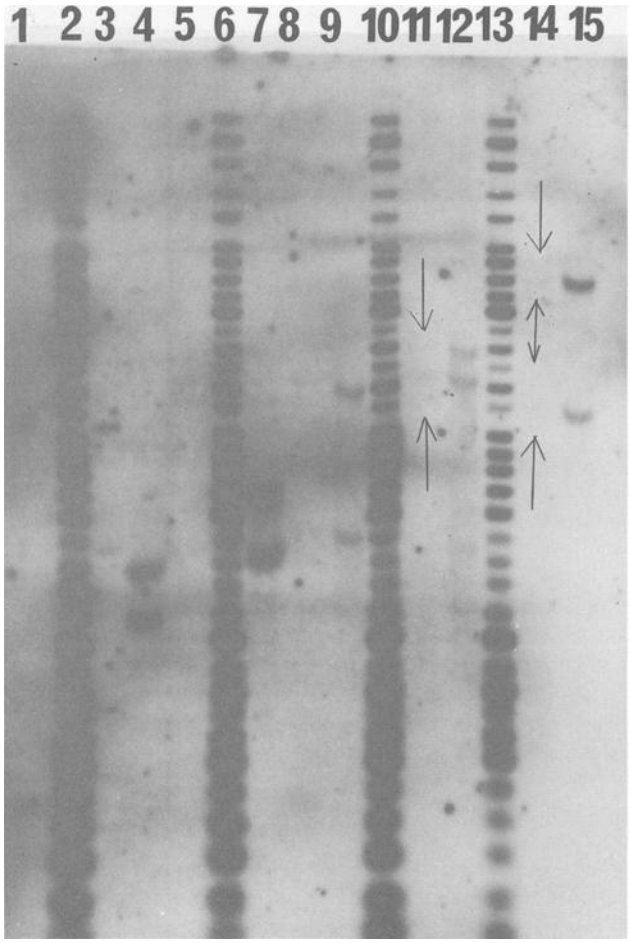


Lane #	Sample	Amount (μL)
1	151u	20
2	23kb sizing std	23
3	Hae III digest cont.	25
4	151b	20
5	152u	20
6	23kb sizing std	23
7	152b	20
8	154u	20
9	154b	20
10	23kb sizing std	23
11	155u	20
12	155b	20
13	23kb sizing std	23
14	156u	20
15	156b	20
16	-	-

FIG. 1—*Lumigram of slot blot analysis.*

Normal Urine Samples

Yield gel analysis of the first batch of samples, ON1–ON10 (15 mL), which had been stored for a period less than one day, revealed that human HMW DNA was obtained in 80% of these samples (8 out of 10). The second batch of samples, N1–N10 (50 mL), which had been stored refrigerated (4°C) for a period of 5 days, yielded HMW DNA in only 30% of the samples (3 of the 10).



Slot Position	Sample	Slot Position	Sample	Slot Position	Sample
1A	0.5 ng Human DNA	3B	147u (0.5 μL)	5A	154u (0.5 μL)
1B	1.0 " " "	3C	148u "	5B	155u "
1C	2.0 " " "	3D	149u "	5B	156u "
1D	5.0	3E	150u "		
1E	10.0 " " "	3F	151u "		
1F	25.0 " " "	3G	152u "		
3A	145u (0.5 μL)	3H	153u "		

Note: Lanes 2, 4, 6 and Positions 1G, 1H, 5D, and 5H are empty.

FIG. 2—*DNA Lumigram of analytical membrane*

PCR-HLA DQα Analysis

Of the 10 fresh casework samples typed, those 5 samples with original volumes of 50 mL were all successfully typed (Table 2). Three of the 5 samples with original volumes of 15 mL were successfully typed. The 10 samples subjected to freezing had 5 samples that yielded positive results. The 7 normal (drug-free) samples, with volumes of 50 mL yielded 6 positive typings. In a study conducted to determine minimum quantities required, samples of 1, 5 and 10 mL starting volumes were all successfully typed. The corresponding blood samples, for each urine sample positively typed, yielded matching DQα types.

Discussion

The individualization of urine specimens is an important area of study now that drug screening of urine has become so important and widespread. Until recently, the only way a questioned specimen could be evaluated was to perform analyses for secreted ABO and Lewis substances or to detect substances in urine that are polymorphic, for example, haptoglobin, orosomucoid or group-specific component, Gc. Unfortunately the concentrations of these substances are often too low to produce positive results. Various methods have been employed to concentrate questioned urine specimens [*21*]. However, even when genotyping is successfully performed, the power of discrimination for these markers is low, and as a result a match between the specimen and the suspected donor is not a conclusive identification.

Over the past few years, various methods have been developed in which genomic DNA can be used to determine the identification of the donor. These include RFLP, PCR-reverse dot blot hybridization, AmpFLP, STR and MVR analyses. The RFLP method developed by Jeffreys [*22,23*] utilizes radiolabelled probes and requires that the specimen to be tested contain a sufficient quantity of HMW DNA. In this study we have used chemiluminescence as a detection method. Sensitivity of the detection method is an additional factor that can influence the outcome of the analysis. We have not compared the sensitivity of chemiluminescence with ^{32}P isotopic detection. Having a sufficient quantity of HMW DNA is generally not a problem when the specimen is fresh and contains a high concentration of nucleated cells. It has been previously demonstrated that high quality DNA can be isolated from fresh casework urine samples and successfully analyzed by RFLP analysis. It appears from our study that there is no correlation between the quality and quantity of the isolated DNA, and any of the specific characteristics detected on the urinalysis strips (see Materials and Methods). Cellular degeneration in a urine specimen begins almost immediately following micturition [*2,3*]. Storage of these specimens can result in changes in factors such as pH. Bacterial contamination may become a significant factor if the specimens are not frozen or chemically preserved. Freezing and thawing may also result in lysis of cells with the concomitant release of nuclear DNA into the urine matrix. In this state, DNA is subject to hydrolysis by various endogenous nucleolytic enzymes that are present in urine. At least

TABLE 2—*Results of HLA DQα typing.*

Sample	Age	Volume	No. Samples	Matched Correctly
Fresh casework	<2 days	50 mL	5	5(100%)
Fresh casework	<2 days	15 mL	5	3(60%)
Frozen casework	6 months	20–50 mL	10	5(50%)
Normal (drug-free)	<2 days	50 mL	7	6(85%)
Min. vol. study	<2 days	1,5,10 mL	3	3(100%)

20 to 50 ng of undenatured HMW human genomic DNA is required for successful RFLP analysis [*11*]. Samples containing lesser amounts of DNA are generally not tested by this method.

Casework urine samples may be stored in various environments and they may also be subjected to freezing and thawing. Sample handling in the laboratory may compound any natural degeneration of nuclear material. Studies of normal urine show that significant breakdown of native DNA had occurred prior to analysis. Only 30% of 5 day old refrigerated samples having a volume of 50 mL produced HMW DNA as compared to 80% of samples less than 1 day old with volumes of between 10 and 15 mL.

Fresh casework samples were selected to serve as baseline controls in an aging study. Evaluation of the resultant analytical lumigrams indicates that there is a low probability of success even for fresh urine specimens (25%). A low probability of success and the knowledge that genomic DNA would rapidly degenerate with time [*1,2,11,12*] precluded any continuation of this study. We therefore conclude that RFLP is not a method of choice for the analysis of urine. PCR HLA DQα analysis of casework urine samples, whether fresh or freeze-thawed, yielded a higher percentage (65%) overall, of successful determinations than RFLP. Thus, PCR initiated analysis is most likely to produce useful results in cases where only a small amount of DNA may be present or where DNA may be somewhat degraded. There is some evidence that an inhibitor of PCR may exist in urine. This may explain why we have observed that PCR-HLA DQα testing does not always produce successful results. The addition of bovine serum albumin to the amplification mixture may improve the efficiency of replication. Dilution of the specimen may also be used to overcome the presence of an inhibitor if present in limited quantity. Further studies are warranted to verify the existence of such an inhibitor and to characterize it if it does indeed exist. PCR-HLA DQα analysis is rapid, easily performed and generally considered to be reliable and accurate. Alternate methods of individualization of urine specimens using DNA such as STR and MVR analysis are promising and merit further study. We therefore conclude that PCR-HLA DQα analysis, despite its limited power of discrimination, is a suitable method in identity determination of urine samples.

References

[*1*] Holland, M. M., Roy, R., Fraser, M. D., and Liu R. H., "Application of Serological and DNA Methods for the Identification of Urine Specimen Donors," *Forensic Science Review,* 1993, Vol. 5, No. 1, pp. 1–14.

[*2*] Graff, L., *A Handbook of Routine Urinalysis,* J. B. Lippincott Co., Philadelphia, PA. 1983.

[*3*] Holley, J. W. and Bradley, M. G., *Urinalysis: An Educational Program.* BMC & ASMT, Research Media, Inc., New York, 1979.

[*4*] Chase, M. G., "ABO Typing Studies on Liquid Urines," *Journal of Forensic Sciences,* Vol. 31, No. 3, July 1986, pp. 881–885.

[*5*] Kotowski, T. M., "Typing of Urine in the ABO and Lewis System," *Journal of Forensic Sciences,* Vol. 32, No. 4, 1987, pp. 1065–1068.

[*6*] Lee, H. C. and Voll, S. M., "Identification of Human Urine Stains," *Serology News,* Vol. 3, No. 6, December 1977, pp. 1–3.

[*7*] Lee, H. C. and Gaensslen, R. E., "ABO Grouping of Urine and Urine Stains," *Forensic Serology Newsletter,* Vol. 6, No. 3, 1980 pp. 1–3

[*8*] Lee, H. C., Gaensslen, R. E., Pagliaro, E. M., and Novitch, B., "Two Dimensional Absorption Inhibition," *Journal of Forensic Sciences,* Vol. 33, No. 5, Sept. 1988, pp. 1127–1138.

[*9*] Roewer, L., Nurnberg, P., Fuhrmann, E., Rose, M., Prokop, O., and Epplen, J. T. "Stain Analysis Using Oligo Nucleotide Probes Specific for Simple Repetitive DNA Sequences," *Forensic Science International,* Vol. 47, 1990, pp. 59–70.

[*10*] Brinkmann, B., Rand, S., and Bajanowski, T., "Forensic Identification of Urine Samples," *International Journal of Legal Medicine,* 1992, Vol. 105, No. 1, pp. 59–61.

[*11*] Kobilinsky, L., "Recovery and Stability of DNA in Samples of Forensic Science Significance," *Forensic Science Reviews,* Vol. 4, No. 1, 1992, pp. 67–87.

[*12*] Baechtel, F. S., "Detection of Amplified Fragment Length Polymorphisms," *The Second International Symposium on the Forensic Aspects of DNA Analysis,* 1993, FBI Academy, Quantico, VA.

[13] FBI Procedures, The Detection of Restriction Fragment Length Polymorphisms in Human DNA, Federal Bureau of Investigation Laboratory, Washington D.C., 1989.
[14] Budowle, B. and Baechtel, F. S., "Modifications to Improve the Effectiveness of Restriction Fragment Length Polymorphism Typing," *Applied and Theoretical Electrophoresis,* Vol. 1, No. 4, 1990, pp. 181–187.
[15] Adams, D. E., "Validation of the FBI Procedure for DNA Analysis: A Summary," *Crime Lab Digest,* Vol. 15, No. 4, 1988, pp. 106–108.
[16] Cesarone, C. F., Bolognesi, C., and Santo, L., "Improved Microflurometric DNA Determination in Biological Material Using 33258 Hoechst," *Analytical Biochemistry,* Vol. 100, 1979, pp. 188–197.
[17] Waye, J. S., Presley, L. A., Budowle, B., Shutler, G. G., and Fourney, R. M., "A Simple and Sensitive Method for Quantifying Human Genomic DNA in Forensic Specimen Extracts," *BioTechniques,* Vol. 7, 1989, pp. 852–855.
[18] Dubitsky, A., Brown, J., and Brandwein, H., "Chemiluminescent Detection of DNA on Nylon Membranes," *BioTechniques,* Vol. 13, No. 3, 1992, pp. 392–399.
[19] Singer-Sam, J. and Tanguay, R., "Use of Chelex to Improve the PCR Signal from a Small Number of Cells," *Amplification, A Forum for PCR Users,* Issue 3, 1989, p. 11.
[20] Cetus Corporation, *Amplitype User Guide—Version 2,* Cetus Corporation, Emeryville, CA, 1990.
[21] Roy, R. "Concentration of Urine Samples by Three Different Procedures: ABO Typing from Concentrated Urine Samples," *Journal of Forensic Sciences,* Vol. 35, 1990, pp. 1133–1137.
[22] Jeffreys, A. J., Wilson, V., Thein, S. L., "Individual-Specific 'fingerprints' of Human DNA," *Nature,* Vol. 316, 1985, pp. 76–79.
[23] Jeffreys, A. J. "Highly variable minisatellites and DNA fingerprints," *Biochemical Society Transactions,* Vol. 15, 1987, pp. 309–317.

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