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Forensic Science International
83 (1996) 167–177

Forensic Science International

Increasing DNA extraction yield from saliva stains with a modified Chelex method

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Received 1 February 1996; revised 12 September 1996; accepted 17 September 1996

Abstract

Recovery, preservation and analysis of body fluid stains is an important aspect of forensic science. PCR-based typing of DNA extracted from recovered stains is often a crucial method to identify a perpetrator or exclude an innocent suspect. This paper reports an improved method of extracting genomic DNA from saliva stains deposited on human skin in simulated bite mark situations. Results of organic (phenol-chloroform) extraction and Chelex extraction were compared to a modified Chelex method developed by the authors. Modifications include pre-extraction preparation with proteinase K and incubations at 56°C and 100°C plus microconcentration of the solution. Quantification results using the classical Chelex extraction method showed that 31.9±4.22% of the deposited DNA was recovered, but using the modified Chelex extraction method DNA recovery was increased to 47.7±6.90%. The quantity and quality of extracted DNA was shown to be adequate for PCR-based typing at two STR loci.

Keywords: DNA extraction; Human bite marks; Forensic DNA; Salivary evidence; Human saliva

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PII S0379-0738(96)02034-8

1. Introduction

Several methods to discriminate the source of a saliva stain to a specific individual have been developed. The analysis of blood cell allotypes, monoclonal antibodies, numerous polymorphic proteins and isoenzymes, isoelectric focusing of complement and subtyping of the transferrin system are a few examples of the numerous forensic identification tests performed on saliva [1–9]. These conventional methods are limited to certain traditional marker systems. But recent studies have shown that saliva can also be isolated from objects and tested using genetic typing systems [10–17]. Genomic DNA has been extracted from saliva found on a variety of surfaces [10] such as chewing gum [R. Fourney, personal communication], cigarette butts [18], postage stamps [19] and human skin [20].

Saliva is deposited on human skin through biting, sucking, licking and kissing, and possibly through other behavior. Stains of dried saliva are invisible, which adds to the difficulty of recognizing and collecting them. The DNA present in saliva on skin is more difficult to collect and extract than similar stains on clothing, paper or other inanimate objects since the substrate on which the saliva is deposited (skin) cannot be submitted directly to extraction procedures. An improved collection method is required because the quantity of DNA in saliva stains on human skin is often minimal. Contamination by DNA from the skin is also a potential problem. The double swab technique which employs a wet cotton swab followed by a dry cotton swab has been shown to increase the amount of salivary DNA recovered from skin when compared to other methods [21].

Following collection of the saliva from the surface of the skin, an efficient method of extracting DNA from the cotton swabs is required. Two methods of DNA extraction from biological samples are commonly used: the organic (phenol-chloroform) method and the Chelex-100 extraction procedure. Because the salivary cells of interest in this study are collected using a cotton swab, the authors hypothesized that a method to remove these nucleated cells from the swab before submitting them to cellular and nuclear lysis protocols would increase DNA yield. Improving the release of cells from the substrate would theoretically increase the DNA available for extraction. A method was devised to wash the swabs in boiling water to release the intact cells. Later these cells were submitted to DNA extraction using the organic method and the Chelex method.

Phenol-chloroform extraction takes a relatively long period of time, is expensive and involves the use of toxic and hazardous chemical reagents but it produces consistent results [22,23]. Chelex is a chelating resin that has a high affinity for polyvalent metal ions. Singer-Sam et al. postulated that boiling a sample in the presence of Chelex prevents the degradation of DNA [24]. Apparently metal ions, which can act as catalysts in DNA breakdown at high temperatures in low ionic strength solutions, are chelated and inhibited from this action. More recently, Walsh et al. reported the use of Chelex-100 as a means of extracting DNA from forensic samples containing whole blood, bloodstains, seminal stains, saliva, hair and post-coital samples [25]. The Chelex technique is simple and rapid. It involves neither the use of toxic organic solvents nor multiple tube transfers for most types

of samples [25–27]. Chelex resin removes impurities from solution and the alkaline pH disrupts the cell membranes resulting in release of DNA.

In the current study, a phenol-chloroform based method [13] and the classical Chelex-100-based method [25] were evaluated to extract DNA from samples with a known concentration of DNA and from expectorated whole saliva. DNA 'extraction' was completed using a reference standard with a known concentration to evaluate the potential loss of DNA through sample manipulation and from whole saliva to determine which extraction method produced the greatest DNA output. The classical Chelex procedure was shown to be the most effective extraction method compared to the phenol-chloroform method. Therefore, the classical Chelex method was studied in a simulated bite mark situation. The double swab technique [21] was used to recover dried saliva from human skin. The extraction results obtained with the classical Chelex technique were compared to results using a modified Chelex method in which the swabs were washed and incubated at different temperatures to increase the release of cells from the swabs.

2. Material and methods

2.1. Sampling

DNA reference standards from K562 cell lines (DNA Typing Grade K562, GIBCO-BRL, 14410-0113) were submitted to DNA extraction using the organic and Chelex methods. The supplied concentration (150 ng/μl) was serially diluted with sterile distilled water to produce a standard working solution containing 10 ng/μl. Thirty aliquots of 2 μl (20 ng of DNA) were submitted to DNA extraction: one-half the samples ($n=15$) using the organic method and one-half ($n=15$) using the Chelex method.

Saliva samples were obtained from five (3 male, 2 female) unrelated individuals. Prior to obtaining the sample, the mouth of each donor was rinsed with clean tap water for 10 s and the water was discarded. After waiting 5 min, each donor expectorated approximately 0.5 ml of saliva which had accumulated in his/her mouth into a clean polypropylene tube; these samples were stored at 4°C. This process was continued for three consecutive days resulting in collection of 15 saliva samples. Two aliquots of 100 μl were removed from each sample and submitted to phenol-chloroform extraction ($n=15$) and Chelex extraction ($n=15$).

2.2. Phenol-chloroform extraction method

DNA from reference standards and from samples of whole saliva were extracted according to the procedure of Walsh et al. [13] with minor modifications. In summary, 100 μl aliquots of whole saliva were centrifuged at 10 000×g for 2 min and the supernatant was discarded. 700 μl of lysis buffer (10 mM Tris–HCl, pH 8.0; 10 mM EDTA; 0.1 M NaCl; 2% SDS) and 35 μl proteinase K (20 mg/ml,

Sigma) were added to the pellet or to aliquots containing a total of 20 ng of DNA taken from the standard working solution. Samples were incubated at 56°C for 10 h before adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v, Sigma P-3803). Following brief agitation and centrifugation at 2500×*g* for 10 min, the layer containing DNA was transferred to a new tube and the extraction process repeated by addition of an equal volume of phenol/chloroform/isoamyl alcohol. Finally, an equal volume of absolute ethanol (−20°C) and 80 μl of 3 M sodium acetate buffer (pH 5.22) were added to precipitate the DNA. The tubes were then stored at −40°C overnight. After centrifugation at 2500×*g* for 10 min the ethanol layer was discarded. The precipitate was washed with 70% ethanol (−20°C) and centrifuged at 2500×*g* for 10 min. The ethanol was slowly discarded and the tube was inverted and allowed to air dry at room temperature for 30 to 60 min. The pellet was resuspended in sterile double distilled water and stored at 4°C pending quantitation.

2.3. Chelex extraction method

DNA from the standard working solution and samples of whole saliva were extracted according to the classical Chelex extraction method [25]. First, 200 μl of 5% Chelex (pH 9.0) (Sigma) were added to tubes containing 100 μl aliquots of whole saliva and to tubes containing a total of 20 ng of DNA from the working solution. These samples were incubated at 56°C for 30 min and at 100°C for 8 min before centrifugation at 2500×*g* for 3 min. Samples were stored at 4°C pending quantitation.

2.4. Modified Chelex extraction method

An experiment was conducted to compare the results of classical Chelex extraction [25] and a modification of the Chelex method on saliva samples collected from human skin. Aliquots of 100 μl of whole saliva were deposited on the forearms of unrelated individuals and allowed to air dry for 20 min. The double swab technique which initially employs a cotton swab immersed in sterile distilled water followed by a dry cotton swab was used to collect these stains [21]. After thorough air drying for at least 30 min, the tips of the two swabs from the double swab technique were combined into a single sample and stored at −20°C pending analysis. When all the samples were collected, the swabs were thoroughly washed in 1.5 ml of sterile distilled water and proteinase K (1 μg/μl). The tubes were agitated for 1 min and then incubated at 56°C for 60 min and 100°C for 8 min. This process was used to liberate salivary cells from the cotton fibers. The swab heads were compressed in a sterile syringe to recover the solution contained in them. This solution and the remainder of the wash solution were transferred to a new polypropylene tube. This was centrifuged at 10 000×*g* for 5 min. The tube containing the pellet was set aside and the supernatant was microconcentrated using Microcon-100 (Amicon) tubes. The concentrated pellet was resuspended in 75 μl of Tris buffer (10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA) and this solution

was transferred to the tube containing the original pellet. The tube was gently agitated to mix the pellet. This sample was submitted to the classic Chelex extraction method mentioned previously to remove and purify DNA from the cells.

2.5. Slot-blot DNA quantitation

The amount of DNA in the samples was quantified using the slot-blot procedure described by Waye et al. [28].

2.6. DNA amplification

DNA extracts obtained using the different methods described in this paper, which contained a minimum of 0.5 ng of DNA, were satisfactorily amplified at two short tandem repeat (STR) loci [29] using previously described protocols for HUMTH01 [30] and HUMVWA [31].

2.7. Statistical analysis

Statistical treatment of the results included use of the Student's *t*-test and linear regression analysis techniques.

3. Results

3.1. Extraction of DNA from reference standards

The phenol-chloroform and the classical Chelex extraction methods were employed to recover DNA from control samples with known concentrations of K562 DNA standard. Table 1 shows the average amount of DNA recovered from a solution containing 20 ng of DNA. The results obtained using each method were compared and statistically significant differences were identified. The average amount of DNA recovered using the classical Chelex method was significantly greater than that recovered using the organic method. The confidence limit of the difference between the averages for both methods (at 99%) ranges from 6.88% to 16.18%.

3.2. Extraction of DNA from saliva

DNA was extracted from 15 different samples of whole saliva following the organic and Chelex extraction protocols described in Material and methods (Section 2). Two aliquots of 100 μl were collected from each sample; extraction of one aliquot was completed using the organic method and of the other using the classical Chelex method. Results of these procedures are expressed as absolute quantities of DNA (ng/μl) in Table 2. They reveal a higher yield for the Chelex

Table 1
Average amount of DNA (ng) extracted from the K562 DNA reference solution (20 ng of DNA) using the organic and Chelex extraction procedures

Sample no.	Organic extraction	Chelex extraction
1	14.6	19.8
2	17.6	19.6
3	18.2	19.4
4	17.0	19.8
5	18.4	19.8
6	18.8	19.0
7	17.4	19.8
8	16.8	20.0
9	16.0	19.8
10	15.6	19.6
11	18.8	19.4
12	18.4	19.8
13	18.6	19.6
14	16.2	19.6
15	17.6	19.6
$\bar{X}\pm$S.D.	17.33±1.3 ng	19.64±0.2 ng

t_{exp}=6.8475 (28 d.f.), $P\leq 0.001$.

method. There is a statistically significant relationship between the two variables [r=0.92851, t_{exp}=9.0168 (13 d.f.), $P\leq 0.001$]. The equation of the linear slope, which indicates the relationship between the organic and classical Chelex extraction methods, is $y=0.6054+0.90899x$.

Table 2
Concentration of DNA (ng/μl) extracted from identical aliquots of saliva using the organic and Chelex extraction methods

Sample no.	Organic extraction	Chelex extraction
1	1.87	2.15
2	2.33	2.73
3	1.90	2.32
4	2.51	2.87
5	2.72	2.90
6	2.15	2.70
7	2.70	3.34
8	1.58	1.84
9	2.90	3.15
10	1.90	2.70
11	2.02	2.51
12	1.87	2.24
13	2.42	2.73
14	3.00	3.41
15	2.87	3.07
$\bar{X}\pm$S.D.	2.32±0.45 ng/μl	2.71±0.44 ng/μl

t_{exp}=2.4099 (28 d.f.), $P\leq 0.05$.

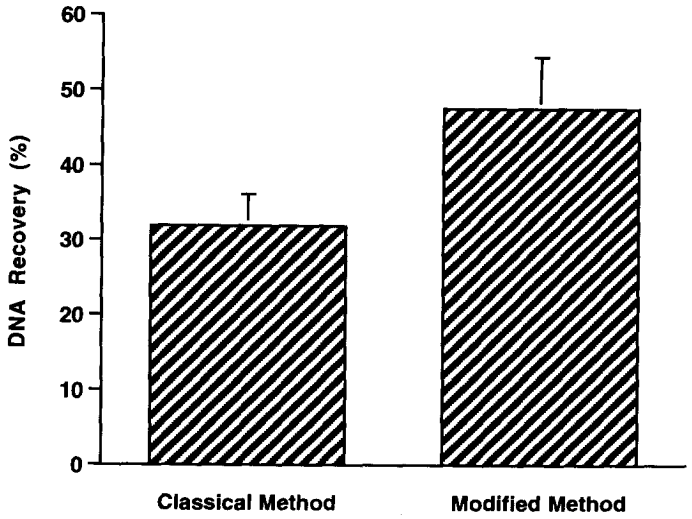

Fig. 1. DNA recovery (%) from 100 μl of whole saliva deposited on skin using the classical Chelex and modified Chelex extraction methods. Deposited DNA concentration was 2.7 ng/μl; therefore, 100% = 270 ng of DNA (t_{exp}=6.4436 (28 d.f.), $P \leq 0.001$).

3.3. Modified Chelex extraction method

In an attempt to closely reproduce the conditions found in an actual human bite mark situation where the amount of DNA is low and may be partially degraded, 100 μl of saliva with a known amount of DNA (2.7 ng/μl) was deposited on the skin and recovered using the double swab technique. Results of DNA extraction using the classical Chelex method were compared to results from Chelex extraction following pre-extraction preparation of the samples using the modified Chelex method (Fig. 1). Results are expressed as the percentage of DNA recovered, where 100% theoretically corresponds to the concentration of DNA present in the aliquots of whole saliva (2.7 ng/μl). A statistically significant difference was found between the two extraction methods. The percentage of DNA recovered using the modified Chelex extraction method was 47.7±6.90%. This was significantly higher than results using the classical Chelex method in which 31.9±4.22% of the DNA was recovered. The differences between the averages varies in a range from 8.9% to 22.6% at a confidence interval of 99%.

4. Discussion

Two methods to extract DNA from saliva were considered: the organic extraction method using phenol-chloroform [13], and the procedure using 5% Chelex-100 resin [25]. Both methods were used to extract DNA from a DNA

reference solution, from whole saliva containing known concentrations of human DNA and from saliva stains on human skin.

Jung et al. completed a large-scale investigation of bloodstains in order to establish adequate results for typing the HLA-DQA1 locus [26]. After analyzing several different variations of the classical extraction techniques, including extraction with organic solvents and filtration with Microcon-100 technology [23], it was concluded that the Chelex procedure is an adequate method for dealing with partially contaminated or degraded remains. It was determined to be possible, if inadequate results were obtained following the first amplification, to purify the extraction solution by additional filtration in Microcon-100 tubes and to submit the sample to a second Chelex extraction.

Jung concluded that the Chelex technique presents three clear advantages over the phenol-chloroform technique [26]. It was determined that Chelex increases the magnitude of DNA amplification by a factor of 6, avoids the use of toxic organic solvents, and involves less time and work. More recently, after completing a comparative study of extraction methods, Wiegand et al. concluded that Chelex extraction is slightly less effective than organic extraction but its rapidity more than makes up for this minor disadvantage [27]. Regardless, in some cases the amount and quality of DNA may be so low that it is necessary to attempt to improve the yield of extraction product. This is particularly true when dealing with saliva stains.

The term 'DNA extraction' is sometimes confusing in the context of forensic haemogenetics because it involves two parallel but different procedures that are not always completed simultaneously. In contrast to other biological disciplines in which nucleated cells, such as whole blood, fresh saliva, cultured cells, etc., are readily submitted to cellular and nuclear lysis to obtain genomic DNA, when dealing with forensic biological evidence the material often arrives at the laboratory as a dry body fluid stain (blood, saliva, semen, etc.) or as a small section of tissue (bone, hairs, teeth, etc.). Therefore, in forensic cases, DNA extraction procedures should accomplish two objectives. First, the procedure should release or wash the cellular material from the substrate to which it is adhering. Second, the procedure should provide a mechanism to liberate the DNA into solution and purify it.

There are some substrate materials — all kinds of clothing, in general, and cotton swabs, in particular — where a rigorous recovery strategy must be employed. This is especially important in cases where minimal amounts of DNA are anticipated, such as in the case of salivary cells recovered after swabbing the skin. The modified extraction method reported here is designed to increase the yield of intact salivary cells from the swab to which they become attached during the swabbing process, hence attempting to improve the initial phase of the DNA extraction procedure.

The classical extraction method for salivary evidence is limited to one direct treatment of the sample with a solution of 5% Chelex-100 [18] or to washing the swab with Tris-EDTA or similar buffers to which proteinase K is added. The incubation time in these processes can vary from 2 h [25] to overnight [13].

The procedure used in this project is in effect a double extraction protocol which involves submitting the cells initially to warm temperatures to liberate the salivary cells adhering to the swab into solution and boiling without Chelex to eliminate nucleases. Subsequently, the samples are boiled with Chelex to extract the DNA from the cells. The results indicate that this modification produces an average recovery of 47.7% of the DNA deposited which is statistically significant (t_{exp}=6.4436 (28 d.f.), $P \leq 0.001$) compared to a recovery of 31.9% of the DNA deposited using previous extraction methods.

In conclusion, extraction of DNA from saliva using Chelex-100 resin is more effective than the organic extraction method using phenol-chloroform. The extraction results are further improved by using the modified Chelex method described in this paper, where the samples are submitted to pre-extraction treatment consisting of washing the swab tips in proteinase K, incubation at 56°C and 100°C, and subsequent micro-concentration of the solution. Therefore, the modified Chelex method is recommended when dealing with small amounts of liquid saliva or saliva stains where minimal amounts of DNA can be expected to be recovered.

Acknowledgments

The valuable assistance of Coroner R.B. Bastien of the British Columbia Coroners Forensic Identification Unit is gratefully acknowledged. This study was supported by a grant from the Ministry of Education and Science of the Government of Spain (DGICYT/PB93-1155B), the Plan Propio de Investigación of the University of Granada, and by the Dentistry Canada Fund/Warner-Lambert Foundation, Ottawa, Canada.

References

[1] A. Clift and C.M. Lamont, Saliva in forensic odontology. *J. Forensic Sci. Soc.*, 14 (1974) 241–245.

[2] E.A. Rutter and P.H. Whitehead, The fractionation of ABH blood group substances in saliva. *J. Forensic Sci. Soc.*, 16 (1977) 241–246.

[3] R.E. Gaensslen, *Sourcebook in Forensic Serology, Immunology and Biochemistry*, National Institute of Justice, Washington, DC, 1983.

[4] B. Brinkmann and K. Henningsen, *Advances in Forensic Haemogenetics*, Springer-Verlag, Berlin, 1985.

[5] A. Fiori, V. Gentile and N. Fucci, Absorption-elution on microplates (AEMP): an improved method for identification of the ABH glycoproteins in saliva and semen of secretors. In W.R. Mayr (ed.), *Advances in Forensic Haemogenetics No. 2*, Springer-Verlag, Berlin, 1988, pp. 429–432.

[6] W. Pflug, G. Bäβler, W. Bruder and B. Eberspächer, Dot blot ELISA on nitrocellulose membranes (NC): a new method for typing Lewis and ABO of body fluids (saliva, semen, vaginal secretions, sweat and urine). In W.R. Mayr (ed.), *Advances in Forensic Haemogenetics No. 2*, Springer-Verlag, Berlin, 1988, pp. 433–438.

[7] K. DeSoyza, Differential detection of A, B, and H antigens in semen and saliva stains detected by an ELISA technique. In H.R. Polesky and W.R. Mayr (eds.), *Advances in Forensic Haemogenetics No. 3*, Springer-Verlag, Berlin, 1990, pp. 167–170.
[8] B. Hoste, R. Merciny, G.G. DeLange and C. Francois-Gerard, Enzyme immunoassay for the typing of the Gm(a) and Gm(f) allotypes of human IgG1 in semen, vaginal secretions and other body fluids. In H.F. Polesky and W.R. Mayr (eds.), *Advances in Forensic Haemogenetics No. 3*, Springer-Verlag, Berlin, 1990, pp. 190–193.
[9] S. Ikemoto, S. Tsuchida, E. Kajii, E. Nozawa and K. Tomita, Distribution of twelve human salivary polymorphisms in Japanese population. In H.F. Polesky and W.R. Mayr (eds.), *Advances in Forensic Haemogenetics No. 3*, Springer-Verlag, Berlin, 1990, pp. 263–264.
[10] C.T. Comey and B. Budowle, Validation studies on the analysis of the HLA DQ alpha locus using the polymerase chain reaction. *J. Forensic Sci.*, 36 (1991) 1633–1648.
[11] I.V. Ovchinnikov, D.K. Gavrilov, V.V. Nosikov and V.G. Debabov, Use of the polymerase chain reaction for typing allelic variants of the human HLA-DQA1 by hybridization with oligonucleotide probes, specific for specific alleles [Russian]. *Molekulyarnaya Biologiya*, 25 (1991) 1266–1272.
[12] Y.A. Berlin and H. Kazazian, Rapid preparation of genomic DNA from dried blood and saliva spots for polymerase chain reaction [Letter]. *Hum. Mutat.*, 1 (1992) 260–261.
[13] D.J. Walsh, A.C. Corey, R.W. Cotton, L. Forman, G.L. Herrin, C.J. Word and D.D. Garner, Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. *J. Forensic Sci.*, 37 (1992) 387–395.
[14] B. Eriksen and O. Svensmark, DNA-profiling of stains in criminal cases: analysis of measurement errors and band-shift. Discussion of match criteria. *Forensic Sci. Int.*, 61 (1993) 21–34.
[15] D.L. Kauffman, P.J. Kelle, A. Bennick and M. Blum, Alignment of amino acid and DNA sequences of human proline-rich proteins. *Crit. Rev. Oral Biol. Med.*, 4 (1993) 287–292.
[16] A.D. Kloosterman, B. Budowle and P. Daselaar, PCR-amplification and detection of the human D1S80 VNTR locus. Amplification conditions, population genetics and application in forensic analysis. *Int. J. Leg. Med.*, 105 (1993) 257–264.
[17] T. Kojima, R. Uchihi, T. Yamamoto, K. Tamaki and Y. Katsumata, DNA typing of the three HLA-Class II loci from saliva stains [Japanese]. *Jpn. J. Leg. Med.*, 47 (1993) 380–386.
[18] M.N. Hochmeister, B. Budowle, J. Jung, U.V. Borer, C.T. Comey and R. Dirnhofer, PCR-based typing of DNA extracted from cigarette butts. *Int. J. Leg. Med.*, 104 (1991) 229–233.
[19] B. Hopkins, N.J. Williams, M.B.T. Webb, P.G. Debenham and A.J. Jeffreys, The use of minisatellite variant repeat-polymerase chain reaction (MVR-PCR) to determine the source of saliva on a used postage stamp. *J. Forensic Sci.*, 39 (1994) 526–531.
[20] D.J. Sweet, *Identificación de manchas de saliva humana mediante el análisis del ADN*, Doctoral Thesis, University of Granada, 1995.
[21] D.J. Sweet, M. Lorente, J.A. Lorente, A. Valenzuela and E. Villanueva, An improved method to recover saliva from human skin: the double swab technique. *J. Forensic Sci.*, (in press March 1997).
[22] J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning — A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
[23] B. Budowle and F.S. Baechtel, Modifications to improve the effectiveness of restriction fragment length polymorphism typing. *Appl. Theor. Electrophor.* 1 (1990) 181–187.
[24] J. Singer-Sam, R.L. Tanguay and A.D. Riggs, Use of Chelex to improve the PCR signal from a small number of cells. *Amplifications: A Forum for PCR Users*, 3 (1989) 11.
[25] P.S. Walsh, D.A. Metzger and R. Higuchi, Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*, 10 (1991) 506–513.
[26] J.M. Jung, C.T. Comey, D.B. Baer and B. Budowle, Extraction study for obtaining DNA from bloodstains for PCR amplification and typing of the HLA-DQ alpha gene. *Int. J. Leg. Med.*, 104 (1991) 145–148.
[27] P. Wiegand, B. Budowle, S. Rand and B. Brinkmann, Forensic validation of the STR systems SE33 and TC11. *Int. J. Leg. Med.*, 105 (1993) 315–320.
[28] J.S. Waye, L.A. Presley, B. Budowle, G.G. Shutler and R.M. Fourney, A simple and sensitive method for quantifying human genomic DNA in forensic science specimen extracts. *BioTechniques*, 7 (1989) 852–855.

[29] D.J. Sweet, J.A. Lorente, A. Valenzuela, M. Lorente and E. Villanueva, PCR-based typing of saliva stains recovered from human skin. *J. Forensic Sci.*, (in press May 1997).
[30] J.A. Lorente, M. Lorente, B. Budowle, M.R. Wilson and E. Villanueva, Analysis of the HUMTH01 allele frequencies in the Spanish population. *J. Forensic Sci.*, 39 (1994) 1270–1274.
[31] J.A. Lorente, M. Lorente, M.R. Wilson, B. Budowle and E. Villanueva, Analysis of the short tandem repeat (STR) VWA (HUMVWA) in the Spanish population. *Forensic Sci. Int.*, 65 (1994) 169–175.