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Extraction strategy for obtaining DNA from bloodstains for PCR amplification and typing of the HLA-DQα gene

Janet M. Jung[1], Catherine T. Comey[1], David B. Baer[2], and Bruce Budowle[1]

[1]Forensic Science Research and Training Center, FBI Academy, Quantico, VA 22135, USA
[2]Florida Department of Law Enforcement, Orlando Regional Crime Laboratory, Orlando, FL 32801, USA

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Summary. A simple, practical approach for the extraction of PCR-amplifiable DNA for the HLA-DQα gene from bloodstains deposited on various substrates is described. DNA from bloodstains is purified using Chelex 100 ion-exchange resin and then amplified. If amplification is not achieved, the extract is washed through a Centricon 100 dialysis/concentration tube. If the second amplification of this extract produces a negative result, the extract is processed with Chelex 100 again. This approach has been found to be reliable, safe, efficient and economical.

Key words: PCR – DQα – DNA extraction

Zusammenfassung. Eine einfache DNA-Extraktionsmethode zur direkten Amplifikation des HLA-DQα-Locus aus auf verschiedenen Spurenträgern angelegten Blutspuren wird beschrieben. Die Extraktion erfolgt mittels des Chelatbildners Chelex 100. Ist die Amplifikation inhibiert, wird das Extrakt in Centricon 100 Mikrokonzentrator-Röhrchen dialysiert. Bleibt auch eine zweite Amplifikation negativ, erfolgt eine erneute Reinigung mittels Chelex 100. Diese Vorgangsweise erwies sich als effizient, zuverlässig und praktikabel.

Schlüsselwörter: Polymerase chain reaction (PCR) – HLA-DQα – DNA-Extraktion

Introduction

In order for PCR amplification to succeed on DNA extracted from forensic bloodstains, inhibitory substances that might co-purify with DNA must be removed [1]. The choice of extraction method(s) can impact on the ability to achieve amplification and, ultimately, the success of dot blot typing of the HLA-DQα gene [2].

This paper describes the results of PCR amplification and dot blot typing of the HLA-DQα gene from DNA recovered using the Chelex and Centricon extraction methods on replicate bloodstains deposited on a variety of substrates. A practical approach for the routine extraction of DNA from forensic bloodstains for PCR analysis is suggested.

Offprint requests to: C. T. Comey

Materials and methods

Sample preparation. Replicate 50-μl bloodstains of HLA-DQα type 1.1,4 were deposited as described by Comey et al. [3] on 16 different materials: scrapings from glass, terrycloth, filter paper, cotton backing from oilcloth, knit backing from vinyl upholstery, cotton sheeting, acrylic sweater, nylon stocking, cotton weave, corduroy, brown paper, suede leather, cotton upholstery, cotton sweatshirt, white denim and blue denim. The fabrics were new and were not washed in the laboratory prior to preparation of the bloodstains.

Extraction. Duplicate stains from each substrate were subjected to modified Chelex [4] or Centricon [5] extraction methods as follows. For the Chelex extraction, a 3 mm × 3 mm size cutting of the bloodstain in 200 μl 5% Chelex (w/v) was incubated at 56°C for 30 min to overnight, depending on the nature of the substrate. Subsequently, the extract was vortexed for 10 s, boiled in a waterbath for 8 min, vortexed again for 10 s, and centrifuged in a microcentrifuge 235C (Fisher Scientific) for 3 min.

Briefly, the Centricon extraction entails placing a 1 cm × 1 cm size cutting of the bloodstain through an organic extraction with phenol/chloroform/isoamyl alcohol (25:24:1) as described by Budowle et al. [6]. The aqueous phase of this extract was subjected to a second extraction with a 250 μl aliquot of phenol/chloroform/isoamyl alcohol and to a third extraction with the same volume of water-saturated *n*-butanol. The aqueous phase was then transferred to a Centricon 100 tube containing 1.5 ml of a soution containing 10 m*M* TRIS and 0.1 *M* EDTA, pH 7.5 (TE buffer). The Centricon tubes are centrifuged at 4000 rpm for 20 min in an IEC Clinical Centrifuge (Fisher Scientific). Fresh TE buffer (2 ml) is then added and the centrifugation is repeated (this constitutes a "wash"). This wash process is repeated three times. The extract is collected by inverting the Centricon tube on the retentate cup and centrifuging it in the IEC Centrifuge at 1200 rpm for 5 min.

A sequential extraction method using Chelex, followed by the Centricon approach (Chelex/Centricon) was performed on six selected substrates by placing the Chelex extract of the bloodstain (3 mm × 3 mm) into a Centricon 100 tube containing 2 ml TE buffer. The wash procedure described above was performed three times and the retentate was collected.

A sequential extraction method using Centricon first followed by the Chelex approach (Centricon/Chelex) was performed on eight selected substrates by subjecting the Centricon retentate of a stain (1 cm × 1 cm) to a Chelex extraction.

A sequential extraction procedure (Chelex/Centricon/Chelex) was performed on one substrate by subjecting the Centricon retentate of the Chelex/Centricon processed stain to an additional Chelex extraction.

The DNA recovered from the stains extracted by the Centricon and Chelex methods was quantified by slot blot analysis as described by Waye et al. [7]. For the Centricon extracts, 2μl of each 20-μl retentate was used for the slot blot and for the Chelex extracts, 20 μl of each 200-μl extract was used.

Amplification and hybridization. The extracted DNA was amplified and typed using the reverse dot blot format of the Amplitype DQα Kit (Cetus; Saiki et al. [8]). The volumes of extractants used for amplification were based solely on the recommended volumes of the individual protocols at the time each portion of the study was conducted and were independent of the quantity of DNA present in the samples. In the case of the sequential extraction procedures, the volume of extractant used for amplification was the recommended volume of the final extraction protocol. These volumes were as follows: 20 μl each of the Chelex extractant, the Centricon/Chelex, and the Chelex/Centricon/Chelex extracts; 5 μl of the Centricon extracts; and 2 μl of the Chelex/Centricon extracts.

Results and discussion

Slot blot results of the DNA recovered by the Chelex and Centricon extraction methods used on bloodstains from the 16 substrates are shown in Fig. 1. As the cutting sizes required for the two protocols vary, the estimated total DNA recovery in nanograms for the Centricon method has been normalized to a 3 mm × 3 mm cutting for each sample. These data are shown in Table 1 and demonstrate that sufficient DNA for amplification and typing purposes was recovered from all extracts. However, the average DNA yield for all the substrates by the Chelex extraction method is 625 ng, while that of the Centricon procedure is 102 ng. Three benefits are realized with the use of the Chelex extraction technique: a 6-fold increase in extraction efficiency, the use of no organic solvents and the expenditure of less analyst time and effort.

The estimated quantity of DNA used for amplification for each substrate processed by the Chelex or the Centricon extraction methods is shown in Table 2. Amplification was conducted on the recommended volume of extract for each extraction protocol irrespective of the quantity of DNA recovered from the sample.

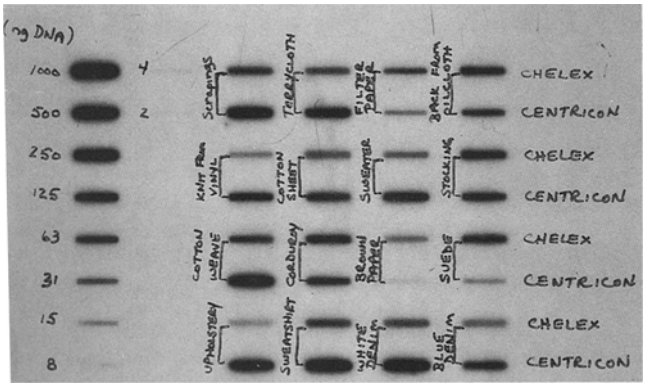

Fig. 1. Autoradiograph of slot blot to determine recovery of human DNA from Chelex and Centricon extracts of bloodstains deposited on various substrates

Table 1. Estimated total DNA recovery (ng) per sample (normalized for cutting size based on 3 mm × 3 mm)

Substrate	Extraction methods	
	Chelex	Centricon
White denim	600	225
Blue denim	400	90
Sweatshirt	600	225
Nylon stocking	1000	90
Sweater	600	108
Cotton sheeting	600	90
Cotton upholstery	200	135
Cotton weave	600	135
Knit back from vinyl	400	90
Corduroy	1000	90
Brown paper	400	7.2
Suede	1000	13.5
Cotton back from oilcloth	800	54
Filter paper	600	13.5
Terrycloth	600	135
Scrapings	600	135

Table 2. Estimated quantity (ng) of DNA which was amplified per sample

Substrate	Extraction methods	
	Chelex	Centricon
White denim	60	56
Blue denim	40	22.5
Sweatshirt	60	56
Nylon stocking	100	22.5
Sweater	60	27
Cotton sheeting	60	22.5
Cotton upholstery	20	34
Cotton weave	60	34
Knit back from vinyl	40	22.5
Corduroy	100	22.5
Brown paper	40	1.8
Suede	100	3.4
Cotton back from oilcloth	80	13.5
Filter paper	60	3.4
Terrycloth	60	34
Scrapings	60	34

The amplification and typing results for the HLA-DQα gene from the DNA recovered by both extraction methods for each substrate together with the results of the combined methods performed on the selected substrates are listed in Table 3. In all samples with positive results, the HLA-DQα 1.1,4 was typed correctly.

Positive typing results were obtained from the Centricon extracts from nine substrates, with one being weak. Hematin has been suggested by deFranchis [9] and Walsh

Table 3. Amplification and typing results for the HLA-DQα gene from DNA extracted from bloodstains deposited on 16 substrates

Substrate	Extraction methods				
	Ch	Ce	Ch/Ce/Ch	Ch/Ce	Ce/Ch
White denim	–	–	+	–	+
Blue denim	–	–	ND	+	–
Sweatshirt	–	+	ND	+	ND
Nylon stocking	WK	+	ND	+	ND
Sweater	WK	+	ND	+	ND
Cotton sheeting	WK	+	ND	+	ND
Cotton upholstery	+	–	ND	ND	–
Cotton weave	+	–	ND	ND	–
Knit back from vinyl	+	WK	ND	ND	+
Corduroy	+	–	ND	ND	+
Brown paper	+	–	ND	ND	+
Suede	+	–	ND	ND	+
Cotton back from oilcloth	+	+	ND	ND	ND
Filter paper	+	+	ND	ND	ND
Terrycloth	+	+	ND	ND	ND
Scrapings	+	+	ND	ND	ND

Ch: Chelex extraction method; Ce: Centricon extraction method; Ch/Ce/Ch: Triple extraction – Chelex + Centricon + Chelex; Ch/Ce: Dual extraction – Chelex + Centricon; Ce/Ch: Dual extraction – Centricon + Chelex; WK: Weak; ND: Not done

et al. [10] to inhibit PCR amplification of DNA extracted from bloodstains. The removal of hematin should be accomplished by passing the aqueous phase of an organic extraction through a Centricon 100 tube. However, DNA extracted from the stains deposited on the cotton upholstery, blue and white denim, cotton weave, corduroy, brown paper and suede leather did not amplify or type.

It is recognized that quantity of DNA template may affect successful amplification and that 2 ng DNA is the recommended threshold quantity for the Amplitype kit. However, the results of this study show that the quantity of sample DNA is not always directly related to the success of amplification. For example, amplification was performed on approximately 34 ng DNA extracted from the scrapings, terrycloth, cotton upholstery and cotton weave. Positive results were obtained form the DNA isolated from the scrapings and terrycloth, while amplification of the DNA extracted from the cotton upholstery and cotton weave produced negative results.

The use of Chelex [11], an ion-exchange resin, has been suggested as a simple approach for the extraction of DNA from a small number of cells for PCR amplification and typing [12] by removing polyvalent iron cations associated with hematin. With the use of Chelex, amplification and typing were obtained for DNA derived from the majority of substrates tested with the exception of the sweatshirt and the blue and white denim fabrics. The negative amplification results obtained from the DNA isolated from these substrates does not appear to be solely a function of quantity for two reasons: other samples produced positive results with similar or smaller quantities of DNA, and the amount of template DNA used for amplification was 20- to 30-fold greater than the recommended threshold for the test.

A positive typing result was obtained from DNA isolated from the sweatshirt by the Centricon method while the DNA isolated by the Chelex method yielded a negative result. Subsequently, the Chelex extract was washed through a Centricon 100 tube and positive amplification and typing results were obtained.

DNA extracted by either the Chelex or the Centricon methods from the blue and white denim did not amplify or type. The Chelex extracts were washed through Centricon 100 tubes and the Centricon extracts were processed with Chelex. The DNA in the Chelex extract from the blue denim, when washed through a Centricon 100 tube, amplified and typed correctly while there was no amplification with the Centricon/Chelex approach. Conversely, the DNA in the Centricon extract from the white denim amplified and typed when processed with Chelex. The Chelex extract from the white denim did not amplify or type when washed through a Centricon 100 tube. However, when this Centricon retentate was processed subsequently with Chelex again (Chelex/Centricon/Chelex), typing results were obtained.

These results suggest that substances which inhibit amplification and typing of HLA-DQα could be extracted from the fabrics and, thus, co-purify with the DNA. Mark et al. [13] and Grayson [14] have detailed the numerous water-soluble compounds, both inorganic and organic, that are used in the manufacture of fibers and textiles. It may be necessary to remove some of these organic and inorganic substances from DNA extracts in order to obtain amplification. The Chelex and Centricon approaches, when used in tandem, appear to achieve this goal.

The amplification and typing results of the DNA isolated by the sequential extraction methods also suggest that the order in which the Chelex and Centricon techniques are used can have an impact upon obtaining results. However, the initial use of a Chelex extraction on those substrates that amplify and type with the Centricon/Chelex format produces no adverse effects.

The most practical and efficient approach to the extraction of DNA from bloodstains for PCR is to process the stain with Chelex and amplify the extracted DNA. If a negative result is obtained, the Chelex extract can then be washed through a Centricon 100 tube with TE prior to re-amplification of the extract. In the instances in which the second amplification produces a negative result, the Centricon extract may be processed again with Chelex and another attempt at amplification can be made.

In conclusion, this protocol is the first to attain the most desirable features of two extraction methodologies; safety, economy, efficiency and success. The elimination of the use of organic solvents increases safety. It is economical: Chelex is inexpensive (less than $0.01 per extraction) and Centricon 100 tubes (approximately $3.00 each) need only be used on selected samples. This approach is efficient since the entire triple extraction, amplification and typing scheme may be accomplished with-

in 2–3 days. Successful use of this procedure has been demonstrated on the variety of substrates described in this study as well as for preliminary data on DNA derived from chewing gum, cigarette butts, envelopes and stamps (manuscripts in preparation).

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