TECHNICAL NOTE

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A Method for the Purification and Recovery of Genomic DNA from an HLA DQA1 Amplification Product and Its Subsequent Amplification and Typing with the AmpliType® PM PCR Amplification and Typing Kit

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ABSTRACT: DNA from plucked single hairs from ten individuals was extracted by two different methods and subsequently amplified and typed using the AmpliType™ HLA DQ alpha Forensic DNA Amplification and Typing Kit. The remaining untyped portions of the DQA1 amplification products were stored refrigerated or frozen for two weeks and subsequently purified using Centricon™ 100 microconcentrators. Genomic DNA was recovered from the DQA1 amplification PCR and used again as a template for a subsequent multiplex PCR. Twenty μL of each retentate were amplified and typed with the AmpliType® PM PCR Amplification and Typing Kit. All typing results were consistent with DQA1 and PM results of control hairs and reference blood samples from the donors and all results were consistent with those obtained when the samples were typed solely for PM. The DQA1-Centricon™ 100-PM approach is useful when the genomic DNA from an evidentiary sample has been used completely for HLA-DQA1 typing, so that only the amplified product is remaining. The typing of five more genetic markers can be achieved from a HLA-DQA1 sample, so additional information for identification purposes could be provided. However, genomic DNA as well as the DQA1 product are recovered and the latter will also serve as a template in the subsequent PM amplifications. Therefore there will be more DQA1 product after the PM amplification than would be expected when only genomic DNA was used as a template. Thus certain practices should be considered when reading the types from PM probe strips if this DQA1-Centricon™ 100-PM approach is used.

KEYWORDS: forensic science polymerase chain reaction (PCR), DNA, genetic typing, human identification, HLA DQA1, ultrafiltration, Centricon™ microconcentrators, LDLR, GYPA, HBGG, D7S8, GC

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HLA-DQA1 typing using the AmpliType™ HLA DQ alpha Forensic DNA Amplification and Typing Kit (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) was the first PCR-based test applied to the analysis of DNA from evidentiary samples [*1,2*], including cigarette butts [*3*] and single hairs [*4*]. In the fall of 1993 the AmpliType® PM PCR Amplification and Typing Kit (Perkin Elmer; Roche Molecular Systems, Inc., Branchburg, NJ) was introduced as an additional commercially available human identity kit for forensic DNA casework analysis. The kit includes reagents that enable the simultaneous amplification of specific regions of six genetic loci. 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.

With the AmpliType™ HLA DQ alpha Forensic DNA Amplification and Typing Kit approximately 0.5–5 ng of genomic DNA is used for typing. Despite the discriminating power of this system applied to some populations is reported to be approximately 0.93 [*5,6*] it provides limited information for forensic casework when an inclusion is found. With the introduction of the AmpliType® PM PCR Amplification and Typing Kit information can now be obtained at six genetic loci simultaneously using essentially the same quantity of genomic DNA.

At times it could be desirable to obtain more information from a previously analyzed sample where the genomic DNA has been used completely for HLA-DQA1 typing and only some of the HLA DQA1 amplification product is remaining. This paper describes a method for the purification and recovery of genomic DNA from an HLA DQA1 amplification product and its subsequent amplification and typing with the AmpliType® PM PCR Amplification and Typing Kit.

Materials and Methods

Repetetive Study

DNA Extraction from Single Hairs and Reference Blood Samples—Two head hairs were plucked from each of ten individuals

and stored in plastic bags at room temperature for two weeks. Each hair had follicular sheath material attached. DNA was isolated from one centimeter of the root portion from ten hairs using method A and from ten hairs using method B (see below).

Method A (Organic extraction with Centricon™ 100 purification): Each hair was incubated over night 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 1000 μL water saturated n-butanol in order to remove traces of phenol. The aqueous phase was then transferred to a Centricon™ 100 microconcentrator tube [*13*] 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.

Method B (Organic extraction with ethanol precipitation): Incubation of the samples was performed as described above. Then the solution was extracted with 500 μL phenol-chloroform-isoamylalcohol (25:24:1). The aqueous phase was transferred to a new 1.5 mL tube, and the DNA was precipitated with cold 100% ethanol followed by a 70% ethanol wash as previously described [*15*]. The DNA was solubilized in 40 μL of sterile water.

In both methods ten percent of the retentate was used to determine the quantity of human DNA by slot blot analysis as described previously [*14*]. Samples containing no hairs served as reagent negative control samples.

Reference blood samples were collected from the ten donors by fingerprick. The blood was placed on cotton cloth, air dried and stored two weeks at ambient temperature prior to analysis. DNA was extracted and quantified by slot-blot analysis as described previously [*14,15*].

Amplification and Typing of the Extracted DNA—Two ng of DNA were amplified using the AmpliType™ HLA-DQ alpha and the AmpliType® PM PCR Amplification and Typing Kits in a Perkin Elmer Thermal Cyler 480 and typed according to the manufacturers protocols [*16,17*], except that each PCR contained 16 μg Bovine serum albumin (BSA, Sigma Cat. No. A 3350)/100 μL PCR rection mix. The AmpliType™ HLA-DQ alpha-, and the AmpliType® PM PCR Amplification and Typing Kit enable the amplification of the following loci: HLA DQA1 (previously referred to as HLA DQ alpha; PCR product size 242/239 bp) [*7*], Low Density Lipoprotein Receptor (LDLR; PCR product size 214 bp) [*8*], Glycophorin A (GYPA; PCR product size 190 bp) [*9*], Hemoglobin G Gammaglobin (HBGG; PCR product size 172 bp) [*10*], D7S8 (PCR product size 151 bp) [*11*], and Group Specific Component (GC; PCR product size 138 bp) [*12*]. A total of 60 amplification and typing reactions were carried out (that is, 10 reference blood samples and 20 head hair samples with each kit).

Purification and Recovery of Genomic DNA from HLA DQA1 Amplification Products/Subsequent Amplification and Typing with the AmpliType® PM PCR Amplification and Typing Kit—In accordance with the recommended protocol, 35 μL of the HLA-DQA1 product from the head hair samples and from the reference blood samples were used for typing. The remaining portion of each sample was stored refrigerated or frozen (4°C; −20°C or −80°C) for 2 weeks. Subsequently the solution was transferred to a Centricon™ 100 microconcentrator tube containing 2 mL sterile water. The sample reservoir was sealed with parafilm and the tube was subjected to centrifugation at 1000 g for 30 min. Two mL of sterile water were added to the retentate and the sample reservoir was sealed with new parafilm. The tube was centrifuged at 1000 g for 30 min or more (up to 60 min) in order to bring the final volume of the retentate to approximately 20 μL. The purified retentate containing the genomic DNA as well as the DQA1 product was recovered by back centrifugation at 1000 g for 5 min. The final sample volume was approximately 20 to 30 μL.

Twenty μL of each retentate were amplified in a 100 μL PCR in a Perkin Elmer Thermal Cyler 480 using the AmpliType® PM PCR Amplification and Typing Kit and typed for six loci according to the manufacturers protocols [*17*], except that each PCR contained 16 μg BSA/100 μL PCR rection mix. One Centricon™-100 tube contained no DNA and was washed with sterile water to serve as a negative control sample. In addition positive and negative amplification control samples were used, and 20 μL of each retentate were amplified and typed. Genomic DNA was thus recovered from HLA DQA1 amplification products from a total of 30 samples (that is, 10 reference blood samples and 20 head hair samples).

Evidentiary Sample

DNA Extraction—The evidentiary sample was a single head hair with attached follicular sheath material that was recovered from under the seat cover of a car seat (1993.93 Q1–2). According to the circumstances of the case, it was possible that this hair could have been deposited in the car by a female victim in September 1990. The evidence was recovered approximately two years after the crime. Several head hairs with attached follicular sheath material (1993.93 K1–5) were plucked from the female victim in September 1990. The evidentiary hair and the reference hairs were stored in paper envelopes at ambient temperature. The DNA from the questioned sample and a reference head hair was extracted in June 1993 using method A and quantified by slot-blot analysis. Nine ng of human DNA were recovered from the single hair from the car seat (1993.93 Q1–2) and twenty ng DNA from a reference head hair (1993.93 K1–5).

Amplification and Typing of the Extracted DNA Using the AmpliType™ HLA DQ Alpha Forensic DNA Amplification and Typing Kit—In June 1993 two ng of DNA from each sample were amplified at the HLA DQA1 locus as described above. From the amplification products 35 μL were used for typing. The remaining portion of each PCR was stored frozen (−80°C) for 6 months until January 1994. All control samples were processed in the same manner.

Purification and Recovery of Genomic DNA from the HLA DQA1 Amplification Products/Subsequent Amplification and Typing with the AmpliType® PM PCR Amplification and Typing Kit—In January 1994 each remaining portion of the HLA DQA1 amplification was purified in a Centricon™ 100 microconcentrator tube and the genomic DNA was recovered as described above. Subsequently, twenty μL of each retentate were amplified in a 100 μL PCR reaction and typed for six loci as described above.

Amplification and Typing of Original Sample DNA Using the

AmpliType® PM PCR Amplification and Typing Kit—In order to compare the results of recovered genomic DNA versus original genomic DNA, two ng of human DNA from the original sample from the single hair from the car seat (1993.93 Q1–2) and from the reference head hair (1993.93 K1–5) were amplified and typed as described above.

Results

Repetitive Study

From the twenty plucked single hairs 3.5 to 145 ng DNA (average 65 ng) were recovered using method A and 8.0 to 200 ng DNA (average 62 ng) using method B. The two extraction methods showed no significant difference in the DNA yield rate or in the amplification and typing success rate. All plucked hair and reference blood samples were successfully amplified and typed at the HLA DQA1 locus using the AmpliType™ HLA DQ alpha Forensic DNA Amplification and Typing Kit and at the LDLR, GYPA, HBGG, D7S8 and GC loci using the AmpliType® PM PCR Amplification and Typing Kit. The "C" dot was clearly visible on all HLA DQA1 strips and the "S" dot was visible on all PM strips. However, at times the "S" dot was very light. All plucked hairs showed the same typing results as the reference blood samples of the respective donors.

Genomic DNA was successfully recovered from all HLA DQA1 amplification products, and amplified, and typed at the LDLR, GYPA, HBGG, D7S8 and GC loci using the AmpliType® PM PCR Amplification and Typing Kit. When subjected to agarose gel electrophoresis, amplification products from the 30 samples were clearly visible and there was an excess of DQA1 product (data not shown). Because the DQA1 locus had been reamplified, the "S" dot on the strips from the DQA1-Centricon™ 100-PM approach was more intense in 26 out of 30 samples of the repetitive study, compared with the samples typed solely for PM. In these samples the intensity of the PM alleles was either lighter than or equivalent to the "S" dot.

All typing results were consistent with the typing results obtained from genomic DNA from the original samples and with the reference blood samples from the respective donors. No extraneous dots, negative results or allele dropouts were observed.

Evidentiary Samples

Nine ng of human DNA were recovered from the questioned sample and twenty ng of human DNA from a chosen reference head hair sample using extraction method A. Genomic DNA from both samples was successfully amplified and typed at the HLA DQA1 locus. The "C" dot was clearly visible on the HLA DQ alpha strips (data not shown). All controls typed correctly. The DQA1 type from the head hair recovered from the car was consistent with the victims reference head hair (1.1,4).

Genomic DNA was successfully recovered from both HLA DQA1 amplification products, and amplified, and typed at the LDLR, GYPA, HBGG, D7S8 and GC loci using the AmpliType® PM PCR Amplification and Typing Kit. When subjected to agarose gel electrophoresis, all six amplification products were clearly visible with an excess of DQA1 product (data not shown). The "S" dot was more prominent than the PM alleles in the evidentiary forensic K and Q samples (Fig. 1).

The genomic DNA from the original evidence and reference sample was successfully amplified and typed at the LDLR, GYPA, HBGG, D7S8 and GC loci using the AmpliType® PM PCR Amplification and Typing Kit. The "S" dot was much lighter than for those samples where the template DNA had been recovered from HLA DQA1 amplification products, but clearly visible (Fig. 2). All controls typed correctly. The head hair recovered from the car again showed the same typing results as the reference head hair (LDLR: AB, GYPA: AB, HBGG: AB, D7S8: BB, GC: CC).

Discussion

The typing of the HLA DQA1 locus has been shown to be a reliable tool for forensic casework [*18*]. However, the frequency of occurrence of a particular HLA DQA1 type might be as high as 1 in 7 [*18*]. Therefore, at times it may be desirable to obtain more information from previous analyzed samples whose genomic DNA has been completely consumed for amplification at the HLA DQA1 locus. Using the described approach the typing of five more genetic markers can be achieved from the very same DQA1 amplified DNA sample after a Centricon™ 100 purification step. This approach was successful on all samples of the repetitive study as well as on the evidentiary and reference sample. No extraneous dots, negative results or allele dropouts were observed.

Centricon™ 100 microconcentrators (100,000 molecular weight cutoff) provide an efficient manner to concentrate and purify small-volume solutions by ultrafiltration through a low-adsorption hydrophilic YM membrane. They are used to purify amplified DNA sequences by removing contaminating primers and dNTPs [*13*]. Therefore, salts, primers and dNTPs can be removed from the sample, while the amplified DQA1 product and the genomic DNA should be retained. When the retentate is subsequently subjected to PCR using the AmpliType® PM PCR Amplification and Typing Kit, six loci will be amplified. However, the DQA1 product also will serve as a template. Thus, there will be more DQA1 product after the PM amplification than would be expected when only genomic DNA was used as a template. The result is that the "S" dot on the PM strip (as a result of the DQA1 product hybridization) at times will be more intense than generally observed.

Comments Regarding the Interpretation of Typing Results Using the DQA1-Centricon™ 100-PM Approach

The "S" dot is generally used as a sensitivity indicator to set limits for avoiding stochastic effects during PCR. Typically, PM allele dots that appear either darker than or equivalent to the "S" dot are considered positive and PM allele dots that are lighter than the "S" dot are not interpreted. However, with the DQA1-Centricon™ 100-PM approach this rule does not apply. In our repetitive study and the evidentiary samples all typing results were consistent with the control samples. Thus, reliable PM results were obtained using genomic DNA recovered from HLA DQA1 amplification products, even when the "S" dot was more intense than the PM allele dots. However, certain practices should be considered to ensure reliable interpretation of the results. First, the original DQA1 result should be evaluated, a "C" dot should be visible and the DQA1 allele dots should be equal to or more intense than the "C" dot. This will address whether or not stochastic effects should be considered. Second, a post amplification product gel should be evaluated, all six amplification products should be detectable and the products from the five PM loci should be similiar in intensity to those from a PM typing using 1–2 nanogram of genomic DNA. The excess of DQA1 product should be visible as a stronger ethidium bromide stained band. Third, when the "S" dot is stronger than the PM allele dots, the PM allele dot intensities should be similar to PM results from a 1–2 nanogram genomic

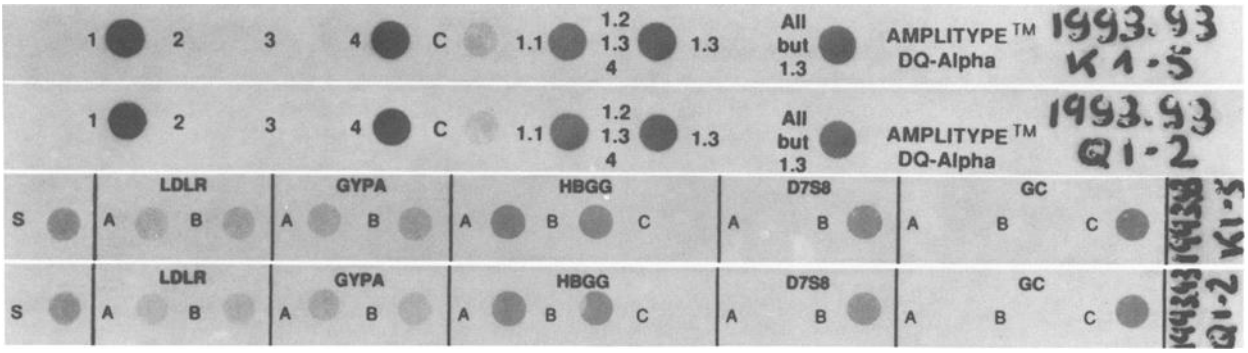

FIG. 1—*HLA DQA1 and Polymarker probe strips with the typing results of the genomic DNA recovered from the DQA1 amplification products of the evidentiary samples 1993.93 K1–5 and 1993.93 Q1–2. The "S" dots on the PM strips are more intense than those for the questioned sample in Fig. 2 (1993.93 Q1–2). The intensity of the PM alleles is either lighter than or equivalent to the "S" dot.*

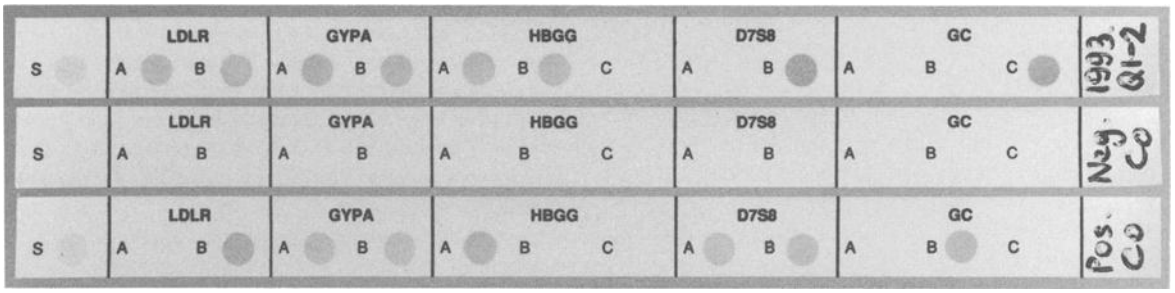

FIG. 2—*Polymarker probe strips with the typing results from the evidentiary sample DNA 1993.93 Q1–2 and control samples. Two ng of genomic DNA from the original sample were typed for PM. The "S" dot is visible and the intensity of the PM alleles is either darker than or equivalent to the "S" dot.*

DNA sample (the current sensitivity limit of the PM typing). A potential requirement for this approach is that the PM dots should be clearly visible on black and white photographs (Figs. 1 and 2) in order to be able to archive and present the typing result.

General Comments Regarding This Approach

Although this approach of recovery and typing genomic DNA from a previous PCR yields reliable results, it is not recommended that this technique be used routinely. Generally, the strategy for performing PCR-based analysis is to separate the PCR setup area from the PCR typing area. This way amplified products will not contaminate the Pre-PCR work area. With the DQA1-Centricon™ 100 method amplified products will be present in a Pre-PCR work area and special precautions will be necessary. Therefore, this approach should be used sparingly and only in special situations, otherwise PCR contamination may become prevalent.

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