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PCR Amplification and Typing of the HLA DQα Gene in Forensic Samples

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ABSTRACT: The polymerase chain reaction (PCR) was used to amplify the HLA DQα gene using DNA recovered from evidentiary samples. Amplified HLA DQα DNA was then typed using sequence-specific oligonucleotide probes. Slight modifications of previously published DNA extraction methods improved typing success of bloodstains and semen-containing material. Evidentiary samples, consisting of 206 known bloodstains, 26 questioned bloodstains, and 123 questioned semen-containing evidentiary materials were analyzed from 96 cases previously analyzed by restriction fragment length polymorphism (RFLP) typing in the FBI Laboratory. Of the known bloodstains, 98.5% yielded DQα typing results. Of the questioned samples, 102 of 149 (24/26 bloodstains and 78/123 semen-containing materials), or 68%, produced typing results. Of the 78 cases that were RFLP inclusions, 59 yielded interpretable DQα results and these were all inclusions. The remaining 19 cases could not be interpreted for DQα. Of the 18 RFLP exclusions, eleven were DQα exclusions, four were DQα inclusions, and three could not be interpreted for DQα. It is expected that because of the difference in discrimination potential of the two methods, some RFLP exclusions would be DQα inclusions. Some samples that failed to produce typing results may have had insufficient DNA for analysis. Employment of a human DNA quantification method in DQα casework would allow the user to more consistently use sufficient quantities of DNA for amplification. It also could provide a guide for determining if an inhibitor of PCR is present, thus suggesting the use of a procedure to improve amplification. This study provides support that the HLA DQα typing procedure is valid for typing forensic samples.

KEYWORDS: pathology and biology, PCR, gene typing procedures

DNA typing of evidentiary material from forensic cases has proven to be a very powerful means of associating or excluding biological evidence with victims, or suspects, or both, in criminal cases. The DNA analysis method that has been used by the FBI Laboratory, as well as by many other forensic laboratories, is restriction fragment length polymorphism (RFLP) typing using a panel of probes homologous with variable number of tandem repeat (VNTR) loci [*1–5*]. Although this approach has been highly successful for the analysis of biological evidence, other DNA typing methods are being examined in order to augment the DNA typing capabilities of forensic laboratories. Methods based on the use of the polymerase chain reaction (PCR) [*6,7*] offer the possibility of increased sen-

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sitivity, allowing analysis of evidentiary material containing less DNA than can be typed using the RFLP method, the analysis of degraded DNA, and increased speed of analysis. Additionally, PCR-based methods enable highly specific analyses and allow for the use of nonisotopic detection techniques.

One PCR-based method that is appropriate for forensic application is the HLA DQα reverse dot blot typing method [*8*]. It has undergone an extensive validation process and has been shown to be reliable on laboratory produced samples [*9–14*], as well as on evidentiary-type samples [*9,11,12,15,16*]. To evaluate the validity and reliability of the test on evidentiary materials, samples from 96 cases, previously analyzed by RFLP typing, were typed using the HLA DQα typing system. Only RFLP cases that were either inclusions at four VNTR loci or exclusions were analyzed for HLA DQα. This article describes the results of HLA DQα analysis of these evidentiary samples.

Materials and Methods

Samples

Cuttings were obtained from 96 cases previously analyzed by RFLP typing in the FBI Laboratory. These cases represent the typical range of cases received by the FBI Laboratory. Once received, the samples were refrigerated or frozen. The storage conditions of samples prior to receipt was unknown. These cases represented either four-locus inclusions (78 cases) or exclusions (18 cases). A total of 206 known bloodstains and 149 questioned samples were analyzed. The questioned samples included 26 bloodstains and 123 semen-containing evidentiary materials, which included 106 semen stains, six vaginal swabs, two vaginal washes, three rectal swabs, two labial swabbings (on gauze), one swabbing of external vaginal area (on gauze), and three cuttings from a condom. Cuttings (3 mm by 3 mm) were taken from stains, condoms, and gauze (three layers of gauze were taken) and used for DNA extractions. One-third portions of vaginal swabs were used for extractions. Stains were made on cotton cloth of the vaginal wash supernatant as well as the sediment, allowed to air dry, and 3 mm by 3 mm cuttings were taken from each. Of the 123 semen-containing questioned samples, 103 were subjected to a differential extraction procedure to separate nonsperm from sperm cells, giving a total of 252 typings of questioned samples (bloodstains and semen-containing materials). Subsequently, three semen stains, not initially subjected to a differential extraction, were extracted differentially, for a final total of 255 typings of questioned samples.

DNA Extraction

DNA was extracted from all samples using the Chelex extraction method [*13,17*] with slight modifications. For cases 1 through 6, all known samples (bloodstains) were extracted as described [*13*]. For cases 7 through 96, a presoak step was added in which the stain was soaked in 1 mL phosphate-buffered saline (PBS) for 15 to 30 min. Following this presoak, the tube containing the stain cutting was subjected to centrifugation in a microcentrifuge (Micro-Centrifuge Model 235C, Fisher Scientific, Pittsburgh, PA) for 1 min. The supernatant was discarded and 200 μL of 5% (w/v) Chelex 100 (BioRad Laboratories, Richmond, CA) was added to the tube containing the cutting. The remaining steps were performed as described [*13,17*]. Twenty semen-containing evidentiary stains in cases 1 through 49 were not extracted in a differential manner. These were from items of evidence other than vaginal swabs or panties. In cases 1 through 40, cuttings from these stains were placed in a tube containing 200 μL of 5% (w/v) Chelex 100 and vortexed briefly, 2 μL proteinase K (Sigma Chemical Company, St. Louis, Missouri) and 20 μL 0.39 M dithiothreitol (DTT, Sigma Chemical Company, St. Louis, Missouri) were added,

the samples were incubated at 56°C for 1 h and then boiled for 8 min. In cases 41 through 49, the addition of Chelex 100 was preceded by a 1 mL PBS presoak as described for bloodstain extractions. For semen-containing evidentiary material in cases 50 through 96, as well as all semen stains on panties and all vaginal swabs in all cases, the differential extraction protocol described [*17*] was performed with the following modifications. The vaginal or rectal swab or stain cutting was initially soaked in 1 mL of PBS instead of water. The initial cell lysis step and the Chelex 100 incubation step were performed at 56°C instead of 37°C. The sperm pellet was washed a total of five times with sperm wash buffer. The term "nonsperm fraction" will be used to describe the DNA extracted in the initial cell lysis step, and "sperm fraction" to describe the DNA from the second lysis step. Microscopic analyses of the fractions were not done.

Amplification and Typing

Extracted DNA samples were amplified using reagents from the AmpliType HLA DQα kit (Perkin-Elmer Corporation, Norwalk, CT) according to the manufacturer's instructions. For bloodstains, 20 μL portions of the Chelex extracts were amplified, and for all other samples, 30 μL portions were amplified. Hybridization of the amplified HLA DQα DNA to the typing strips, as well as color development of the strips, was done according to the manufacturer's instructions.

Centricon Washes

The 14 samples that yielded no typing results were further subjected to Centricon dialyses and concentration [*13*]. The remaining portion of the Chelex extract and 1.5 mL TE buffer (consisting of 10 mM tris(hydroxymethyl)aminomethane (Tris)/chlorine (C1) and 0.1 mM ethylenediaminetetraacetic acid (EDTA), at pH 8.0) were added to a Centricon 100^{R} device (Amicon, Danvers, MA). Following centrifugation of the device at approximately 1000 times g for 20 min., two 2 mL washes with TE were done. A 4 μl portion of approximately a 20 μL Centricon retentate was amplified.

Interpretation of Dot Blots

Results were categorized by judging the intensities of the typing dots (1 ,2, 3, and 4) and the subtyping dots (1.1, [1.2, 1.3, 4], 1.3, [All but 1.3]) relative to the C dot. The C dot is designed to be less intense than the other dots in an unmixed sample (with the exception of the All but 1.3 dot in [1.3, 4] and [4, 4] types). For classification in this study, if no dots were seen on a typing strip, a sample was classified as no result (NR). Samples that showed three or more alleles or samples in which typing and subtyping dots were apparent, but the C dots were not, were classified as inconclusive (inc). Otherwise, a sample was classified as having a positive DQα typing result.

Results and Discussion

DNA Extraction Methods

DNA was extracted from 12 known bloodstains from the first six cases as described [*13*]. No DQα typing results were obtained from four of the 12 extracts from known bloodstains. Because the possibility existed that an inhibitor in the extracts could be a cause of the failure to obtain results, a reduced quantity of the extracts (5 μL instead of 20 μL), and thus a reduced amount of a potential inhibitor, were then subjected to amplification and typing. One of the samples then yielded a result. Subsequently, new

cuttings were taken from the known bloodstains from the first six cases and subjected to either a water or a PBS soak prior to Chelex extraction. All of these samples yielded typing results. Consequently, a PBS presoak was performed on the remainder of the known bloodstains and all but two of the questioned bloodstains. One of these exceptions was a very light stain, and the other consisted of scrapings from an item of evidence. Both were successfully typed without presoaking.

Studies of DNA extractions from vaginal swabs, both postcoital vaginal swabs and vaginal swabs to which semen was applied, revealed that two slight modifications of the procedure reported by Walsh et al. [*17*] led to improved typing results. The first modification involved replacing the water presoak with a PBS presoak of the swab. This led to more intense typing dots in the nonsperm DNA fraction. Nonsperm cells may lyse in a water presoak, resulting in a loss of DNA prior to the Chelex extraction of the nonsperm fraction. The second modification involved using a total of 5, instead of 2 or 3, washes of the sperm pellet with sperm wash buffer. This generally yielded sperm fractions free of DNA containing the victim's DQα alleles and gave rise to cleaner typing results without significantly increasing the labor or time of the extraction procedure. This procedure was used for all differential extractions reported in this study.

Semen stains from items of evidence other than vaginal swabs, panties, or other items that would have been in close contact with the vaginal area were not extracted differentially in cases 1 through 49. Four semen stains from among cases 1 through 40 that failed to give typing results were re-extracted using a PBS presoak. Three of the four then yielded typing results. Thus, the PBS presoak step was added to nondifferential semen stain extractions in cases 41 through 49. Some of the semen stains from cases 1 through 49 that were not differentially extracted contained DQα alleles shared by the victim. Thus, all semen stains were extracted differentially for cases 50 through 96.

Results of Known Bloodstains

Of the 206 known bloodstains analyzed, 198 gave DQα results on the first attempt (96%). Four of the eight stains that did not give DQα results were from cases 1 through 6; three of these were re-extracted using the PBS presoak and subsequently gave typing results. The fourth was not retested because it was from a suspect who was also a suspect in another case and typing results were already obtained for that individual. Another sample that initially did not give a typing result (initially extracted using a PBS presoak) subsequently yielded results when a new cutting was taken and extracted exactly as it had been on the first attempt. Another sample subsequently yielded results when the Chelex extract was subjected to Centricon dialysis. The two remaining samples that initially gave no typing results were stains derived from the serum portion of whole blood and were the only samples available for testing. Thus, 203 of 206 known bloodstains (98.5 percent) yielded typing results.

Results of Questioned Samples

A total of 149 questioned samples (26 bloodstains and 123 semen stains) from the 96 cases were analyzed. Initially, 89 of the 149 questioned samples produced DQα typing results. Twenty-eight questioned samples that initially failed to produce a DQα typing result were reanalyzed (either a smaller quantity of extract was amplified, a new extraction was performed using PBS presoak, a new extraction was performed with no modifications, or a Centricon dialysis of the Chelex extract was performed. The remaining 32 samples that yielded no typing result were not reanalyzed. Reanalyses involving the first three methods listed above were done early in the study prior to regular use of PBS presoaks. Reanalyses involving Centricon dialyses were done later in the study after it was learned

that this method could improve amplification success). Thirteen of these retests subsequently yielded typing results. Table 1 lists the samples tested by type and categorizes the DQα results into four groups (see Materials and Methods for description of result classification): (1) a positive result, either for both fractions if sample was differentially extracted or for the only fraction if not differentially extracted; (2) a positive result on the sperm fraction of a differentially extracted sample and an inconclusive or negative result on the nonsperm fraction; (3) a positive result on the nonsperm fraction of a differentially extracted sample and an inconclusive or negative result on the sperm fraction; and (4) inconclusive or negative results on both fractions of a differentially extracted sample or on the only fraction if not differentially extracted. The results were characterized in this way because generally it is the sperm fraction of a semen stain that is the probative evidence in a case. After retests, a total of 102 of the 149 questioned samples (68.4%) produced DQα typing results for nondifferentially extracted samples or for the sperm fraction or both fractions of differentially extracted samples.

Comparison of RFLP and DQα Results

This study was designed to evaluate the validity of the DQα amplification and typing system on evidentiary material by determining whether interpretations were consistent between the two test methods. The cases selected for this study had samples that had sufficient DNA to yield RFLP results. Thus, the DQα results cannot be interpreted as being predictive of the success rate of the DQα typing system on samples with amounts of DNA less than that necessary to produce RFLP results.

It might be expected that because these cases had sufficient DNA to yield RFLP results, the samples should have enough DNA for DQα typing (2 ng of genomic DNA can readily be typed using this system). However, DQα typing results were not obtained from all questioned samples from which RFLP results had previously been obtained. Of 186 samples that gave RFLP results, 28 did not yield DQα results. Two explanations can account for this. First, there may have been insufficient DNA remaining for DQα typing. Because the best portions of the stains were used for RFLP analysis, the remnants of stains extracted for DQα typing may not have had the same proportions or quantities of female epithelial cells and sperm cells. In effect, these samples were slightly different evidentiary samples than those used for RFLP analysis. Second, sufficient DNA may have been extracted from the cuttings, but the extract may have contained an inhibitor of PCR. Because there was some success in obtaining DQα typing results following reextraction of stains or by subjecting Chelex extracts to Centricon washes, failure to obtain DQα results for some samples may have been caused by PCR inhibition. Knowledge of the amount of DNA present in a sample prior to PCR amplification would help to distinguish among possible causes for failure to obtain typing results. A DNA quantification method, such as slot blot hybridization of sample extracts with a human alphoid DNA probe [*18*], is recommended for use in casework to allow the user to ensure that at least 2 ng of genomic DNA are subjected to amplification. If there is insufficient sample DNA, it would be known in advance that DQα typing would be fruitless. Failure to obtain typing results using sufficient DNA, suggesting the possible presence of an inhibitor of PCR, would then suggest use of a procedure to attempt to effect amplification, such as Centricon washes of Chelex extracts [*13*], Chelex extraction of these Centricon washes [*13*], or addition of bovine serum albumin to the amplification reaction [*11,19*]. The use of slot blot quantification, along with these methods for improving amplification, could improve the overall success of amplifying forensic samples as well as laboratory efficiency since analysis of samples shown to have insufficient DNA will be terminated.

A total of 74 of the 96 cases (77%) had sufficient information from DQα results to interpret whether questioned samples could be associated with known samples. Table 2

TABLE 1—*DQα results of 149 questioned samples.*

Result	Vaginal swab	Semen stain		Vaginal wash	Rectal swab	Vaginal swabbing	Labial swabbing	Condom	Bloodstain	Totals
		Non-diff	Diff							
+ both fxn/ only fxn	3	8	28	1		1	2	2	24	69
inc/nr ns + s	1		31		1					33
+ ns inc/nr s	2		13		2			1		18
inc/nr both/ only fxn		9	17	1					2	29
Totals	6	17	89	2	3	1	2	3	26	149

Calls on 149 Q samples: 102/149 = 68.4%.
fxn = DNA extract fraction.
ns = non-sperm fraction of a differentially extracted sample.
s = sperm fraction of a differentially extracted sample.
inc = inconclusive DQα result.
nr = no DQα result.
+ = DQα result.

TABLE 2—*Calls on cases.*

DQα Results	RFLP Inclusions (78)	RFLP Exclusions (18)
DQα Inclusion	59	4
DQα Exclusion	0	11
DQα Inconclusive	5	0
DQα No Result	14	3

shows the number of interpretations that could be made on cases based on the DQα results. There were no inconsistencies in interpretations when profiles were obtained for both RFLP and DQα, even though different cuttings were analyzed. Of the 78 RFLP matches, 59 could be interpreted for DQα. All of these were DQα inclusions. Of the 18 RFLP exclusions, 15 could be interpreted for DQα. All DQα exclusions were RFLP exclusions. Four of the 15 RFLP exclusions that could be interpreted for DQα, however, were DQα inclusions. Given the relative discrimination potential of the two methods (the probability of discrimination for DQα is in the range of 0.90, varying slightly among different population groups), it is to be expected that some DQα inclusions would be RFLP exclusions.

Selected Cases

Selected cases that are informative as examples are detailed as follows. The typing results from these cases are shown in Fig. 1.

Inclusion (Case 48)—In this rape case, cuttings from three different condoms were subjected to differential extraction and DQα analysis. These samples demonstrate the differences which can be seen in the sperm and nonsperm fractions of differentially extracted specimens. In sample 48-1, the nonsperm fraction (sample 48-1F) is consistent with the victim's profile (DQα type 1.1, 4, sample 48-4) and also shows the presence of a 3 allele, consistent with the suspect's profile (DQα type 1.2, 3, sample 48-5). The presence of the victim's 4 allele masks the possible presence of the suspect's 1.2 allele. The sperm fraction (sample 48-1M) is consistent with the suspect's profile (sample 48-5). In sample 48-2, both fractions (samples 48-2F and 48-2M) are consistent with the suspect's profile. The DQα alleles consistent with the suspect in the nonsperm fraction of sample 48-2 (sample 48-2F) could have arisen from sperm that have lysed prior to DNA extraction or from nonsperm DNA arising from the perpetrator. In sample 48-3, the nonsperm fraction (sample 48-3F) is consistent with the victim's profile while no result was obtained for the sperm fraction (sample 48-3M).

Exclusion (Case 42)—Two questioned samples were typed for DQα in this rape/homicide case, a semen stain on a bathrobe, which was not differentially extracted (sample 42-1), and a vaginal swab, which was differentially extracted (sample 42-2). The semen stain (sample 42-1) exhibits 3 DQα alleles, 2, 3, and 4. The 2 and 4 alleles are consistent with the victim's DNA (DQα type 2, 4, sample 42-3), but the 3 allele is not consistent with either the suspect's (DQα type 1.2, 4, sample 42-4) or victim's DNA. The differentially extracted vaginal swab (sample 42-2) shows cleanly separated sperm and nonsperm fractions, showing a pattern consistent with the victim's in the nonsperm fraction (sample 42-2F), and a 3, 4 pattern in the sperm fraction (sample 42-2M). Note that the DQα type of the contributor to the nondifferentially extracted semen stain (sample 42-1, DQα alleles 2, 3, and 4) which is not consistent with the victim cannot be unambiguously

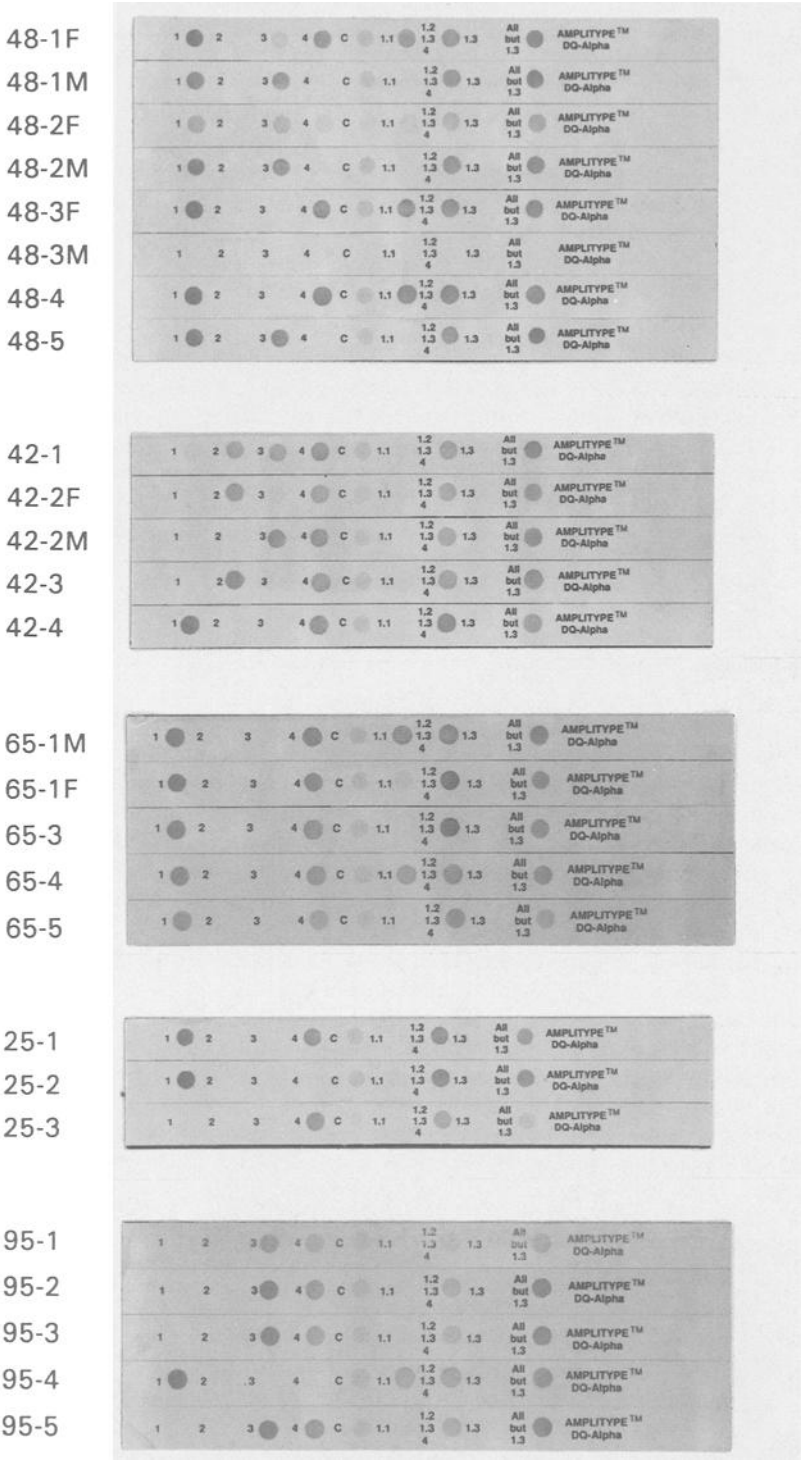

FIG. 1—*DQα typing results from forensic cases.*

determined (if there is only one other contributor, the DQα type could be [2, 3], [3, 3], or [3, 4]).

Masked 1.2 allele (Case 65)—In this rape case, where there are two suspects, the victim and one suspect share the same DQα types (1.2, 4; samples 65-3 and 65-5, respectively), while the other suspect (sample 65-4) is DQα type 1.1, 4. The sperm fraction from the semen stain on a pair of panties (sample 65-1) shows the 1.1 and 4 alleles, which is consistent with one suspect (sample 65-4). However, since a 1.2 allele could be present in the sperm fraction of the questioned sample (sample 65-1M) and masked by the 4 allele, the other suspect (sample 65-6) cannot be excluded as a contributor.

Mixed sample (Case 25)—This case is a rape/homicide in which a semen stain found on a bed sheet was analyzed. The semen stain (sample 25-1) was not differentially extracted and exhibits the 1.2 and 4 alleles. The victim's DQα type is a 1.2, 1.2 (sample 25-2), while the suspects's type is a 4, 4 (sample 25-3). The presence of the 1.2 allele in the semen stain is consistent with the victim's DNA, while the presence of the 4 allele includes the suspect as being a potential contributor. Differential extraction of the stain could have helped to clarify the results. However, RFLP analysis excluded this suspect as a contributor to this sample.

Variations in dot intensities (Case 95)—In this murder case, DQα results were obtained for three bloodstains found at the crime scene. The suspect (DQα type 3, 4, sample 95-5) is included as a contributor of the questioned bloodstains (samples 95-1, 95-2, 95-3). It is interesting to note that in the questioned bloodstains, as well as in the known sample from the suspect, the 3 dot is more intense than the 4 dot. At times the 3 dot in heterozygotes is fainter than the 1, 2, or 4 dot [20]. The identified subtypes of the 4 allele would not account for the 4 dot being lighter, as they each completely match the 4 probe. Additional causes for the observed intensity variations may be due to variations in probe strip lots which affect the relative intensities of the dots, or, possibly, the 4 allele in these particular samples may contain a base pair mismatch with the 4 probe which could decrease hybridization to the probe. Whatever the cause, the intensity variations are consistent with the strips showing the suspect's type and the strips showing the questioned samples' types.

General Comments Regarding Variations in Dot Intensities

It was apparent from the analysis of the DQα typing strips that variations in dot intensities exist, even in known (unmixed) samples. The "All but 1.3" dot is considerably less intense in 1.3, 4, and 4, 4 samples, because this probe has a 1 base pair mismatch with the 4 allele [*20*]. The variation also is present in other types, apparently depending on whether one or both alleles of a heterozygote perfectly match this probe. For example, the All but 1.3 dot can be lighter in a 3, 4 than in a 1.1, 2 on blots where the nominal probe typing dots (probes 1 to 4) are of equivalent intensities. Variation also is evident in the 1.2, 1.3, 4 dot, perhaps also dependent on whether one or both alleles in a sample are homologous to the probe. Additionally, the 1.2, 1.3, 4 dot can be lighter if a sample has a 4.2 or 4.3 allele rather than the more common 4.1 allele because the probe has a single base pair mismatch with the 4.2 and 4.3 alleles [*20*]. In contrast, the 4 probe is equally homologous to all 3 of the 4 subtypes [*8,20*]. Although information provided with the typing kit suggests the 3 probe can be weaker than the other typing probes in heterozygotes, variation in relative intensity also was observed. Sometimes the 3 was slightly less intense, sometimes equal, and sometimes slightly more intense than the other nominal allele probes in a heterozygote (known sample). It is important to note, however, that these variations do not prevent interpretation of a DQα type but rather indicate

that care must be taken when evaluating dot intensities as a means of determining whether or not a sample may be a mixture.

Conclusions

This study provides additional support that the HLA DQα typing procedure is a valid procedure for typing forensic samples. All interpretations for cases were compatible with interpretations using the RFLP procedure. Although most samples amplify and type with a standard extraction procedure, use of procedures to improve amplification can help increase the success rate [*11*,*13*]. Employment of a human DNA quantification method will allow the user to amplify sufficient quantities of DNA and provide a guide for possibly determining whether an inhibitor may be present.

Care must be taken when interpreting DQα typing results and determining the statistical weight of an inclusion. When determining the statistical weight of an inclusion, the proper legal question is "what is the likelihood that someone other than the defendant could have left the crime scene sample?" [*21*]. The frequency of occurrence of the DQα type should be based on all available relevant, general databases (for example, Caucasians, African Americans, Southeastern Hispanics, and Southwestern Hispanics), and the jury can make a decision regarding the most pertinent database(s) and values based on all relevant information pertaining to a case [*21*]. There are two ways of interpreting the evidence: (1) the evidentiary type was derived from one individual, or (2) the evidentiary type was derived from a mixture of two or more individuals. If there is no information indicating that more than one probative contributor exists, the frequency calculation for a DQα typing pattern is in most casework a straightforward frequency taken directly from the genotype frequencies of the population databases. In some evidentiary samples there may be an expected or known mixture of contributors who cannot be separated and for these samples the frequency estimate for all possible contributors can be summed. These interpretational issues are not matters for consideration in an admissibility hearing, but rather these are issues for a jury to decide based on the evidence presented in a trial.

The HLA DQα typing system has been shown to be a valid and reliable approach for analysis of biological evidence. It is anticipated that the increased sensitivity of the DQα test will provide results in some situations in which there is insufficient DNA for RFLP analysis. With proper care in interpretation, the HLA-DQα typing system should serve well as an adjunct to other DNA typing methods.

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