TECHNICAL NOTE

Ann Marie Gross,[1] M.S. and Richard A. Guerrieri,[2] M.S.

HLA DQA1 and Polymarker Validations for Forensic Casework: Standard Specimens, Reproducibility, and Mixed Specimens

REFERENCE: Gross AM, Guerrieri RA. HLA DQA1 and Polymarker validations for forensic casework: Standard specimens, reproducibility, and mixed specimens. J Forensic Sci 1996;41(6): 1022–1026.

ABSTRACT: This study describes the testing performed by the Minnesota Forensic Science Laboratory (MFSL) to validate the Amplitype® DQA1 and Amplitype® Polymarker (PM) PCR Amplification and Typing Kits before implementation for casework.

All studies were based on the analysis of mock forensic case samples, which were assembled from various biological samples from individuals at the MFSL.

To address the validation of standard specimens, DNA was isolated from semen, vaginal secretions, saliva, urine, and blood samples. Typing results from all tissues from a particular individual yielded the same typing results using both the DQA1 and PM systems.

Reproducibility between laboratories was evaluated by having duplicate samples analyzed by a second laboratory. The Roche Biomedical Laboratories (RBL) were sent a duplicate set of mock cases and all analyses including extraction, quantitation, amplification, and typing were performed at the RBL using their established testing procedures. All typing results for both laboratories, from the approximate 30 single source samples analyzed, were in agreement.

Mixed specimens were evaluated by examining the results obtained from semen/vaginal, semen/saliva, semen/blood, semen/urine, and semen/vaginal/blood mixtures. All typing results of these mixtures for both laboratories were in agreement. It was determined that by incorporating a wash step of the sperm cell pellet, a complete separation of the nonsperm cell fraction was more likely to be attained.

After completing the above studies, as well as population studies, environmental insult studies, and proficiency testing, the MFSL determined that both kits were suitable for use on forensic casework.

KEYWORDS: forensic science, DNA, polymerase chain reaction, validation, HLA-DQA1, Amplitype PM, LDLR, GYPA, D7S8, HBGG, GC

The validation of DNA analytical procedures is essential to ensure that testing procedures perform appropriately under forensic casework situations. Guidelines for the validation of DNA analysis procedures have been recommended by the Technical Working Group on DNA Analysis Methods (TWGDAM) (1). Powerful DNA typing systems utilizing the polymerase chain reaction (PCR) based technology have been developed by Roche Molecular Systems. The first system, the Amplitype® HLA DQA1 PCR Amplification and Typing Kit, has been validated by many investigators (2–7), and is well accepted in the forensic arena. The second system, the Amplitype Polymarker (PM) PCR Amplification and Typing Kit, provides for the typing of five sequence variable genetic markers; Low Density Lipoprotein Receptor (LDLR); Glycophorin A (GYPA); Hemoglobin G Gammaglobin (HBGG); D7S8; and Group Specific Component (GC) (8,9).

Validation studies were designed to compare matches obtained using the single system HLA DQA1 typing method with matches obtained by the multiple loci PM system. Restriction fragment length polymorphism (RFLP) data were also used to help evaluate interpretation of mixed stains.

Two laboratories, the Minnesota Forensic Science Laboratory (MFSL), St. Paul, Minnesota and the Roche Biomedical Laboratory (RBL), Research Triangle Park, North Carolina, analyzed samples to evaluate the following criteria: (a) Comparison of typings obtained from semen, vaginal epithelial cells, saliva, and urine to typings obtained from corresponding known bloods. (b) Concordance studies between the two laboratories. (c) Evaluation of extraction methods used for mixed stains. (d) Comparison of data obtained using the DQA1 and PM kits to the corresponding RFLP results. The results from these validation studies will show that both the DQA1 kit and the PM kit are suitable for the typing of forensic casework samples.

Materials and Methods

Sample Set-up

Four duplicate sets consisting of nine simulated sexual assault cases were prepared at the MFSL. The cases were prepared in such a manner as to mimic actual casework samples. The set-up of each mock case is summarized in Table 1. Three sets of cases were analyzed by the MFSL and one set of cases was analyzed by RBL. A summary of the extraction methods and loci analyzed is shown in Table 2.

DNA Extraction Methods

The DNA was extracted by four different methods which have been previously described (10–13). Modifications to these methods are listed below.

[1]Forensic scientist, Minnesota Forensic Science Laboratory, 1246 University Ave, St. Paul, Minnesota.

[2]Assistant director of the Forensic Identity Laboratory, Roche Biomedical Laboratories, Research Triangle Park, North Carolina (currently employed as a Forensic DNA Examiner, FBI Laboratory, Washington, DC).

Received for publication 17 Nov. 1995; revised manuscript received 16 Feb. 1996; accepted for publication 27 March 1996.

Copyright © 1996 by ASTM International

TABLE 1—*Summary of the controls and samples used in the set-up of nine mock forensic cases. Each set consisted of the nine cases listed below. Three sets were analyzed by the MFSL and one set was analyzed by the RBL.*

Case #	Controls	Swab #1	Swab #2	Swab #3	Swab #4
1	B_1, B_2	V_2	VM_2	$V_2 + S_1$	S_1
2	B_1, B_2	V_2	VM_2S_1†	$O_2 + S_1 + B_2$	S_1
3	B_1, B_2	V_2	$VM_2 + S_1$	$O_2 + S_1$	S_1
4	B_1, B_2	V_2	I	X	S_1
5	B_1, B_2	V_2	I	$O_2 + S_1$	S_1
6	B_1, B_2, B_3	$V_2 + S_1$	$VM_2 + S_1$	$V_3 + S_1$	—
7	B_1, B_2, B_3	A_1	U_2	$A_2 + S_3$	$U_2 + S_3$
8	B_1, B_2, B_3	$A_2 + S_1 + S_3$	$A_2 + S_1 + S_3$	$A_2 + S_1 + S_3$	—
9	B_1, B_2	S_2^*	S_2^*	S_1^*	—

B = Blood.
S = Semen.
S* = Semen from vasectomized individual.
V = Semen free vaginal swab.
VM = Semen free vaginal swab taken during menstrual cycle.
I = Vaginal swab after vaginal intercourse.
O = Oral swab.
X = Oral swab after oral sex.
A = Semen free anal swab.
U = Urine.

TABLE 2—*Summary of extraction methods and the loci examined by the Minnesota Forensic Science Laboratory (MFSL) and by the Roche Biomedical Laboratories (RBL), in the analysis of four duplicate sets of nine simulated sexual assault cases.*

		Extraction Method		Loci Examined		
Set	Lab	Organic	Chelex	DQA1	PM	RFLP
I	MFSL	X		X		X
II	MFSL		X	X		
III	MFSL	X			X	
IV	RBL	X		X	X	X

Method I—MFSL Organic Extraction—The DNA from the samples of Set I was organically extracted (10). For the differential extraction of the swabs, once the nonsperm cell fraction was removed, the swab material was placed back in the tube with the sperm cell pellet.

Method II—MFSL Chelex 100® Extraction—The DNA from the samples of Set II was extracted using Chelex 100® (11,12). The pellets were washed three to four times with 0.5 mL of sperm wash buffer (10mM Tris, 10mM EDTA, 50mM NaCl, 2% SDS, pH 7.5).

Method III—MFSL Modified Organic Extraction—The DNA from the samples of Set III was organically extracted as described in Method I except for the following modifications. During the initial incubation period, tubes were sonicated for 15 to 20 s to facilitate the removal of cells from the material. Using the piggy-back centrifugation technique (10), the swab material was removed. The sperm and nonsperm cell fractions were then separated. The sperm cell pellets were washed two to three times with 0.5 mL of sperm wash buffer. The sperm cell pellets, the swab material, and the nonsperm cell fraction were carried through the remainder of the extraction procedure separately.

Method IV—RBL Organic Extraction—The DNA from the samples of Set IV was organically extracted followed by purification using Centricon® 100 filters (13). Following three to five washes of the sperm cell pellets with TNE (10mM Tris, 100mM NaCl, 1.0mM EDTA), the swabs were placed back in the tube for the remainder of the extraction.

DNA Quantitation

The extracted DNA was quantitated by slot blot hybridization with the primate-specific probe p17H8 (locus D17Z1) previously described by Waye et al. (14). DNA extracted by Method I was quantitated using a radiolabeled (^{32}P) probe (14). DNA extracted by Method II was quantitated using the commercially available ACES™ Human Quantitation Kit (Gibco BRL, Bethesda, MD) (15). DNA extracted by Methods III and IV was quantitated using the commercially available Quantiblot Kit™ (Perkin Elmer, Emeryville, CA) (16).

HLA DQA1 and Polymarker Amplification

DNA was amplified using the AmpliType HLA DQA1 and AmpliType PM PCR Amplification and Typing Kits commercially available from Perkin Elmer. Manufacturer's recommended guidelines were followed. Approximately 5 ng of DNA were amplified. A Thermal Cycler 480 and Geneamp® PCR System 9600 were used at the MFSL for DQA1 and PM amplification respectively. A Thermal Cycler 1 (TC1) was used at the RBL.

HLA DQA1 and Polymarker Amplification Verification

An evaluation of the DQA1 PCR product was performed by analyzing 10 μL of each sample on a 10 by 16 cm, 2% agarose gel prepared with 1X Tris · Acetate · EDTA (TAE)(0.04 M Tris · Acetate, 0.001 M Ethylenediaminetetraacetic acid) containing ethidium bromide (1 μg/mL). Sizing markers made up of DNA fragments in the size range of 50 to 1000 bp (US Biochemical Corporation, Cleveland, OH) or in the size range of 100 to 1500 bp (Gibco BRL) were loaded into the outer wells of each gel. Electrophoresis proceeded at 200 V for 30 min in 1X TAE buffer. The PCR products were examined under ultraviolet light. Photographs were taken for permanent record. An evaluation of the PM

PCR product was performed as described above except that 6 μL of each sample was loaded on a 4% gel containing the same buffer and size markers.

HLA DQA1 and Polymarker Hybridization and Typing

Amplified DNA samples were hybridized to strips containing the allele-specific oligonucleotide probes using the reverse dot blot procedure as outlined in the inserts of the AmpliType HLA DQA1 and Amplitype PM PCR Amplification and Typing Kits. Wet strips were wrapped in Glad® Cling Wrap and photographed. The photographs were compared with the strips to ensure that all dots visible on the strips were also visible on the photograph. Interpretation of the DQA1 and PM alleles were made by reading the pattern of dots on the photograph as described in the AmpliType Users Guide and the AmpliType PM PCR Amplification and Typing Kit insert.

DQA1 and Polymarker Results

Three identical sets of nine mock sexual assault cases were evaluated using DQA1 typing utilizing Extraction Methods I, II, and IV to extract one set of samples each. Two identical sets of nine mock sexual assault cases were evaluated using PM typing using Extraction Methods III and IV to extract one set of samples each.

Bloodstains—Extracts from three sets of 21 bloodstains were typed using DQA1 and extracts from two sets of 21 bloodstains were typed using PM. The DQA1 and PM typing results obtained for all corresponding samples were the same.

Semen (neat)—Extracts from three sets of five semen containing swabs were typed using DQA1 and extracts from two sets of five semen containing swabs were typed using PM, and the results compared with DQA1 and PM results from corresponding known bloodstains. No discrepancies between DQA1 and PM types obtained from the semen and blood were observed for any of the samples. Both the sperm and nonsperm cell fractions of the swabs were typed. DQA1 types were detected in both fractions for 13 of the 15 swabs. No DNA was obtained in the nonsperm cell fraction of the remaining two swabs. PM types were detected in both fractions for all of the swabs.

Extracts from three sets of three swabs containing seminal fluid from vasectomized males were typed using DQA1 and extracts from two sets of three swabs typed using PM, and the results compared with DQA1 and PM results from corresponding known bloodstains. Sufficient DNA for amplification was obtained from the nonsperm cell fraction for seven of the nine swabs for DQA1 and for five of the six swabs for PM. No discrepancies between DQA1 and PM types obtained from the semen and blood were observed for any of the samples that contained sufficient DNA for amplification. No DNA was obtained from the sperm cell fraction of any of the swabs.

Vaginal (neat)—Extracts from three sets of six vaginal swabs were typed using DQA1 and two sets of six vaginal swabs were typed using PM and results compared with DQA1 and PM results from corresponding known bloodstains. No discrepancies between DQA1 and PM types obtained from the vaginal secretions and blood were observed for any of the samples. For DQA1, DNA was not obtained from one swab because of tube failure.

Mixed Stains—DQA1 and PM—Extracts from 12 mixed stains were typed and compared with DQA1 and PM results obtained from corresponding known bloodstains. The mixtures contained various combinations of vaginal secretions, semen, blood, and saliva. The performance of the differential extraction procedures on these 12 mixed stain samples is summarized in Table 3. The fractions were graded using the following procedure: (a) "Clean" indicates there was no carry over of sperm cell or nonsperm cell to their respective fractions. (b) "Not Determinable" indicates that the allele combination of the components of the mixture did not give rise to being able to detect mixtures.

Mixed Stains—DQA1—In addition to the 12 mixtures described above, the following mixtures were also evaluated. One anal swab was extracted by Methods I, II and IV, however, insufficient DNA was obtained for amplification. Four anal swabs were spiked with semen and analyzed using DQA1 typing. Results were obtained for three of the four swabs (sperm fraction) extracted by Method IV. No typing results from the semen on the anal swabs were obtained using Method I or Method II, although sufficient DNA was obtained for all eight samples. The DNA extracted using Method II (MFSL chelex) was subjected to a Centricon 100 clean-up, (3,17,18) as well as addition of bovine serum albumin (BSA) (3,5,18–20), however, the samples failed to amplify. Increasing the amount of Taq may have overcome the inhibition, but this strategy was not attempted. The entire extract from Method I was consumed in the initial amplification, therefore, no additional treatment could be performed on these samples. Three additional anal swabs spiked with semen were extracted using the MFSL modified organic procedure. Initial amplification failed, however, results were obtained for all three swabs after the samples were subjected to a Centricon 100 clean-up. All typing results obtained corresponded to the donor of the semen on the swab and not from the donor of the fecal material.

For the swabs that had urine applied to them and utilizing DQA1 typing, a very weak result (no "C" dot) for the nonsperm cell fraction was obtained for the DNA extracted by Method I. Insufficient DNA was obtained by Methods II and IV. For the swabs with urine and semen on them, DQA1 typing results for the semen were obtained by Methods I, II and IV. No typing results were indicated to have been contributed by the urine on the swabs. For PM typing, one anal swab and one urine containing swab were extracted by Methods III and IV. Slot blot analysis indicated that no DNA was recovered, so amplification was not performed.

Extracts from two sets of four anal swabs to which semen had been applied were typed and compared with PM results obtained from corresponding known bloodstains. PM results were obtained for two of the four swabs extracted by Method III. The PM results were consistent with having come from the semen applied to the swab and not from the epithelial cells from whom the swab was taken. PM typing results were obtained for three of the four swabs extracted by Method IV. Again, PM results corresponded to the semen applied to the swab and not from the epithelial cells from whom the swab was taken.

The extract from the one swab to which urine and semen had been applied was typed and compared with PM results obtained from the corresponding bloodstains. PM typing results were obtained which corresponded to the donor of the semen on the swab and not from the donor of the urine on the swab.

TABLE 3—*The comparison of three different differential extraction methods to resolve mixtures when using DQA1 typing and the comparison of two different differential extraction methods to resolve mixtures when using PM typing.*

	DQA1 Typing			PM Typing	
	Method I	Method II	Method IV	Method III	Method IV
Mixture in nonsperm fraction	7/12	5/12	3/12	11/12	5/12
"Clean" nonsperm fraction‡	0/12	2/12	4/12	0/12	6/12
Not Determinable§	5/12	5/12	5/12	1/12	1/12
Mixture in sperm fraction	9/12*	0/12	3/12†	0/12	2/12
"Clean" sperm fraction	1/12	11/12	9/12	12/12	9/12
No results	2/12	1/12	0/12	0/12	1/12

*Different dot intensities were noted on four of the nine strips.
†Different dot intensities were noted on two of the three strips.
‡"Clean" indicates there was not any carry over of the sperm or nonsperm sample to the nonsperm or sperm fractions, respectively.
§"Not Determinable" indicates that the allele combination of the components of the mixture did not give rise to being able to detect mixtures.

RFLP Comparison

Twelve semen/vaginal mixtures were analyzed for RFLP, DQA1 and PM. The PCR results obtained from the sperm cell fraction of the mixed samples were compared with the RFLP results on the basis of match or nonmatch. The 12 sperm cell fractions would have been called "matches" for all three systems (RFLP, DQA1 and PM).

Discussion

These validations demonstrate that correct DNA typing results can be obtained from DNA isolated from different tissues from the same individual using both the Amplitype DQA1 and PM PCR Amplification and Typing kits. It also shows that there is inter- and intra-laboratory consistency in the typing results. This was demonstrated not only with bloodstains, but also with combinations of mixtures of body fluids. Of particular interest was the ability of these two systems to type correctly the DNA obtained from the semen of vasectomized males.

For the 127 specimens analyzed for DQA1, all typings were the same as those obtained from the corresponding blood samples. Likewise, for the 96 specimens analyzed for PM, all typings were the same as those obtained from the corresponding blood samples. For PM, it is important to note that for two samples even though an "S" dot was not present, the same PM typings were indicated as were obtained from the corresponding blood samples.

Sufficient DNA was obtained from the neat stains (blood, semen, and vaginal secretions) by all extraction methods. However, comparison of the extraction methods for mixed stains indicated that washing of the sperm pellet is important to achieve a complete separation of the sperm and nonsperm components of a mixture. This becomes important when analyzing vaginal swabs from an alleged sexual assault in forensic casework. Comparison of the results from DQA1 typing of the sperm and nonsperm fractions of the swabs indicated that a better separation of the components was achieved through washing of the sperm pellet. This washing step was incorporated into the MFSL organic protocol as reported under Method III and had been used for both DQA1 and PM analysis with Method IV.

For samples that contain inhibitory substances, such as anal swabs, it was found that if the samples were subjected to a "clean up" with a Centricon 100 filter following the organic extraction, the potential for amplification was increased as compared to Chelex 100 extracted samples or organically extracted samples.

Conclusion

In this study, validations were completed that address issues pertaining to using PCR DNA kits for the analysis of forensic samples. These validations demonstrated that the commercially available Amplitype HLA DQA1 PCR Amplification and Typing kit and the Amplitype PM PCR Amplification and Typing kit provide reliable PCR results for DNA isolated from various biological specimens. Inter- and intra-laboratory studies show that reproducible results can be obtained when using these kits. The results presented in this paper are in agreement with other published studies which indicate that the DQA1 kit (2–7) as well as the PM kit (8,9) are suitable for the typing of forensic casework samples.

Acknowledgments

The authors would like to express their gratitude to Terry L. Laber, Joseph M. O'Connor, Staci A. Bennett, and Charles Barna for reviewing and commenting on the manuscript.

References

(1) Guidelines for a quality assurance program for DNA analysis. Crime Lab Digest 1991;18(2):44–75.
(2) Blake E, Mihalovich J, Higuchi R, Walsh PS, Erlich H. Polymerase chain reaction (PCR) amplification and human leukocyte antigen (HLA)-DQα oligonucleotide typing on biological evidence samples: casework experience. J Forensic Sci 1992;37:700–26.
(3) Comey CT, Budowle B. Validation studies on the analysis of the HLA DQα locus using the polymerase chain reaction. J Forensic Sci 1991;36:1633–48.
(4) Comey CT, Budowle B, Adams EW, Baumstark AL, Lindsey JA, Presley LA. PCR amplification and typing of the HLA DQα gene in forensic samples. J Forensic Sci 1993;38:239–49.
(5) Hochmeister MN, Budowle B, Jung J, Borer UV, Comey CT, Dirnhofer R. PCR-based typing of DNA extracted from cigarette butts. Int J Legal Med 1991;104:228–33.
(6) Presley LA, Baumstark AL, Dixon A. The effects of specific latent fingerprint and questioned document examinations on the amplification and typing of the HLA DQ alpha gene region in forensic casework. J Forensic Sci 1993;38:1028–36.
(7) Walsh PS, Fildes N, Louis AS, Higuchi R. Report of the blind trial of the Cetus AmpliTaq HLA DQα forensic deoxyribonucleic acid (DNA) amplification and typing kit. J Forensic Sci 1991;36:1551–6.
(8) Herrin G, Jr, Fildes N, Reynolds R. Evaluation of the AmpliType™ PM DNA test system on forensic case samples. J Forensic Sci 1994;39:1247–53.
(9) Budowle B, Lindsey JA, DeCou JA, Koons BW, Giusti AM, Comey CT. Validation and population studies of the loci LDLR, GYPA, HBGG, D7S8 and Gc (PM loci) and HLA-DQα using a multiplex amplification and typing procedure. J Forensic Sci 1995;40:45–54.

(10) Laber TL, Giese SA, Iverson JT, Liberty JA. Validation studies on the forensic analysis of restriction fragment length polymorphism (RFLP) on le agarose gels without ethidium bromide: Effects of contaminants, sunlight, and the electrophoresis of varying quantities of deoxyribonucleic acid (DNA). J Forensic Sci 1994;39:707–30.
(11) Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 1994;10:506–13.
(12) Amplitype™ Users Guide, Version 2, Cetus Corporation, 1991.
(13) McNally L, Shaler RC, Baird M, Balazs I, DeForest P, Kopilinsky L. Evaluation of DNA isolated from human bloodstains exposed to UV light, heat, humidity, and soil contamination. J Forensic Sci 1989;34:1059–69.
(14) Waye JS, Presley LA, Budowle B, Schutler GG, Fourney RM. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. Biotechniques 1989;7:852–5.
(15) Carlson D, Horton M, Klevan L. An improved assay for quantitating human DNA. American Academy of Forensic Sciences 46th Annual Meeting; 1994 Feb 14–19; San Antonio (TX).
(16) Walsh PS, Varlaro J, Reynolds R. A rapid chemiluminescent method for quantitation of human DNA, Nucl Acids Res 1992;20:5061–5.
(17) Akane A, Shiono H, Matsubara K, Nakamura H, Hasegawa M, Kagawa M. Purification of forensic specimens for the polymerase chain reaction (PCR) analysis. J Forensic Sci 1993;38:691–701.
(18) Comey CT, Koons BW, Presley KW, Smerick JB, Sobieralski CA, Stanley DM et al. DNA extraction strategies for amplified fragment length polymorphism analysis. J Forensic Sci 1994;39:1254–69.
(19) Hagelberg E, Kykes B, Hedges R. Ancient bone DNA amplified. Nature 1989;342:485.
(20) Paabo S, Gifford JA, Wilson AC. Mitochondrial DNA sequences from a 7000-year old brain. Nucl Acids Res 1988;16:9775–87.

Address requests for reprints or additional information to
Ann Marie Gross
Minnesota Forensic Science Laboratory
Biology Section
1246 University Avenue
St. Paul, MN 55104