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Summary of Validation Studies from Twenty-Six Forensic Laboratories in the United States and Canada on the Use of the AmpliType® PM PCR Amplification and Typing Kit

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ABSTRACT: A cooperative study was undertaken to collect and summarize the results of validation studies from forensic laboratories in the United States and Canada on the use of the AmpliType® PM PCR amplification and typing kit for genetic typing of forensic biological evidence. This report compiles data from 26 laboratories on: 1) reproducibility studies on DNA extracted from various samples, 2) genetic typing of DNA extracted from a variety of biological samples on various substrates, 3) the effects of exogenous chemicals, materials, and environmental factors on test results, 4) sensitivity studies to determine the least detectable amount of extracted genomic DNA that can be reliably typed, 5) analysis of mixtures containing two sources of genomic DNA, 6) cross-hybridization with DNA extracted from various nonhuman species, and 7) evaluation of assay performance on parallel studies with other genetic typing systems on proficiency test panels, mock cases, and adjudicated/nonprobative casework. Equivalent results were obtained by each laboratory that supplied data, demonstrating the reliability and consistency of the test. Overall, it can be concluded from this study that the AmpliType PM PCR amplification and typing kit meets the guidelines of the Technical Working Group on DNA Analysis Methods (TWGDAM) and there is general scientific acceptability of this kit for forensic DNA testing.

KEYWORDS: forensic science, polymerase chain reaction, DNA typing, PM, polymarker, LDLR, GYPA, HBGG, D7S8, GC

In 1990, the AmpliType HLA DQα forensic DNA amplification and typing kit (HLA DQα kit), a non isotopic forensic DNA test based on the polymerase chain reaction (PCR), was introduced and made commercially available by the Cetus Corporation and was qualified for forensic testing (1). This test permitted amplification and detection of six out of eight major alleles at the DQα locus on human chromosome six. The HLA DQα assay format, frequently described as a "reverse dot blot", is based on detecting alleles in the amplified DNA by hybridization to sequence-specific probes that are immobilized on a membrane (2). In late 1993, the AmpliType PM PCR amplification and typing kit (PM kit) developed by Roche Molecular Systems (3) became commercially available. The PM kit has the same format as the HLA DQα kit, but it has been designed to amplify six loci simultaneously and to detect two or three alleles at five different loci. This kit detects genetic polymorphisms at the low density lipoprotein receptor (LDLR) on chromosome 19, glycophorin A (GYPA) on chromosome four, hemoglobin G gammaglobin (HBGG) on chromosome 11, D7S8 on chromosome seven, and group specific component (GC) on chromosome four.

For admissibility of forensic test results in a court of law, it is important to demonstrate general acceptability of the test in the appropriate scientific community. Criteria for acceptability of a DNA-based forensic test by the forensic scientific community include reproducibility and consistency in test results obtained by forensic laboratories on similar test materials. In 1994, a cooperative study was initiated that involved the compilation of data from 26 working forensic laboratories that were conducting validation and population studies with the PM Kit. The purpose of this report is to evaluate and compare PM test results from 26 of these laboratories that had used a standardized set of commercially available reagents and were guided by a standardized protocol that included directions for amplification, hybridization, typing, and interpretation.

Prior to this report, the PM kit was evaluated in a field trial by seven laboratories (4). The FBI Forensic Science Research and Training Center also reported results of its validation study on this test (5). Additionally, reports have appeared on the use of the PM kit with forensic casework (6) as well as reports of other validation studies (7–11). Finally, Hochmeister et al. have reported the use of this test to analyze genomic DNA recovered from samples previously amplified with the HLA DQα kit (12).

The evaluation and analyses presented in this report have been based on tabulated summaries of data provided to us for analysis from the 26 separate laboratories. Data were submitted from the following types of studies: 1) reproducibility studies on DNA extracted from various samples, 2) genetic typing of DNA extracted from a variety of biological samples on various substrates, 3) the effects of exogenous chemicals, materials, and environmental factors on test results, 4) sensitivity studies to determine the least detectable amount of extracted genomic DNA that can be reliably typed, 5) analysis of mixtures containing two sources of genomic DNA, 6) cross-hybridization with DNA extracted from various nonhuman species, and 7) evaluation of assay performance on parallel studies with other genetic typing systems on proficiency test panels, mock cases, and adjudicated/nonprobative casework.

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A separate report (in preparation) will present the results of allele and genotype frequencies of the five PM loci in different populations (13,14). This report presents data which support the conclusion that the PM kit yields reliable and consistent results and fulfills TWGDAM requirements for a forensic genetic typing test (15).

Materials and Methods

Samples

Blood and biological samples for mock casework were obtained with informed consent from laboratory personnel and, in some cases, families. The proficiency panels were obtained from Collaborative Testing Services, the College of American Pathologists, and Cellmark Diagnostics. Casework samples were nonprobative samples previously analyzed and/or adjudicated cases available to the laboratories participating in this study.

DNA Extraction

DNA was extracted from blood, stains, and biological samples by one of two methods: One method used to extract DNA was the Chelex® extraction method as described by Walsh et al. (16). In samples containing mixtures of spermatozoa and other biological fluids, the pellet from the spermatozoa was collected by centrifugation prior to extraction with Chelex. A second method originally described by Gill et al. (17) used to extract DNA is based on a method that uses detergents, proteolytic enzymes, and organic solvents (18). Briefly, the sample was treated with a lysis buffer containing SDS detergent and Proteinase K to lyse all cells and release the DNA. The DNA was then purified by a series of extractions in phenol, phenol/chloroform, isobutanol, and/or isoamyl alcohol. The organic solvents were separated from the DNA either by washing on a Centricon 100 filter (Amicon, Bedford, MA) with water, TE buffer, or both, or by precipitation with ethanol or isopropanol followed by resuspension in distilled water. In samples containing mixtures of spermatozoa and other types of cells, after the initial lysis step, a second pellet was obtained by centrifuging the Proteinase K/SDS treated cells. The DNA was released from the sperm by treating this pellet with DTT/Proteinase K/SDS (and in some cases, sarkosyl), then incubating for several hours to overnight at 56°C to 65°C, and purified as described above. Some laboratories washed the sperm pellet three to five times prior to the second digestion.

Overall, the procedures used by all participating laboratories were similar, but there were some variations in methodology from laboratory to laboratory. In every case, however, the methods used had been previously validated with other DNA testing systems, had been described in published reports, or both (4–12, 19–21). In the studies described in this report, six laboratories used only a Proteinase K/organic solvent extraction procedure, 10 laboratories used only the Chelex extraction procedure, and 10 laboratories had both procedures available and used either the Proteinase K/organic solvent method or the Chelex extraction method.

DNA Quantitation

Three commercially available human DNA quantitation kits and three standardized methods were used to quantify the extracted DNA. The kits used were the QuantiBlot™ Kit (Perkin Elmer Applied BioSystems), the ACES Kit (Gibco Life Technologies), and the Nanoblot Kit (Lifecodes). Some laboratories also used the D17Z1 probe 32P-radiolabeled or suitably labeled for detection by chemiluminescence, as described by Waye et al. (22). One laboratory quantitated the DNA by UV spectroscopy and one used the yield gel quantitation method (19).

Amplification and Hybridization and Interpretation

All laboratories used the AmpliType PM PCR Amplification and Typing Kit (Perkin Elmer Applied Biosystems, Norwalk, CT) according to instructions provided by the manufacturer in the package insert. Likewise, all laboratories interpreted the results based on guidelines detailed in the package insert. All PCR amplifications were performed in either a DNA Thermal Cycler Model 480 or 9600 Gene Amp PCR instrument system (Perkin Elmer Applied Biosystems, Norwalk, CT). All chemicals and reagents were obtained as molecular biology grade from standard sources. Waterbaths, ovens, electrophoresis equipment, power supplies, gel boxes, and centrifuges were also obtained from standard sources. Data were reviewed, analyzed, and interpreted according to procedures and protocols adapted by each laboratory for the PM kit.

Results

Study Design

To fulfill the requirement for reproducibility and acceptability, this study has been designed to address whether correct results can be obtained by forensic laboratories that are doing PCR-based DNA typing with protocols they have adapted or developed in their respective laboratories for performing and interpreting PM test results. Laboratories were requested to provide, in a standardized format, summaries of results obtained with the PM kit when they used the standardized set of reagents and followed their own laboratory's protocol for amplification, hybridization/typing, and interpretation. All data were reviewed, analyzed, and interpreted in the respective laboratories prior to our evaluation for consistency and reproducibility. Because the ability of a laboratory to obtain and interpret results was a critical part of the study design, we did not ask for nor did we examine any typing strips of individual test results, nor did we review policy guidelines developed by each laboratory as to what are the criteria for reporting a result obtained with the PM test.

Twenty-six laboratories contributed data for the validation studies summarized in this report. Based on the TWGDAM guidelines for a PCR-based DNA forensic test, we identified eight categories of experiments which would constitute validation studies on the PM test. These included measurement of reproducibility of typing results on replicate or the same DNA samples, the effect of various substrates on the ability to obtain an interpretable result, the effect of environmental and chemical contaminants on typing results, the lowest concentration of DNA that could be typed, an analysis of mixtures of DNA derived from two different individuals, whether DNA obtained from non human species yielded and interpretable typing result, and the performance characteristics of the PM kit with proficiency panels, mock case and nonprobative/adjudicated casework in parallel studies with other genetic typing systems. Not all 26 laboratories submitted data in all categories. Rather, the data summarized in Tables 1 through 5 and 7 represent our summary of the data submitted by the 26 laboratories.

Biological Samples Analyzed

The types of biological samples that were extracted and typed from 26 laboratories with the PM kit are summarized in Table 1.

TABLE 1—*Types of biological samples tested.*

Sample	Number Tested	Number with Results	Percent Typed§
Liquid Blood	204	202	99%
Blood Stains*	1115	1071	95.8%
Liquid Saliva	11	11	100%
Oral Swabs	331	330	99.7%
Saliva Stains	3	3	—
Cigarettes	65	59	90.7%
Stamps	31	25	80.6%
Envelopes	12	8	66.7%
Liquid Semen	2	2	—
Semen Stains	4	4	—
Hair	129	102	79.1%
Tissue†	39	37	94.9%
Tissue‡	17	17	100%
Vaginal Swabs	29	29	100%
Bone	14	13	92.8%
Tooth	1	1	—
Urine	31	7	22%
Nasal Secretions	13	13	100%
Finger Nails	8	8	—
Ear Wax	8	7	—
Toothbrush	8	8	—
Chewing Gum	2	2	—
Sweat (stain)	1	1	—
Serum	2	0	—
Shoe Liners (inside)	3	0	—
Cap Liners (cloth)	4	0	—
Total	2088	1956	93.7%

The concentration of template DNA amplified varied from 1 to 10 ng.
*Blood stains were made on various types of cloth or filter paper.
†Includes skin, organs, and fetal tissue.
‡Tissue sections, formalin fixed and embedded in paraffin blocks.
§No percentage was calculated when the number of samples tested was <10.

TABLE 2—*Effect of exogenous materials on test results with AmpliType PM kit.*

Contaminant	Blood Stains	Semen Stains
Dirt	8/11*	
Hand Soap	2/2	2/2*
Hand Lotion	1/1	
Liquid Cleaning Solution	1/1	
Ammonia	1/1	
Bleach	3/3	1/1
Motor Oil	1/1	1/1
Gasoline	2/2	1/1
Cola	1/1	
Whiskey	1/1	
Beer	1/1	
Microorganism		
C. albicans	1/1	
E. coli	2/2	1/1
Contraceptive Gel		1/1
Contraceptive Foam		1/1
Baby Oil		1/1

In these experiments, the blood or semen was added to the substrate before extracting the DNA.
The concentration of template DNA amplified was 1 to 4 ng.
*Number of Results/Number of samples tested.

TABLE 3—*Results of dilution of DNA on AmpliType PM results.*

Amount of DNA Tested	Dots Present	All Expected Dots Present	S Dot Present
≥2 ng	Yes	Yes	Yes
1 to 0.1 ng	Variable	Variable	Variable
<0.1 ng	Yes	No	No

TABLE 4—*Cross-reactivity with other species observed with the PM kit.*

Higher Order Primates
Chimpanzee and Gorilla: Reactivity noted at all loci and S dot.
Orangutan, Gibbon: Reactivity with S dot and 4 of 5 loci.
Lower Order Primates—New World
Tufted Capuchin, White-throated Capuchin, White fronted Capuchin, Spider monkey, Squirrel monkey: Reactivity only with S dot.
Lower Order Primates—Old World
Pigtail Macaque, Cyanomologous Macaque, Assamasse Macaque, Stumptail Macaque, Olive Baboon, African Green Monkey, Rhesus Monkey: Reactivity with S dot, HBGG, D7S8 and GC, variable reactivity with LDLR.
Nonprimate Species
Pig: Reactivity with GC; no S dot seen.
Cat: Reactivity with D7S8; weak reactivity with LDLR, GYPA, and GC; no S dot seen.
Pronghorn antelope: Weak reactivity with HBGG and D7S8; no S dot seen.
Snake: Reactivity with D7S8; no S dot seen.
No reactivity with DNA isolated from blood of the following species: Rabbit, Squirrel, Crocodile, Bear, Deer, Dog, Elephant, Goat, Grey Seal, Hawk, Horse, Otter, Raccoon, Sheep, Coyote, Guinea Pig, Fish (Blue Gill), Black Bear, Mule, Deer, Sheep, and Ostrich.
Prokaryotic Organisms
No reactivity with DNA from the following microorganisms: *Staphylococcus aureus, Streptococcus agalactiae, Pseudomonas aeruginosa, Escherichia coli, and Leishmania species.*
Between 1 and 10 ng of template DNA was used.

A result was scored if there was a visible S dot. The success rate at typing a wide variety of samples was between 100% and 90% for those samples in which sufficient nuclear DNA was expected to be present. The samples for which a lower success rate was obtained were those in which there may often be few cells present in the original sample (e.g., hairs, urine), the extraction process may not be optimized and/or there might be inhibitors of the Taq DNA polymerase present (e.g., stamps and envelopes), or where there was a high likelihood of degraded DNA being present. No samples were reported as giving an incorrect type including samples with a partial result and samples in which no S dot was observed. A total of 2088 amplifications were performed and 1956 yielded interpretable results which gives an overall rate of 93.7% typed. The success rate may be higher than observed with casework samples because individual laboratories used different criteria for selecting samples for this study. For example, some laboratories chose to use isolated DNA that was known to produce results with the HLA DQα kit.

Reproducibility

Reproducibility was determined by repetitive typing on blood stains, hair, saliva stains, semen stains, and other biological samples. Typings which involved amplification and hybridization on replicate samples obtained from multiple individuals were done

TABLE 5—*Analysis of mixtures of DNA: Varying the ratio of major to minor DNA samples.*

Category I, detection of the presence of dots from the minor type when the major type was homozygous.

Total Concentration of DNA (ng) in initial dilution	Dilution									
	1:1	1:2	1:3/1:4	1:5	1:6/1:7/1:8	1:10	1:12.5/1:15	1:20	1:50	1:100
100	+	+	NT	+	NT	+	NT	+	+	+
25	+	+	NT	+	NT	+	NT	+	+	±
20	+	+	+	+	+*	+	+	NT	NT	NT
10–12.5	+	+	+	+	+	+	NT	+	−	−
4	+	+	NT	NT	NT	NT	NT	NT	NT	NT
2.5	+	+	NT	+	NT	+	NT	+	±	−

+ = dots present from the minor type; − = no dots present from the minor type; NT = Not Tested; 9 laboratories reporting.
*One laboratory obtained no results beyond a 1:8 dilution at this concentration of DNA in Category I.

Category II, detection of darker dots from the minor type when the major type was heterozygous.

Total Concentration of DNA (ng) in initial dilution	Dilution									
	1:1	1:2	1:3	1:4	1:5	1:7	1:8	1:10	1:15	1:16
100	+	+	NT	NT	−	−	−	−	−	−
25	+	+	NT	NT	−	−	−	−	−	−
20	+	+	NT	+	−	−	−	−	−	−
10	+	+	+	±	−	−	−	−	−	−
4	+	+	NT	NT	NT	−	NT	NT	NT	NT
2.5	+	+	NT	NT	−	NT	NT	NT	NT	NT

+ = intensity difference detected; − = no intensity difference noted; NT = Not Tested; 7 laboratories reporting.

by 24 of 26 laboratories. Identical results were obtained on the replicate samples. Typings obtained from blood and compared to other tissues (hair, saliva and semen) from the same individual were done by 24 of 26 laboratories. The typing results were identical for each sample derived from the same individual. Furthermore, the method of extraction of DNA had no effect.

Effects of Exogenously Added Material

The effect of exogenous materials on the ability to type DNA extracted from dried blood or semen stains with the PM kit is summarized in Table 2. Some of these studies were performed as described by Laber et al. (19). Briefly, the exogenous material to be tested was applied to a piece of cloth and dried. The liquid blood or semen was added to the stain and dried prior to extraction of the DNA. Three laboratories performed this kind of study. No studies were done on the stability of biological samples incubated for varying lengths of time with these exogenous materials. Generally, the exogenously added materials had no effect on the ability to extract, or amplify and hybridize DNA with the PM kit. Samples that were mixed with dirt yielded variable results (8 out of 11 blood stains gave results). No other materials tested had any significant effect on the ability to obtain an interpretable and correct result.

Analytical Sensitivity Studies

Studies were done to determine the lowest concentration of DNA that could be typed by the PM kit. Twenty-four laboratories conducted this type of experiment. These results are summarized in Table 3. All laboratories obtained results when the concentration of template DNA was 2 ng or greater. Variable results were obtained when the concentration of template DNA ranged from 1.0 to 0.1 ng. No laboratory reported obtaining an interpretable type when less than 0.1 ng of input template DNA was used for amplification.

One laboratory studied the effects of digestion with the endonuclease DNAse I on human DNA. DNA degraded to limited (23 kb size or smaller) or moderate (10 kb size or smaller) amounts could be successfully amplified and typed when less than 2 ng of template DNA was used. Highly degraded DNA (1 kb size or smaller) could be amplified and typed when 2 ng or greater were used.

Based on the DNA sequences of the alleles detected using the PM kit, no results should be obtained with HaeIII digested DNA. For HinfI digested DNA, results for the GYPA, D7S8, and GC loci should be obtained and no results for the LDLR and HBGG loci should be obtained (Rebecca Reynolds, Roche Molecular Systems, personal communication). It should also be noted that unless the DNA is totally digested with the restriction enzyme at a particular allele, any undigested DNA may be amplified and a result may be obtained at that locus. These predicted results were confirmed by several laboratories participating in this study.

The PM kit contains primers for amplification at the DQA1 locus; the kit used in this study did not contain the strips for typing the DQA1 alleles. However, the results of studies by one laboratory participating in this study showed that the DQA1 types can be obtained from as little as 0.4 ng of genomic DNA amplified with the PM kit by using the PM hybridization protocol and DQA1 strips from the DQα kit. Similar results have been reported by the FBI Laboratory (5). The combined Amplitype PM + DQA1 PCR

amplification and typing kit is now available which allows for typing at all 6 loci.

Nonhuman DNA

A variety of studies were undertaken with the PM kit to determine the ability to obtain a reaction with nonhuman DNA following the standard amplification and hybridization procedures. The results of these studies are summarized in Table 4. As expected, some cross-reactivity was detected with the nonhuman primates. In other species, when dots were observed at certain loci, no S dot was observed. These results indicate that, other than high nonhuman primate DNA, DNA from other nonhuman species will not type in this test. Ten laboratories conducted this type of test.

Mixtures of DNA

There are two ways that the PM kit can potentially detect mixtures of DNA derived from two or more sources. First, when 3 alleles are detected at the HBGG and/or GC loci, in the absence of hybridization artifacts, this is definitive evidence of a mixture of DNA derived from two or more sources. Second, mixtures of DNA derived from two or more sources is also indicated when there is distinctly unbalanced dot intensity of two alleles at the same locus. The reason for this is that the PM kit has been designed so that the intensity of the reaction for each allele at a given locus will be equivalent or balanced with heterozygous types when a single source of DNA is used (23). Furthermore, if a sample contains two or more sources of DNA that differ in concentration and also in genotype at any of the 5 loci detected, the intensity of the reaction will be greater for the DNA sample that is present in the highest amount.

A study was performed to determine at what concentration a second source of DNA could be detected when the alleles (or types) differed for the two sources at one or more loci. There were two parts to this experiment. In the first part (Category I), a sample that was homozygous at a given locus (e.g., AA) was held constant and the second sample that was homozygous for a different allele (e.g., BB) or heterozygous (e.g., AB) was diluted from 1:1 to 1:100. In Category I, laboratories recorded at what dilution the presence of the "extra dot" from the second source (e.g., B in the example cited above) could still be detected. Overall, in Category I, the second source of DNA could be detected at a 1:20 dilution and variably at dilutions of greater than 1:20. In the second part (Category II), a sample that was heterozygous at a given locus was diluted 1:1 to 1:16 with a sample that was homozygous at that locus. In Category II, laboratories recorded at what dilution a more intense reaction at the homozygous allele could still be detected. In Category II the second source was detected at the 1:1 and 1:2 dilutions and variably at dilutions of 1:4 or greater. Varying amounts of DNA (2.5–100 ng) were amplified in all studies. Not all laboratories tested all concentrations or all dilutions. The composite results from nine laboratories are summarized in Table 5.

One laboratory (Suffolk County Crime Laboratory, Hauppauge, NY) did an extensive study on dot intensities in mixtures of DNA derived from two different sources. They prepared mixtures of 12 different phenotypes comprising DNA from six different individuals (see Table 6A) to concoct a variety of homozygous/homozygous and homozygous/heterozygous combinations. (See Table 6B). A total of 4 ng of mixed DNA from the 10 different phenotype combinations in the following ratios were amplified: 8:1 (3.5 + 0.5 ng), 4:1 (3 + 1 ng), 1:1 (2 + 2 ng), 1:4 (1 + 3 ng), and 1:8 (0.5 + 3.5 ng). These investigators then determined at what dilution

TABLE 6A—*Results of typing of mixtures of DNA with the AmpliType PM kit. The typings of the individuals are summarized as follows.*

	PM Type				
Individual	LDLR	GYPA	HBGG	D7S8	GC
#1	B	A	AC	A	AB
#2	AB	AB	B	B	A
#3	B	AB	AB	B	B
#4	A	A	A	A	BC
#5	AB	A	BC	AB	AC
#6	AB	B	B	A	C

TABLE 6B—*Mixtures of the six individuals prepared to give combinations of phenotypes.*

Phenotype	LDLR	GYPA	HBGG	D7S8	GC
A/B	#4/#3	#4/#6	#4/#6	#4/#3	#2/#3
A/C	—	—	—	—	#2/#6
B/C	—	—	—	—	#3/#6
A/AB	#4/#6	#4/#3	#4/#3	#6/#5	#2/#1
B/AB	#3/#6	#6/#3	#6/#3	#3/#5	#3/#1
A/AC	—	—	#4/#1	—	#2/#5
C/AC	—	—	—	—	#2/#5
B/BC	—	—	#6/#5	—	#3/#4
C/BC	—	—	—	—	#6/#4

TABLE 6C—*A total of 12 mixtures of DNA was amplified and typed. Five dilutions of any two individuals were prepared containing mixtures in the ratios of 1:8, 1:4, 1:1, 4:1, and 8:1. The results are summarized as follows.*

		Dilution				
Mixture	Category	1:8	1:4	1:1	4:1	8:1
#3:#4	I	+	+	+	+	+
	II	+	+	+	+	+
#6:#4	I	+	+	+	+	+
	II	−	+	+	−	−
#1:#4	I	+	+	+	+	+
	II	+	+	+	+	−
#1:#3	I	+	+	+	+	+
	II	−	+	+	+	−
#2:#3	I	+	+	+	+	+
	II	−	+	+	−	−
#2:#6	I	+	+	+	+	+
	II	NA	NA	−	−	−
#3:#6	I	+	+	+	+	+
	II	−	−	+	+	−
#2:#1	I	+	+	+	+	+
	II	−	+	+	+	−
#2:#5	I	+	+	+	+	+
	II	−	+	+	+	−
#6:#5	I	+	+	+	+	+
	II	−	+	+	NA	NA

+ = mixture detected.
− = mixture not detected.
NA = no heterozygote in the sample of highest concentration.
Mixtures of #2 and #6 and #4 and #6 were not detectable at ratios of 4:1 and 1:1, respectively.

TABLE 7—*Summary of AmpliType® PM testing of proficiency panels, mock casework, and genuine casework.*

Case Type	Sample Type	Inconclusive§	Inclusion‖		Exclusion¶		Mixtures**	Totals
			Victim	Suspect	Victim	Suspect		
Mock Cases*	Differential Extraction							
	Epithelial Cells	2	14	7	2	1	16	42
	Sperm Cells	0	1	37	0	1	2	41
	Blood	1	0	9	0	2	0	12
	Other	0	0	0	0	0	0	0
Nonprobative Adjudicated Cases†	Differential Extraction							
	Epithelial Cells	1	22	2	0	0	8	33
	Sperm Cells	1	0	23	0	21	8	54
	Blood	3	34	9	1	13	0	60
	Other	0	22	12	0	3	1	38
Proficiency Cases‡	Differential Extraction							
	Epithelial Cells	1	4	4	0	0	2	11
	Sperm Cells	1	0	12	0	0	0	13
	Blood	0	5	10	2	6	1	24
	Other	1	0	2	0	0	0	3
Totals	Differential Extraction							
	Epithelial Cells	4	40	13	2	1	28	86
	Sperm Cells	2	1	72	0	22	10	107
	Blood	4	39	28	3	21	1	96
	Other	1	22	14	0	3	1	41

*Mock cases included 2 homicides, 25 sexual assaults and 8 paternity studies (family studies).
†Nonprobative/adjudicated cases included 25 homicides, 1 sexual assault/homocide, 48 sexual assaults, 7 disputed paternities, 1 criminal paternity, 1 paternity/missing person, 1 disputed pathology specimen, 4 identification of a body, 1 identification of a blood sample, 3 physical assaults, and 2 burglaries.
‡Proficiency panels included 18 homicides, 13 sexual assaults and 1 disputed paternity.
§Inconclusive was scored when there was no S dot but there was reactivity at one or more loci.
‖An inclusion for one person in a case was not scored as an exclusion for another individual in a case; some cases involved more than two individuals.
¶An exclusion for one person in a case was not scored as an inclusion for another individual in a case; some cases involved more than one individual.
**A mixture was scored when the results indicated there were two or more sources of DNA.

the presence of an extra dot from the second source could be detected (Category I, see above) and at what dilution a given heterozygote with a more intense reaction than a homozygote was also detected (Category II, see above). The results of this study are summarized in Table 6C. The investigators in this laboratory reported detecting mixtures at all concentrations in Category I. In particular, they always detected mixtures when there were three alleles present at the HBGG and/or GC loci. In Category II, mixtures could only be routinely detected when the two DNA samples were present in equal amounts. Mixtures of homozygotes of the same type could not be detected, mixtures of homozygotes of different types were clearly distinguishable unless the mixture was comprised of equal quantities of both components, and homozygous/heterozygous mixtures were detected even when the minor heterozygous component was 1/8 of the total.

Test Results with Proficiency Panels, Mock Casework, and Adjudicated/Nonprobative Casework

Data obtained with the PM kit on samples from proficiency testing panels, mock cases, and previously analyzed and/or adjudicated cases from 21 laboratories were provided. A total of 162 cases were analyzed. The numbers and types of samples tested were as follows: reference blood samples (liquid and stains), 444; evidence blood stains, 63; differential extractions of sexual assault samples containing nonsperm and sperm cells, 114 (resulting in 91 nonsperm cell fractions and 97 sperm cell fractions); other (including tissue hair and bone), 34. Some samples were typed multiple times. In one instance, a series of five mock sexual assault cases were tested by five laboratories as part of a cooperative study on reproducibility and all laboratories testing this panel of mock cases obtained the same results. Overall, out of 779 typings performed on the 162 cases, 765 yielded interpretable results; the 779 typings included the 444 reference blood samples, 442 of which yielded interpretable results. A noninterpretable result was reported when no S dot was present. No laboratory reported an incorrect result. Different samples were used in this part of the study than those yielding the data presented in Table 1.

An analysis was performed on the data to determine the rate of exclusion and inclusion that was observed with the PM kit. The data are summarized in Table 7. Overall, in the proficiency tests, mock casework, and nonprobative/adjudicated casework examples, a total of 52 exclusions and 229 inclusions for either the victim or the suspect were reported. It should be emphasized that the rates of exclusion and inclusion found in this study would not necessarily represent routine forensic casework. The reported cases were selected either to provide a basis for evaluating assay performance or to show that the increased discrimination power afforded by using 5 separate loci may lead to exclusions that otherwise might be missed with a test with a lower power of discrimination. Also, it should be noted that the total number of cases does not correlate with the total number of samples tested nor with the number of exclusions and inclusions. The reasons for this include: (i) cases often consisted of multiple suspects and/or victims, (ii) no results were obtained on reference blood samples and/or evidence in some instances, (iii) some cases often had multiple pieces of evidence, (iv) all exclusions and inclusions were recorded even in cases in which one piece of evidence included an individual as a potential donor and another piece of evidence excluded another individual as the donor of the extracted DNA, and (v) in sexual assault cases, most laboratories analyzed two fractions, the nonsperm and sperm fractions.

The paternity tests and family studies were designed to demonstrate that all markers followed the expected pattern of Mendelian inheritance. Additionally, the power of exclusion of the PM test

would indicate that it could be useful in excluding paternity or maternity and/or establishing potential family relationships in cases in which the relationships of the subjects is of importance. A total of 23 paternity and family studies were done and there was one homicide in which the issue of paternity was part of the analysis. The mean combined paternity index in these studies was 6.1 with a range from 1.1 to 23.6. In all family studies, the expected pattern of Mendelian inheritance was observed.

A variety of genetic typing tests were used in parallel studies to determine whether the results of testing (i.e., inclusion or exclusion) with the PM kit correlated with results obtained with these other forensic analyses. Twenty-one of 26 laboratories provided results. Confirmatory systems that correlated 100% with results of PM kit testing included correlation with ABO red cell typing and PGM, ESD, ADA, and AK serological test results reported for proficiency panels obtained from the Collaborative Testing Service Proficiency Test results) (2 cases); analysis with the AmpliFLP D1S80 system (26 cases); a PCR method for typing ABO blood group substances (3 cases); the STR CYP-19 system (5 cases), and the STR quadruplex VWA, THO1, F13A, FES/FPS (1 case).

In those cases in which the HLA DQα test was also done, there was correlation in 120 out of 126 cases with the PM kit results. In 6 cases the DQA1 locus included a suspect but PM test results excluded the individual; the exclusions were confirmed by RFLP analysis in 5 of the cases. These results were not unexpected because the 5 loci detected by the PM kit may have an increased power of discrimination over DQA1 alone (23,24). In cases in which RFLP analyses were done as a confirmatory test, there was correlation in 72 out of 73 cases. In that one case the PM kit test results included an individual, but RFLP test results excluded the individual. RFLP testing, due to the high degree of heterozygosity at each locus, has a higher power of discrimination compared with the PM kit. In previously reported studies on the PM kit (25,26), one laboratory compared results from DNA samples from Desert Storm casualties using the HLA DQα1 kit, the PM kit, several STRs, RFLP, and minisatellite variant repeat sequences (MVR) (27). Similar findings to those presented here were obtained.

Discussion

When a new test is introduced for forensic testing, usually a series of validation studies are undertaken to ascertain the performance characteristics of the test prior to introducing it as part of the forensic testing services. In the application of DNA-based genetic analyses to biological evidence that is part of a forensic case, the requirements in a validation study are two-fold: (i) to provide a valid test result that is totally accurate in the identification of genetic types in all individuals and evidence, and (ii) to be able to reliably use the typing results to compare individuals to the biological evidence in terms of the genetic marker types detected. These criteria are derived from general testing guidelines that have been articulated for introducing new assays for use in medical diagnostic testing (28,29) and have been refined to specific requirements for DNA-based forensic testing in the TWGDAM guidelines (see Discussion below). The data that have been obtained from the validation studies independently undertaken by 26 forensic laboratories who contributed to results summarized in this report permit an estimate of the reliability, reproducibility, and usefulness of the PM kit for forensic testing.

The data obtained on reproducibility and sensitivity can be used to estimate quantitative aspects of assay performance of the PM kit. Repeat typing of the same individual always yielded the same type regardless of the tissue analyzed or DNA extraction procedure used. Studies with nonhuman DNA samples confirmed that the only nonhuman samples that could be detected (e.g., there was a reaction with the S dot and all loci) were the higher nonhuman primates DNA samples in which there is extensive homology with human DNA. In general, the sensitivity studies were performed with high quality DNA extracted from liquid blood or blood stains, and the smallest amount of DNA typeable with the PM kit was established to be approximately 0.1 ng of template DNA. Although for highly degraded samples, the minimal amount of template DNA required might be expected to be several fold higher, one laboratory found that 2 ng of DNA highly degraded with DNAse I could be typed. The methods used in this study were not that sensitive to detecting small changes in quality and quantity of DNA, and may explain why variable results on the presence of an S dot was observed for DNA concentrations reported in the range of 1 to 0.1 ng of DNA (see Table 3). Overall, these studies establish that the analytical sensitivity was between 1.0 and 0.1 ng of DNA, whether the DNA was from a single source (Table 3) or a mixture (Table 5). This observed value is consistent with the specifications of the manufacturer (23). Similar results have been reported in the PM kit validation study reported by Budowle et al. (5).

Studies on DNA extracted from cellular material deposited and dried on different substrates also gave high rates of success and were in agreement with the results of testing blood stains, semen stains, and tissues even when the biological sample was treated with chemicals or exposed to environmental factors. In some instances, no reportable type was obtained. There are three possible explanations for these observations: (i) it was due to an insufficient concentration of DNA being extracted from the substrate, (ii) there was failure to amplify the DNA extracted from these samples due to the presence of Taq DNA polymerase inhibitors in the samples, and/or (iii) the DNA was too degraded to support amplification. Several strategies and techniques have been suggested for overcoming inhibition of the Taq DNA polymerase, including additional washing of the extracted DNA on a Centricon filter, addition of Bovine Serum Albumin (BSA) to the amplification mixture (30), addition of up to 10 U of Taq DNA polymerase, and the treatment of the DNA extracts with thiopropyl Sepharose (31). A strategy suggested for amplifying degraded DNA is to increase the concentration of template DNA. Overall, the results of these studies support the conclusion that a reliable and consistent result with the PM kit will be obtained if human DNA can be isolated from a sample free of inhibitors and is in a state in which degradation has not proceeded beyond the size of the targeted sequences.

The results obtained from the sensitivity studies and from the mixture studies provide information regarding the ability to detect the presence of a second source of DNA in a sample (e.g., sperm on a vaginal swab, mixed blood stains, contaminants). If the amount of the second source of DNA in a sample is below the threshold of detection (hundreds of pg of DNA for intact DNA up to 2 ng or more for degraded DNA), then no results will be observed for the second source of DNA. The PM types for only the primary source of DNA will be observed (i.e., the results will type as a single-source sample). If, however, the secondary source of DNA is present in a sample at a level above the threshold of detection of the PM kit, then the presence of the secondary source of DNA will only be detected when the ratio of the secondary:primary DNA is in the appropriate range. The appropriate ratio of secondary:primary DNA will vary from sample to sample depending on the particular genotypes present at each locus for each of the two DNA sources. "Extra" alleles (category I in Results and Table 5)

may be detected at a level above the "S" dot when the secondary source is present at a 1/20th or greater of the primary source. Below a 1:20 dilution, the extra alleles may be observed, but the intensity would generally be below the "S" dot and the results may or may not be interpretable. For samples that have overlapping alleles at every locus (category II in Results and Table 5), the presence of the secondary source will only be observed when it is in an amount similar (1:1 to 1:3) to the primary source. For this category, DNA sources present in lower amounts simply will not be observed.

Based on these studies, the PM kit can be expected to detect readily mixtures of DNA that fall within the criteria listed above and the studies support the notion that relative dot intensities are a linear function of the number of copies of alleles contributed by each individual in the mixture. The ability to interpret the results from mixed samples will vary from sample to sample depending on the ratio of the two DNAs and on the genotypes at each locus for each sample. In some cases, the primary DNA types will be predominant and may be interpretable. For such cases, results obtained with other PCR-based typing systems (e.g., D1S80, HLA DQA1, STRs) may be used in conjunction with the PM results to determine the minimum number of sources of DNA present in a sample for the interpretation of the mixed sample.

The evaluation of assay performance with casework undertaken by the laboratories resulted in a wide array of different types of data. The data included results from analyses of proficiency panels, mock casework, and adjudicated/nonprobative casework. The distribution was approximately one third for each case category. It is significant that none of the results from parallel studies with other DNA testing methods on cases in which the correct result was known in advance indicated the PM kit misidentified or incorrectly typed a sample. No failures or errors were reported in the data from all the cases which comprise 779 separate isolations, amplifications, and hybridizations. The results of the studies on proficiency panels and mock cases corroborated the results from the reproducibility studies demonstrating that the ability of the PM kit to always obtain the correct type is 100%.

This lack of detectable error in typings with the PM test is distinguished from the limited cases in which the laboratories routinely noted both inclusions and exclusions of individuals as potential sources of DNA extracted from evidence when several typing systems were used. In those instances in which correlation was not observed between PM kit results and the test results of other DNA testing methods, it was found that the test results from the more discriminating test resulted in an exclusion. In this study, no case was observed in which a less discriminating test excluded and the PM kit included an individual as a source of DNA extracted from evidence. It should be emphasized that there is always the possibility that a less discriminating test can distinguish two differing samples that type identically with a more discriminating test. Overall, the test results indicate that the performance characteristics approach the ideal for a diagnostic test. When properly performed, the PM kit gives reliable and consistent results in forensic analysis and there is a high likelihood of excluding an individual who is falsely accused of being the source of the DNA extracted from the biological sample.

In summary, the data presented in this report support the following conclusions about the PM kit: (1) DNA extracted with either the Chelex ion exchange resin or with organic solvents can be amplified and typed, (2) the typings are reproducible when either replicate samples or different types of samples from the same individual are typed, (3) the substrate, chemical substances, and environmental factors generally had little effect on the ability to amplify and type a sample, (4) as little as 100 pg of template DNA can be amplified and typed, (5) other than high nonhuman primates, only human DNA is detected, (6) mixtures of DNA of two or more individuals can be detected, (7) the collective studies on proficiency panels, mock cases, and adjudicated/nonprobative casework in particular demonstrate that reliable and interpretable results can be obtained whenever it is possible to obtain sufficient quantity and quality of DNA from an item containing biological evidence, and (8) all laboratories obtained essentially equivalent results using the kit.

Finally, the Technical Working Group on DNA Analysis Methods (TWGDAM) has established guidelines for any PCR-based test that is to be used for forensic genetic analysis. The data obtained in these validation studies demonstrate that the AmpliType PM PCR amplification and typing kit has met the guidelines and performance requirements of TWGDAM. More important, every laboratory obtained identical or equivalent results in all aspects of this collaborative study. Thus it can be concluded that not only does the PM kit meet the regulatory requirements for a DNA-based test, it has also fulfilled the requirement of general scientific acceptability of the test for forensic DNA testing.

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