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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

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ABSTRACT: Studies were performed to evaluate the forensic applicability of multiplex amplification of the loci low density lipoprotein receptor, glycophorin A, hemoglobin G gammaglobin, D7S8, and group-specific component (PM loci) and simultaneous typing of these loci using a reverse dot blot approach where allele specific oligonucleotide probes are immobilized on a nylon membrane strip. These results were obtained by using the AmpliType® PM PCR Amplification and Typing Kit. The experiments included: mixed body fluid studies; chemical contaminant effects on the DNA in body fluid samples; the effect of typing DNA from body fluid samples deposited on various substrates; the effect of microorganism contamination on typing DNA derived from blood and semen; the effect of sunlight and storage conditions on DNA typing; determination of the sensitivity of detection of the PM test kit; determination of cross-reactivity of DNA from species other than human; typing DNA derived from various tissues from an individual; and an evaluation of the hybridization temperature of the assay. The data demonstrate that DNA exposed to a variety of environmental insults yields reliable PM typing results. Allele and genotype frequencies for six loci (PM loci and HLA-DQα) were determined in African Americans, Caucasians, southeastern Hispanics, and southwestern Hispanics. All loci meet Hardy-Weinberg expectations and there is little evidence for association of alleles between the loci. The frequency data can be used in forensic analyses and paternity tests to estimate the frequency of a multiple locus DNA profile in various general United States populations.

KEYWORDS: pathology and biology validation studies, polymerase chain reaction, population databases, LDLR, GYPA, HBGG, D7S8, Gc, HLA-DQα, multiplex amplification

The detection and typing of DNA derived from forensic biological materials have been facilitated by the use of the polymerase chain reaction (PCR) [*1–15*]. HLA-DQα is the most characterized PCR-based forensic system [*2–6,8,9,12,14,16–19*], and the test can be performed using a commercially available kit. Additional PCR-based systems would be desirable to increase the discrimination power of the DNA analysis.

Using the AmpliType® PM PCR Amplification and Typing Kit (Perkin Elmer Corporation, Norwalk, CT), it is possible to amplify simultaneously six loci: HLA-DQα [*20*], low density lipoprotein receptor (LDLR) [*21*], glycophorin A (GYPA) [*22*], hemoglobin G gammaglobin (HBGG) [*23*], D7S8 [*24*], and group-specific component (Gc) [*25*]. Subsequently, LDLR, GYPA, HBGG, D7S8, and Gc (PM loci), are typed simultaneously, in a manner similar to that of HLA-DQα [*26*], using a reverse dot blot approach where the allele specific oligonucleotide probes are immobilized on a nylon membrane strip. The nylon strip contains a probe designated as an "S" which is specific for the HLA-DQα locus. The S dot is designed to be the lightest dot on the nylon strip and is intended to act as a threshold for evaluating stochastic effects. With this kit LDLR, GYPA, and D7S8 each have two detectable alleles, designated A and B; while HBGG and Gc each have three alleles that can be typed, designated A, B, and C. The advantage of a multiplex system is that less template DNA is consumed than when analyzing each locus independently.

Before a technique can be used to analyze evidentiary samples, it should be evaluated for its forensic applicability. While the effect of environmental insults on DNA is well-understood [*1–15*], the limitations of the use of a new test may need some definition. In addition, for the use of genetic markers in identity testing, it is desirable to collect allele/genotype frequency data from relevant population(s) so that the forensic scientist can provide an estimate of the rarity of a genetic profile. This paper presents the results from a forensic validation study on the typing of PM loci and the allele/genotype frequency data for the HLA-DQα, LDLR, GYPA, HBGG, D7S8, and Gc loci for United States African American, Caucasian, and Hispanic population samples.

Materials and Methods

Sample Preparation

The source and extraction of DNA from whole blood from the population samples were described previously [*27,28*]. The validation samples used in this study were a subset of those analyzed previously in a D1S80 validation study (see Baechtel et al., in preparation). Tissue samples (blood, bone, kidney, liver, muscle,

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and spleen) from four individuals were kindly provided by A.G. Kasselberg (Vanderbilt University, Nashville, TN). DNA was extracted from the tissues according to the method described by Comey et al. [*30*]. The quantity of DNA in each sample was estimated using the slot-blot procedure described by Waye et al. [*29*]. Generally, two-to-five ng of DNA were amplified by PCR.

Validation Study

For the validation study body fluid samples from eight different donors were used. The PM loci types of the donors are shown in Table 1. All PM alleles were represented.

The validation study consisted of the following analyses: 1) sensitivity and detection of mixed samples; 2) chemical contaminant effects on the DNA in body fluid samples; 3) the effect of typing DNA from body fluid samples deposited on various substrates; 4) the effect of microorganism contamination on typing DNA derived from blood and semen; 5) the effect of sunlight and storage conditions on DNA typing (see Table 2 for the number of samples analyzed); 6) determination of the sensitivity of the PM test kit by typing genomic DNA from two samples at different quantities. The quantities of DNA amplified for these two samples were 5 ng, 2 ng, 1 ng, 500 pg, and 250 pg; 7) cross-reactivity of DNA from species other than human. DNA from the following organisms was analyzed: gorilla, Japanese macaque, orangutan, spider monkey, Celebes ape, Debrazza monkey, pig, sheep, burro, horse, goat, dog, chicken, cat, rabbit, steer, Candida albicans and Escherichia coli. These samples were analyzed twice using template DNA ranging from five to 60 ng; and 8) DNA derived from various tissues, including blood, bone, kidney, spleen, liver, and muscle from four individuals was typed.

In addition, this study examined the effect of varying the hybridization temperature of the assay. Four different DNA samples were typed for PM loci at different hybridization temperatures. Using a NIST traceable thermometer, the temperature in the hybridization water bath was adjusted from 52 to 59°C in one degree increments.

Typing

The PM loci were typed using the AmpliType® PM PCR Amplification and Typing Kit (Perkin Elmer Corporation, Norwalk, CT). The amplification conditions were those recommended by the manufacturer, except that 16 μg of bovine serum albumin (Sigma, catalog #3350) were added to the PCR [*6,31*]. Amplification was carried out in a Perkin-Elmer DNA Thermal Cycler 480.

The population samples also were typed using the AmpliType® HLA-DQα Forensic DNA Amplification and Typing Kit (Perkin Elmer Corporation, Norwalk, CT) by following the manufacturer's recommended protocol. The HLA-DQα PCR product was derived from the PM multiplex amplification.

TABLE 1—*PM marker genotypes of body fluid donors for validation study.*

LDLR	GYPA	HBGG	D7S8	Gc
AA	AB	AB	AB	BC
AB	BB	AB	AB	BC
AB	BB	AB	AB	AA
AB	BB	BB	AB	CC
BB	AA	AB	AA	BC
AB	BB	AB	AB	CC
AA	AB	AB	AB	AC
BB	AA	AA	AA	BC

TABLE 2—*Results of environmental insult validation study.*

Validation samples*	No. of samples analyzed	No. of samples successfully typed
Mixtures[b]—blood/blood,	5	5
Blood/semen,	20	18[c]
Saliva/semen	20	20
Oil/blood	2	2
Oil/saliva	1	1
Oil/semen	2	2
Gasoline/blood	2	2
Gasoline/saliva	1	1
Gasoline/semen	2	2
Dirt/blood	1	1
Dirt/saliva	1	1
Dirt/semen	2	2
Detergent/saliva	1	1
Detergent/semen	2	2
0.4N NaOH/saliva	1	1
0.4N NaOH/semen	2	1[d]
5% Acetic acid/blood	1	1
5% Acetic acid/saliva	1	0[d]
5% Acetic acid/semen	2	2
Candida albicans/blood	2	2
Candida albicans/semen	2	1[d]
Staphylococcus epidermis/blood	2	2
Staphylococcus epidermis/semen	2	1[d]
Escherichia coli/blood	2	2
Escherichia coli/semen	2	1[d]
Bacillus subtilis/blood	2	2
Bacillus subtilis/semen	2	2
Blood/sun 20 weeks	1	1
Blood/shade 20 weeks	1	1
Blood/RT 20 weeks	1	1
Semen/sun 2 weeks	1	1
Semen/sun 4 weeks	1	0[d]
Semen/shade 8 weeks	1	1
Semen/shade 10 weeks	1	1
Semen/shade 12 weeks	2	2
Semen/shade 14 weeks	2	2
Semen/shade 20 weeks	1	1
Semen/RT 16 weeks	1	1
Semen/RT 20 weeks	1	1
Carpet/blood	1	1
Carpet/semen	1	1
Denim/blood	1	1
Denim/semen	1	1
Nylon/blood	1	1
Nylon/semen	1	1
Wallboard/blood	1	1
Wallboard/semen	1	1
Wood/blood	1	1
Wood/semen	1	1
Leather/blood	2	1[e]
Leather/semen	2	2
Totals	113	104

[a]Validation samples were a subset of samples derived from a D1S80 validation study (Baechtel, et al., in preparation).

[b]Ratios of body fluid mixtures ranged from 1:1 to 1:20.

[c]Seven of the blood/semen mixture typed samples initially had no S dot. In these samples the quantity of template DNA used for the PCR was less than 2 ng. There was sufficient DNA (2 ng) in five of the seven samples to perform the PCR again. When typed again, the S dots were visible.

[d]= The sample that was not successfully typed had no visible S dot.

[e]= The sample that was not successfully typed did not exhibit any PCR amplification products.

Statistical Analysis

The frequency of each allele for each locus was calculated from the numbers of each genotype in the sample set. Unbiased estimates of expected heterozygosity were computed as described by Edwards et al. [*32*]. Possible divergence from Hardy-Weinberg expectations (HWE) was determined by calculating the unbiased

estimate of the expected homozygote/heterozygote frequencies [*33–35*], the likelihood ratio test [*32,36,37*], and the exact test [*38*]. An interclass correlation criterion [*39*] was used for detecting disequilibrium between loci. Dependence across the PM markers, HLA-DQα, and D1S80 [*28*] also was determined by examining whether or not the observed variance of the number of heterozygous loci in the population sample is outside its confidence interval under the assumption of independence [*40,41*]. When appropriate, the Bonferroni procedure [*42*] was used to correct for multiple analyses to determine whether or not HWE or equilibrium between loci holds in the population.

The G-statistic [*42,43*] was used to test for homogeneity (1000 shuffling experiments) between various population samples. The program was kindly provided by G. Carmody (Carleton University, Ottawa, Canada).

Results and Discussion

Environmental Insults

The approach for the validation study on PM loci was similar to that reported by Comey and Budowle [*3*]. However, since there exists now a good understanding of the reliability of typing DNA derived from environmentally-insulted samples [*1–15*], a less extensive validation study was performed. The purpose of the study was to define the limitations of PM typing on biological materials.

DNA extracted from blood, semen, and saliva exposed to a variety of environmental insults can be amplified by multiplex PCR and typed successfully at the PM loci LDLR, GYPA, HBGG, D7S8, and Gc. These results are summarized in Table 2. Types were obtained for 104 of the 113 samples. Of the nine samples that could not be typed eight samples had no visible S dot. One blood sample on leather did not amplify; however, three other samples on leather were typeable. Since sufficient DNA was available for analysis, this amplification failure may be due to some inhibitor of PCR.

Tissue Study

DNA extracted from liver, bone, muscle, spleen, and kidney from four different individuals (a total of 40 samples was analyzed in duplicate) was typed for the PM loci. All samples were typeable and consistent with the blood exemplars from the individual donors. Figure 1 shows an example of PM typing of DNA extracted from different tissues.

Species Cross-Reactivity

While cross-reactivity of a DNA typing test with DNA from species other than humans (or higher primates) does not invalidate the use of a forensic DNA test, it is important to determine whether or not DNA from other species can yield positive results. With PM loci typing typeable allele dots were observed for gorilla (LDLR, HBGG, and Gc), Japanese macaque (all PM loci), Debrazza monkey (all PM loci), and orangutan (LDLR, D7S8, and Gc). S dots were visible for gorilla, Japanese macaque, spider monkey, and orangutan. The PM allele dots were very weak for the other primates. There were no positive PM allele dots for any non-primate species other than horse (at 10 ng template DNA – LDLR and HBGG) and goat (at 40 ng template DNA – GYPA, HBGG, D7S8, and Gc) (Fig. 2). The typing strips for horse and goat had no visible S dot. The allele dot intensities for horse and goat were extremely weak and less intense than the intensities of S dots obtained from 2 ng human genomic template DNA samples.

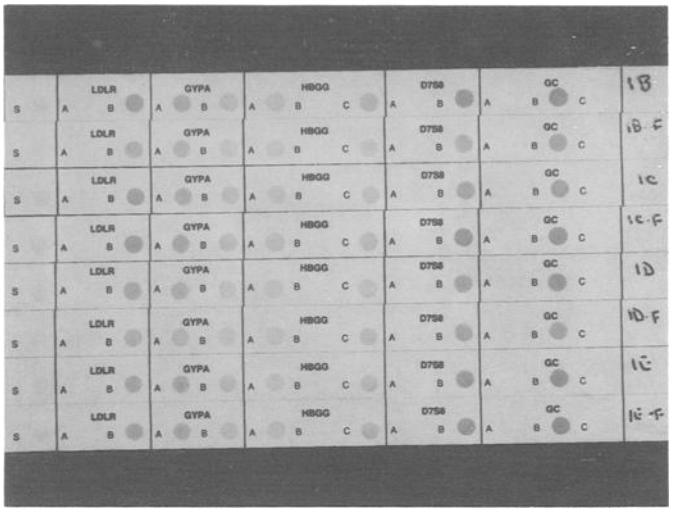

FIG. 1—*PM loci typing of DNA from various tissues from one individual. Samples 1B and 1B-F are from liver, 1C and 1C-F are from bone, 1D and 1F are from muscle, and 1E and 1E-F are from spleen.*

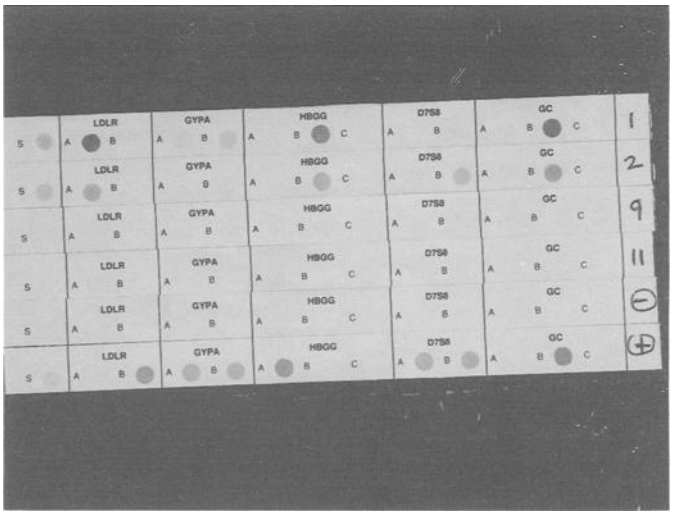

FIG. 2—*PM loci typing of DNA from different non-human species.*

Performance Parameters

The data above demonstrate that PM loci typing of biological materials yields reliable results. However, throughout the course of the PM loci validation study observations were made concerning the performance of the PM loci typing system.

Sensitivity Study

Typing of HLA-DQα has been possible with as little as 250–500 pg of genomic template DNA [*3*]. Genomic DNA at quantities of 5 ng, 2 ng, 1 ng, 500 pg, and 250 pg from two different individuals was typed for the PM loci at a hybridization temperature of 55°C. Based on the presence of a sensitivity dot (or S dot) on the PM nylon strip, at least 1 ng of genomic DNA was required for successful typing. When no PM PCR products appeared on the post-amplification yield gel, PM typing was not possible, because there were no visible S dots and the PM allele dots were weak or not detected. The S dot (as well as the C dot on an HLA-DQα typing

strip) is used as an indicator for consideration of stochastic effects when too little template DNA is used for the PCR. Based on the number of template molecules required before stochastic effects are considered, the S dot sensitivity could be increased comparable to that of the C dot on an HLA-DQα typing strip without any loss in the reliability regarding stochastic effects with PM marker typing.

Hybridization Temperature

During the course of the PM validation study, it was observed on a few occasions that the Gc allele dots were weaker than other PM loci allele dots. Based on the sequence of Gc [*25*], this phenomenon would be expected if the hybridization temperature were too high. The manufacturer's protocol recommends using a hybridization temperature of 55°C for PM loci typing. Four different samples were typed at different hybridization temperatures ranging from 52–59°C. The allele dot intensity for the PM markers was more intense at 54°C than at 55°C (Fig. 3). Furthermore, the intensity of the allele dots decreased noticeably at 56°C. At a hybridization temperature of 53°C there was very weak hybridization at the HBGG A allele (when no A product was present) and at 52°C cross-hybridization was observed at the HBGG A and C alleles and at the Gc B alleles (when these PCR products were not present). The cross-hybridization signals at 52°C and 53°C were less than the S dot and did not interfere with PM typing. Based on these observations, a hybridization temperature of 54°C is recommended to achieve a greater sensitivity of detection. However, a hybridization control sample which is an HBGG B homozygote should be considered to monitor cross-hybridization at the HBGG A and C alleles when the hybridization temperature is too low. With proper quality assurance and quality control measures to monitor the water bath temperature, it should be possible to maintain a 54°C hybridization temperature. Alternatively, a 55°C hybridization temperature may be desirable for quality control and quality assurance considerations. Minor hybridization cross-reactivity (that is, below the S dot) did not compromise the PM typing results (the alleles were typed correctly).

Effect of Multiplex Amplification on HLA-DQα Typing

The AmpliType® PM PCR Amplification and Typing Kit (Perkin Elmer Corporation, Norwalk, CT) enables the multiplex amplification of the PM markers, as well as HLA-DQα. The five loci LDLR, GYPA, HBGG, D7S8, and Gc are typed simultaneously on one nylon strip, while HLA-DQα is typed using a separate nylon strip. Therefore, six different loci possibly could be typed from a single DNA sample of at least 1 ng. When the HLA-DQα products from the multiplex amplifications were typed, the HLA-DQα allele dots were more intense than when similar quantities of DNA were amplified and typed singly using the AmpliType® HLA-DQα Forensic DNA Amplification and Typing Kit (Perkin Elmer Corporation, Norwalk, CT). In contrast, the C dots from the HLA-DQα products from the multiplex amplification were less intense. In fact the C dots tended to be less intense than the S dots in PM loci typing from the same multiplex amplification samples. However, the intensity of the PM allele dots can be used to indicate whether or not an HLA-DQα C dot typing would be visible from a PM multiplex amplification product.

Regardless, the absence of a visible C dot from an HLA-DQα product from a PM multiplex amplification should not necessarily preclude typing the HLA-DQα locus. The S dot from the PM typing strip can be used to evaluate whether or not stochastic effects should be considered. If the HLA-DQα allele dots are more intense than those observed from typing a 500 pg – 1 ng sample, then it would be appropriate to proceed with HLA-DQα profile interpretation.

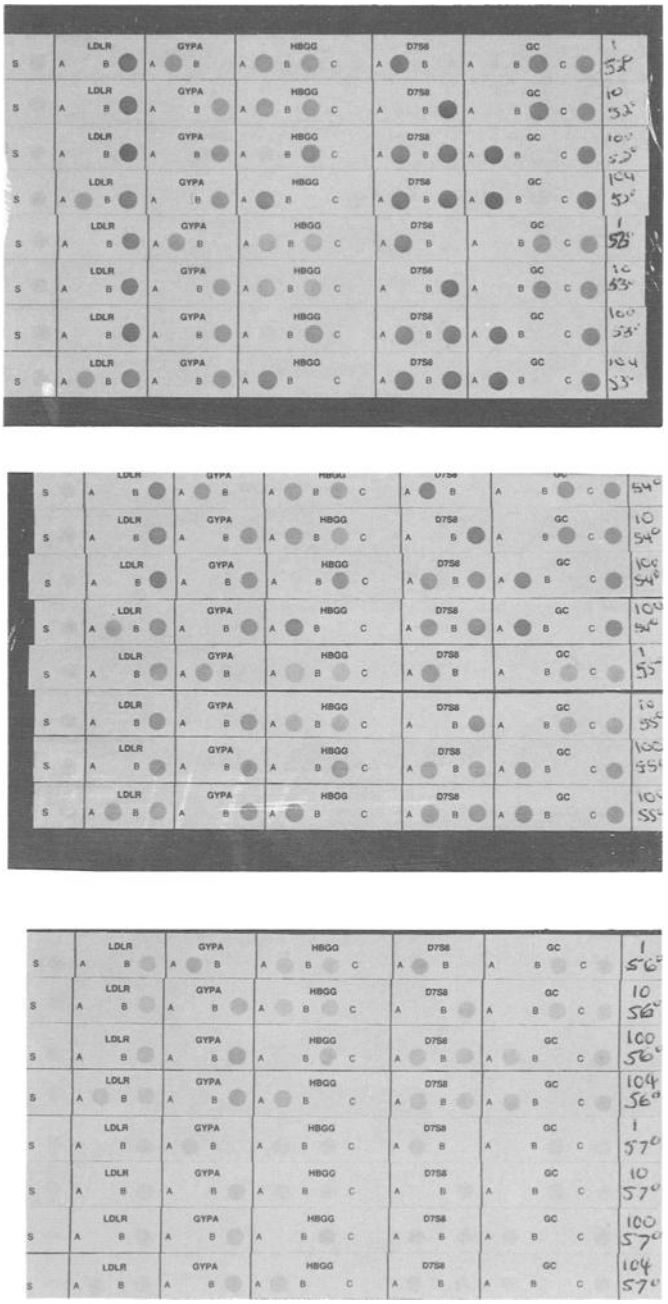

FIG. 3—*The effect of hybridization temperature on PM loci typing of DNA from four different individuals. From top-to-bottom the first four samples were hybridized at 52°C, the second four samples were hybridized at 53°C, the third four samples were hybridized at 54°C, the fourth four samples were hybridized at 55°C, the fifth four samples were hybridized at 56°C, and the last four samples were hybridized at 57°C.*

Balanced Alleles

Generally, the allele dot intensities across a PM typing strip are balanced. In other words, the allele dot intensities of a heterozygote at one locus are comparable to those of a heterozygote profile at

another locus. Additionally, the allele dot of a homozygous profile is more intense than the dots of a heterozygous profile (Fig. 4, sample #1341). However, there were a few samples where the allele dots were not balanced (Fig. 4). These samples were population database samples and therefore generally can be presumed to be unmixed samples. An example of unbalanced dots are samples 1119 and 1127. In these samples, the HBGG B dot is more intense than the HBGG C dot and the HBGG A dot is less intense than the HBGG C dot, respectively. In sample 1123, the HBGG alleles have different intensities and the HBGG C allele is more intense than the homozygous profiles at the other four loci. Also, in sample 1181, the GYPA and the HBGG heterozygous profiles are unbalanced. The exact percentage of samples that exhibit unbalanced allele dot intensities is difficult to determine, because the determination of unbalanced intensity is somewhat subjective.

In addition, there can be an intensity difference for the LDLR locus dots compared with the other PM loci dots (Fig. 4). For example, in the positive control sample the LDLR B dot is no more intense than the heterozygous dots at GYPA and D7S8 and less intense than the homozygous dots at HBGG and Gc. This phenomenon with the LDLR locus is the most prevalent example of unbalanced alleles and appears to be related to the quality of the DNA sample. Generally, the weak dots for LDLR are correlated with the reduced product on the the test gels. Hochmeister, et al. (in preparation) have shown that Chelex-extracted DNA tends to exhibit a reduced LDLR dot intensity compared with organically extracted DNA that has been washed using a Centricon 100 microconcentrator device. Importantly, none of these observations of unbalanced alleles resulted in an incorrect type; though it must be considered different intensities of allele dots should not be problematic.

Body Fluid Mixtures

The presence of two or more contributors to a sample generally is inferred by the presence of unbalanced dots and/or as well as extra dots in HBGG and Gc. Figure 5 shows some examples of mixtures on PM typing strips. Some mixtures are readily detected (samples 5–38, 5–39, 5–42, and 5–43) and others are such that the minor component is too low to be detected (samples 5–29 and 5–31). As a general rule, samples with two contributors of known PM types displayed PM types as predicted. Each contributed to the profile and when the samples shared alleles in common the dot intensities were increased compared to alleles that were not shared. The minor component of a 1:20 mixture of two samples was barely detectable, and the allele dot for the minor component was less intense than the S dot.

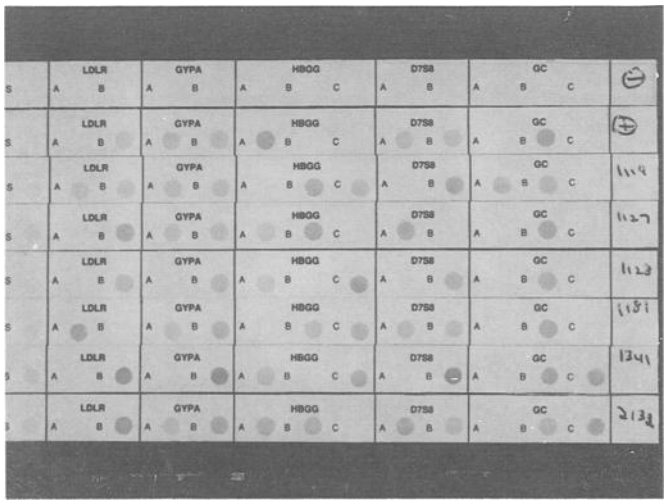

FIG. 4—*PM loci typing of samples displaying unbalanced alleles.*

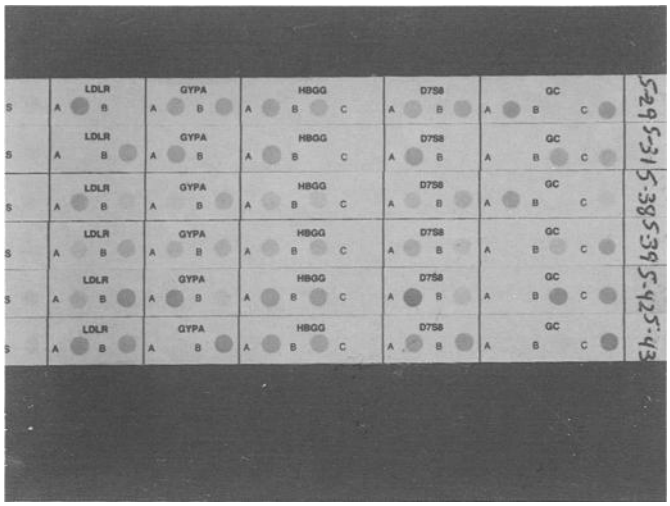

FIG. 5—*PM loci typing of DNA from mixed body fluid samples. All samples were mixtures of saliva and semen in the following ratios: 5-29 is 1:20; 5-31 is 1:20; 5-38 is 20:1; 5-39 is 20:1; 5-42 is 5:1; and 5-43 is 5:1.*

Population Data

The distributions of observed genotype and allele frequencies for the five PM loci are polymorphic in African Americans, United States Caucasians, southeastern Hispanics, and southwestern Hispanics (Tables 3 and 4). There is no detectable deviation from HWE for these loci in the four population groups based on the homozygosity test, likelihood ratio test, and the exact test (Table 5).

Since HLA-DQα was coamplified with the PM loci, the population samples also were typed for HLA-DQα. The HLA-DQα genotype and allele frequency distributions for the previously PM typed African American, United States Caucasian, southeastern Hispanic, and southwestern Hispanic population samples are shown in Tables 6 and 7. There is no detectable deviation from HWE for HLA-DQα in these population samples based on the homozygosity test, likelihood ratio test, and the exact test (Table 6).

Estimating the frequency of a DNA profile containing all six loci by the direct count method with databases of the sizes reported in our study generally does not provide a reliable estimate of the rarity of a multiple locus DNA profile. This is exacerbated when additional PCR-based loci are considered, such as D1S80. Except for GYPA and Gc, which both are on chromosome 4, the PM, HLA-DQα, and D1S80 loci are on different chromosomes and, therefore, it would be expected that their frequencies are independent. Regardless, analyses were performed to determine whether or not there were any detectable associations between any of the PM, HLA-DQα, and D1S80 loci. These samples were typed previously for D1S80 [*28*]. An interclass correlation test [*39*] analysis demonstrated that there is little evidence for correlation between the alleles at any of the pairs of loci (Table 8). There were four examples of deviation out of a total of 84 interclass correlation tests, which is approximately 5% of the comparisons. Thus, the

TABLE 3—*Observed frequency distributions of PM loci genotypes.*

Genotype	African American (N = 145)[a]	Caucasian (N = 148)[a]	Southeastern Hispanic (N = 94)[a]	Southwestern Hispanic (N = 96)[a]
LDLR AA	0.048	0.176	0.191	0.313
LDLR AB	0.352	0.554	0.447	0.500
LDLR BB	0.600	0.270	0.362	0.188
GYPA AA	0.228	0.351	0.330	0.448
GYPA AB	0.503	0.466	0.404	0.417
GYPA BB	0.269	0.182	0.266	0.135
HBGG AA	0.262	0.223	0.160	0.135
HBGG AB	0.172	0.493	0.521	0.365
HBGG BB	0.062	0.277	0.266	0.406
HBGG AC	0.317	0.000	0.011	0.052
HBGG BC	0.097	0.000	0.043	0.042
HBGG CC	0.090	0.007	0.000	0.000
D7S8 AA	0.338	0.358	0.340	0.458
D7S8 AB	0.552	0.514	0.489	0.448
D7S8 BB	0.110	0.128	0.170	0.094
Gc AA	0.014	0.054	0.053	0.083
Gc AB	0.131	0.074	0.181	0.083
Gc BB	0.517	0.047	0.043	0.063
Gc AC	0.048	0.331	0.266	0.292
Gc BC	0.248	0.176	0.181	0.208
Gc CC	0.041	0.318	0.277	0.271

[a] N refers to the number of individuals in the database.

TABLE 4—*Observed allele frequency distributions for PM loci.*

Allele	African American (N = 145)[a]	Caucasian (N = 148)[a]	Southeastern Hispanic (N = 94)[a]	Southwestern Hispanic (N = 96)[a]
LDLR A	0.224	0.453	0.415	0.563
LDLR B	0.776	0.547	0.585	0.438
GYPA A	0.479	0.584	0.532	0.656
GYPA B	0.521	0.416	0.468	0.344
HBGG A	0.507	0.470	0.426	0.344
HBGG B	0.197	0.524	0.548	0.609
HBGG C	0.297	0.007	0.027	0.047
D7S8 A	0.614	0.615	0.585	0.682
D7S8 B	0.386	0.385	0.415	0.318
Gc A	0.103	0.257	0.277	0.271
Gc B	0.707	0.172	0.223	0.208
Gc C	0.190	0.571	0.500	0.521

[a] N refers to the number of individuals in the database.

amount of deviation was no more than expected. A Bonferroni procedure [*42*] was used as a correction when multiple tests are performed on a population sample. After correction, the data support that for the seven PCR-based loci the populations meet expectations of independence (P = 0.0006 is the rejection level).

The effect on a frequency estimate of a detectable two-locus deviation from independence can be demonstrated by two examples in Table 9. The impact of a two-locus DNA profile estimate for LDLR and GYPA in southeastern Hispanics (the lowest P value in the interclass correlation tests) and LDLR and HBGG in Caucasians (the lowest P value in the Caucasian interclass correlation tests) can be examined by comparing the two locus genotype frequencies derived by the counting method (observed two-locus genotype frequency) with those by the multiplication rule under the assumption of independence (that is, the product rule). Those estimates in Table 9 marked by an asterisk are situations where the product rule yielded a lower frequency estimate than the counting method. For the southeastern Hispanics the counting method never produced an estimate that was more than 2.6 fold greater than the product rule approach (LDLR AA and GYPA AA genotype). In fact, it is the LDLR AA and GYPA BB genotype that contributes most to the two-locus deviation, and in this case the product rule would contribute a significantly more common estimate of the two-locus genotype than the counting method. As an additional example, the counting method for the Caucasian LDLR/HBGG two-locus deviation from independence did not yield an estimate that was more than 1.6 fold greater than the product rule (LDLR AA and HBGG BB genotype). The two locus genotype that contributed the most to a significant deviation from independence was LDLR AA and HBGG AA, again where the counting method yielded a lower frequency than did the product rule. These examples demonstrate that the use of the product rule would provide a

TABLE 5—*Tests for independence on PM loci.*

	African American	Caucasian	Southeastern Hispanic	Southwestern Hispanic
LDLR	(N = 145)	(N = 148)	(N = 94)	(N = 96)
Obs. Homozygosity	64.8%	44.6%	55.3%	50.0%
Exp. homozygosity[a]	65.1%	50.3%	51.2%	50.5%
Homozygosity test[b]	0.945	0.167	0.423	0.918
Likelihood ratio test[b]	1.000	0.199	0.532	1.000
Exact test[b]	1.000	0.199	0.532	1.000
GYPA				
Obs. homozygosity	49.7%	53.4%	59.6%	58.3%
Exp. homozygosity[a]	49.9%	51.3%	49.9%	54.7%
Homozygosity test[b]	0.951	0.606	0.062	0.468
Likelihood ratio test[b]	1.000	0.768	0.103	0.497
Exact test[b]	1.000	0.639	0.069	0.497
HBGG				
Obs. homozygosity	41.4%	50.7%	42.6%	54.2%
Exp. homozygosity[a]	38.1%	49.9%	47.9%	48.9%
Homozygosity test[b]	0.422	0.858	0.298	0.302
Likelihood ratio test[b]	0.421	1.000	0.343	0.365
Exact test[b]	0.360	0.887	0.368	0.407
D7S8				
Obs. homozygosity	44.8%	48.7%	51.1%	55.2%
Exp. homozygosity[a]	52.4%	52.5%	51.2%	56.4%
Homozygosity test[b]	0.067	0.351	0.981	0.811
Likelihood ratio test[b]	0.063	0.382	1.000	0.812
Exact test[b]	0.063	0.382	1.000	0.812
Gc				
Obs. homozygosity	57.2%	41.9%	37.2%	41.7%
Exp. homozygosity[a]	54.5%	42.0%	37.3%	38.5%
Homozygosity test[b]	0.504	0.986	0.988	0.521
Likelihood ratio test[b]	0.786	0.393	0.249	0.643
Exact test[a]	0.658	0.364	0.254	0.561

[a]Expected homozygosity is an unbiased estimate.
[b]These values are probability values.

TABLE 6—*Distribution of observed HLA-DQα genotype frequencies in several United States general population groups Caucasians.*

Genotype	African American[a] (N = 145)[e]	Caucasian[b] (N = 148)[e]	Southeastern Hispanic[c] (N = 94)[e]	Southwestern Hispanic[d] (N = 96)[e]
1.1–1.1	0.021	0.014	0.064	0.021
1.1–1.2	0.034	0.068	0.064	0.052
1.1–1.3	0.000	0.007	0.011	0.000
1.1–2	0.028	0.041	0.053	0.031
1.1–3	0.028	0.054	0.032	0.042
1.1–4	0.103	0.047	0.074	0.115
1.2–1.2	0.110	0.020	0.032	0.021
1.2–1.3	0.062	0.000	0.021	0.010
1.2–2	0.048	0.061	0.032	0.031
1.2–3	0.062	0.068	0.064	0.083
1.2–4	0.200	0.115	0.064	0.052
1.3–1.3	0.000	0.014	0.011	0.000
1.3–2	0.014	0.007	0.032	0.000
1.3–3	0.000	0.014	0.032	0.031
1.3–4	0.034	0.027	0.043	0.021
2–2	0.007	0.020	0.043	0.010
2–3	0.007	0.041	0.043	0.021
2–4	0.083	0.047	0.074	0.083
3–3	0.007	0.061	0.032	0.042
3–4	0.048	0.135	0.149	0.198
4–4	0.103	0.142	0.032	0.135

[a]African Americans – Observed Homozygosity = 0.248; Expected Homozygosity (unbiased) = 0.243; HWE – Homozygosity Test (P = 0.870), Likelihood Ratio Test (P = 0.313), Exact Test (P = 0.402).
[b]Caucasians – Observed Homozygosity = 0.270; Expected Homozygosity (unbiased) = 0.213; HWE – Homozygosity Test (P = 0.087), Likelihood Ratio Test (P = 0.158), Exact Test (P = 0.094).
[c]Southeastern Hispanics – Observed Homozygosity = 0.213; Expected Homozygosity (unbiased) = 0.175; HWE – Homozygosity Test (P = 0.341), Likelihood Ratio Test (P = 0.662), Exact Test (P = 0.486).
[d]Southwestern Hispanics – Observed Homozygosity = 0.229; Expected Homozygosity (unbiased) = 0.233; HWE – Homozygosity Test (P = 0.926), Likelihood Ratio Test (P = 0.776), Exact Test (P = 0.741).
[e]N refers to the number of individuals in the database.

TABLE 7—*HLA-DQα observed allele frequencies in several United States general population groups.*

	African Americans (N = 145)	Caucasians (N = 148)	Southeastern Hispanics (N = 94)	Southwestern Hispanics (N = 96)
Allele 1.1	0.117	0.122	0.181	0.141
Allele 1.2	0.314	0.176	0.154	0.135
Allele 1.3	0.055	0.041	0.080	0.031
Allele 2	0.097	0.118	0.160	0.094
Allele 3	0.079	0.216	0.191	0.229
Allele 4	0.338	0.328	0.234	0.370

TABLE 8—*Two locus inter-class correlation test for HLA-DQα, PM, and D1S80 loci for several United States population samples.*

	African Americans (N = 145)	Caucasians (N = 148)	Southeastern Hispanics (N = 94)	Southwestern Hispanics (N = 96)
LDLR/GYPA	0.649	0.409	0.000[a]	0.737
LDLR/HBGG	0.144	0.016[a]	0.851	0.916
LDLR/D7S8	0.577	0.247	0.520	0.594
LDLR/Gc	0.586	0.829	0.605	0.747
LDLR/DQα	0.262	0.121	0.363	0.297
GYPA/HBGG	0.644	0.240	0.616	0.537
GYPA/D7S8	0.616	0.778	0.139	1.000
GYPA/Gc	0.935	0.884	0.439	0.251
GYPA/DQα	0.623	0.149	0.683	0.653
HBGG/D7S8	0.145	0.236	0.802	0.749
HBGG/Gc	0.268	0.150	0.761	0.275
HBGG/DQα	0.695	0.031[a]	0.212	0.426
D7S8/Gc	0.700	0.698	0.970	0.256
D7S8/DQα	0.253	0.730	0.380	0.896
Gc/DQα	0.303	0.506	0.961	0.301
D1S80/LDLR	0.846	0.919	0.187	0.059
D1S80/GYPA	0.998	0.741	0.004[a]	0.462
D1S80/HBGG	0.547	0.564	0.134	0.572
D1S80/D7S8	0.672	0.498	0.879	0.682
D1S80/Gc	0.816	0.374	0.946	0.307
D1S80/DQα	0.632	0.679	0.674	0.374

[a] = deviation at P = 0.05 level; with Bonferroni procedure level of rejection is P = 0.0006.

[b]D1S80 population data from Budowle et al. [*28*].

TABLE 9*a*—*Frequencies for the two-locus genotypes for LDLR and GYPA in 94 unrelated southeastern Hispanics.*

Genotypes	GYPA AA	GYPA AB	GYPA BB
LDLR AA	0.049[a]	0.086	0.038
	(0.128)[b,c]	(0.064)	(0.000)
LDLR AB	0.138	0.242	0.106
	(0.128)	(0.181)	(0.138)[c]
LDLR BB	0.097	0.170	0.075
	(0.074)	(0.160)	(0.128)[c]

[a]Values without parentheses are two-locus genotype frequency estimates based on the product rule.

[b]Values in parentheses are two-locus genotype frequency estimates based on the counting method.

[c]Those product rule frequency estimates that are less than the counting method.

TABLE 9*b*—*Frequencies for the two-locus genotypes for LDLR and HBGG in 148 unrelated United States Caucasians.*

Genotypes	LDLR AA	LDLR AB	LDLR BB
HBGG AA	0.045[a]	0.110	0.066
	(0.014)[b]	(0.142)[c]	(0.074)[c]
HBGG AB	0.101	0.245	0.147
	(0.074)	(0.277)[c]	(0.142)
HBGG BB	0.056	0.136	0.082
	(0.088)[c]	(0.135)	(0.054)

[a]Values without parentheses are two-locus genotype frequency estimates based on the product rule.

[b]Values in parentheses are two-locus genotype frequency estimates based on the counting method.

[c]Those product rule frequency estimates that are less than the counting method.

valid estimate of a multiple loci frequency for forensic purposes. Moreover, since product rule estimates for some of the single locus patterns in a multiple loci profile will be greater than those obtained by the counting method, any potential wrongful bias at one locus or two loci will tend to be minimized across several loci.

To confirm that there is little deviation from expectation when using the product rule to derive a multiple locus frequency estimate, an additional test for association that addresses all seven PCR-based loci at one time was considered. The test examines whether or not the observed variance (s_k^2) of the number of heterozygous loci in a population sample is outside its confidence interval under the assumption of independence using the procedure described by Brown et al. [*40*]. While this test criterion results in some loss of information in summarizing multiple locus genotype data, it is more powerful than the classical goodness-of-fit χ^2 test [*41*]. There was no evidence of association for the seven loci described in our African American, Caucasian, southeastern Hispanic, and southwestern Hispanic sample populations using the s_k^2 criterion ($s^2_{k\text{AFRICAN AMERICAN}}$ = 1.512, 95% confidence interval of variance is 1.186–1.840; $s^2_{k\text{CAUCASIAN}}$ = 1.751, 95% confidence interval of variance is 1.237–1.907; $s^2_{k\text{SOUTHEASTERN HISPANIC}}$ = 1.598, 95% confidence interval of variance is 1.114–1.935; $s^2_{k\text{SOUTHWESTERN HISPANIC}}$ = 1.620, 95% confidence interval of variance is 1.151–1.983).

All multiple locus PM and HLA-DQα profiles were compared among the 483 total samples in African Americans, Caucasians, southeastern Hispanics, and southwestern Hispanics for matching genotypes. There were 20 pairs of samples that had identical PM and HLA-DQα types; of these no more than two individuals shared the same six locus genotype. Each match pair was different at the D1S80 locus.

Conclusion

Multiplex amplification by PCR of DNA exposed to a variety of environmental insults yields reliable PM typing results. There was no evidence of false positive or false negative results. Generally, the intensity of the PM allele dots are balanced within a typing strip, but this is not always the case. Therefore, allele dots with different intensities may not be indicative the presence of a body fluid mixture in a biological sample. Because of the potential for unbalanced allele dot intensities and the limitations for detecting some mixed samples containing equivalent amounts of DNA, caution should be exercised when interpreting evidentiary samples that potentially may be from more than one donor.

Sensitivity of detection of PM allele dots was increased by lowering the hybridization temperature to 54°C. However, to carry

out PM analysis more effectively for quality control and quality assurance, a 55°C hybridization temperature is recommended.

The distribution of the genotype frequencies for the various PM loci (as well as HLA-DQα) meet HWE, and there is little evidence for association of alleles across loci (for the PM loci, HLA-DQα, and D1S80) for our African American, Caucasian, southeastern Hispanic, and southwestern Hispanic population databases. The data demonstrate that valid estimates of a multiple locus profile frequency can be derived for identity testing purposes using the product rule under the assumption of independence.

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