## TECHNICAL NOTE

*Nicola Fildes,*[1] *B.S. and Rebecca Reynolds,*[1] *Ph.D.*

# Consistency and Reproducibility of AmpliType® PM Results Between Seven Laboratories: Field Trial Results

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**ABSTRACT:** The AmpliType® PM Field Trial was designed to assess the ability of forensic laboratories to obtain the correct results from samples commonly encountered in forensic casework. The seven forensic laboratory participants of the AmpliType® PM Field Trial each performed four studies. Samples were analyzed using components of the AmpliType® PM PCR Amplification and Typing Kit. Laboratories were also provided with DNA probe strips to type the DQA1 locus. Of the 381 PM and 325 DQA1 DNA probe strip results obtained from DNA-containing and non-DNA-containing samples, 98.2% and 95.7% showed the correct result for PM and DQA1 types, respectively. No samples were typed incorrectly. The remaining small percentage of samples were either uninterpretable due to the presence of a mixture, or no result was obtained due to insufficient DNA. The Field Trial demonstrated that laboratories can easily implement the AmpliType® PM system to analyze DNA-containing samples and controls successfully for forensic casework applications.

**KEYWORDS:** forensic science, AmpliType® PM, field trial, validation

An important development in the field of forensic science has been the use of DNA typing to analyze biological evidence [*1,2*]. The AmpliType® HLA DQα Forensic DNA Amplification and Typing Kit (HLA DQα kit) was the first forensic kit based upon the polymerase chain reaction (PCR) process [*3*]. This kit determines the HLA DQα genotype of DNA samples. Since its release by Cetus Corporation in February, 1990, the HLA DQα kit has been implemented by over a hundred laboratories worldwide [*4–6*]. The HLA DQα kit, which employs the reverse dot blot technology [*7*], has undergone an extensive validation process and has been accepted in courtrooms throughout the world [*4,6,8*]. Roche Molecular Systems, Inc., (RMS) has developed an additional kit for determining individual identity based on the combined PCR and reverse dot blot technologies. The AmpliType® PM PCR Amplification and Typing Kit (PM kit) provides an increase in the power of discrimination available from a single test because multiple polymorphic loci are amplified and typed at the same time instead of only one. As with the HLA DQα kit [*6,9,10*], the PM kit is capable of rapidly analyzing samples that cannot be typed by other methods, including samples containing minute amounts of DNA and very old and/or degraded DNA.

The AmpliType® PM PCR Amplification and Typing Kit includes PCR amplification reagents that direct the simultaneous amplification of six genetic loci using twelve locus specific primers. The six loci amplified are HLA DQA1 (formerly called HLA DQα) [*11*], low density lipoprotein receptor (LDLR)[*12*], glycophorin A (GYPA) [*13*], hemoglobin G-gamma globin (HBGG) [*14*], D7S8 [*15*], and group specific component (GC) [*16,17*]. As with the HLA DQα kit, typing is performed by hybridization of the amplified products to sequence-specific probes that have been immobilized on a nylon membrane strip at specific locations [*7*]. PCR products hybridizing to complementary DNA probe sequences, which correspond to different alleles, are visualized upon enzymatic conversion of a colorless substrate to a blue precipitate. The genotype for each locus is determined from the pattern of blue dots on the strip. The intensities of the typing dots are compared to a control dot on the same strip. The AmpliType® PM kit contains detection reagents and DNA probe strips for typing LDLR, GYPA, HBGG, D7S8 and GC. The HLA DQA1 locus is typed using a separate DNA probe strip.

The LDLR, GYPA and D7S8 loci each have two alleles (designated "A" and "B") distinguished by the AmpliType® PM DNA Probe Strip. Three possible genotypes can be obtained from each of these loci. A third GYPA allele, differing from the "A" allele by a single nucleotide in the probe region, has been identified in African American individuals [*18*]. However, the AmpliType® PM kit was designed to type this variant as the more common "A" allele. The HBGG and GC loci each have three common alleles (designated "A", "B", and "C") that determine six possible genotypes for each locus. The power of discrimination [*19*] of the PM system (excluding DQA1) was calculated for an African American population ($n$ = 191), a U.S. Caucasian population ($n$ = 182) and a U.S. Hispanic population ($n$ = 100) using Hardy Weinberg frequencies and found to be 0.9949, 0.9953, and 0.9962, respectively. When the HLA DQA1 locus is also included, the power of discrimination values are increased to 0.9997, 0.9998, and 0.9998 for these same populations (RMS population database).

Before a new test is adopted for forensic casework, it should be characterized according to the validation guidelines set forth

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[1] Scientist and Section Manager, respectively, Department of Human Genetics, Roche Molecular Systems, Inc., Alameda, CA.

by the Technical Working Group for DNA Analysis Methods (TWGDAM) [20]. In accordance with TWGDAM guidelines, the consistency and reproducibility of a new system should be evaluated both within a laboratory and between different laboratories. To address these guidelines, the AmpliType® PM system was evaluated prior to its commercial release by seven forensic laboratories in a structured Field Trial to assess their ability to obtain the correct AmpliType® PM results on samples typically encountered in their laboratories. Additionally, several studies in the Field Trial were designed to reproduce experiments already performed at RMS addressing other criteria outlined in the TWGDAM guidelines.

## Materials and Methods

### *Study Design*

Seven laboratories participated in the Field Trial: Department of Justice, California DNA Laboratory, Berkeley, California; Center for Blood Research, Boston, Massachusetts; Chicago Police Department, Chicago, Illinois; Federal Bureau of Investigation, Quantico, Virginia; Regional Crime Laboratory at Indian River Community College, Ft. Pierce, Florida; Georgia Bureau of Investigation, Decatur, Georgia and Roche Biomedical Laboratories, Research Triangle Park, North Carolina. Each laboratory performed a total of four studies. The first study represented the training phase and was designed to introduce the participants to the AmpliType® PM kit using different types of simulated forensic casework samples. Field laboratories were required to complete the Training Study and obtain the correct types without contamination before they were sent materials for the remaining studies. The three remaining studies were designed to address issues outlined in the TWGDAM guidelines: the Tissue Comparison Study compared results obtained from different tissues (blood, hair, saliva, semen) from the same individual; the Single versus Coamplification Study demonstrated equivalent typing performance for each locus whether it was amplified alone or as part of the coamplified AmpliType® PM system; the Simulated Sexual Assault Case Study evaluated simulated sexual assault samples supplied in a case format (three reference bloodstains and a postcoital swab). Additionally, laboratories were requested to repeat the analysis of the simulated sexual assault samples on a separate day or by a second operator to evaluate the reproducibility of the procedures within each laboratory.

### *Samples Supplied*

Each of the laboratories was supplied with a total of 19 DNA-containing samples of known genotype for the four studies in addition to control DNA. These samples included seven bloodstains, three hairs, three saliva stains, one semen stain, three postcoital swabs, and two purified DNAs (see Table 1). Blood, saliva, and semen stains were spotted onto sterile cotton cloth. Dacron swabs were used for postcoital samples. Hairs were plucked and examined for the presence of sheath material. After air drying, all samples were stored at −20°C until use. Purified DNAs extracted from buffy coats by a "salting out" procedure [21] were quantitated by D17Z1 probe hybridization as described by Walsh et al. [22] using the QuantiBlot™ Human DNA Quantitation Kit (Perkin Elmer, Norwalk, CT) and diluted to 0.1 ng/μL. Except for the purified DNAs and hairs, the laboratories were instructed to cut samples into thirds. One-third of each sample was extracted by the Chelex method [23,24] and analyzed. The Chelex procedures used were identical to the ones outlined in the AmpliType® User Guide with the following modifications: (1) for the postcoital swab extractions, the cell debris pellet was resuspended in TE buffer, not $H_2O$, prior to epithelial cell digestion, (2) two microliters of 10 mg/mL Proteinase K was added to 200 μL 5% Chelex during the 56°C incubation of the hair, and (3) for all extracted samples 20 μL was added to the PCR. A repeat analysis with a second portion of the sexual assault samples was performed on a separate day or by a second operator as mentioned above. The postcoital swabs were separated into sperm and epithelial cell (E cell) fractions during extraction using a differential lysis protocol [24], and were analyzed and counted as separate samples. For the hairs, the 0.5 cm root end was Chelex-extracted and analyzed. Laboratories were also provided with the appropriate substrate negative controls (three cloth controls and two swab controls) to be analyzed in parallel with DNA-containing samples. Each of the four studies also included a reagent control (no DNA added to the PCR mix) and a positive control (amplification of 2 ng purified DNA).

Before the samples were sent to the Field Trial laboratories, the Field Trial studies were completed by two internal RMS sites. The internal sites determined that the samples were free from contaminants and that they contained the expected types to which the results reported by the Field Trial laboratories would be compared.

TABLE 1—*Summary of samples provided and analyzed by each Field Trial laboratory.*

| Samples provided | # Amplifications | # PM strips | # DQA1 strips |
|---|---|---|---|
| 7 bloodstains[a] | 10 | 10 | 10 |
| 3 hairs | 3 | 3 | 3 |
| 3 saliva stains | 3 | 3 | 3 |
| 1 semen stain | 1 | 1 | 1 |
| 3 postcoital swabs[a] | 8[b] | 8 | 8 |
| 2 purified DNAs[c] | 14 | 12 | 4 |
| 1 positive control | 6 | 6 | 6 |
| 3 cloth controls[a] | 4 | 4 | 4 |
| 2 swab controls[a] | 3 | 3 | 3 |
| Reagent control[d] | 6 | 6 | 6 |
| | 58[e] | 56[e] | 48[e] |

[a]Three of the bloodstains, 1 of the postcoital swabs, 1 of the cloth controls and 1 of the swab controls were extracted and amplified twice in the Simulated Sexual Assault Case Study.

[b]From the 3 postcoital swabs there were a total of 4 sperm and 4 E cell fractions extracted and amplified because one swab was analyzed twice in the Simulated Sexual Assault Case Study.

[c]In the Single versus Coamplification Study, each of the 2 purified DNAs was amplified in premixes containing single locus primers (6 premixes) and typed on 5 PM and 1 DQA1 DNA probe strips. Each of the 2 DNAs was also amplified in a PM premix containing all twelve primers and typed on a PM and a DQA1 DNA probe strip.

[d]The reagent control was not provided to the laboratories. They were instructed to use 20 μL autoclaved distilled or ultra-filtered water.

[e]One laboratory did not repeat the analysis of the Simulated Sexual Assault Case samples; therefore, only 47 amplifications were performed and 45 PM DNA probe strips and 37 DQA1 DNA probe strips were typed by this laboratory.

### *Sample Analysis*

Following extraction, each sample was amplified in a Thin-Walled GeneAmp™ Reaction Tube for 32 cycles (94°C, 1 min, 60°C, 30 s, 72°C, 30 s), with a final 7 min extension at 72°C in a Perkin Elmer DNA Thermal Cycler 480 (TC 480). One laboratory performed the amplifications in a Perkin Elmer DNA Thermal Cycler, not the TC 480 recommended for this study. Each reaction

contained 40 μL AmpliType® PM PCR Reaction Mix, 40 μL 6 mM $MgCl_2$, 2 drops mineral oil and 20 μL DNA. The amplified products were analyzed on a 3% NuSieve GTG/1% Seakem GTG (FMC, Rockland, ME) agarose gel to determine which loci had amplified and then typed on PM and DQA1 DNA probe strips following protocols identical to those in the AmpliType® PM package insert. The laboratories were instructed to interpret and record results from the wet strips. The gels and typing strips were then photographed in duplicate as a permanent record.

The results from each study were recorded by the analyst on forms supplied by RMS. A second person reviewed the strips and recorded their results on identical forms. The two sets of result sheets were then compared and a consensus result sheet was completed by one of the reviewers. All forms and one set of photographs were submitted to RMS for review.

## Results

The results of the AmpliType® PM Field Trial are reported and described as DNA probe strip results because it was difficult to summarize the results by the number of amplified samples. For example, all of the samples in the Simulated Sexual Assault Study were amplified twice and there were two portions extracted and amplified from each postcoital swab. Additionally, PM and DQA1 DNA probe strips were not typed for every amplification in the Single versus Coamplification Study.

Six of the laboratories reported results from 56 PM DNA probe strips and 48 DQA1 DNA probe strips. The seventh laboratory did not repeat the analysis of the Simulated Sexual Assault samples; therefore only 45 PM and 37 DQA1 DNA probe strip results were reported by this laboratory (Table 1). In total, there were 381 PM and 325 DQA1 DNA probe strip results reported for the AmpliType® PM Field Trial. For the 381 PM DNA probe strip results, 294 were obtained from DNA-containing samples and 87 from non-DNA-containing samples (substrate and reagent controls). Similarly, the 325 DQA1 DNA probe strip results were obtained from 238 DNA-containing samples and 87 non-DNA-containing samples (Table 2).

The consensus results reported by the laboratories were grouped into four categories defined by the Field Trial reviewers: "Correct result reported," "Incorrect result reported," "No result reported" or "Uninterpretable result reported." The "Correct result reported" category included DNA-containing samples and controls where the result was consistent with the expected type. Typing results were reported for 97.6% of the PM DNA Probe Strips and 94.1% of the DQA1 DNA Probe Strips from DNA-containing samples. All of the typing results reported were correct (Table 2). The "Correct result reported" category also included non-DNA-containing samples (substrate and reagent controls) from which no typeable result was obtained. In the Field Trial, strips that did not have an "S" or "C" dot visible were defined as not typeable. There were no typeable results reported for any of the PM and DQA1 DNA probe strips from a total of 87 non-DNA-containing samples (Table 2). The "S" and "C" dots are designed to give the weakest probe signals on the PM and DQA1 DNA probe strips, respectively. The AmpliType® PM standard probe "S" is identical in sequence to the AmpliType® HLA DQα (DQA1) "C" probe. It is recommended in the AmpliType® package inserts that typing dots that appear either darker than or equivalent to the "S" or "C" dot are considered positive. Dots that are lighter than the "S" or "C" dot should be interpreted with care because they may indicate the presence of either a mixture or cross-hybridization. Figure 1 shows an example of correct results obtained in the Training Study on AmpliType® PM DNA Probe Strips.

A consensus result would be included in the "Incorrect result reported" category if the result reported for the DNA probe strip from a DNA-containing sample was not consistent with the expected type. Also, a non-DNA-containing sample would be included in this category if it had a typeable signal (visible "S" or "C" dot). There were no incorrect results reported.

The results from DNA-containing samples were placed in the "No result reported" category if both laboratory reviewers agreed that no result could be called (no "S" or "C" dot visible on the wet strip). There were 4 PM and 5 DQA1 DNA probe strips that were classified as "No result reported." One laboratory did not obtain a DQA1 DNA probe strip result for a saliva stain; faint typing dots were visible but there was no "C" dot. The same laboratory did not obtain a PM or DQA1 result for two hairs (No amplified DNA was detected on the agarose gel or DNA probe strips for these hairs). Another laboratory did not obtain a PM or DQA1 result for the sperm fraction of the postcoital swab provided for the Simulated Sexual Assault Study. The PM and DQA1 DNA

TABLE 2—*Summary of combined results from all samples amplified in the Field Trial.*

| Typing Result Categories | PM[a] | DQA1[a] |
|---|---|---|
| DNA-containing Samples | | |
| Correct Result Reported | 287 | 224 |
| Incorrect Result Reported | 0 | 0 |
| No Result Reported | 4 | 5 |
| Uninterpretable Result Reported | 3 | 9 |
| Non-DNA-containing Samples | | |
| Correct Result Reported[b] | 87 | 87 |
| Incorrect Result Reported | 0 | 0 |
| No Result Reported | 0 | 0 |
| Uninterpretable Result Reported | 0 | 0 |
| Total number of strips | 381 | 325 |

[a]Number of DNA probe strips.
[b]For these samples, a "correct result" is defined as no typeable result.

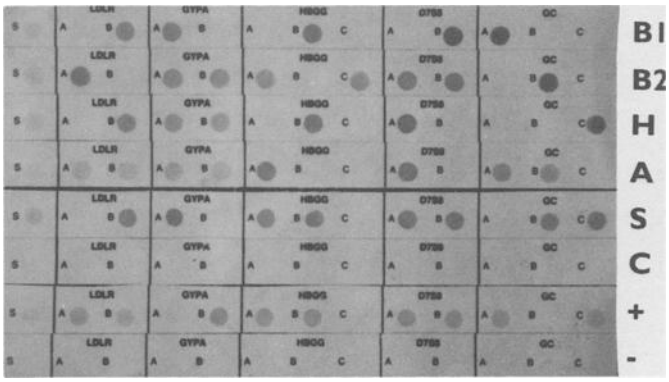


FIG. 1—*AmpliType® PM DNA Probe Strip results from the Training Study. These samples were amplified and typed by one of the Field Trial laboratories: B1 and B2 = bloodstains from different individuals, H = hair, A = saliva stain, S = semen stain, C = cotton cloth control, "+" = 2 ng purified DNA control and "−" = reagent control (no DNA added to the reaction).*

probe strips did not have a visible "S" or "C" dot and there was no DQA1 product observed on the agarose gel for this sample. However, the PM DNA probe strip had dark typing dots visible for the five PM loci corresponding to the expected alleles. This laboratory obtained a correct PM and DQA1 result for the E cell fraction of this sample. In the repeat analysis of this postcoital sample (the second part of this study) the laboratory obtained typeable, correct PM and DQA1 results from the sperm fraction. Correct PM results were obtained from the E cell fraction, but there were no visible dots on the DQA1 DNA probe strip and the background was very white compared to the other strips in the typing tray. There was "No result reported" from this DQA1 DNA probe strip because the laboratory did not retype this sample, even though the DQA1 product band was present on the gel and the "S" dot was visible on the PM DNA probe strip. A third laboratory had a postcoital swab result for which the PM DNA probe strip for the sperm fraction was categorized as "No result reported" because the "S" dot was not visible. Also, the typing dots were faint on this strip. The corresponding DQA1 DNA probe strip had the correct typeable result (dark typing dots and the presence of a faint "C" dot). It was difficult to correlate the results of gel analysis with the typing strip results for this sample since the gel electrophoresis conditions used did not give clear resolution of the amplified products.

A result was placed in the "Uninterpretable result reported" category if interpretation of the strip was prevented by extraneous dots on either DNA probe strip or by imbalanced dots on the PM DNA probe strip that were greater in intensity than the "S" and/or "C" dots. A result was also placed in this category if there was no consensus agreement on the result between the reviewers. Extraneous dots are defined as signals indicating the presence of more than two alleles on the DQA1 DNA probe strip or at the HBGG or GC locus on the PM DNA probe strip. In the Field Trial, these dots prevented interpretation of the strip if they were darker than the "S" or "C" dots. Imbalanced dots on the PM probe strips generally indicate that a mixture is present. If the weaker dot of the imbalanced signal is greater than the "S" dot, it is difficult to determine the major DNA type. However, if the imbalanced intensities are disparate enough that the signal of the minor component is below the intensity of the "S" dot, then the major type can usually be resolved, particularly with sexual assault mixed samples.

All "Uninterpretable results reported" were consistent with incomplete separation of the sperm and E cell fractions from the postcoital swab extractions. There were 3 out of 54 PM DNA probe strips and 9 out of 54 DQA1 DNA probe strips for which an uninterpretable result was reported (Table 3). Results from seven E cell fractions could not be interpreted due to the presence of sufficient sperm DNA to produce extraneous and/or imbalanced probe signals greater than the "S" or "C" signals. Of the seven E cell fractions, two were uninterpretable on both PM and DQA1 DNA probe strips. From the remaining five fractions, only the PM DNA probe strips were interpretable. Results from two sperm fractions could not be interpreted due to the presence of sufficient E cell DNA to produce extraneous and/or imbalanced probe signals greater than the "S" or "C" signals. Of the two sperm fractions, one was uninterpretable on both the PM and DQA1 DNA probe strip. From the other fraction, only the PM DNA probe strip was interpretable. In total, there were six instances in which the PM DNA probe strips were interpretable, even in the presence of the mixture, but the corresponding DQA1 DNA probe strips were uninterpretable. However, there were no samples for which PM DNA probe strips were uninterpretable, when the corresponding DQA1 DNA probe strips were interpretable.

TABLE 3—*Summary of "Uninterpretable Results Reported."*

| DNA Probe Strips | Uninterpretable Probe Strips | |
|---|---|---|
| | E Cell Fraction | Sperm Fraction |
| PM only | 0 | 0 |
| DQA1 only | 5 | 1 |
| PM and DQA1[a] | 2 and 2 | 1 and 1 |
| Total # strips | 9 (from 7 fractions) | 3 (from 2 fractions) |

[a]A result placed on the "PM and DQA1" line indicates that both the PM and DQA1 DNA probe strips for a particular sample were uninterpretable.

The Single versus Coamplification Study compared the typing performance for each locus whether it was amplified alone or as part of the coamplified AmpliType® PM system (Fig. 2). All laboratories obtained the correct type for a locus whether it was amplified alone or as part of the PM system demonstrating that no information is lost by coamplifying multiple markers.

The instances of contamination of samples analyzed in the Field Trial were minimal. Out of 294 PM DNA probe strips and 238 DQA1 DNA probe strips typed from DNA-containing samples there were only three probe strips that exhibited contamination. There was one DQA1 DNA probe strip from a positive control DNA that showed faint "4" and "1.2, 1.3, 4" probe dots that could not be attributed to the expected type, cross-hybridization or DXα [25]. The intensity of the two contaminant dots was not greater than the "C" dot and therefore did not interfere with the correct type of the sample. The PM DNA probe strip from this sample showed only the expected typing dots. There were 2 PM DNA probe strips typed from DNA-containing samples that showed contamination. For one of these 2 PM DNA probe strips, the contaminant was revealed by imbalance between the A and B probe intensities at the GYPA locus. The GYPA B probe intensity was lighter than the intensity of the A probe and the "S" dot. The expected GYPA genotype of this sample was AA. This sample was re-extracted, amplified and typed again. No evidence of contamination was observed from the re-extracted sample or on the original DQA1 DNA probe strip. The contaminant on the second PM DNA probe strip was revealed by the presence of three alleles at the GC locus. The GC A and C probe dots were much darker than the B dot. The DQA1 DNA probe strip from this sample did not show any extraneous dots. This sample was reamplified and typed. Only the expected GC A and C probe dots were visible. There were 4 PM and 1 DQA1 DNA probe strips analyzed from non-DNA-containing samples that showed contamination, but these amplified samples did not produce visible "S" or "C" dots.

The laboratories were required to repeat the analysis of samples in the Simulated Sexual Assault Study to evaluate the reproducibility of the procedures within their own laboratory. Six of the seven laboratories completed this section. Four of the six laboratories obtained the same results for both analyses (100% reproducibility). The other two laboratories obtained 100% PM/ 82% DQA1 and 91% PM / 82% DQA1 reproducibility of results. For these two laboratories, the irreproducibility in results between the two analyses was due to the laboratory obtaining a "correct" result during one analysis and either an "uninterpretable" or "no result" for the same sample in the other analysis. Lack of reproducibility was not due to either laboratory obtaining an incorrect result but rather to variability between their postcoital swab extractions, as described in the Discussion.

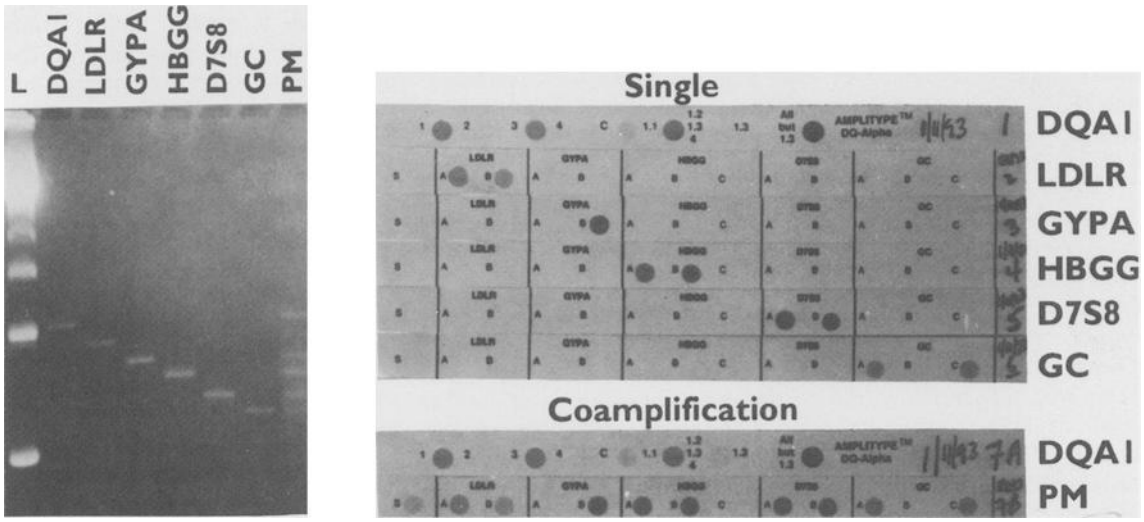


FIG. 2—*Gel electrophoresis and DNA typing results obtained from one laboratory in the Single versus Coamplification Study. PCR product generated from 2 ng purified DNA from premixes containing primers either from only one locus or for all 6 loci (PM) are shown on the gel* (left). *The lane labeled "L" contains a 123 bp molecular weight marker. The PM and DQA1 DNA probe strip results from the individually amplified loci* (top right) *were compared to the results obtained from the coamplified loci* (bottom right).

## Discussion

There were 374 of 381 AmpliType® PM DNA probe strips from DNA-containing and non-DNA-containing samples for which a correct result was reported (98.2%). Of the 325 DQA1 DNA probe strips obtained from DNA-containing and non-DNA-containing samples, 311 had the correct result reported (95.7%). There were no incorrect results reported for the Field Trial (Table 2). Results other than "correct" were due to strips that were "uninterpretable" or "no result" was obtained. All uninterpretable results (3 PM DNA probe strips and 9 DQA1 DNA probe strips; Table 2) were due to incomplete separation of sperm and E cell DNA during the extraction of postcoital swabs. All laboratories were able to determine the type of the major contributor to a mixture if the intensity of the typing dots corresponding to the minor component were less than the "S" or "C" dot.

The AmpliType® PM DNA Probe Strips were developed so that the probe dots within a locus would be balanced in the presence of DNA heterozygous for that locus. The Field Trial laboratories communicated that balanced signals on the typing dots of the PM DNA probe strips aided in determining whether a mixture was present. The importance of balanced probe signals within each locus in detecting mixtures and the ability to resolve the DNA types based on relative signal intensities is shown in Fig. 3*a–c*. In Fig. 3b, the E cell fraction shows faint dots on the GYPA A and GC B probes on the PM DNA probe strip that are less intense than the "S" dot and much less intense than the other probe signals at these loci. The imbalanced intensities within a locus and the presence of three alleles for GC indicated that a mixture was present. There was also the presence of a faint dot on the "1.2, 1.3, 4" probe of the DQA1 DNA probe strip that was less intense than the "C" dot. The type of the major DNA contributor could be determined because the corresponding probe signals were much darker than the signals from the minor contributor. The major genotype was consistent with the female reference sample and the fainter signals were consistent with the type of the sperm donor (Fig. 3*a*). The laboratory was able to type the PM and DQA1 DNA probe strips correctly. In Fig. 3*c*, a mixture was again detectable on

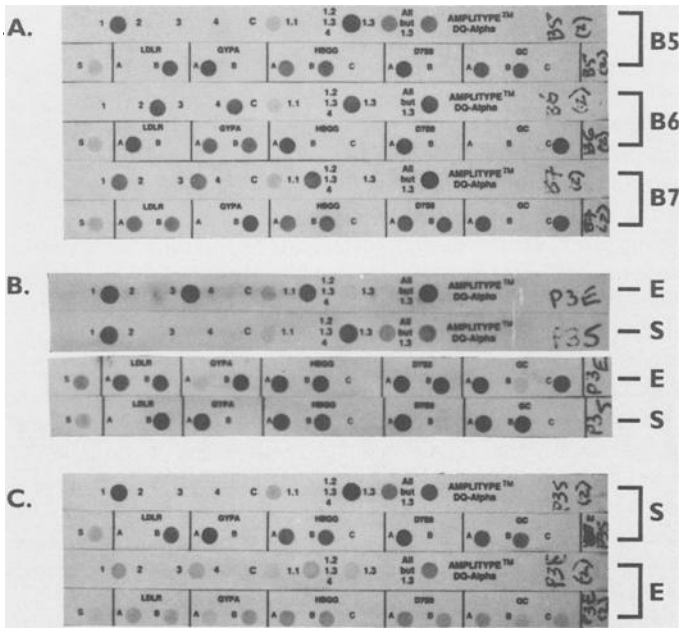


FIG. 3—*Simulated Sexual Assault Case Study results from two laboratories showing interpretable and uninterpretable DNA probe strips. 3A. Samples B5 and B6 are suspect reference bloodstains and sample B7 is the female reference bloodstain. 3B. E and S are the E cell and sperm fractions from the postcoital swab. The PM and DQA1 DNA probe strips from the E cell fraction show a mixture. The significantly darker typing dots on both DNA probe strips correspond to the female reference type: LDLR AB; GYPA BB; HBGG AB; D7S8 AB, GC AC, and DQA1 1.1, 3. The type of the sperm fraction is LDLR BB, GYPA AA, HBGG AB, D7S8 AA, GC AB, and DQA1 1.2, 1.3, which is consistent with sample B5. 3C. The PM and DQA1 DNA probe strips from the E cell fraction show a mixture. For this sample, the major and minor types cannot be resolved because the type from the minor DNA contributor is greater than the "S" and "C" dots.*

the E cell fraction PM DNA probe strip based on the imbalance seen in the typing dots at the GYPA, D7S8, and GC loci and the presence of three alleles at the GC locus. There was also a signal present on the DQA1 "1.2, 1.3, 4" probe for the same fraction. All probe dot intensities were reported as greater than or equal to the "S" and "C" dots. Consequently, the genotypes of the major and minor DNA contributors could not be resolved and the laboratory called these strips "uninterpretable." On DQA1 DNA probe strips, the probes are not as well balanced and the signal intensities cannot be used as easily to associate alleles from different sources. The Field Trial results suggest that it was easier to determine the major genotype on the PM DNA probe strips than the DQA1 DNA probe strips because the relative intensities of the dots could be compared. There were 6 DQA1 DNA probe strips that were uninterpretable while the corresponding PM DNA probe strips were interpretable, even in the presence of a mixture. In contrast, there were no uninterpretable PM DNA probe strips for which the corresponding DQA1 DNA probe strip was interpretable. The potential for sample mixtures in forensic casework analysis has always required careful and thoughtful interpretation. Individual laboratories will need to develop their own policies for the interpretation of mixtures based on their experience and case history information.

In addition to the effects of mixtures on relative probe dot intensities, the probe dots within a locus may become imbalanced in the presence of variant alleles or non-optimal assay conditions. Presently, several variants of the GYPA locus have been identified that cause an imbalance in the typing dots at that locus [*18*]. The presence of a variant allele should be considered any time an imbalance is observed at only one locus on a PM DNA probe strip. The source of the imbalance in signals within a locus on a PM DNA probe strip can be further investigated using an additional marker, such as DQA1 or D1S80, to determine whether it is due to a mixture of DNAs or possibly to a variant allele. Additionally, if the stringency of the hybridization and washing steps of the typing procedure (for example, waterbath temperature, salt concentration of buffers, time of incubation) falls outside of the parameters recommended in the AmpliType® PM package insert, the balance of the probe intensities within a locus can be affected. If the assay conditions are affecting the dot balance of the DNA probe strip, the control DNA will usually exhibit the same probe dot imbalance. Therefore, comparing any probe dot imbalances observed to the typing results obtained from other samples and control DNAs typed under the same conditions will aid in identifying the source of the imbalanced signals. In summary, imbalance observed in dot intensities within a locus should be evaluated on a case by case basis with comparisons to control samples since it can be attributed to several factors.

All of the Field Trial laboratories had previous experience with the AmpliType® HLA DQα kit. They were aware of the sample handling techniques required to minimize the chances of contamination [*24,26*] and had set up their laboratories accordingly. Unlike the DQα Field Trial, there was no single laboratory that had increased levels of contamination compared to the others [*27*]. Rather, contamination was rare and sporadic, with only 8 out of 395 amplifications showing the presence of a contaminant. Five of the instances of contamination appeared on DNA probe strips developed from non-DNA-containing samples, indicating a low level of contamination of either the single reaction tube or of a reagent. The appearance of faint dots on the control DNA probe strips does not necessarily mean that results obtained for the associated DNA-containing samples are incorrect, as discussed in Section 4.2 of the AmpliType® User Guide [*24*]. However, individual laboratories may decide to conduct further testing of casework materials if their controls show contamination. The level of contamination observed in this study never compromised the ability of the laboratories to determine the correct genotype of the sample.

The presence or absence of PCR product bands on the postamplification agarose gel can be a good indicator of whether or not a locus will have visible dots on the DNA probe strip. In general, if a locus had a product band on the gel, it also had a visible probe dot. If a locus did not have a visible product band on the gel, a typeable dot was usually not observed on the DNA probe strip. However, it was apparent from the photographs returned to RMS that the type of gel electrophoresis box used and the size of the wells formed affected the detection and resolution of the six PM product bands. It is important for laboratories to use the gel electrophoresis system and protocols recommended in the AmpliType® PM kit package insert or to make the effort to optimize conditions for their existing gel electrophoresis equipment so that valuable information is not lost. If typing dots for a locus are absent on the DNA probe strip and the amplification has not been confirmed on a gel, the operator will not know if the absence of signal is due to lack of amplification or a problem during the hybridization and color development steps. The absence of PCR product from some but not all loci occurs most frequently in the presence of an inhibitor or degraded DNA.

Two of the hair samples analyzed in the Field Trial showed no amplified product on the PCR product gel and no visible typing dots on the PM and DQA1 DNA probe strips. This lack of amplification was most likely due to insufficient DNA being extracted from the hairs. Five other DNA probe strips were also categorized as "No Result Reported" because they had faint typing dots but no visible "S" or "C" dot on the PM and/or DQA1 DNA Probe Strips (with the exception of the extremely white strip previously discussed in the Results section). These results may also be attributed to a low amount of input DNA since the "S" and "C" dots are designed not to be visible if the amount of template DNA is less than approximately 0.3 to 0.5 ng. Quantitation of the amount of DNA in a sample (for example, using the D17Z1 probe-based QuantiBlot™ Kit) prior to amplification allows the analyst to amplify the optimal amount of DNA for the test (2 to 40 ng of DNA is recommended in the AmpliType PM Package Insert). The Field Trial laboratories were not instructed to quantitate their samples. However, results may have been obtained if samples that did not produce a typeable result had been quantitated and reanalyzed. If quantitation revealed the Chelex extracts contained insufficient DNA to produce a typeable result, the extracts could have been concentrated by using a Centricon 100 microconcentrator (Amicon, Danvers, MA) and reamplified [*28*]. If quantitation showed sufficient DNA in the sample extracts, then the untypeable result was most likely due to the presence of a PCR inhibitor or degraded DNA. The addition of more *Taq* DNA polymerase or dilution of sample extract can aid in obtaining a PM result when inhibitors are present. Increasing the amount of DNA amplified with the AmpliType® PM kit can increase success in analyzing degraded samples (Fildes, unpublished data).

The reproducibility section of the Simulated Sexual Assault Case Study demonstrated that most laboratories (four out of six) were able to obtain the same correct result when a sample was extracted and analyzed in duplicate. The two laboratories that obtained different results for the duplicate analyses did not obtain incorrect results. Instead, the discrepancies were due to one of the duplicate analyses producing either a strip that was uninterpretable due to a mixture of sperm and E cell DNA, or no DNA type being

obtained on PM or DQA1 DNA probe strips for one fraction of the postcoital swab. The other duplicate analysis generated the correct result. These results reflect variability in the DNA extraction procedure, including the portion of the sample chosen for extraction, rather than the PM amplification or typing procedures. The postcoital swab extraction procedure involves many manipulations of the sample; the length of time samples are digested and the number of washes performed (ranges are suggested in the AmpliType® User Guide [*24*]) can affect the level of separation of sperm from E cell DNA.

All of the laboratories used the Chelex-extraction methods described in the Materials and Methods section of this paper for extraction of the samples provided in the AmpliType® PM Field Trial. The results obtained from these Chelex-extracted samples are summarized in this report. However, two of the laboratories did additional analysis on the provided Field Trial samples using other sample extraction methods. One laboratory used an organic extraction procedure to extract a separate portion of all the samples that were provided in the Field Trial [*29*] and they reported equivalent results using either method. Another laboratory re-extracted only the postcoital swabs provided in the Field Trial using the Lifecodes organic extraction procedure [*30*]. After extracting the swabs using the organic procedure, they reported to us that they were able to obtain an interpretable, correct result for all E cell and sperm fractions. Previously, "uninterpretable results" were obtained for 1 out of 8 PM DNA probe strips and four out of eight DQA1 DNA probe strips from postcoital swabs extracted using the Chelex method and analyzed by this laboratory. The results of the additional testing that was performed by these two laboratories are not included in the summary presented in this paper but suggest that alternate extraction methods can be used successfully with the AmpliType® PM system.

The AmpliType® PM Field Trial demonstrated the ability of forensic laboratories to analyze samples and obtain the correct results using the AmpliType® PM PCR Amplification and Typing Kit. These results confirm that the analysis of six genetic markers using the PM kit meets the TWGDAM consistency and reproducibility guidelines. The participants were able to incorporate the AmpliType® PM system into their laboratories easily because they had all previously performed validation studies with the AmpliType® HLA DQα kit. The technology, protocols, reagents and equipment are shared between the two AmpliType® systems. Without additional training, the laboratories had a very high success rate obtaining a correct typing result with minimal procedural problems or instances of contamination. The balance of the dots within a locus on the PM DNA probe strip proved to be a valuable asset of the system for the analysis of mixtures. This feature is an important benefit of the PM system since a high percentage of forensic casework involves the analysis of sexual assault samples. The AmpliType® PM kit clearly increases the options currently available to the forensic community for analysis of limited or degraded DNA samples and offers a high power of discrimination. Not only does the AmpliType® PM system provide a simple, rapid assay that can be used to screen samples, the results can be combined with results from other DNA typing and serological tests to further increase the discriminatory power of forensic genetic marker analysis.

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Address requests for reprints or additional information to
Nicola Fildes
Dept. of Human Genetics
Roche Molecular Systems, Inc.
1145 Atlantic Ave.
Alameda, CA 94501