TECHNICAL NOTE

Cecelia A. Crouse,[1] Ph.D.; Daniel C. Nippes,[2] M.S.; and Earl L. Ritzline,[2] B.S.

Confirmation of PM Typing Protocols for Consistent and Reliable Results

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ABSTRACT: A recent report in the Perkin Elmer "Forensic Forum" bulletin described a modification to the previously published PM typing protocol indicating that in order to obtain consistent and reliable PM and DQA1 typing results, disodium EDTA should be added to the post-amplification mixture before denaturation of the DNA fragments. The analysis and validation of this suggestion is described in the accompanying paper. We report the evaluation of this additional step when typing for PM alleles and conclude that the standard operating procedures currently enforced at the Palm Beach County Sheriff's Office and Indian River crime laboratories do not necessitate the need for the addition of disodium EDTA to the PM amplified products prior to the heat denaturation step. Further, depending on an individual laboratory's PM protocol, the recommendation by Perkin Elmer to add disodium EDTA to PM amplified products before typing has merit and should be carefully considered when determining laboratory PM typing protocols.

KEYWORDS: forensic science, DNA, polymerase chain reaction, HLA-DQA1, LDLR, GYPA, HBGG, D7S8, GC, validation, AmpliType PM, reverse dot blot

The polymerase chain reaction (PCR) has been used to amplify DNA polymorphic loci for the purpose of individual identification in forensic and paternity casework for many years. The Perkin Elmer AmpliType PM kit employs PCR methods and reverse dot-blot technologies to identify the LDLR, GYPA, HBGG, D7S8, GC and HLA DQA1 alleles. PM validation studies have provided valuable information regarding the use and interpretation of typing results (1,2). An important step in the PM typing protocol is to denature the PM PCR products using a 95°C heating block followed by dispensing 20 μL of the amplified fragments into the hybridization solution with the PM typing strips. In the following paper by Grow et al., it is reported that consistent and reliable results are obtained if disodium EDTA is added to the PCR products before the amplified reaction is denatured and applied to the typing strips (3). The impetus for this change in protocol was the observation that the PM GC "B" allele-specific probe and the HLA DQA1 "4.1" allele-specific probe was less intense than the other probes on these typing strips "when heat-denatured, amplified PCR product was allowed to cool significantly prior to being added to the hybridization mixture" (3). Investigation into the intensity difference phenomenon at these alleles by Roche Molecular Systems offered the following explanation. The location of the probe complementary sequence for the GC "B" and DQA1 "4.1" alleles are immediately adjacent to the primer binding site of these loci. If sufficient cooling of the amplified product occurs, the Taq polymerase will polymerize short DNA fragments which will remain annealed to the complementary strand thus decreasing the amount of available amplified product that will anneal to the typing strip GC "B" and DQA1 "4.1" probes. As a result, less intense probe-signals may be observed on the PM and DQA1 typing strips.

The purpose of this report is to 1) demonstrate reliable PM typing results for the GC "B" allele-specific probe utilizing standard operating procedures 2) duplicate the PM GC "B" "less intense" dots phenomenon described in the accompanying paper and 3) evaluate the effect of EDTA on PM and amplified products prior to typing.

Materials and Methods

DNA Extraction and PM Amplification and Typing

The Palm Beach County Sheriff's Crime Laboratory (PBSO) and the Indian River Crime Laboratory (IRCL) investigated DNA samples representing all possible GC genotypes. DNA samples from the PBSO population database were organically extracted as previously described (4). DNA samples from IRCL casework were extracted using the Chelex method (5). The PBSO DNA samples were quantified using the QuantiBlot System (Perkin Elmer, Norwalk, CT). Amplification for the PM loci (LDLR, GYPA, HBGG, D7S8 and GC) was conducted as per manufacturers protocol using the AmpliType PM kit (Perkin Elmer, Norwalk, CT). PBSO amplified 5 ng of DNA. In lieu of DNA quantitation, the IRCL used analyst's experience and confidence that the amount of template DNA amplified falls within the expected concentration and as a result, 20 μL of Chelex extracted samples was consistently used in the amplification reaction. The DNA samples were amplified using a PE 480 (IRCL) or the GAPS 9600 (PBSO) thermal cycler.

The amplified PM products were typed by the following methods: 1) using current PBSO and IRCL standard operating procedures, that is, immediate dispensing of denatured amplified product

[1]Senior Forensic Scientist, Palm Beach County Sheriff's Office Crime Laboratory, West Palm Beach, FL.

[2]Lab Director and Forensic Scientist, respectively, Regional Crime Laboratory at Indian River Community College, Fort Pierce, FL.

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into tray wells with hybridization solution, 2) heating tubes to 95°C followed by holding the tube for 20 seconds prior to dispensing the amplified product or 3) adding 5 μL of 200mM disodium EDTA to each PCR reaction prior to heating the tube, then holding the tube 20 seconds before dispensing amplified products into the tray wells. In addition, PM amplified products which had been stored in the refrigerator at 4°C for 6 months were retyped for PM loci by 1) laboratory standard operating procedure and 2) by the addition of 5 μL of 200mM disodium EDTA to the tubes, holding the heated-tubes for 20 seconds before dispensing amplified products into the tray wells.

Results and Discussion

The standard operating procedure for the initiation of PM typing methods currently used at the PBSO and IRCL laboratories states that the denatured PM amplified products should be added "immediately" to the hybridization solution with the PM typing strips. For both laboratories, this translates into approximately 7 seconds from the time the test tube is removed from the 95°C heating block until the amplified product is placed into the appropriate typing strip well. The GC "B" signal intensity phenomenon described by Grow et al. (3) was tested by the PBSO and IRCL laboratories using the scheme described in Fig. 1. The experimental design tests the effects of the addition of disodium EDTA and "significant cooling" of PM amplified products prior to typing for the PM loci. Hand holding the tubes for 20 seconds was considered a significant cooling period as this would be a major deviation from the PBSO and IRCL protocol.

Results from representative DNA samples analyzed at the PBSO and IRCL are shown in Figs. 2 and 3. When PBSO PM amplified products were typed as per laboratory protocol, that is, amplified products heat denatured followed by "immediately" adding the fragments to the PM typing strips, there was little (H613) or no (H600, H664, H670) signal intensity differences observed at the GC "B" allele specific-probe (Fig. 2 "protocol"). When the test tubes were hand-held for 20 seconds before the denatured PCR products were added to the typing strips, all of the PBSO DNA

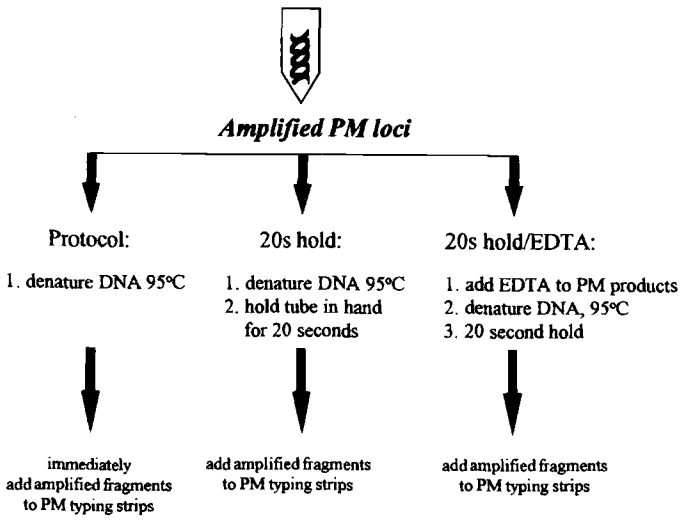

FIG. 1—*Amplified PM DNA products were 1) immediately added to the PM typing strips as per PBSO and IRCL laboratory protocol, or 2) held for 20 seconds before addition to the PM typing strips or 3) EDTA was added to the PM PCR products prior to denaturation followed by a 20 second hold and finally addition to the PM typing strips.*

samples demonstrated some degree of GC "B" probe intensity difference when compared to the GC "A" or "C" allele specific probe intensity (Fig. 2, "20s Hold"). Signal intensity differences were eliminated when the addition of disodium EDTA was added to the amplified reaction before denaturation (Fig. 2, "20s Hold/EDTA"). The IRCL DNA samples included the six possible GC genotypes. Samples IRCL-2 and IRCL-5, which are heterozygous for the GC "B" allele show definitive signal intensity differences when the samples are "cooled" for 20 seconds (Fig. 3). As with the PBSO samples, the addition of EDTA eliminated these intensity differences. The effect of disodium EDTA was duplicated at both the PBSO and IRCL laboratories. The PM typing strips maintained balanced signals at all other PM loci regardless of experimental treatment of the amplified products prior to typing.

The "less intense" signal phenomenon with the HLA DQA1 "4.1" probe and the benefits for the addition of disodium EDTA was not investigated by the PBSO and IRCL laboratories due to the fact that HLA DQA1 strips with the 4 allele subtypes are not commercially available.

The PBSO laboratory has stored PM amplified products from population samples in anticipation of typing the HLA DQA1 loci when the typing strips become commercially available. There were no GC "B" allele intensity differences when the samples were initially typed for the PM loci (data not shown). The amplified PM DNA has been stored at 4°C for the past six months. Seven amplified PM products were retyped for the PM loci in order to determine if there were any signal intensity differences with the GC "B" allele after long term storage. Figure 4 shows the re-typing of these seven samples by the PBSO "immediate" and "20s Hold/EDTA" protocol. It was observed that for 5 of the 7 samples there were signal intensity differences at the GC "B" allele when compared to the GC "A" and "C" probe signals even when the denatured PM products were dispensed immediately (Fig. 4, samples PBSO-3 through PBSO-7). Since the signal intensity was observed with the 7 second protocol, a 20 second hold test was not necessary. The addition of disodium EDTA prior to denaturation followed by a 20 second hold did not mitigate the signal intensity difference. This may be due to the fact that following the initial PM typing of the samples six months prior, the remaining sample cooled to room temperature over a 60 minute period at which time the polymerization of shorter DNA fragments could have occurred. This lengthy cooling period most likely provided an excess of PCR short fragments that would readily hybridize to the complementary strand even when held only 7 seconds. Since the shorter fragments have already been amplified, addition of disodium EDTA to the stored denatured PCR products would not alter the already present "less intense phenomenon." Further, the addition of EDTA may not have an effect simply because after six months the Taq polymerase is no longer a fully functional protein and amplification of additional DNA fragments during the 95°C denaturation/cooling period does not occur. There appear to be unbalanced signal intensity differences at other PM loci for these stored amplified PM products, such as the PBSO-4 HBGG locus, although this is not as predictable as the GC "B" allele signal intensity difference.

Although the PBSO and IRCL do not store casework amplified products, laboratories that have a policy of storing PM PCR fragments for retyping, possibly for the defense if a sample stain has been consumed during DNA extraction procedures, should be aware that intensity differences may occur that may not have originally been observed. Further, these laboratories may want to consider the addition of disodium EDTA to the PM amplified

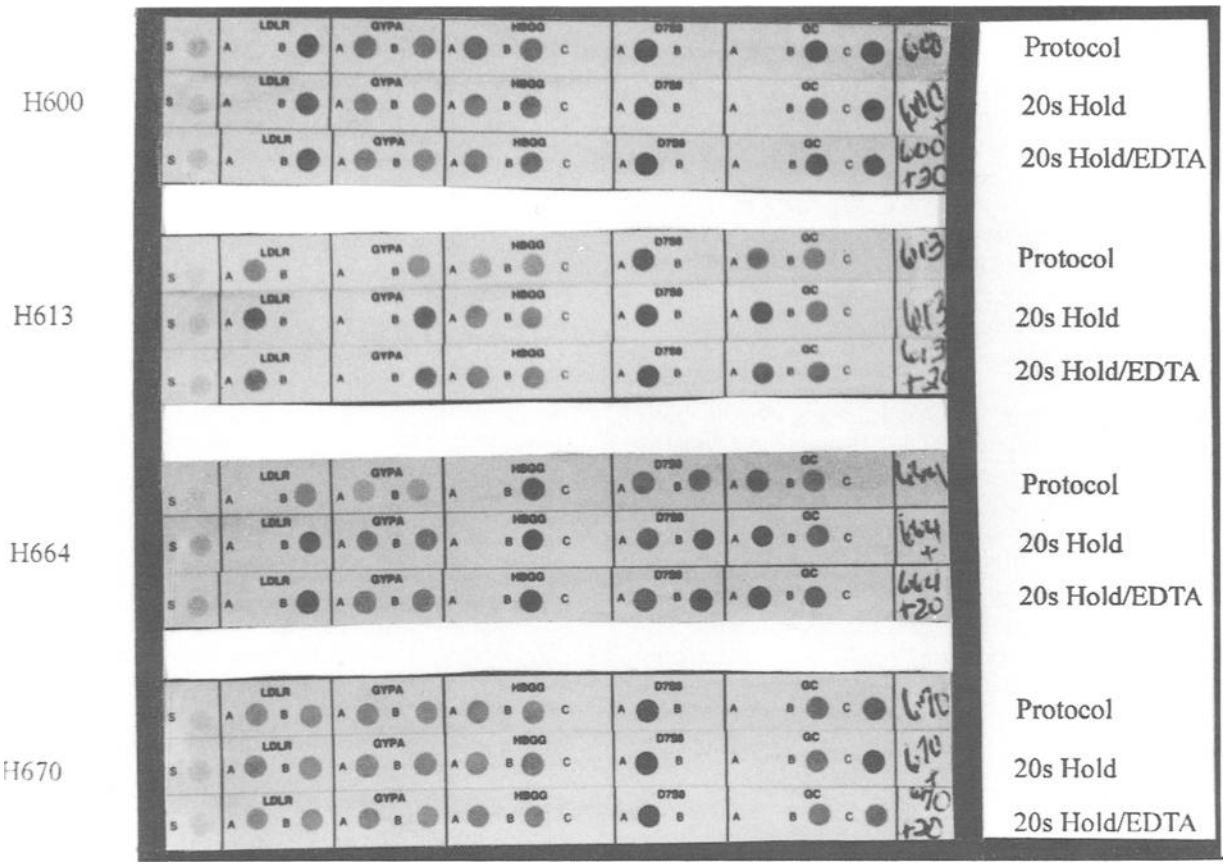

FIG. 2—*DNA samples were amplified for PM loci and typed as described in Fig. 1.*

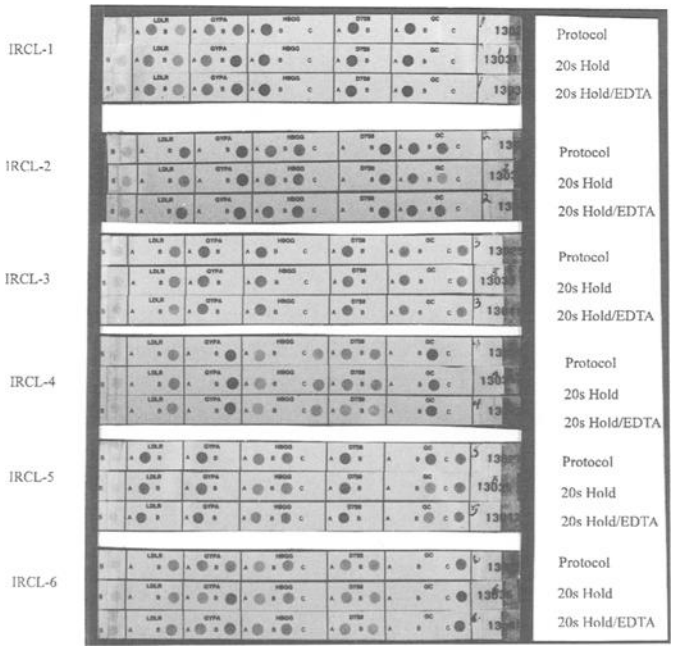

FIG. 3—*DNA samples representing all six of the PM GC locus genotypes were amplified for all PM loci and typed as described in Fig. 1.*

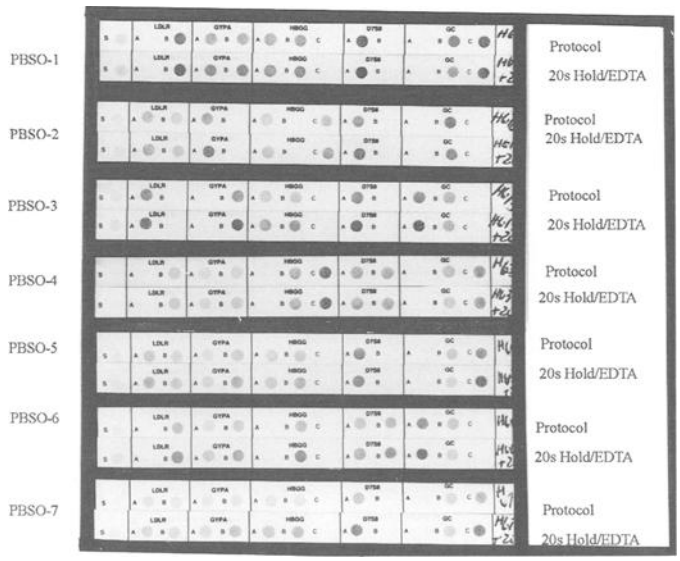

FIG. 4—*DNA samples that had previously been amplified and typed for PM loci and stored at 4°C for six months were re-typed for PM loci for these studies. The PBSO protocol and the 20 second hold with EDTA were evaluated.*

products if retyping is a possibility regardless if the laboratory uses an "immediate" protocol.

In addition to the work presented here, it is interesting to note that recent published manuscripts reporting PM results indicate in the Materials and Methods section that the PM typing procedure was carried out as per manufacturers' recommendation (1,2). The PM typing strips presented in these articles do not show GC "B" signal intensity differences. Further, the PBSO laboratory has accumulated population data for 117 Caucasians, 101 African Americans, 103 Hispanic and 78 Haitian individuals, a total of 798 alleles, which have been analyzed for the PM loci. When the Polaroid archival photographs of the PM typing strips for these populations were reanalyzed, of the 372 individuals known to have the GC "B" allele, only three PM typing strips showed any signal intensity differences between the GC "B" allele-specific probe and the GC "A" and "C" allele-specific probes. Although the GC "B" signal is less intense than the other GC allele-specific probes, they are definitely not weak or ambiguous signals and the samples could not be mistyped. A similar review of two years of forensic casework data by IRCL showed no GC "B" intensity differences in samples where no indications of mixtures were identified.

In summary, when PM amplified products are immediately pipetted into the tray wells with the PM strips, there are no apparent visual differences between the signal intensities of the GC alleles. Since the "less intense phenomenon" is readily observed on a PM typing strip, each laboratory should re-visit archival photos of PM strips to evaluate the GC "B" allele intensity. If a laboratory has a protocol whereby the denatured PM amplified products are allowed to cool "significantly," this will be evident on the PM typing strips. It is important that laboratories have clear PM and DQA1 typing protocols to accommodate the "less intense signal phenomenon" especially if the laboratory differentiates between signal intensity differences for mixed stains in casework samples, or if PM PCR products are stored for potential retyping in the future.

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Address requests for reprints or additional information to
Cecelia A. Crouse, Ph.D.
Palm Beach County Sheriff's Office Crime Laboratory
3228 Gun Club Road
West Palm Beach, FL 33406