TECHNICAL NOTE

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Post-Amplification Primer Extension of Heat-Denatured AmpliType® PCR Products: Effects on Typing Results

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ABSTRACT: Alleles of the HLA DQA1, LDLR, GYPA, HGBB, D7S8 and GC loci, which are amplified using the AmpliType® PM PCR Reaction Mix and Primer Set, can be detected using sequence-specific oligonucleotide probes immobilized on a nylon membrane strip. Using reagents supplied in AmpliType PCR Amplification and Typing Kits, patterns of blue dots corresponding to particular alleles are visualized on the DNA probe strips. Frequently, the correct interpretation of typing results is dependent not only on the presence of probe signals but also on their relative intensities. The relative probe signal intensities obtained from an undegraded DNA sample extracted from a single individual will be different from those obtained from degraded DNA and from samples containing DNA from more than one source. Because probe signal intensity is an essential consideration for interpretation, factors that can influence it need to be identified. Clearly, the time and temperature of the assay steps and the salt concentration in the typing solutions can affect probe signal intensity. Also, if heat-denatured PCR products are allowed to cool for several minutes, the strands will reanneal and become unavailable for binding to the probes immobilized on the strips. However, the selective loss of GC B and HLA DQA1 4.1 probe signals observed after shorter cooling times cannot be explained by these factors. We demonstrate that following heat denaturation of PM PCR products there is sufficient residual *Taq* DNA polymerase activity to extend primers as the solution cools and that this primer extension occurs at a more rapid rate than PCR product reannealing. Primer extension across probe binding sites will prevent hybridization of the PCR product to complementary probes on the strip. The extent of signal reduction is dependent on the position of the probe binding site relative to the 3′ ends of the primers and on the strand to which the probe is complementary. We recommend a simple modification to the AmpliType typing protocol to ensure all probe binding sites will be available for hybridization to PM and HLA DQA1 DNA probe strips.

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

AmpliType® PM PCR products, consisting of amplified regions of LDLR, GYPA, HBGG, D7S8 and GC, and HLA DQA1 PCR products are typed using sequence specific oligonucleotide (SSO) probes immobilized on strips of nylon membrane. The HLA DQA1 PCR product is typed on a single strip containing 11 probes. The five PM PCR products are typed on a separate strip containing probes immobilized at 13 positions. To obtain the PM and HLA DQA1 types of the sample, PCR products are heat-denatured and then hybridized to probes containing complementary sequences. Following a streptavidin-horseradish peroxidase enzyme conjugate binding step and a stringent wash step, specifically bound PCR products are visualized by the addition of a 3,3′,5,5′-tetramethylbezidine (TMB) solution that is converted to a blue precipitate in the presence of H_2O_2 and horseradish peroxidase.

The intensity of the blue dots can be affected by several factors, including amount of PCR product added, waterbath temperature during wash steps, concentration of salt in wash solutions, and time and temperature of the color development step. The effects of these variables have been well characterized and have been described elsewhere (1–3). Another variable that can affect the intensity of probe signals is the time between heat denaturation of the PCR products and addition of the denatured PCR products to the typing tray well, which contains hybridization solution and a DNA probe strip. If this time period is too long, the single stranded PCR products begin to renature and become unavailable for binding to the immobilized probes, resulting in an overall reduction in probe signal intensity. Specification of a 20–30 second window by the manufacturer for the addition of denatured product to the typing tray well ensures that the PCR product will remain single stranded and be able to hybridize to complementary probes.

An unusual PM typing result obtained during a PCR training workshop and independent assay validation experiments performed with the new HLA DQA1 DNA probe strips indicated that the GC B probe signal and the HLA DQA1 4.1 probe signal were particularly sensitive to the length of time between heat denaturation of the PCR products and addition to the typing tray well. The specific loss of signal intensity at these two probes can result in ambiguous and, potentially, incorrect results. The mechanism for this specific effect and a simple protocol modification to eliminate loss of signal are described in this paper.

Materials and Methods

DNA Extraction

DNA was isolated from buffy coats using the salting-out method described by Miller et al. (4) followed by phenol/chloroform extraction and ethanol precipitation. The OD 260/280 value for the purified DNA samples was 1.8–1.9. The concentrations were

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determined by UV spectroscopy and D17Z1 probe hybridization using the QuantiBlot™ Human DNA Quantitation Kit (Perkin Elmer, Norwalk, CT).

PCR Amplification and Typing

AmpliType® PM PCR amplification and typing were performed as described by Herrin et al. (5) with the following exception. In selected experiments the heat-denatured PM PCR products were allowed to cool at room temperature for varying lengths of time prior to addition to the hybridization solution in the typing tray. All protocol modifications are noted in the Results and Discussion section and in the figure legends.

Preparation of 200mM EDTA Solution

To prepare the 200m*M* EDTA solution, 0.5*M* EDTA (formulated according to the AmpliType PM kit package insert) was diluted with deionized water (4 mL 0.5*M* disodium EDTA added to 6 mL deionized water). The 200m*M* EDTA solution was filtered through a 0.2 μm Nalgene® filter prior to use.

Results and Discussion

The first post-amplification typing step for AmpliType PM and HLA DQA1 PCR products is heat denaturation at 95°C for 3–10 minutes. The denatured products are transferred to the typing tray well, which contains hybridization solution and a DNA probe strip, within 20 seconds. This time period between heat denaturation and addition to the hybridization solution was chosen to minimize PCR product renaturation and therefore maximize the quantity of PCR product available for binding to complementary probes on the strips.

At a PCR training workshop, one participant repeatedly observed an imbalanced GC type with known heterozygous DNA samples containing a GC B allele; all other participants using the same DNA samples observed balanced dot intensities at all loci. The only apparent difference between this individual and the other participants was the organization of the typing space. At this participant's station, the GeneAmp PCR System 9600 (GAPS 9600) was not located next to the area designated for the typing tray and rotating water bath. Consequently, following heat denaturation in the GAPS 9600, the sample was carried to another location before it was added to the hybridization solution in the typing tray. This step took longer than the recommended 20 seconds for AmpliType PM PCR products.

The decrease in the GC B signal could not be attributed solely to PCR product renaturation because the signal intensities of other alleles were not significantly affected by the delay in adding the denatured PCR products to the tray wells. This observation prompted a more systematic study of the effect of transfer time on GC B probe intensity. Following heat denaturation of PCR products containing either a GC AB or GC BC type, the tubes were kept at room temperature for "0" seconds (that is, transferred to the typing tray as quickly as possible), 30 seconds, 1 minute, and 2 minutes before adding 20 μL of the reaction to the hybridization solution in the typing tray well. The typing results in Fig. 1 show that the GC B signal decreases rapidly as the transfer time is increased. The signal is no longer visible in the 1.5 ng DNA input samples after 1 minute of cooling. Some of the other PM alleles are affected (for example, HBGG A) but their signals are still visible after 1 minute at room temperature. Independent but concurrent assay validation studies with the HLA DQA1 DNA probe

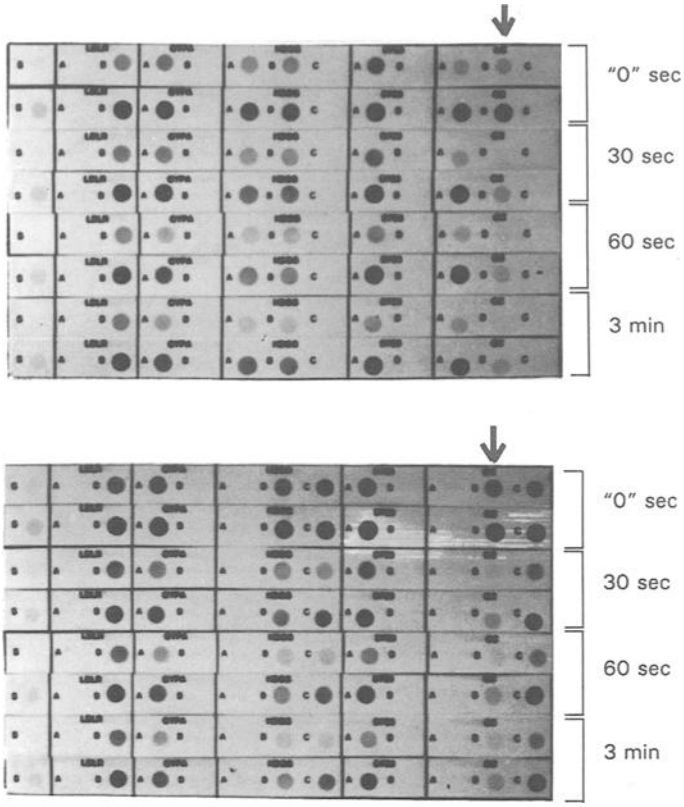

FIG. 1—*Reduction of GC B probe signal intensity as cooling time is increased. Two DNA samples heterozygous at the GC locus (GC AB and GC BC) were amplified with AmpliType PM PCR Reaction Mix. The PCR products were heat-denatured and then allowed to cool at room temperature for 30 seconds, 60 seconds, or 3 minutes before being added to the hybridization solution in the typing tray wells. The PCR products hybridized to the strips labeled "0" sec were added to the typing tray wells as quickly as possible (approximately 5 seconds). For each pair of strips, PCR products amplified from 1.5 ng and 30 ng DNA were hybridized to the top and bottom strips, respectively. The* arrow *indicates the GC B probe.*

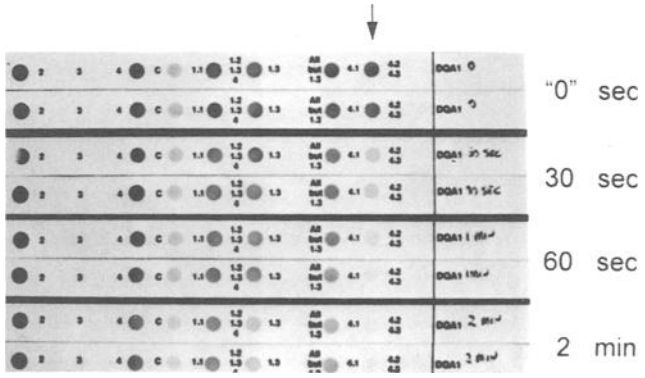

FIG. 2—*Reduction of HLA DQA1 4.1 probe signal intensity as cooling time is increased. Control DNA 1 from the AmpliType PM kit was amplified with the PM PCR Reaction Mix. The PCR products were heat-denatured and then allowed to cool at room temperature for 30 seconds, 60 seconds, or 2 minutes before being added to the hybridization solution in the typing tray wells. Duplicate strips were typed for each timepoint. The PCR products hybridized to the strips labeled "0" sec were added to the typing tray wells as quickly as possible (approximately 5 seconds).*

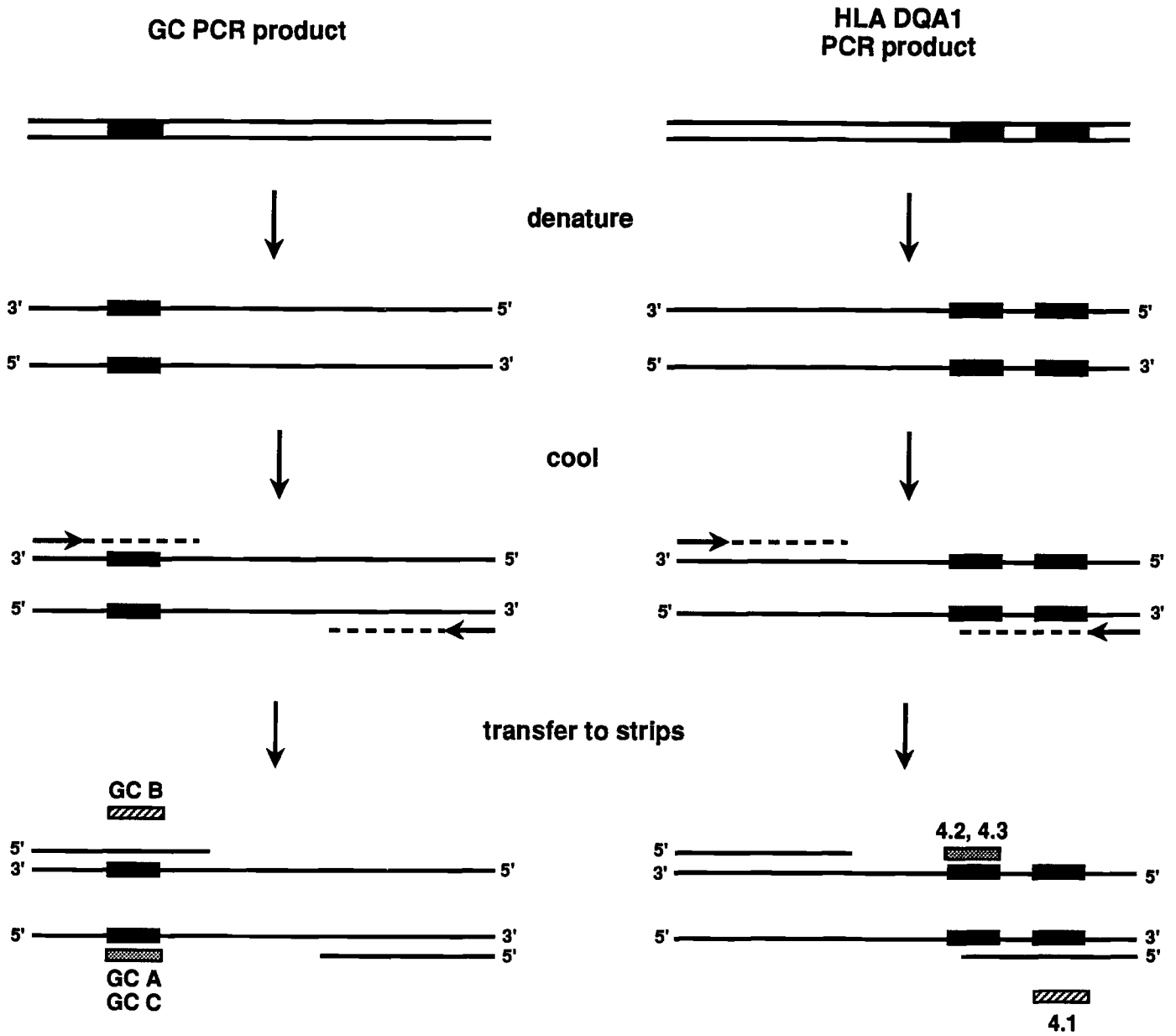

FIG. 3—*Mechanism of signal intensity reduction at GC B and HLA DQA1 4.1 probes. In this scheme, PCR product is heat-denatured prior to hybridization to probes on the DNA probe strips. As the solution slowly cools, primers can anneal and be extended. The greater the time of cooling, the farther the primers will be extended. Probe binding sites that are close to the 3′ end of one of the primers may become double-stranded and incapable of binding to complementary probes immobilized on the DNA probe strip. The effect of primer extension through a probe binding region on signal intensity depends on which strand of the PCR product is complementary to the probe. The solid boxes on the double- and single-stranded PCR products indicate the approximate locations of the probe binding sites for the GC A, B, and C polymorphisms* (left diagram) *and the HLA DQA1 4.1, 4.2 and 4.3 polymorphisms* (right diagram). *Primers are shown as* arrows *and the* dotted lines *indicate extension of the primers. The shaded boxes represent sequence-specific probes that are attached to the strips. In the GC system, the B probe binds to one strand of the GC PCR product and the A and B probes bind to the same region on the opposite strand. In the HLA DQA1 system, the 4.1 probe binds to one strand of the HLA DQA1 PCR product and a single probe on the opposite strand binds to both 4.2 and 4.3 alleles. The 4.2/4.3 probe binding region does not overlap the 4.1 probe binding region as indicated.*

strips revealed a similar effect on the DQA1 4.1 probe signal (Fig. 2). The "1.2,1.3,4" probe signal also appears to decrease but at a significantly slower rate.

After comparing all of the PM and HLA DQA1 probe sequences and the locations of the probe binding sites within the PCR products relative to the 3′ ends of the primers, the following mechanism was proposed to explain the specific loss of the GC B and HLA DQA1 4.1 probe signals. The probe binding regions for the three GC alleles (A, B, and C) and the HLA DQA1 4.1 allele are located nearer to the 3′ end of one of their respective primers than any of the other PM and HLA DQA1 alleles. The beginning of the GC probe binding region is 3 nucleotides from the 3′ end of the "left" GC primer and the beginning of the DQA1 4.1 probe binding region is 9 nucleotides from the 3′ end of the "right" HLA DQA1 primer. The distances between 3′ ends of primers and probe binding regions for the other PM alleles range from 20 nucleotides to over 100 and for the HLA DQA1 alleles they range from 40 nucleotides to over 150. During the time period between heat denaturation

and addition to hybridization solution, primers that were not incorporated during the amplification step can anneal to the denatured PCR products and be extended a short distance by *T. aquaticus* DNA polymerase (*Taq* DNAP), which retains some activity following the 3–10 minute 95°C step. Given the proximity of the GC and DQA1 4.1 probe binding regions to the 3′ ends of the primers, it is conceivable that the primers can be extended across these regions (Fig. 3). As a result of primer extension, the probe binding sites on the strands to which the GC B and DQA1 4.1 probes are complementary become unavailable (Fig. 3). Consequently, fewer single-stranded PCR products will be available to bind to these particular probes, resulting in lower signal intensities relative to other probe signals on the same strip. Note that the GC A and C probes are complementary to the opposite strand of the GC PCR product relative to the GC B probe. The probe binding region on this strand of the PCR product is not blocked by extension of either GC primer (Fig. 3). Similarly, note that the DQA1 4.1 probe is complementary to the opposite strand of the HLA DQA1 PCR product relative to the DQA1 4.2/4.3 probe and that the DQA1 4.2/4.3 probe binding region on this strand is not blocked by extension of either HLA DQA1 primer (Fig. 3).

This model for specific signal reduction is dependent on *Taq* DNAP activity. Therefore, to test the model, a series of experiments were performed in which the enzyme was inactivated. *Taq* DNAP can be inactivated by extensive heat treatment and/or protease digestion, by extreme pH conditions, and by chelation of magnesium ions. To test the effect of different conditions on GC B and DQA1 4.1 probe signal intensities, PCR products were denatured for 5 minutes at 95°C following the DNA polymerase inactivation treatment. The tubes containing denatured PCR products were transferred to room temperature for 1 minute and then 20 μL of each reaction were added to hybridization solution in the typing tray wells. All subsequent typing steps were performed as recommended by the manufacturer for AmpliType PM except that both PM and HLA DQA1 DNA probe strips were used.

Heat inactivation studies indicated that the amplified samples need to be heated at 99°C for 10 minutes to prevent the reduction of signal intensity at the GC B and DQA1 4.1 probes. Heating the amplified samples at 98°C for 10 minutes was not sufficient to inactivate the enzyme; reduced signal intensities were observed (data not shown). Given the stringent heating requirements for inactiviation of *Taq* DNAP, this approach is not a reliable method for preventing probe-signal reduction. The simplest and most reproducible approach to prevent the reduction of signal intensity at the GC B and the DQA1 4.1 probes is to add EDTA to the amplified sample prior to the heat denaturation step. The results of adding EDTA to final concentrations ranging from approximately 5 to 50m*M* on GC B and DQA1 4.1 signal intensity are shown in Figs. 4 and 5, respectively. All concentrations of EDTA were sufficient to restore both probe signals completely. In addition, other probe signals that were light but still visible were increased to the intensity observed when the heat-denatured PCR product is added to the tray within 20 seconds (compare to "0" seconds samples in Figs. 1 and 2).

Some laboratories add BSA to PCR amplification reactions to overcome inhibition of *Taq* DNAP (3,6). BSA at a final concentration of 160 μ/mL in the AmpliType PM reaction did not interfere with the effect of EDTA on *Taq* DNAP activity (Figs. 4 and 5). To keep the protocol modification as simple as possible, we recommend that 5 μL of a 200m*M* EDTA solution be added to the 100 μL of PCR products in each reaction tube following amplification and prior to heat denaturation (final concentration

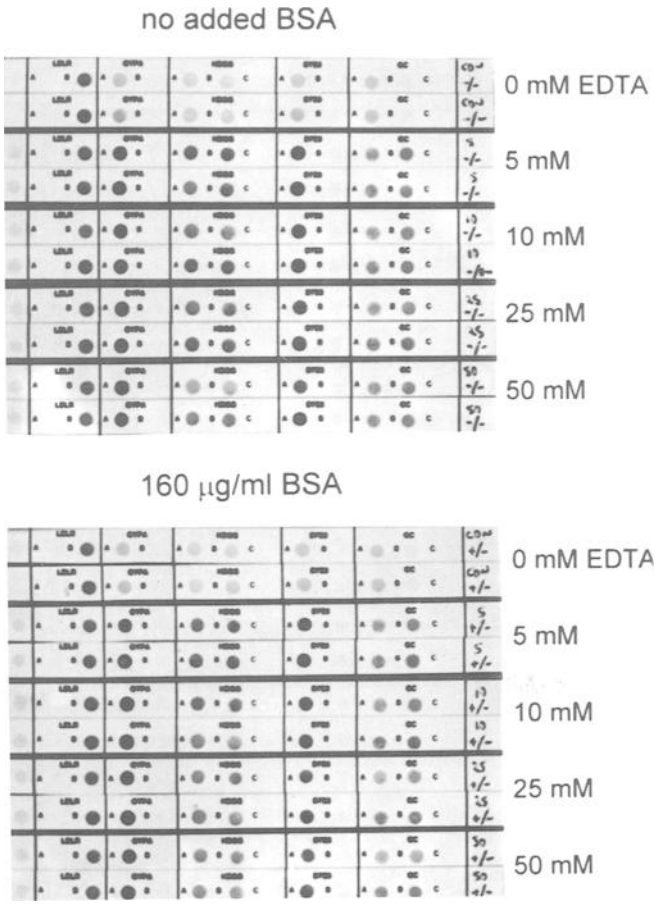

FIG. 4—*Effect of EDTA on signal intensity reduction on AmpliType PM DNA Probe Strips. DNA was amplified with the AmpliType PM PCR Reaction Mix in the absence* (A) *and presence* (B) *of 160 μg/mL BSA. Following amplification, disodium EDTA was added to individual tubes to final concentrations of approximately 5, 10, 25 and 50mM (actual concentrations are somewhat less because of the small volume change upon addition of the concentrated EDTA solution). PCR products hybridized to the "0 mM" strips did not receive EDTA. The amplified samples were heat-denatured for 5 minutes at 95°C and then transferred to room temperature for 1 minute before being added to the hybridization solution in the typing tray wells. Duplicate strips were typed for each concentration of EDTA.*

in the solution is 9.5m*M*). This protocol modification has been implemented at Roche Molecular Systems for all validation studies and quality testing involving PM and/or HLA DQA1 DNA probe strips. As discussed below, this modification is not necessary when the original HLA DQα DNA probe strips are used because there is no DQA1 4.1 probe on the strip.

The addition of EDTA as recommended has absolutely no effect on amplification of the PM and HLA DQA1 loci because it is performed post-amplification. However, it was important to determine if the addition of EDTA adversely affects gel electrophoresis, hybridization, or storage stability of the PM PCR products. The PCR product gels in Fig. 6 demonstrate that the addition of EDTA does not interfere with gel electrophoresis using the conditions described in the AmpliType PM package insert. With regard to any effect the EDTA may have on the hybridization, wash, and color development steps, the strips in Figs. 4 and 5 show that the same PCR product gives the same result at all concentrations of EDTA, which vary by an order of magnitude. To address the effect

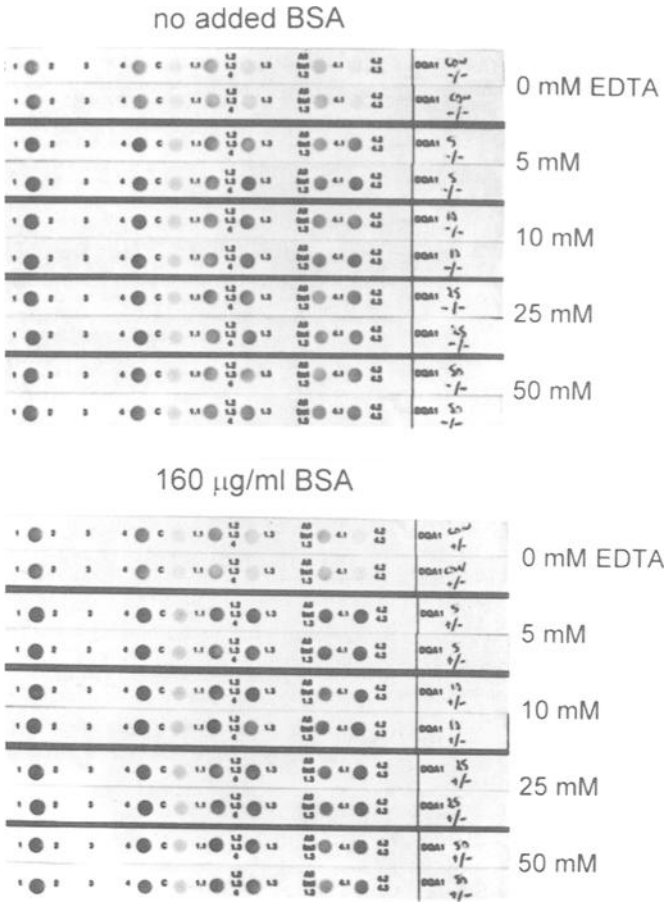

FIG. 5—*Effect of EDTA on signal intensity reduction on AmpliType HLA DQA1 DNA Probe Strips. DNA was amplified with the AmpliType PM PCR Reaction Mix in the absence* (A) *and presence* (B) *of 160 μg/mL BSA. Following amplification, disodium EDTA was added to individual tubes to final concentrations of approximately 5, 10, 25 and 50 mM (actual concentrations are somewhat less because of the small volume change upon addition of the concentrated EDTA solution). PCR products hybridized to the "0 mM" strips did not receive EDTA. The amplified samples were heat-denatured for 5 minutes at 95°C and then transferred to room temperature for 1 minute before being added to the hybridization solution in the typing tray wells. Duplicate strips were typed for each concentration of EDTA.*

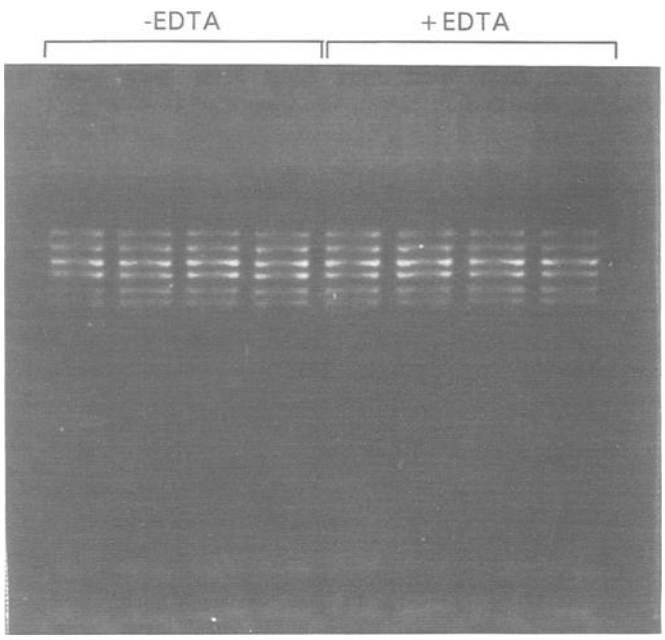

FIG. 6—*Effect of EDTA on gel electrophoresis of PM PCR products. DNA was amplified with the AmpliType PM PCR Reaction Mix. Following amplification, EDTA was added to four tubes of PCR products to a final concentration of 9.5 mM (5 μL of 200 mM EDTA added to 100 μL of PM PCR products). An aliquot from each tube containing EDTA as well as aliquots from tubes to which EDTA was not added were loaded on a 3% NuSieve/1% agarose gel cast and run in 0.5X TBE as described in the AmpliType PM package insert. The gel was stained with ethidium bromide. The six PM PCR products appear equivalent in all lanes.*

of EDTA addition on the storage stability of PCR product, multiple tubes of PM PCR product amplified using the DNA Thermal Cycler 480 and the GeneAmp PCR System 9600 were stored at 2–8°C and at ambient temperature (~22°C). EDTA was added to a final concentration of 9.5 mM to one half of the tubes prior to storage. After 16 weeks, DNA probe strips hybridized with denatured PCR products stored in the presence and absence of EDTA were indistinguishable (Fig. 7). The results obtained from PCR products stored at 2–8°C were comparable to results obtained from PCR products stored at room temperature (Fig. 7).

Many laboratories are already using AmpliType PM and the original AmpliType HLA DQα kits for casework (3,5,7–13). The reduction of the GC B and DQA1 4.1 probe signals due to excessive cooling after the heat denaturation step is not relevant to the HLA DQα kit because those DNA probe strips do not contain the DQA1 4.1 probe and the kit reagents amplify only the HLA DQA1 locus. The nominal DQA1 4 probe found on both HLA DQα and HLA DQA1 strips is not affected because the probe binding site on the PCR product is over 100 nucleotides from the 3′ end of both primers. Consequently, EDTA does not have to be added to PCR products typed with the original HLA DQα DNA probe strips.

Laboratories that have already validated the AmpliType PM kit for casework can implement this protocol modification into their procedures without having to repeat their validation studies. The most critical step in PCR-based DNA typing is the amplification of the extracted DNA, and most validation studies are designed to assess the effects of different substrates and environmental conditions on the amplification step, not the hybridization and color development steps. Therefore, the addition of EDTA after the amplification step does not compromise the results of these studies. Also, most individuals will find that they are able to add denatured PCR product to the tray in less than 20 seconds (see Crouse et al., this issue). Consequently, studies conducted previously are not likely to have been compromised by excessive signal reduction of the GC B probe. To assuage concerns that previous studies are not valid, the GC results on the strips from all of the validation studies can be reviewed. If there are any results that appear ambiguous, the PCR product can be typed again after EDTA is added to the reaction tube. In addition, if a laboratory had been systematically reducing or eliminating the GC B signal, the population data generated by that laboratory would signal the problem. First, the GC locus would not be in Hardy-Weinberg equilibrium if the B allele was not being detected in all samples containing the GC B allele. Second, the frequency of the GC B allele obtained with the PM system, which corresponds to the Gc 1F allele as defined by isoelectric focusing, would not be comparable to the vast amount of published allele frequency data generated from Gc protein typing.

In conclusion, we have identified a mechanism by which the

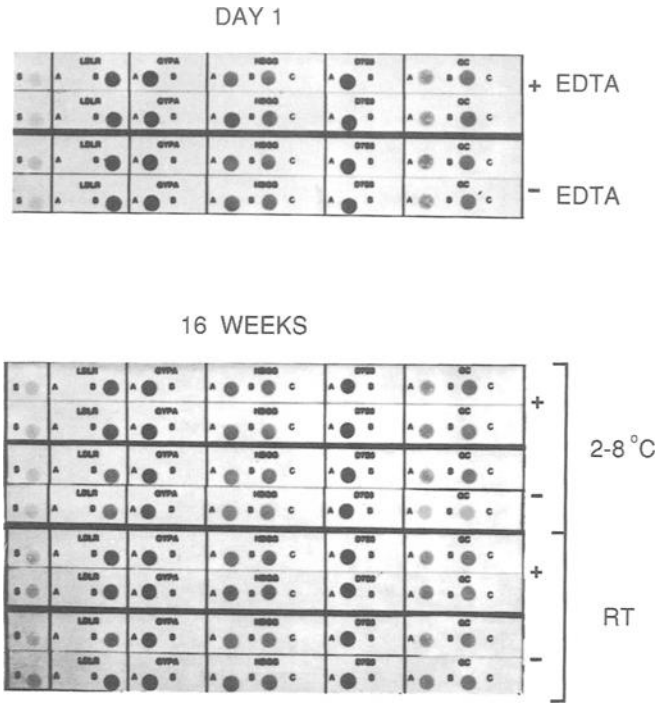

FIG. 7—*Effect of EDTA on PM PCR product storage stability. DNA was amplified with the AmpliType PM PCR Reaction Mix. Following amplification, the PCR products were pooled and distributed into Thin-Walled tubes. EDTA was added to half of the tubes to a final concentration of 9.5 mM. Tubes with and without added EDTA were put into storage at 2–8°C and room temperature. On the day of amplification (DAY 1) PCR products with and without added EDTA were hybridized to PM DNA probe strips (this development lot of PM DNA probe strips had a weak GC A probe signal). After 16 weeks of storage at 2–8°C and room temperature (RT), PCR products with (+) and without (−) added EDTA were hybridized to PM DNA probe strips.*

intensities of the GC B and HLA DQA1 4.1 probes can be selectively reduced or eliminated and have determined that the addition of EDTA to a final concentration of 9.5 mM prior to heat denaturation of the PCR products prevents loss of signal intensity at all PM and HLA DQA1 probes. While there is little chance that an experienced user will take longer than 20 seconds to transfer denatured PCR product to the typing tray well, we are recommending a simple procedure to ensure that selective probe signal loss will not occur. By implementing this safeguard, observation of a weak GC B signal relative to GC A and/or C signals on the same strip cannot be attributed to a procedural error on the part of the user. Rather, the user would be able to conclude with confidence that the weak GC B signal indicates a second contributor to that particular sample, which can be a very important piece of information for that case. Similarly, the addition of EDTA would allow a user to interpret a weak DQA1 4.1 probe signal correctly. Crouse, et al. in the accompanying paper describe approaches for evaluating existing PM validation and population data. In addition, they discuss the importance of establishing standard operating procedures that ensure reliable PM typing results will be obtained.

Acknowledgments

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