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TECHNICAL NOTE

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Recovery of Genomic DNA from Archived PCR Product Mixes for Subsequent Multiplex Amplification and Typing of Additional Loci: Forensic Significance for Older Unsolved Criminal Cases*

ABSTRACT: A method for genomic DNA recovery from different types of PCR product mixes suitable for multiplex amplification and typing using the Profiler Plus™ STR typing system has been investigated. The application of this method is of significance in cases where the original DNA samples have been exhausted due to repeated typing analyses in an effort to maximize their evidentiary value. Such cases typically involve samples analyzed using the available DNA typing systems of the time which gave a markedly lower power of discrimination, either alone or in combination, compared to that of modern multiplex STR typing systems. It was found that an effective method for recovering genomic DNA from HLA-DQA1+PM and CTT triplex amplification mixes, suitable for reproducible achievement of the complete Profiler Plus™ profile, involved the use of Amicon Microcon-100 microconcentrators. Interestingly, this method was not required to achieve the complete nine STR profile using D1S80 amplification mixes.

KEYWORDS: forensic science, DNA typing, genomic DNA, polymerase chain reaction, multiplex amplification, short tandem repeat loci, HLA-DQA1, LDLR, GYPA, HBGG, D7S8, Gc, CSF1PO, THO1, TPOX, D1S80, F13A, FES, D3S1358, D5S818, D8S1179, vWA, D13S317, D21S11, FGA, D7S820, D18S51, amelogenin

For the majority of casework involving DNA profiling, all Australian forensic biology laboratories now routinely use the Applied Biosystems AmpFℓSTR® Profiler Plus™ PCR Amplification Kit together with automated fluorescence-based allele detection. This multiplex PCR system, also used in most U.S. forensic laboratories, simultaneously amplifies nine short tandem repeat (STR) loci and a gender-typing locus. Its major advantage over previously used PCR-based typing systems is its very high discriminatory power; the use of Profiler Plus™, alone, enables an "effective individualization" to be made depending on the size of the defined population. Because there is no statistical proof of certainty, it cannot be scientifically stated that a particular DNA profile comes from only a single individual. However, in the context of the current Australian population of 19 million, this nine loci STR profile is considered sufficient for effective individualization as the probability of a random match between two unrelated people in this population is calculated to be not less than 1 in 300 million. This figure is based on the Queensland Caucasian database consisting of 553 individuals as of December, 1999 (Forensic Biology Laboratory, Queensland Health Scientific Services, QHSS, Australia). Also, statistical analysis shows that >99% of all profiles have an expected random match probability of not less than 1 in 1 billion (Janet Chaseling, personal communication).

Prior to the use of Profiler Plus™, other PCR-based typing systems used in the QHSS Forensic Biology Laboratory included HLA-DQA1+PM, D1S80, and two multiplex systems: CTT triplex (CSF1PO; THO1; TPOX), and Quadruplex (consisting of two sets of separately amplified duplexes, F13A/FES and vWA/THO1). None of these typing systems, either alone or in combination, gave a sufficiently high power of discrimination to enable effective individualization.

For some older casework investigations, biological material collected from crime scenes and the corresponding DNA extracts became exhausted for a number of reasons. These included repeated samplings from the original DNA extracts for use in a number of typing systems in an effort to improve the evidentiary value of the DNA results, and the availability of only limited quantities of DNA for analysis due to environmental degradation or to the small quantity of biological material available for testing. In such instances, the only material remaining was the unused portions of amplification product mixes made using one or

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more of the PCR-based typing systems, which have now been superseded by Profiler Plus™.

Additional loci could be examined, and also compared with contemporary reference and other crime scene samples, if the genomic DNA present in these amplification product mixes could be recovered and successfully used for multiplex amplification with the current Profiler Plus™ system. Such examinations would likely strengthen the evidentiary value of the DNA analysis. The ideal approach would involve a size-exclusion method to enable the separation of remaining non-amplified genomic DNA from amplified PCR products and reaction components. However, while the sizes of genomic DNA and PCR products are sufficiently different to allow such separation in principle, three considerations impact on method design. First, for many forensic casework samples, the genomic DNA may be severely degraded making the separation of this DNA from the PCR products difficult. Second, commercially available ultrafiltration devices such as the range of Microcon microconcentrators (Amicon) do not enable size separation of genomic DNA from PCR products, and were not designed to do so, but will remove amplification reaction components such as unincorporated primers and deoxynucleoside triphosphates. Third, the question arises as to whether the separation of genomic DNA from amplified products generated by older typing systems is, in fact, necessary prior to amplification and typing using Profiler Plus™. The presence of these amplified products in Profiler Plus™ PCR reaction mixes would seem unlikely to interfere with this multiplex profiling since the Profiler Plus™ Primer Set is designed to anneal only to specific target sequences. With the exception of the vWA locus, these target sequences are not present in the amplification products from the older PCR-based typing systems examined in this study.

For each of four now-superseded PCR-based typing systems, the issue of whether or not any pretreatment of their amplification product mixtures is needed prior to successful multiplex amplification using the Profiler Plus™ system is addressed in this Technical Note. Where a pretreatment was found to be necessary, the method that gives reproducible achievement of the complete nine loci STR profile plus gender typing from the recovered DNA is described. Similar studies have been previously reported (1–3) but none of these examined suitability of recovered DNA for multiplex amplification and typing of STR loci using the Profiler Plus™ system.

The significance of the work described here for older unsolved criminal cases is also addressed.

Materials and Methods

Sample Collection

Twenty-four original DNA extracts from sets of blood samples (taken from the mother, child, and suspected father) in eight completed cases of disputed paternity were retrieved from −70°C storage at the QHSS Forensic Biology Laboratory. In all cases, DNA had been extracted using 20% (w/v) Chelex as described by Walsh et al. (4). These samples were selected on the basis that they had been previously profiled using one or more of three typing systems (HLA-DQA1+PM, D1S80, and CTT triplex) and their corresponding amplification product mixtures were available for retrieval from −20°C storage where they had been located since 1995. In addition to these samples from laboratory archives, fresh blood samples were collected from two unrelated Caucasian volunteers. The DNA was immediately extracted using 20% (w/v) Chelex, and the quantity of DNA in pooled extracts from each volunteer estimated using the GIBCO-BRL ACES™ 2.0 system. Each of these DNA samples was then analyzed using two typing systems (HLA-DQA1+PM and Quadruplex) with typings performed in duplicate for each system.

DNA Typing

HLA-DQA1+PM loci were analyzed using the Applied Biosystems AmpliType® PM+DQA1 PCR Amplification and Typing Kit according to the manufacturer's recommendations. The D1S80 locus was typed using the Applied Biosystems AmpliFLP™ D1S80 PCR Amplification Kit as per the manufacturer's protocol. The CSF1PO, TPOX, and THO1 loci (CTT triplex) were analyzed using the Promega GenePrint™ STR system according to the recommended protocol. Due to evidence of preferential amplification of smaller loci in the Quadruplex typing system (data not shown), two separate reactions to amplify each pair of loci (FES/F13A and vWA/THO1) were used. Both reactions were in 50 μL volumes containing 3.5 μL of forward and fluorescently-tagged reverse primer sets (except, for vWA, the forward primer rather than the reverse primer is fluorescently-labelled; ABI PRISM™ STR Primer Set: PCR Amplification and Typing protocol, part #903223), 20 μL PCR reaction mix, and 1 μL of a 1/4 dilution of AmpliTaqGold™ (1.25 units). Thermal cycling was performed using an ABI GeneAmp system 9600 with the following parameters: 95°C for 9 min; 95°C for 1 min, 54°C for 1 min, 72°C for 1 min (28 cycles); 72°C for 10 min. Aliquots (5 μL) from each of the respective amplification product mixtures were combined prior to electrophoresis on an ABI Prism® 377 DNA Sequencer, and analysis performed according to the recommended protocol. The nine STR loci and amelogenin sex-typing locus of the Applied Biosystems AmpFℓSTR® Profiler Plus™ system were amplified and typed according to the manufacturer's recommended protocol. A master mix containing, for each sample, 21 μL of AmpFℓSTR® PCR Reaction Mix, 11 μL of Profiler Plus™ Primer Set, and 1 μL AmpliTaqGold™ (5 units) was prepared, and 30 μL of this dispensed into each tube in preparation for the addition of DNA sample to a final reaction volume of 50 μL. Thermal cycling was performed using an ABI GeneAmp system 9600 with the following parameters: 95°C for 11 min; 95°C for 1 min, 54°C for 1 min, 72°C for 1 min (28 cycles); 72°C for 10 min. Samples were prepared for electrophoresis on an ABI Prism® 377 DNA Sequencer by combining 2 μL of amplification product mix with 2.5 μL of loading mix (30 μL Genescan ROX-500 internal size standard and 150 μL of dye mix, the latter prepared as a stock solution by mixing 1 mL of deionized formamide with 300 μL dextran blue). After denaturation of samples at 95°C for 1 min, they were snap-cooled on ice prior to gel loading. Data was analyzed using the Genescan analysis software, Version 3.1, and Genotyper software, Version 2.5.

Recovery of DNA from Amplification Product Mixes

Microcon-100 microconcentrators (Amicon) with a nominal molecular weight cut-off of 100 kDa, corresponding to 300 nt of single-stranded DNA or 125 bp of double-stranded DNA, were used to recover DNA from amplification product mixes generated by four different PCR-based typing systems. Since amplified PCR products from these systems are all larger than 125 bp, this method was not able to separate unused genomic DNA templates from amplified DNA products, but was able to remove amplification reaction components such as unincorporated primers and deoxynucleoside triphosphates. For each amplification product mixture, a Microcon-100 sample reservoir was placed into a microcentrifuge tube and the reservoir filled with 400 μL TE (10 mM Tris-HCl

pH8.0, 0.1 mM EDTA) and up to 50 μL of amplification product mixture. After centrifugation at 500 g for 15 min in an Eppendorf Model 5415C microcentrifuge at room temperature, another 400 μL TE was added to the sample reservoir and centrifugation continued until the volume in the retentate cup was reduced to about 20 μL (approximately 15 min). To recover purified DNA, the reservoir was removed and inverted into a new microcentrifuge tube then centrifuged at 500 g for 2 min.

DNA Quantitation

Quantitation of recovered DNA was not carried out, and is not recommended for the reasons given in Results and Discussion (see *Quantitation of Recovered DNA is Unnecessary*).

Results and Discussion

HLA-DQA1+PM Amplification Product Mixes

Original DNA extracts from 12 individuals involved in four completed cases of disputed paternity, together with their corresponding HLA-DQA1+PM amplification product mixes, were retrieved from −70°C and −20°C storage, respectively, for analysis using Profiler Plus™. An aliquot from each amplification product mix was then divided equally, with half receiving no further treatment and the other half treated with a Microcon-100 microconcentrator to recover the DNA. Equivalent aliquots from each of these were then amplified and typed using the AmpFℓSTR® Profiler Plus™ system. In contrast to the untreated samples, which resulted in the detection of only small spurious peaks, none of which corresponded to the alleles detected using Profiler Plus™, analysis of every Microcon-100 treated sample resulted in a complete nine STR loci profile plus the amelogenin gender-typing (see Fig. 1). Further, these profiles were identical to those obtained when the corresponding original DNA extracts were amplified and typed using Profiler Plus™ (data not shown), although the RFU (relative fluorescence unit) values for the detected peaks were reduced, as expected (see *Theoretical Considerations* below), by about 2.5-fold in comparison with those obtained for the original DNA extracts.

These results indicate that no typing errors were caused by either Microcon-100 treatment nor the presence of HLA-DQA1+PM amplification products in the Profiler Plus™ PCR reaction mix. They also suggest that the use of Microcon-100 microconcentrators to recover DNA from HLA-DQA1+PM amplification product mixes enables reproducible, reliable, and accurate typing of recovered genomic DNA using Profiler Plus™, and is an essential step for successful typing.

D1S80 Amplification Product Mixes

Original DNA extracts from these same 12 individuals, together with their corresponding D1S80 amplification product mixes which had been stored at −20°C, were similarly analyzed. Irrespective of whether a Microcon-100 microconcentrator treatment was used or not, complete nine STR loci profiles with similar RFU values, together with the amelogenin gender-typing, were obtained (Fig. 2). As before, these profiles matched those obtained when the original DNA extracts were typed using Profiler Plus™ (data not shown). In comparison with STR profiles of the original DNA extracts, the expected reduction in RFU values (by about 2 fold) of the detected peaks was observed for both untreated amplification product mix samples and samples treated with Microcon-100 microconcentrators prior to Profiler Plus™ typing (data not shown).

CTT Triplex Amplification Product Mixes

Original DNA extracts from another 12 individuals in four other completed cases of disputed paternity, together with their corresponding CTT triplex amplification product mixes retrieved from −20°C storage, were analyzed using the same approach. Only two of the untreated amplification product mix samples gave complete profiles using Profiler Plus™ typing while the remaining untreated samples resulted in partial STR profiles ranging from 3–7 loci; those loci not detected for a given sample always corresponded to the larger molecular weight loci (see Fig. 3). In contrast, all 12 of the Microcon-100 treated amplification product mix samples resulted in complete nine loci STR profiles (see Fig. 3), with each one having the same DNA profile as that obtained for the corresponding original DNA extract using Profiler Plus™ typing (data not shown). Again, an expected modest reduction (~3-fold) in RFU values of the detected peaks, in comparison with those of the corresponding original DNA extracts, was observed for CTT triplex amplification product mix samples treated with Microcon-100 microconcentrators prior to Profiler Plus™ typing (data not shown).

These results suggest that the use of Microcon-100 microconcentrators to recover DNA from CTT triplex amplification product mixes markedly improves the capacity to obtain reproducible, reliable, and accurate typing of recovered genomic DNA using Profiler Plus™.

Quadruplex Amplification Product Mixes

Extracted DNA from two unrelated Caucasian volunteers was amplified using the Quadruplex system consisting of separate FES/F13A and vWA/THO1 duplex reactions. Each amplification product mixture was then divided equally into three sample volumes. The first sample was not treated with Microcon-100, nor amplified using the AmpFℓSTR® Profiler Plus™ system. Rather, an aliquot was taken from the FES/F13A amplification product mix, electrophoresed (without any further treatment) directly on the ABI Prism® 377 DNA Sequencer, and analyzed using the Profiler Plus™ matrix. This resulted in the detection of the exact size and location of FES/F13A alleles (Fig. 4). Consequently, in cases where recovered DNA from stored FES/F13A amplification product mixes is amplified and typed using Profiler Plus™, any STR alleles which overlap with these "known" FES/F13A alleles could not be unambiguously interpreted. Nevertheless, despite these circumstances, mis-typing is not possible.

For the remaining two samples from each FES/F13A amplification product mixture, only one was treated with Microcon-100 to recover the DNA prior to equivalent aliquots of both being amplified and typed using the Profiler Plus™ system. In both cases, alleles detected at any particular locus were the same and also identical with those detected by amplification and typing of the corresponding DNA extracts (data not shown). These results indicate that no typing errors were caused by either Microcon-100 treatment nor the presence of components from the FES/F13A amplification product mixture in the Profiler Plus™ PCR reaction mix. However, whether or not Microcon-100 treatment was used, only partial STR profiles, ranging from amelogenin plus 4–8 loci were obtained using Profiler Plus™ typing; those loci not detected for a given sample always corresponded to the larger molecular weight loci (data not shown). Further, duplicate aliquots from the Microcon-100 treated FES/F13A amplification product mixes did not give the same partial STR profiles: identical numbers of detected loci were not always obtained for each sample of a duplicate (data not shown), suggesting that reproducibility is questionable

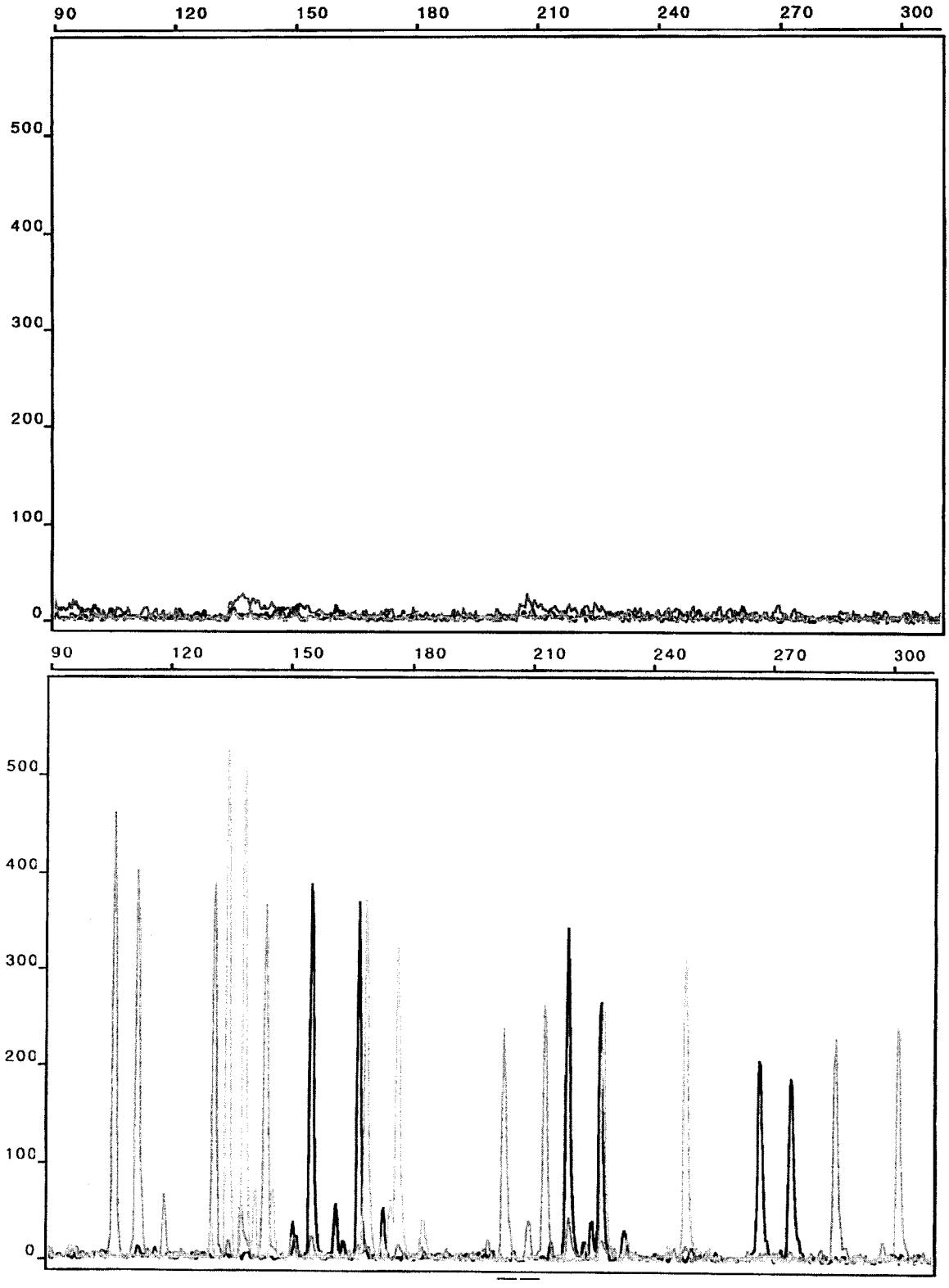

FIG. 1—*Profiler Plus™ electropherograms of DNA from a HLA-DQA1+PM amplification product mix. Prior to amplification and typing using Profiler Plus™, one aliquot of this mix received no further treatment (upper panel) while a second equivalent aliquot was treated with a Microcon-100 microconcentrator to recover the DNA (lower panel). The numbers on the X-axis are sizes in bp, while the numbers on the Y-axis are RFU values.*

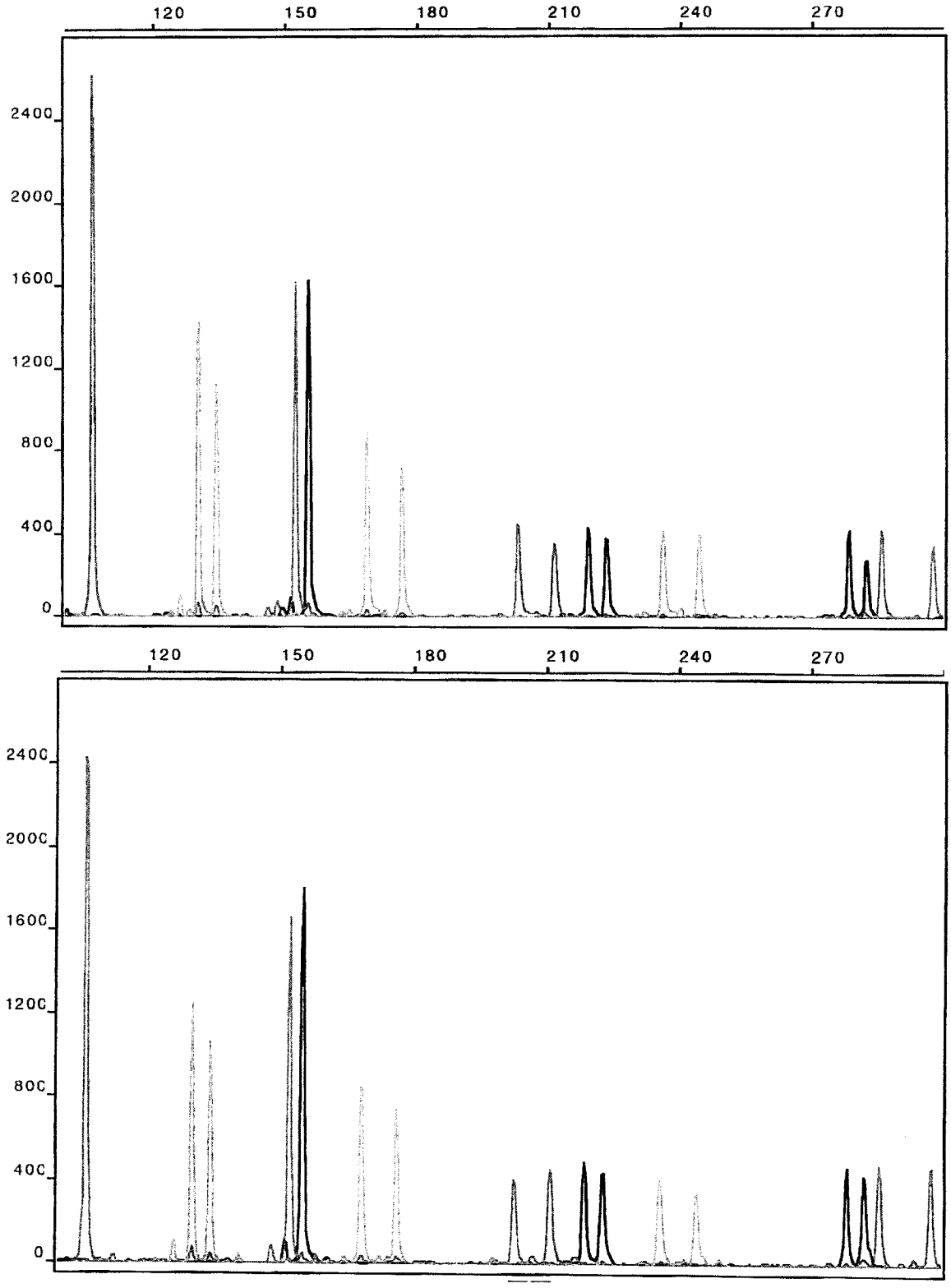

FIG. 2—*Profiler Plus™ electropherograms of DNA from a D1S80 amplification product mix. Prior to amplification and typing using Profiler Plus™, one aliquot of this mix received no further treatment (upper panel) while a second equivalent aliquot was treated with a Microcon-100 microconcentrator to recover the DNA (lower panel). Numbers on the X- and Y-axes are as described in the legend to Fig.1. Comparison of the profile of the original DNA extract (reference sample; data not shown) with the profile in the lower panel revealed that all identified alleles were identical and that the imbalance among loci seen in the lower panel was the same for the reference sample.*

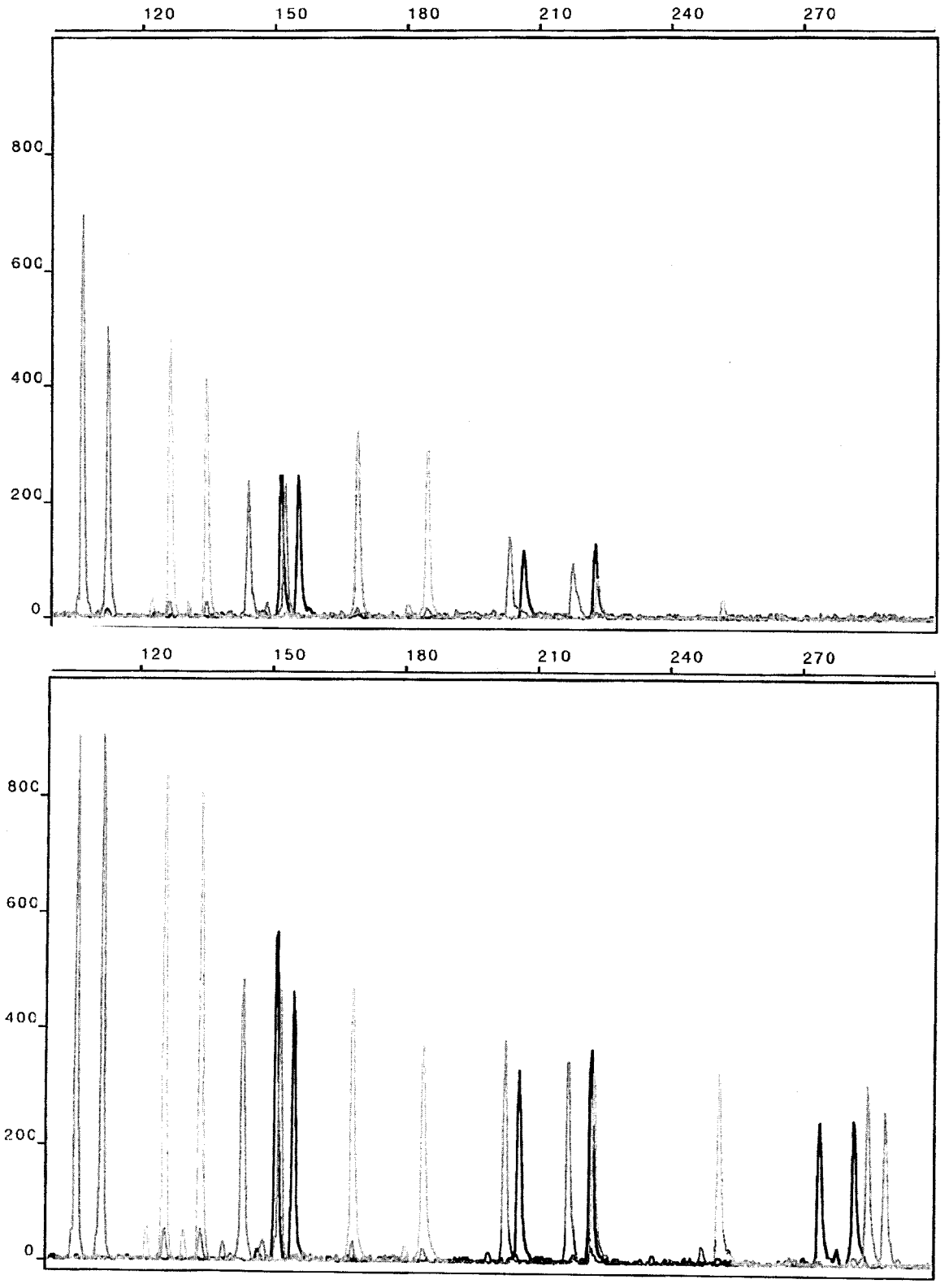

FIG. 3—*Profiler Plus™ electropherograms of DNA from a CTT triplex amplification product mix. Prior to amplification and typing using Profiler Plus™, one aliquot of this mix received no further treatment (upper panel) while a second equivalent aliquot was treated with a Microcon-100 microconcentrator to recover the DNA (lower panel). Numbers on the X- and Y-axes are as described in the legend to Fig.1. Comparison of the profile of the original DNA extract (reference sample; data not shown) with the profile in the lower panel revealed that all identified alleles were identical and that the imbalance among loci seen in the lower panel was the same for the reference sample.*

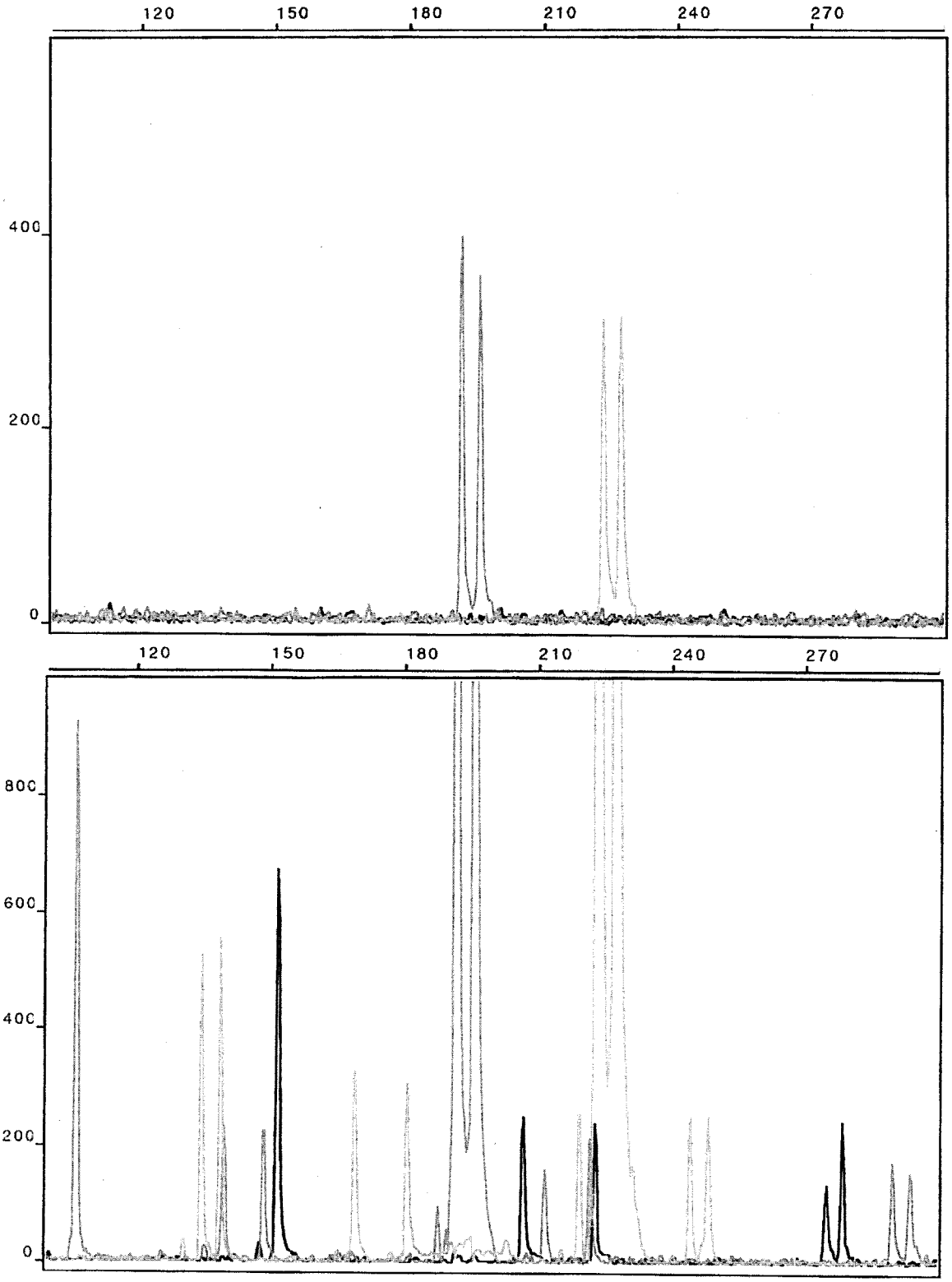

FIG. 4—*Profiler Plus™ electropherograms of DNA from a FES/F13A amplification product mix. One aliquot of this mix was not treated with a Microcon-100 microconcentrator, nor amplified using the Profiler Plus™ system, prior to gel electrophoresis (upper panel); the Profiler Plus™ matrix enables detection of the FES and F13A alleles. Another equivalent aliquot was treated with a Microcon-100 microconcentrator to recover the DNA prior to amplification and typing using the Profiler Plus™ system (lower panel). Numbers on the X- and Y-axes are as described in the legend to Fig.1. Comparison of the profile of the original DNA extract (reference sample; data not shown) with the profile in the lower panel revealed that all identified alleles were identical and that the imbalance among loci seen in the lower panel was the same for the reference sample.*

when FES/F13A amplification product mixes are used as the source material for Profiler Plus™ typing. For example, regarding any set of duplicates, sometimes a partial STR profile of amelogenin plus six loci was obtained for one of the duplicates while amelogenin plus eight loci was obtained for the other duplicate; the six loci common to both duplicates, however, gave the same DNA profile (data not shown). Nevertheless, despite this reproducibility limitation, additional loci were typed using this approach, and this may be useful in some circumstances.

Because the original DNA extracts were still available in these cases (as this DNA was obtained from two unrelated Caucasian volunteers; see above), the amount of DNA used in each of the original FES/F13A amplification mixes could be estimated (using the GIBCO-BRL ACES™ 2.0 system); this DNA quantity was estimated to be 4 ng. To mimic the situation with stored amplification product mixes (where only about 20 μL could be recovered; see *Theoretical Considerations*), only 10 μL of each original amplification mix was used for Profiler Plus™ amplification and typing since a comparison was sought between untreated and Microcon-100 treated samples. Thus, because the volume of these FES/F13A amplification mixes was 50 μL, use of a 10 μL aliquot results in the maximum amount of recovered template DNA present in the Profiler Plus™ mix being 0.8 ng. While this amount of template DNA falls within the range for successful amplification and typing using Profiler Plus™ (see *Theoretical Considerations*), it is conceivable that recovery of template DNA from an FES/F13A amplification product mix would be less than optimum. This, in turn, suggests that the lack of reproducibility in Profiler Plus™ STR profiles between duplicate aliquots from the same Microcon-100 treated FES/F13A amplification product mixes may be due to stochastic effects.

In contrast to the FES/F13A results, Profiler Plus™ typing of Microcon-100 treated vWA/THO1 amplification product mixes did not result in identifiable STR alleles due to the appearance of large, spurious peaks with RFU values around 6000 in the region spanning the expected location of vWA alleles (Fig. 5). These peaks were presumably caused by amplification of the vWA locus common to both Profiler Plus™ and the vWA/THO1 duplex; the vWA/THO1 amplification product mixture contains both remaining genomic DNA and already amplified vWA products, which both serve as templates for vWA amplification. The likely high ratio of vWA template to genomic template for other loci has presumably caused preferential amplification of the vWA locus suggesting that any amplification product mixtures sharing a locus/loci with Profiler Plus™ are not suitable candidates for subsequent typing with Profiler Plus™.

Theoretical Considerations

An expected limitation of the genomic DNA recovery method described here was the amount of template DNA in each type of amplified product mix which would be available for Profiler Plus™ amplification.

In contrast to those casework samples where the original biological material and corresponding DNA extracts have been exhausted, the original DNA extracts corresponding to the stored amplification product mixes used in this study were still available. Thus, the amount of template DNA added to each original amplification reaction mix could be readily verified for any particular sample using the GIBCO-BRL ACES™ 2.0 system. For the HLA-DQA1+PM amplification mixes, the D1S80 amplification mixes, and the CTT triplex amplification mixes, template DNA in the range of 2.5–5ng was used; for both the FES/F13A and vWA/THO1 duplex amplification mixes, the template DNA used was estimated to be 4 ng.

However, the application of the method reported herein is in cases where only the amplification product mixes remain in storage as a source for recovery of template DNA. In such cases, we do not recommend quantitation of recovered DNA (see *Quantitation of Recovered DNA is Unnecessary*). While the total volume of each of the amplification mixes from these older typing systems was 50 μL (except for HLA-DQA1+PM, which was 100 μL), the remaining volume of each mix recovered from storage was only around 20 μL. In evaluating the outcome of Microcon-100 treatment in terms of profiles obtained after amplification and typing with Profiler Plus™, we also profiled an equal volume of untreated aliquots of each amplification mix for comparison. As a result, only 10 μL of each original amplification mix was amplified and typed using Profiler Plus™ for both untreated and Microcon-100 treated samples. Thus, for every sample, a maximum of 20% of the template DNA added to each original amplification reaction mix was amplified and typed using Profiler Plus™. Two predictions can be made based on this limitation. First, the RFU values of the detected peaks from Profiler Plus™ typing of both untreated and Microcon-100 treated amplification mix aliquots would be expected to be less than those obtained from typing original DNA extracts as controls, since the exact amount of available template DNA in the former would be hard to quantify but would be less than optimum. Indeed, modest RFU reductions of 2–3 fold were obtained for all amplification product mixes typed using Profiler Plus™. Second, since the optimal amount of template DNA for a Profiler Plus™ amplification is in the range of 0.5–1.0 ng then, theoretically, a minimum of 2.5 ng template DNA would need to have been present in each amplification reaction mix of the now-superseded typing systems to ensure a complete profile using Profiler Plus™ is obtained. Since the detection limit for HLA-DQA1 typing, for example, is 0.5 ng, then samples that typed weakly in this system would likely not be successfully typed using Profiler Plus™ following the DNA recovery method described in this paper, despite the sensitivity of this multiplex STR method.

Quantitation of Recovered DNA is Unnecessary

While 0.5–1ng template DNA is optimal for Profiler Plus™ amplification and typing (see above), up to 2.5 ng can be used without compromising the quality of the DNA profile obtained (unpublished data). Thus, it may be suggested that determination of the amount of DNA recovered from amplified product mixtures using Microcon-100 microconcentrators would be important prior to amplification and typing using Profiler Plus™. However, this quantitation is not necessary nor indeed is it recommended for the reasons described below.

For those older case work investigations in which both the original biological material and corresponding DNA extract have been exhausted, the only remaining source of template DNA is the unused portion of appropriately stored amplification product mixtures made using one or more of the now-superseded PCR-based typing systems. In these systems, template DNA in the range of 2.5–5 ng had been used in the original PCR mixtures. Even if recovery of purified template DNA using the method described herein was 100% efficient, then it is possible to add a maximum of only 1–2 ng of this original template DNA in the Profiler Plus™ amplification and typing reaction since only 20 μL of the typical 50 μL volume of original amplification product mix was recoverable from storage (see *Theoretical Considerations*). Further, it would be undesirable to use all of this recovered DNA for Profiler Plus™ typing since it

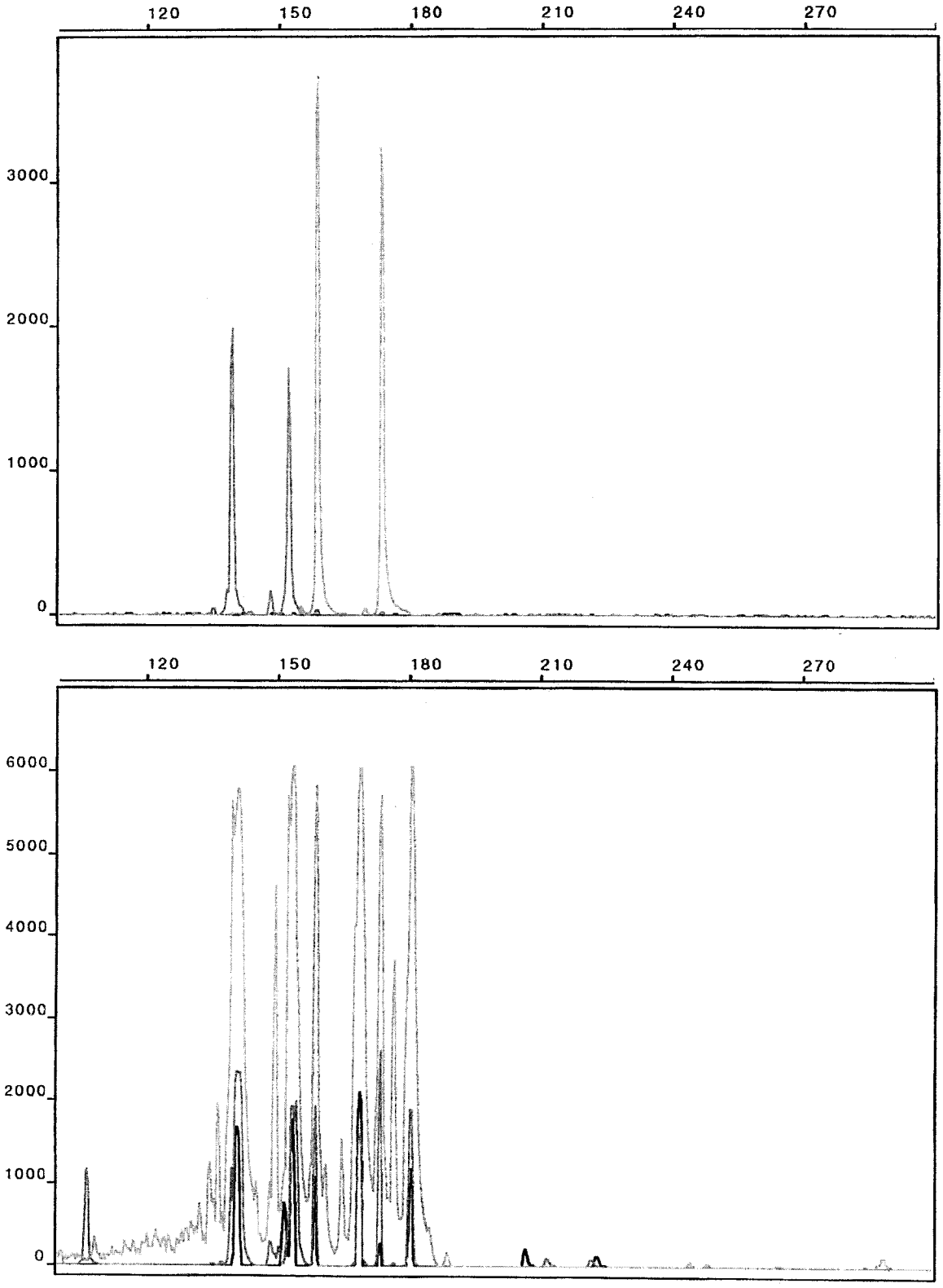

FIG. 5—*Profiler Plus™ electropherograms of DNA from a vWA/THO1 amplification product mix. One aliquot of this mix was not treated with a Microcon-100 microconcentrator, nor amplified using the Profiler Plus™ system, prior to gel electrophoresis (upper panel); the Profiler Plus™ matrix enables detection of the vWA and THO1 alleles. Another equivalent aliquot was treated with a Microcon-100 microconcentrator to recover the DNA prior to amplification and typing using the Profiler Plus™ system (lower panel). Numbers on the X- and Y-axes are as described in the legend to Fig. 1.*

is preferable to retain some DNA (say 50%) so, for example, it can be made available for independent testing by the Defense. In these circumstances, it follows that 0.5–1 ng of recovered DNA can only be used per reaction; thus, it is not possible to add an excessive amount of recovered DNA (i.e., more than 2.5 ng; see above), which would most likely compromise the quality of the DNA profile obtained after Profiler Plus™ amplification and typing. In these circumstances, quantitation of recovered DNA is unnecessary. On the other hand, and particularly if the quantity of DNA template used in the original amplification reaction was relatively low, it is possible to add an amount of recovered DNA that may be significantly less than the recommended optimum template amount for Profiler Plus™ amplification and typing. However, this circumstance provides an even more compelling reason not to quantitate the amount of recovered DNA since the potential problem of using sub-optimal amounts of DNA for Profiler Plus™ amplification and typing would be compounded by use of a portion of this recovered DNA for quantitation purposes.

Contamination Considerations

In all forensic laboratories that routinely perform DNA profiling, there are strict protocols to prevent contamination of preamplification reaction components with amplified product. In our laboratory, for example, in addition to the use of a designated reagent preparation room, there is another room ("pre-PCR") specifically designated for the addition of template DNA to the pre-amplification reaction components. Amplified products are kept physically separate by performing PCR reactions in another designated room ("post-PCR"). Tubes containing both pre-amplification reaction components and template DNA are not opened prior to amplification in this "post-PCR" room.

In the method described in this paper, recovered DNA for Profiler Plus™ amplification and typing consists of both genomic DNA and amplified products from PCR mixtures produced using one or more of four superseded typing systems. Thus, in view of our laboratory protocols, it was not appropriate to add this DNA to the Profiler Plus™ pre-amplification reaction components in the "pre-PCR" room even though the amplified products present in the recovered DNA from PCR product mixtures produced using HLA-DQA1+PM, CTT triplex, and D1S80 are from loci which are not analyzed with the now routinely used Profiler Plus™ system. However, products from vWA loci originally typed using the Quadruplex system were present in these recovered DNA samples, which represented a contamination threat in the preparation of standard Profiler Plus™ reactions. For both these reasons, it was considered more appropriate to add this DNA to the Profiler Plus™ preamplification reaction components in the "post-PCR" room. In this "post-PCR" room, a microcentrifuge was dedicated for use in recovery of DNA from amplification product mixtures. The preamplification reaction components, without this DNA, were prepared in the "pre-PCR" room as usual, then taken into the "post-PCR" room; here, each tube was briefly uncapped to add the recovered DNA sample, then re-capped and amplified. This minimized the time the preamplification mixture was exposed to possible aerosol contamination from unrelated Profiler Plus™ amplification products.

As such, this protocol did not pose a contamination threat to those unrelated standard reaction mixtures consisting of both preamplification reaction components and template DNA (without the additional presence of amplified DNA products) because these tubes were not opened prior to amplification in this "post-PCR" room. Further, to counter the contamination threat from amplified DNA present in the "post-PCR" room to the profiles obtained following Profiler Plus™ amplification and typing of recovered DNA, two controls were utilized. First, Profiler Plus™ reagent blanks (negative controls) containing all preamplification reaction components, except DNA, which had been subjected to the same protocol using Microcon-100 microconcentrators, did not reveal the presence of any alleles following Profiler Plus™ amplification and typing (data not shown). Second, Profiler Plus™ profiles of original DNA extracts (positive controls), known to be from single contributors (see Materials and Methods), were identical to those obtained from corresponding DNA recovered from stored amplification product mixtures. No extra alleles were present in the latter (data not shown) as would be expected if contaminated with amplification products from unrelated reactions or from other DNA present as a result of "carryover contamination" (see below). Thus, the Profiler Plus™ profiles obtained from recovered DNA reported herein are therefore valid.

However, in circumstances where stored amplification product mixtures remain but corresponding reference samples are not also available, the use of such positive controls is not possible. In these cases, it follows that the presence of any extra alleles from recovered DNA amplified and typed using Profiler Plus™ may not necessarily indicate contamination; rather, the possibility of a mixed DNA sample cannot be excluded. The chance of more than one contributor to the DNA sample in the original amplification reaction remains a possibility even if results from the superseded typing system previously used suggested only a single DNA contributor; this is due to the much improved sensitivity of the Profiler Plus™ system compared to these superseded typing systems.

Contamination is an alternative possibility in this case even if the Profiler Plus™ reagent blanks show no detected alleles. For example, the presence of any contaminating DNA in either the original HLA-DQA1+PM reagents or in the corresponding amplification product mixes which may not have been detected by HLA-DQA1+PM amplification and typing could, in principle, be detected as a minor component by Profiler Plus™ amplification and typing, as it would be recovered along with the template DNA using the Microcon-100 treatment reported herein. Since Profiler Plus™ reagent blanks would not detect such "carryover contamination," it may be suggested that additional negative controls are essential. These controls would correspond to reagent blanks from the original amplifications (e.g., HLA-DQA1+PM), which had been subjected to Microcon-100 treatment prior to amplification and typing using Profiler Plus™. However, two factors impact on this suggestion. First, if the Profiler Plus™ profile obtained from recovered DNA is consistent with that of a single contributor, the chance of carryover contamination is precluded since preferential recovery of any contaminating DNA over template DNA from these amplification product mixes is not possible. Second, if the profile of the recovered DNA is consistent with more than one contributor, this profile should be interpreted from the perspectives of being either a mixture or containing contaminating DNA from the original amplification product mix or reagents. (This interpretation presumes that the Profiler Plus™ reagent blanks do not detect any alleles.) To distinguish between these two possibilities, the original reagent blanks (e.g., from the HLA-DQA1+PM analysis) may be amplified and typed using Profiler Plus™ (following Microcon-100 treatment). Absence of detected alleles from these reagent blanks would confirm the recovered DNA was a mixture rather than containing carryover contamination and, as such, provides the best demonstration for lack of such contamination.

Also, in the interests of using a cautious approach to interpretation, profile comparisons of at least one unrelated positive control

together with its corresponding recovered DNA sample following Profiler Plus™ amplification and typing is suggested.

Conclusions

In summary, in circumstances where the original DNA extracts and biological source material are no longer available, it may be possible to type nine additional loci using the Profiler Plus™ system, providing that corresponding amplification product mixtures made using now-superseded typing systems are still available. Further, while no treatment of D1S80 amplification product mixtures is needed in order to obtain complete Profiler Plus™ STR profiles, treatment with Microcon-100 microconcentrators is essential when only HLA-DQA1+PM and/or CTT triplex amplification product mixtures are available to obtain complete Profiler Plus™ STR profiles. In contrast, Quadruplex amplification product mixtures do not give the nine additional STR loci whether treatment with a Microcon-100 microconcentrator is used or not. However, the use of FES/F13A amplification product mixes, whether treated or not, in Profiler Plus™ typing does give some additional STR loci, but our results suggest that reproducibility and the capacity for unambiguous interpretation of profiles are significant problems in this instance.

The question arises as to why untreated aliquots containing genomic and amplified DNA from product mixtures produced using these four superseded typing systems give such different outcomes when amplified and typed using Profiler Plus™. These variable outcomes may be caused by alterations to the optimal final concentrations of Profiler Plus™ reaction mix components due to the addition of untreated aliquots of these original amplification product mixtures. If true, then because the composition of these mixtures differs depending on which of the four superseded typing systems was used to produce them, these variable outcomes using the Profiler Plus™ typing system are not unexpected. However, a comparison of the compositions of the amplification product mixtures from these typing systems does not provide any clear correlation between the nature of the components and the extent of interference with Profiler Plus™ amplification and typing.

For D1S80 amplification product mixtures, it is clear that carryover of their reaction components into Profiler Plus™ PCR reaction mixes does not interfere with efficient amplification and typing since no pre-treatment with Microcon-100 microconcentrators is necessary. Also, since the stored D1S80 amplification product mixes contain approximately twice the amount of added template DNA compared to that of other superseded typing systems, it is possible that there is sufficient DNA present in the "untreated" D1S80 amplification product mix to obviate the need for Microcon-100 treatment prior to successful amplification and typing using Profiler Plus™.

For HLA-DQA1+PM and CTT triplex amplification product mixtures, the requirement for treatment with Microcon-100 microconcentrators suggests that either carryover of reaction components from their untreated amplification product mixtures does interfere with efficient amplification and typing using Profiler Plus™ and/or that the concentration of template DNA using Microcon-100 treatment is essential prior to successful amplification and typing using Profiler Plus™.

Forensic Significance

Providing relevant amplification product mixtures are still available in the laboratory under appropriate storage conditions, the application of this method enables DNA typing of an additional nine (STR) loci despite exhaustion of the original DNA extracts and corresponding source biological material. Its significance is two-fold. First, in relation to older unsolved criminal cases where DNA evidence obtained using now-superseded typing systems may not have been compelling, even in combination, this method provides the means to strengthen the evidentiary value of the DNA data as the discriminatory power can be dramatically increased. For example, the Profiler Plus™ profile of recovered DNA from a stored amplification product mix (which had been previously produced using a superseded typing system and an original crime scene sample) can be compared with a suspect's DNA profile (from a newly acquired sample typed using Profiler Plus™). Second, so that contemporary reference and crime scene samples can be compared to older samples from unsolved crime scenes using Profiler Plus™ typing, the method reported here will be useful for the many older crime scene samples, typed using now-superseded systems, which have had their original DNA extracts exhausted. This would allow, for example, links to be established between previously unrelated crime scenes.

Indeed, application of this method has resulted in the re-opening of a number of such cases for further examination in the QHSS Forensic Biology Laboratory.

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