TECHNICAL NOTE

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Sequential Multiplex Amplification: Utility in Forensic Casework with Minimal Amounts of DNA and Partially Degraded Samples

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ABSTRACT: Since its introduction, PCR has become a widely-used, routine technique in forensic laboratories. A number of PCR protocols that were developed originally are now being replaced by more powerful approaches, particularly those based on multiplex amplification of short tandem repeat (STR) loci.

One alternative form of multiplex PCR amplification, called Sequential Multiplex Amplification (SMA), was designed to amplify a single locus and then recover and reuse the remaining genomic DNA as a template for subsequent PCR. The SMA process could be repeated several times. SMA has proven to be useful in typing genomic DNA contained in stored PCR samples and analyzing samples of limited quality and/or quantity for multiple loci. The efficacy of the use of SMA for actual typing of casework samples permitted typing for a second locus 98.11% of the samples considered; 70.75% were typeable for a third locus, and 16.98% for a fourth locus.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, sequential multiplex amplification, short tandem repeats, HLA-DQA1, D1S80, HUMTH01, HUMVWA, HUMCSF1PO, HUMTPOX

Typing polymorphic loci at the DNA level has become a routine procedure in the identity testing field. DNA samples are amplified by the polymerase chain reaction (PCR) and subsequently typed; the remainder of the untyped amplified product is generally not used or discarded.

To obtain more genetic information from a sample therefore several distinct loci can be amplified in one PCR simultaneously (1,2). Lorente et al. (3,4) proposed an alternate multiplex PCR approach called sequential multiplex amplification (SMA) that could be useful in some situations, as considered in the discussion. Sequential multiplex amplification utilize the same template DNA for several sequential PCR amplifications. The DNA is amplified for a particular target sequence; after typing the locus, the remaining PCR sample is washed using a Microcon-100 filtration device; the recovered DNA then is amplified for another locus and the process can be repeated, if desired.

In this study, up to four different loci (D1S80, HUMTH01, HUMVWA, HLA-DQA1) have been amplified by SMA from an average of 3 ng of DNA recovered from forensic specimens (hairs, bloodstains, cigarette-butts, and bones). These samples had been amplified previously for only one locus (usually HLA-DQA1 or D1S80) and were maintained frozen at −40°C in the amplification tubes. The efficacy of the SMA method for typing forensic samples at multiple PCR-based loci is discussed.

Materials and Methods

For this study, SMA was performed using a number of DNA samples from evidentiary material that had been typed for only one locus (usually HLA-DQA1 or D1S80) between 1990 and 1994. The remaining PCR samples had been maintained frozen at −40°C in the amplification tubes. At the time of the original analysis, some samples could not be typed further because the DNA may have been partially degraded or because there were minimum amounts of template (less than 10 ng).

The general procedure for SMA typing was as follows (see Fig. 1):

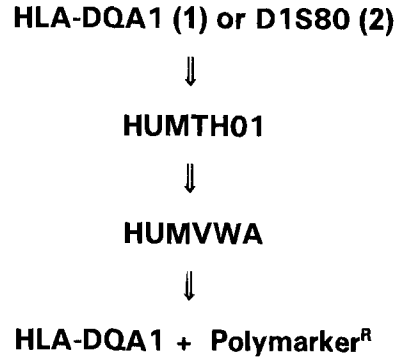

FIG. 1—*Recommended hierarchy for SMA amplification. (1) If D1S80 or HLA-DQA1 were amplified previously, then amplify the loci in the following order: HUMTH01, HUMVWA, HLA-DQA1 + Polymarker. (2) If beginning SMA from an extract in which no PCR has been carried out initially, the largest locus (D1S80) should be amplified first, then the loci should be amplified in descending order by size with HLA-DQA1 + Polymarker amplifications performed the last.*

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1. PCR samples, containing former amplification products for the D1S80 or HLA-DQA1 locus, were thawed in a refrigerator (4°C). If starting with a DNA sample (that has not been subjected to PCR previously) DNA must be extracted with standard techniques (organic or chelex) and proceed to step #2.
2. The quantity of DNA was determined with a slot-blot procedure using a human specific alphoid probe (5).
3. Amplify and type DNA, for example, for the D1S80 locus (6) using all the former amplification product remaining after quantitation.
4. Recover the genomic DNA from the remaining D1S80-PCR solution by filtration through a Microcon −100 (Amicon, Beverly, MA) (3,4).
5. Amplify and type the recovered DNA for the HUMTH01 locus (7).
6. Repeat step 4 and amplify and type for HUMVWA (8).
7. Repeat step 4 and amplify and type for HLA-DQA1 (9).

Results and Discussion

A total of 106 samples from cases submitted to the University of Granada-Laboratory of Criminalistics, between 1990 and 1994, were analyzed by SMA. Initially, because of the laboratory policy and protocol and the quantity and/or quality of the DNA, only one locus could be amplified and typed at that time (usually D1S80 or HLA-DQA1). The 106 evidentiary samples were from blood-stains (39), semen-stains (12), hairs (22), cigarette butts (15), stamps or envelopes (10), and bones (8). Since whole genomic DNA was extracted and its quantity determined a number of years ago (the amounts of DNA used in the original amplifications ranged between 2 and 10 ng), in this particular study the SMA procedure started at step #4 of the Materials & Methods described above.

Out of 106 samples, 102 could be amplified and typed after SMA for a second locus. For a third locus, positive amplification and correct typing was possible in 59 out of the 102 samples that yielded a result for the second locus. It should be noted that two out of the four samples that originally did not amplify for a second locus were typeable for the third locus, probabily because of specific degradation for this particular second locus. Further, 18 samples out of 61 of these samples were amplified and typed for an additional fourth locus, and 16 out of the 43 samples negative for the second locus, were typeable for the fourth marker (Table 1).

SMA enabled amplification of additional loci from PCR samples that had been stored up to six years after initial analysis. Therefore, a higher degree of discrimination was obtained from a small quantity of sample, than was previously possible. The power of discrimination (PD) for each locus (0.921 for the D1S80 locus, 0.933 for the HUMTH01 locus, 0.941 for the HUMVWA locus, and 0.934 for the HLA-DQA1 locus) increased to 0.99998 when all four loci are typed (10). If Polymarker loci (DP = 0.995) are included, the DP can be increased to 0.9999999 (Table 2).

When using the SMA procedure with the Perkin-Elmer Polymarker or HLA-DQA1 kits, special attention should be given to the interpretation of the stochastic control-dots (11). When a PCR sample is first amplified for the HLA-DQ1 locus and is subsequently subjected to Polymarker amplification, the HLA-DQA1 amplicons and the genomic DNA serve as template DNA. Since the "S" dot probe on the PM strip is composed of an HLA-DQA1 specific probe, the "S" dots may be more intense than expected (11), and therefore type must be done with caution.

With this procedure, it is also possible to recover template DNA from PCR samples that yielded no amplification product or typeable results initially. Such DNA from evidentiary samples that might be too degraded for a given locus, but could be amplified for some other loci with smaller sized amplicons can be generated. As described in this study, some samples that did not yield results initially for a particular locus could be typed subsequently.

The SMA approach can be compatible with any of the multiplex amplification approaches, and good results have been obtained using the Promega CTT Triplex (HUMCSF1PO, HUMTPOX, HUMTH01) after a D1S80 amplification and just before using the HLA-DQA1 kit (results not shown).

As a general rule, amplification of the loci should proceed by the following hierarchy (Fig. 1): length polymorphisms first (in descending order by size), followed by sequence polymorphisms (currently detected by sequence-specific oligonucleotide probes). The reason for this hierarchy is two-fold. First, because residual amplicons from preceding PCRs are present, it is desirable to amplify the locus with the largest fragments first and with the smallest locus last. By typing the largest length polymorphisms initially, heteroduplex bands, if presents, from smaller loci will not interfere with DNA profile interpretation. Therefore, length polymorphism loci that do not overlap in size should be employed. Second, sequence-specific oligonucleotide probe detection will not be affected by the presence of length polymorphism amplicons in subsequent amplifications.

In conclusion, SMA enables: 1) typing of several loci using only one DNA sample without requiring all the loci to be amplified under one set of PCT conditions (4); 2) retained PCR samples can be typed, particularly when then original sample was "consumed" entirely for the initial PCR; and 3) for situations where the first

TABLE 1—*Resume of the results obtained after analyzing 106 samples. Following this protocol, the probability of discrimination was increased in 104 samples.*

Total Number of Samples Amplified	Number of loci Amplified	Percent of Samples Typed
106	1	100
104	2	98.11
75	3	70.75
18	4	16.98

TABLE 2—*Probabilities of discrimination (PD) that can be reached using the SMA procedure (PD values calculated based upon a Spanish Caucasian population database (10)).*

AMPLIFIED LOCI	PD
D1S80	0.9208
HLA-DQA1	0.9343
D1S80 + HLADQA1	0.9952
D1S80 + HUMTHO1	0.9951
HLADQA1 + HUMTHO1	0.9956
D1S80 + HLADQA1 + HUMTHO1	0.9996
D1S80 + HLADQA1 + HUMTHO1 + HUMVWA	0.99998
D1S80 + HLADQA1 + HUMVWA	0.9997
D1S80 + HLADQA1 + HUMTHO1 + HUMVWA + PM®	0.9999999

analysis is negative, recovery of the template DNA for potential typing at another locus.

The SMA approach should be used sparingly. Because amplified materials are being handled, SMA should proceed with extreme caution, and laboratories conducting this type of analysis should have designated extraction and amplification areas specifically for reamplification or recovered DNA template, using designated primer sets, dNTP's and Taq, and following the same rules and caution than when performing nested-PCR or when re-amplifying allelic ladders (12).

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