C_038_PF: INVESTIGATION OF LONG PRIMER TARGET-ENRICHMENT COMBINING WITH STR TYPING FOR DEGRADED DNA ANALYSIS

Pattranit Srisuwanuntanakul,[1,*] Nathinee Panvisavas,[2] Punchapat Sojikul[3]

[1]Forensic Science Unit, Faculty of Science, Mahidol University.
[2]Department of Plant Science, Faculty of Science, Mahidol University.
[3]Department of Biotechnology, Faculty of Science, Mahidol University.
*e-mail: pattranit.sri@student.mahidol.ac.th

Abstract:

This study aimed to develop an alternative for the analysis of degraded DNA samples by long primer target-enrichment combining with short tandem repeat (STR) typing. The CSF1PO locus was selected for the investigation. This locus is one of the large STR locus and often being the first locus to be drop out from the STR profiles of degraded DNA. Analysis results by agarose gel electrophoresis showed that the 344-bp PCR product of was amplified from the artificially degraded DNA samples that were amplified by CSF1PO long primers prior to the single-locus CSF1PO-STR typing, but not those without the long primer amplification step. These suggested the promising application of long primers in the target-enrichment step prior to STR typing. Further investigation was conducted by using the automated capillary electrophoresis, the routinely used instrument in forensic DNA typing. Optimizations were carried out by reducing concentration of long primers to eliminate the non-target peaks from being excessive. Results also showed the expected CSF1PO peak was detected from the artificially degraded DNA sample that was pre-amplified using the long primers prior to STR typing, but not from the artificially degraded sample that was directly typed. Although, non-target peaks of smaller sizes were reduced, they were still present in the electropherogram. In summary, experiments demonstrated that CSF1PO long primer amplification could help to restore the dropped-out locus in STR profiles of degraded DNA samples by enriching the target locus prior to STR typing. Further optimizations in the long primer amplification step and its application to STR typing is suggested.

Introduction:

Forensic analysis of degraded DNA samples remains challenging nowadays. Forensic scientists often deal with human remains and biological specimens that undergone extreme conditions from criminal activities, natural disasters, terrorist attacks, etc. In these cases, biological specimens are directly exposed to factors such as heat, moisture, UV, DNase, etc., which accelerate degradation of the samples and their DNA unpredictably. As a result, either incomplete or null DNA profiles would be generated, causing difficulties or failure in the human identification process. Although, there are many techniques for human identification, DNA-based human identification still plays the key role [1].

Currently, DNA profiles from degraded DNA can be generated by mini-Short tandem repeat (STR) typing and analysis of single nucleotide polymorphisms (SNPs) [2]. Mini-STR typing can be used to type DNA template with smaller fragment size. Due to the smaller number of loci being analyzed, the power of discrimination would be lower

when compare with the 16-loci STR typing kit [3]. This technique improved the DNA typing results of degraded samples by reducing the length of PCR target, therefore the PCR product decreased. However, DNA typing of highly degraded biological samples with fragmented DNA sizes less than 200-bp long is still a limitation for mini-STR analysis. For SNPs analysis, the technique allows the analysis of single nucleotide polymorphisms present in shorter DNA fragments. However, it is suggested that analysis of 40-60 SNPS is recommended to equivalent the same power of discrimination for human identity testing using 16 STR-loci that is routinely used [4, 5].

Muatner, Santangelo and Corti (2017), reported the development of long primer pairs or 'superprimers' for degraded DNA analysis. These long ssDNA polynucleotides are designed to anneal closer to the target repeat sequences which would reduce the requirement of the actual length of intact DNA. Thus, allowing the analysis of smaller amplicon size variations being analyze in a similar way with mini-STRs. However, the size of the amplified product would also be large enough for the routine DNA fragment analysis by automated capillary electrophoresis [6, 7].

Because the nature of forensic biological evidence is often present in very small amount and being degraded (or low quality), the use of long primer to enrich the DNA target in degraded samples prior to the routine STR-typing protocol is proposed. In this study, the CSF1PO locus, which is categorized as a large STR fragment in the STR profile and often drop-out in the analysis of degraded DNA samples, was investigated. It is expected that the success of target enrichment by long primer amplification could restore the locus from being drop-out from the STR-profile generated by the validated STR typing kit used routinely.

Methodology:

DNA preparation

Whole blood was centrifuged at 2,500 g for 10 min to separate 3 layers of blood components; plasma, buffy coat (white blood cells and platelets) and red blood cells. The buffy coat which the source of DNA materials was extracted using QIAamp® DNA mini kit (QIAGEN, 2016). DNA concentration was estimated by spectrophotometry (Nanodrop™, USA). The DNA extract was then stored at -20 °C.

To prepare artificially degraded DNA, an aliquot of 100 µL of 5 ng/µL DNA extract was put into an ultrasonic bath. The sonication time was varied to 30, 45, 60, 75, 90, 105, and 120 min to generate different ranges of DNA fragment size (or different degree of degradation).

DNA amplification

For standard STR-typing, the DNA profile was generated by using AmpFLSTR® Identifiler® Plus PCR Amplification kit (Applied Biosystem, USA), which composed of multiplex primers for 15-STR loci and a sex determination locus (AMEL). Amplification was carried out in a total volume of 12.5 µL, containing 5 µL of the AmpFLSTR® Identifiler® Plus master mix, 2.5 µL of AmpFLSTR® Identifiler® Plus primer set, DNA template and sterile double distilled water. Amplification was performed in GeneAmp 9700 thermocycler (Applied Biosystem, USA) using the following condition; initial denaturation at 95 ºC for 11 min following with 28 cycles of denaturation at 94 ºC for 20 seconds, annealing and extension at 59 ºC for 3 min, then the final extension at 60 ºC for 30 min. (Applied Biosystem, 2015). The size of detected fragments was used for grouping the different levels of degraded sample with the manner of sonicated time and fragment sizes.

For CSF1PO single-locus typing, amplification was carried out in a total reaction volume of 25 µL, the reaction consists of 1X PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1 unit of AmpliTaq gold® DNA polymerase, 10 pmole of each forward and reverse CSF1PO-STR primers and DNA template. Primer sequences [forward primer: 5'- CCG GAG GTA AAG GTG TCT TAA AGT -3'; reversed primer: 5'- ATT TCC TGT GTC AGA CCC TGT T -3'] were obtained

from PowerPlex® 16 Primer Pairs [8]. Amplification was performed in Gene Amp 9700 thermocycler (Applied Biosystem, USA) using the following condition; initial denaturation at 95 ºC for 5 min following with 28-34 cycles of denaturation at 94 ºC for 1 min, annealing at 64 ºC for 1 min and extension at 72 ºC for 1 min, then the final extension at 60 ºC for 30 min. [9]

Amplification using the CSF1PO long primers prior to the STR-typing was carried out in a total reaction volume of 20 µL, the reaction consists of 1X PCR buffer, 1.5 mM of MgCl2, 200 µM of dNTPs, 1 unit of GoTaq® DNA polymerase, 0.5 pmole of each forward and reverse primer and 5 ng of DNA template. CSF1PO long forward primer sequence was 5'- CGG AGG TAA AGG TGT CTT AAA GTG AGA AAG AAT AAC TGC ATC TTA ACC TAT TGG GAG GTC ATT GTA AAG AGG AGA GTG ATG GGG TCA GAT TGT ACA GAG GAG GCA CTT CGT GGT GGT CAG GAG CAC ACA CTC CAG GGC AGT GTT CCA ACC TGA GTC TGC CAA GGA CTA GCA GGT TGC TAA CCA CCC TGT GTC TCA GTT T-3' and CSF1PO long reverse primer sequence was 5'- ATC TCC TGG TGC ACA CTT GGA CAG CAT TTC CTG TGT CAG ACC CTG TTC TAA GTA CTT CCT-3' [6]. Amplification was performed in Gene Amp 9700 thermocycler (Applied Biosystem, USA) using the following condition; initial denaturation at 94 ºC for 5 min following with 35 cycles of denaturation at 94 ºC for 10 seconds, annealing and extension at 72 ºC for 1 min, unless otherwise stated. Then, long primer amplified mixture was used for STR-typing according to previously described protocols.

DNA separation and detection

For DNA separation and detection by agarose gel electrophoresis, PCR products were separated in 2%(w/v) agarose/Tris-borate-EDTA (TBE) buffer. Agarose gel was prepared by weighing 1.4 g of agarose powder, then dissolved in 70 mL of 1X of TBE buffer and stained with 10 mg/mL of ethidium bromide. An aliquot of 10 µL of PCR product was mixed with 2 µL of gel-loading dye and loaded into the well. The 100-bp DNA ladder was used as DNA marker of. Separation was carried out 75 voltages for 90 min. After that, the separated DNA bands were visualized under UV light and photographed using a gel documentation system (BIORAD CO., USA). [10]

For DNA separation and detection by automated capillary electrophoresis, an aliquot of 1 µL of PCR product was mix together with 10.7 µL of hi-di formamide (Applied Biosystem, USA) and 0.3 µL of LIZ internal size standard (Applied Biosystem, USA) in an Eppendorf tube. The tube was heated at 95 °C for 5 min to separate double-stranded DNA into single-stranded. The tube was then immediately chilled on ice for 2 min before loading the samples into the sample tray of the ABI PRISM 310® Genetic Analyzer (Applied Biosystem, USA). Samples were injected into capillary by electrokinetic injection. Electrophoresis was carried out using the POP-4™ polymer (Applied Biosystem, USA), which is recommended for electrophoresing DNA under denaturing conditions. Injection time was allowed for 5 sec, and the run time for data collection was 24 min for each sample. the module and matrix files were set according to the ABI PRISM 310® Genetic Analyzer User's Manual. At the end of the run, the samples were automatically analyzed by the GeneMapper® Analysis Software. The electropherograms could then be viewed in the result control window. (Applied Biosystem, USA)

Results and Discussion:

STR typing of artificially degraded DNA

Artificially degraded DNA samples, generated by using different sonication time, were typed by AmpFLSTR® Identifiler® Plus PCR Amplification kit. STR loci and their allele size ranges of the AmpFLSTR® Identifiler® Plus PCR Amplification kit are showed in Table 1. Results in table 2 showed that full STR profile was only obtained from the 30-min sonicated DNA sample. Other sonicated samples gave partial STR profiles. Results also showed that larger STR loci dropped out before smaller ones. Allele and loci with amplified product sizes larger than approximately 300 bases were absent (or drop-out) when sonication time was 45, 60, and 75 min. Less than approximately 150 bases fragments were present when sonication time was 90 and 120 min. Thus, longer sonication time resulted in an

increase of loci drop-outs. It is also noted that the smallest fragment, which is the 106-bp AMEL locus, was amplified in all samples, implying that approximately DNA fragments of 100 bp-long were present when 120-min sonication time was applied.

When DNA samples are sonicated, the ultrasound breaks the H-bond and C-O bond in the DNA molecule in random positions, resulting in fragmented DNA of different sizes [11]. In addition, these random fragmentations of the DNA molecule could result in the loss of primer binding sites [12]. Longer sonication time would then increase the number of fragmentation event, as well as the loss of primer binding sites. Hence, no PCR product would be amplified. Table 3 summarized the sonication time and fragment size ranges of the artificially degraded DNA generated. These suggested that 3 different levels of degraded DNA were generated in this study.

Table 1. STR loci present in AmpFLSTR® Identifiler® Plus PCR Amplification kit (Applied Biosystem, USA) and their fragment size ranges.

STR locus name	Size ranges of STR fragment (bases)
AMEL	106-112
D19	92-150
D3	97-145
D8	123-175
D5	130-178
vWA	152-212
TH01	160-204
D21	138-256
D13	193-241
TPOX	209-257
FGA	196-352
D7	253-293
D16	248-296
D18	264-351
CSF1PO	301-345
D2	291-359

Table 2. The STR profiles generated from different time of sonication samples by size.

Sonicated time (min)	AMEL	D19	D3	D8	D5	vWA	TH01	D21	D13	TPOX	*FGA*	D7	D16	D18	CSF1PO	D2
0	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
30	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
45	√	√	√	√	√	–	√	×	√	–	×	×	–	×	×	×
60	√	√	√	√	–	×	√	×	×	×	×	×	×	×	×	×
75	√	√	√	–	√	–	√	×	√	–	×	×	×	×	×	×
90	√	–	×	×	×	×	×	×	×	×	×	×	×	×	×	×
105	√	×	–	×	×	×	×	×	×	×	×	×	×	×	×	×
120	√	–	–	×	×	×	×	×	×	×	×	×	×	×	×	×

√ complete locus

× locus dropout

– allele dropout

Table 3. Different time of sonication generated ranges of fragment sizes.

Sonicated time (min)	Fragment size[a] (bp)
≤ 30	> 300
45-75	150-300
≥90	< 150

[a] Fragment sizes of target product which generated the STR profile using AmpFLSTR® Identifiler® Plus PCR Amplification kit (Applied Biosystem, USA).

Investigation of CSF1PO long primers-coupled with single-locus CSF1PO locus typing

The human c-fms proto-oncogene for CSF-1 receptor gene (CSF!PO) locus is one of the core STR loci in the Combined DNA Index System (CODIS) database. Allele for this locus contain 5-16 repeat units of tetranucleotide AGAT, giving amplification product size 317-361 bp [13]. So, CSF1PO locus was selected for the investigation because the size of its amplification product is a large and reported to be drop out in the analysis of degraded DNA samples [14]. Firstly, primers were tested. Amplification using 10 pmole of CSF1PO-STR primers for single-locus analysis [9] and 5 pmole of CSF1PO long primers [6] gave a 344- and 370-bp product, respectively. However, amplification using

CSF1PO long primers according to [6] showed a smaller-size band at the lower part of the agarose gel, which may have been excess primers or shorter oligonucleotides. Further optimizations were made to eliminate this band by reducing the primer concentration from 5 to 2.5 pmole, and the thermocycling condition was changed to 2-steps amplification at 72°C. Then, these primers were used to amplify different levels of artificially degraded DNA samples. Results showed that the 344-bp PCR product of the CSF1PO-STR primers (figure 1a) was detected only form the 30-min-sonicated DNA sample (lane 3). No PCR products were detected in other lanes. For the amplification by the CSF1PO long primers, the 370-bp product was detected from 30- and 45-min sonicated DNA samples (lane 3 and 4 in figure 1b). Results suggested that long primer showed the advantage of detecting the target CSF1PO locus from a higher-level of artificially degraded DNA sample. The CSF1PO long primers was previously developed by extending the primer length from the standard primers of 20-25 bp to 60-200 bp. Binding site of the CSF1PO long primers was shifted closer to the STR region than the CSF1PO-STR primers but still generate the same large fragment size as STR typing kit [6]. These features increased the chance of target annealing and amplification.

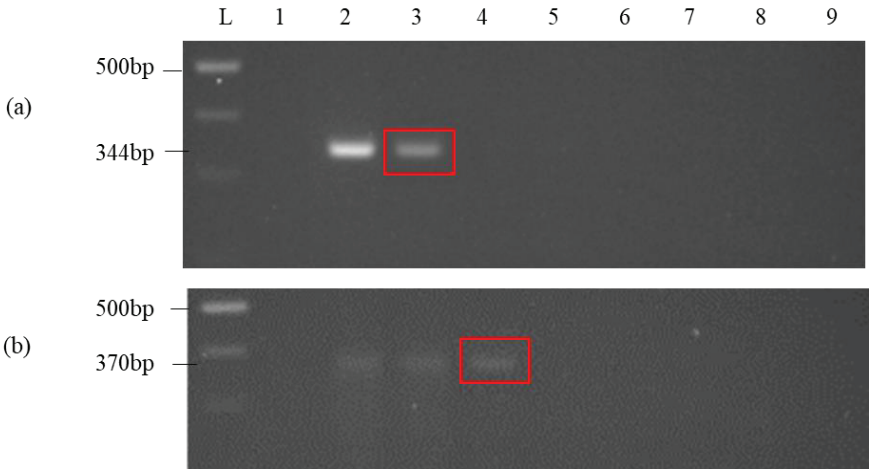

Figure 1. Amplification of artificially degraded DNA samples using (a) CSF1PO primers for single-locus analysis, and (b) CSF1PO long primers (2.5 pmole). Lane L: 100-bp DNA ladder. Lane 1: negative PCR control, lane 2: positive PCR control (non-degraded DNA sample), lane 3-9: artificially degraded DNA samples (sonication time was 30, 45, 60, 75, 90, 105, and 120 min, respectively)

Then, the CSF1PO long primer was applied for target enrichment prior to STR amplification. After artificially degraded DNA was amplified by long CSF1PO long primers, 1 µL of the amplification mix was used as template for the single-locus CSF1PO-STR amplification. Amplification results were showed in figure 2, the 344-bp PCR products were clearly detected from all artificially degraded DNA samples. The CSF1PO long primer amplification served as a target-enrichment step prior to the single-locus CSF1PO-STR typing. Intensities of the 344-bp DNA band became fainter when the DNA template was sonicated for a longer time. This may be due to a smaller number of target molecules were present in those samples being longer sonicated. The presence of an unexpected 200-bp DNA band was clearly noted throughout all lanes in figure 2. Intensities of these 200-bp DNA bands increased according to the level of DNA degradation, suggesting that these may be due to excess amount of the CSF1PO long primer in the target-enrichment step. Although the correct PCR product size could be detected from all artificially degraded DNA samples, further optimization of target-enrichment step by long primer amplification is needed to eliminate the unexpected 200-bp DNA band. Further optimization was then carried out for DNA separation and detection by

automated capillary electrophoresis (CE), which is the routine method used for STR-typing. This separation and detection platform are more sensitive than agarose gel electrophoresis.

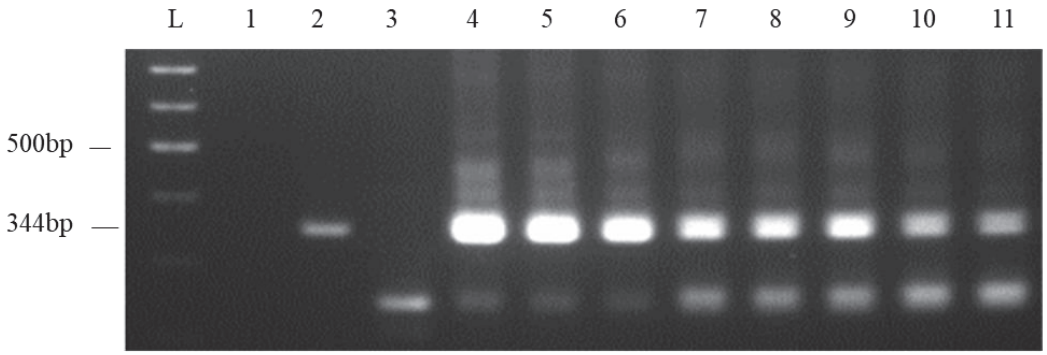

Figure 2. Amplification of artificially degraded DNA sample using CSF1PO long primers-coupled with CSF1PO-STR primers. L: 100-bp ladder. Lane 1 and 2: negative and positive PCR controls for CSF1PO-STR primers; Lane 3 and 4: negative and positive controls for CSF1PO long primer amplification followed with CSF1PO-STR primers using artificially degraded DNA samples as templates (sonication time was 30, 45, 60, 75, 90, 105, and 120 min, respectively).

Detection of amplification product CSF1PO long primers-coupled with CSF1PO single-locus typing by automated capillary electrophoresis

DNA fragment analysis by automated capillary electrophoresis is the routinely used technique in forensic laboratories for casework. Though in the previous section target enrichment by using 2.5 pmole of CSF1PO long primers and followed by the single CSF1PO-single locus STR typing using 28 cycles showed satisfying amplification results for degraded DNA samples in the agarose gel, but it still needs further optimization to eliminate those non-target DNA bands which might interfere the STR profiles if this approach is applied to enrich target locus prior the routine 16-loci STR typing.

To reduce or eliminate the non-target peaks, concentration of CSF1PO long primers for target-enrichment step was reduced from 2.5 to 1, 0.5 and 0.25 pmole. No DNA amplification using the 3 different long primer concentrations were carried out prior to CSF1PO single-locus typing. In figure 3, non-target peaks were detected in these no DNA controls electropherograms. Height of these non-target peaks reduced as the concentration of the CSF1PO long primers reduced to 1 and 0.5 pmole (Figure 3a and 3b), and no non-target peak was observed when the CSF1PO long primer concentration was 0.25 pmole (Figure 3c). Therefore, the long primer concentration of 0.25 pmole was applied for target enrichment.

Next, 0.25 pmole of CSF1PO long primers were applied for target-enrichment and followed by CSF1PO single-locus typing. As showed in figure 4, amplification products were detected from the non-degraded DNA sample, and allele 12 of the CSF1PO locus was correctly typed from the non-degraded DNA sample (figure 4a). In contrast, no amplification product was detected when tested with the 30-min-sonicated DNA sample (figure 4b). This may be due caused by too less DNA template was present for CSF1PO single-locus typing.

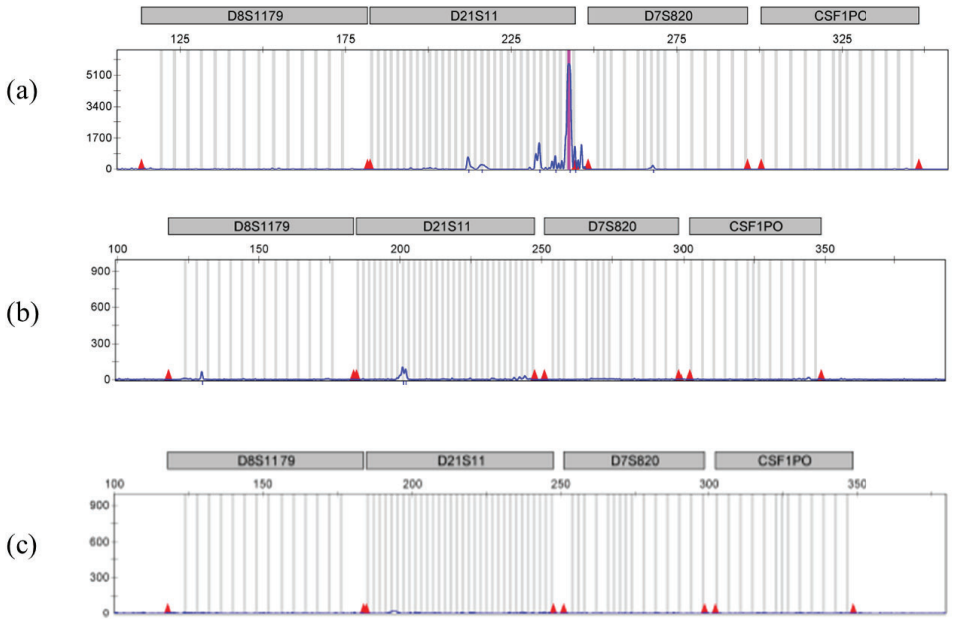

Figure 3. Electropherograms obtained from CSF1PO single-locus typing of 'no DNA' CSF1PO long primer amplification (negative controls) using 3 different long primer concentrations for target-enrichment, i.e., 1 pmole (figure a), 0.5 pmole (figure b), and 0.25 pmole (figure c). Heights of the primer-dimers (non-target peaks) reduced when primer concentrations were lower. Using 0.25 pmole CSF1PO long primer gave a flat base-line.

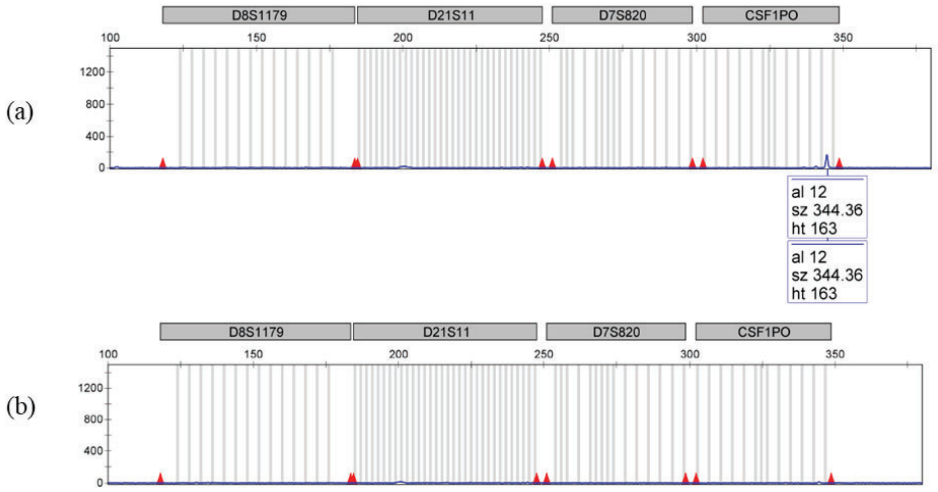

Figure 4. Electropherograms obtained from 28-cycle CSF1PO single-locus typing of target-enriched samples; non-degraded DNA sample (figure a) and 30-min sonicated DNA sample (figure b). Target-enrichment was carried out using 0.25 pmole of CSF1PO long primer prior to single-locus typing.

Then, the amplification condition for single-locus typing was increased from 28 to 34 cycles. Increase of thermocycling cycle from 28 to 34 is an alternative for low template DNA amplification [17], as the amplification product would be increased. Non-degraded and 30-min sonicated (degraded) DNA samples were typed, using 34 cycles. Results showed that the target CSF1PO product (or peak) was present in both target-enriched samples (Figure 5a and b) and without target enrichment amplification for non-degraded sample (Figure 5c). No peak was present in the electropherogram of the degraded DNA sample without the long primer target enrichment step (figure 5d). Results demonstrated that the application of CSF1PO long primer for target enrichment prior to single-locus CSF1PO-STR typing could restore the locus from drop-out.

In addition, peak heights of the amplified target product obtained from single-locus typing of long primer target-enriched DNA samples (Figure 5a and b) are higher than those obtained from single-locus typing without long primer target-enrichment step (Figure 5c and d), and higher than peaks obtained from 28-cycle amplifications. However, non-target peaks were also present in the electropherograms obtained from long primer target-enriched samples. The presence of these non-target peaks in the electropherogram can interfere the interpretation of the 16-loci STR profiles or DNA profiles from any multiplex typing.

There are alternative techniques for degraded DNA analysis such as mini-STR typing kit, SNPs, and long primer typing panel. However, laboratories additionally implementing these techniques would require more investments in chemicals (kits) and facilities. Whereas the amplification of degraded sample using long primers-coupled with STR typing in this study demonstrated the potential that this could simply generate the target product from highly degraded samples, and there's no requirement for special facilities. Although the correct amplification product could be obtained from degraded DNA analysis by using long primers-coupled with STR typing the further optimizations of the long primer amplification for target-enrichment and optimization for STR typing application is suggested.

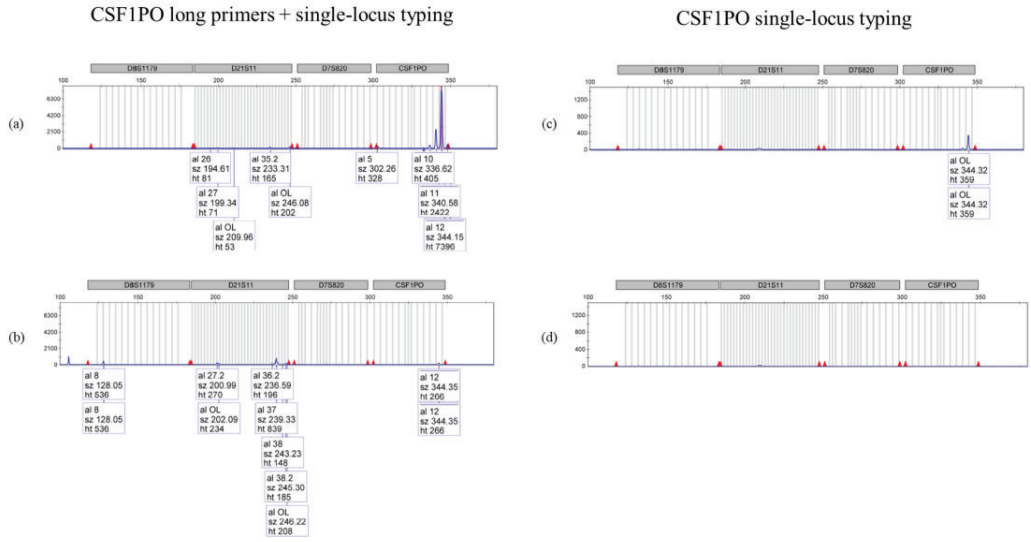

Figure 5. Electropherograms obtained from 35-cycle CSF1PO single-locus typing of target-enriched samples, i.e., non-degraded DNA sample (figure a) and 30-minute sonicated DNA sample (figure b); compared with 35-cycle CSF1PO single-locus typing (without target-enrichment) i.e., non-degraded DNA sample (figure c) and 30-minute sonicated DNA sample (figure d). Results showed that the target CSF1PO peak was present in the CSF1PO target-enriched degraded sample (figure b), but no peak was obtained from the single-locus typing without target-enrichment by long primers. However, non-target peaks were also present in the electropherogram.

Conclusion:

In this study, it has been demonstrated that target-enrichment by long primer amplification could restore the locus in degraded DNA that was drop-out from STR profile generated by a standard protocol. This provided an alternative strategy to generate DNA profiles from degraded DNA samples. However, further optimization of the long primer target-enrichment step and its application in STR typing is suggested.

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References:

1. Hughes-Stamm S, Ashton K, Daal A. Int J Leg Med. 2011;125:341–348.
2. Senge T, Madea B, Junge A. Leg Med. 2011;13:68–74.
3. Coble M, Butler J. Forensic Sci. 2005;50:43-53.
4. Butler J, Coble M, Vallone P. Forensic Sci Med Pathol. 2007;3:200-205.
5. Senge T, Madea B, Junge A. Leg Med. 2011;13:68-74.
6. Muatner M, Santangelo A, Corti Bielsa A. PLOS ONE. 2017:1-20.
7. Muatner M, Caputo M, Corach D. Forensic Sci Int Genet. 2017;6:448–450.
8. Elsner H, Lindblad E. DNA. 1989;697-701.
9. Thamnurak C, Bunakkharasawat W, Riengrojpitak S, Panvisavas N. Forensic Sci Int Genet. 2011:524-525.
10. Sambrook J, Russell DW. Molecular Cloning. 2001.
11. Krenke B, Tereba A, Anderson S. Forensic Sci. 2002;47:773-785.
12. Butler J, Shen Y, McCord B. Forensic Sci. 2003;48:1055-1064.
13. Butler J. Forensic Sci. 2006;51:253-265.
14. Vernarecci S, Ottaviani E, Agostino A. Forensic Sci Int Genet. 2015;16:77-85.
15. Balding D, Buckleton J. Forensic Sci. Int. Genet. 2009;4:1–10.