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Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci

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SUMMARY

In this study, we have evaluated the efficacy and the validity of the AmpFlSTR® SGM plus™ multiplex PCR typing system when Low Copy Number (LCN) amounts of DNA are processed. The characteristics of SGM plus profiles produced under LCN conditions were studied on the basis of heterozygote balance, between loci balance and stutter proportion based on profiles that were obtained from a variety of mock casework samples. These experiments clearly showed that LCN DNA profiles carry their own characteristic features, which must be taken into account during interpretation. Herewith, we confirmed the data of recent other studies that a comprehensive interpretation strategy is dependent upon multiple replication of the PCR using the same extract together with the proper use of extraction and amplification controls.

The limitations of LCN DNA analysis were further studied in a series of single cell PCR experiments using an amplification regime of 34 PCR cycles. The allele dropout phenomenon was demonstrated to its full extent when single cells were analysed. However, the "consensus profile" which was obtained from separate single cell PCR experiments matched the actual profile of the cell donor. Single cell PCR experiments also showed that a further increase of the number of PCR cycles did not result in enhanced sensitivity and had a highly negative effect on the balance of this multiplex PCR system which hampered correct interpretation of the profile.

Also, the potential of LCN typing in analysing mixtures of DNA was investigated. It was clearly shown that LCN typing had no advantages over 28 cycles amplification in the detection of the minor component of DNA-mixtures.

In addition to the 34 cycles PCR amplification regime, the utility of a new approach that involved re-amplification of the 28 cycle SGM plus PCR products with an extra 6 PCR cycles after the addition of fresh AmpliTaq Gold® DNA Polymerase was investigated. This approach provides the scientist with an extra typing result that enhances the reliability of the consensus profile, which is commonly retrieved from two separate 34 cycle PCR results. Furthermore, the 28 + 6 cycles approach may be used to screen LCN samples for their potential to produce a 34 PCR cycle profile. Finally and as a last resort the 28 + 6 cycles approach can be used in those cases where no further extract from the crime sample is available.

Finally, the potential of LCN typing was demonstrated in typing samples from non-probative and actual casework examples. From a high proportion of samples that failed to demonstrate SGM plus typing results using the standard protocol of 28 cycles, at least partial profiles could be obtained after LCN methods were used. For example, LCN typing was applied in a case where 10-year old samples from bones and teeth that were retrieved from a mass grave had to be identified. This study resulted in the positive identification of a number of victims by comparing the LCN DNA profiles with the profiles from putative relatives. The value of LCN DNA typing was further demonstrated in a strangulation case. The throat of the victim was sampled and only after 34 PCR cycles were we able to reveal that the evidential sample contained a distinct mixture of the victim's own DNA and the DNA of the defendant.

RÉSUMÉ

Efficacité et limites du génotypage d'échantillons d'ADN en petit nombre de copies par PCR (Polymerase Chain Reaction) multiplex de loci STR (Short Tandem Repeats)

Nous avons évalué l'efficacité et la validité du typage PCR à l'aide du kit commercial AmpF1STR®, SGM plus™, lorsque le nombre de copies d'ADN disponible est faible (Low Copy Number = LCN).

Dans les profils SGM+ en conditions LCN, l'équilibre entre allèles d'un locus, l'équilibre entre locus et la proportion d'erreurs ont été comparés à ceux d'échantillons artificiellement mélangés en proportions variées.

Ces expériences ont montré que chaque profil d'ADN LCN comporte ses propres particularités qui doivent être prises en compte lors de l'interprétation. Nous confirmons les données d'études récentes, selon lesquelles une stratégie d'interprétation générale dépend de répétitions multiples des PCR à partir du même extrait, accompagnées de contrôles d'extraction et d'amplification selon les protocoles classiques.

Les limites de l'analyse de l'ADN LCN ont aussi été étudiées dans une série d'expériences sur cellule isolée, avec un régime d'amplification de 34 cycles de PCR. Le phénomène de perte d'allèle s'est alors révélé dans toute son ampleur. Cependant le « profil consensus », obtenu à partir d'une série d'analyses PCR sur cellules isolées identiques, coïncidait avec le profil réel de la cellule donneuse. Les expériences de PCR sur cellule isolée ont également montré qu'augmenter le nombre de cycles au delà de 34 ne donnait pas une meilleure sensibilité, mais avait un effet négatif important sur l'équilibre du système PCR multiplex, au détriment d'une interprétation correcte du profil.

L'efficacité du typage LCN de mélanges D'ADN a également été étudiée. Au delà de 28 cycles d'amplification, le typage LCN ne permet pas de détecter des composants mineurs dans les mélanges.

À côté du régime d'amplification par 34 cycles de PCR, on a tenté de cerner l'utilité d'une nouvelle approche comportant une réamplification des produits de la SGM à 28 cycles à l'aide de 6 nouveaux cycles effectués après addition d'une nouvelle dose d'ampli-Taq gold® DNA polymerase. Cette approche fournit un typage supplémentaire qui augmente la fiabilité du profil consensus, par rapport à l'approche habituellement utilisée, qui comporte 2 PCR indépendantes à 34 cycles. De plus la stratégie 28 + 6 cycles peut servir à cribler des échantillons LCN, pour déterminer ceux qui sont susceptibles de fournir un profil lisible par PCR à 34 cycles. Enfin, en dernier ressort, la stratégie 28 + 6 cycles peut être mise en oeuvre dans le cas où un seul échantillon criminel est disponible.

Enfin, le potentiel du typage LCN a été démontré sur des échantillons non probatoires ou des échantillons de cas criminels réels. Dans une proportion élevée d'échantillons qui ne donnaient lieu à aucun résultat SGM Plus avec le protocole standard de 28 cycles, des profils partiels ont pu être obtenus grâce aux méthodes LCN. C'est le cas par exemple d'échantillons osseux et dentaires vieux de dix ans provenant d'une tombe collective. L'étude a permis d'identifier avec certitude un certain nombre de victimes en comparant les profils LCN à des profils de parents présumés. L'intérêt du typage LCN a également été démontré dans un cas de strangulation. L'échantillon prélevé sur la gorge de la victime a révélé clairement, mais seulement après 34 cycles, un mélange de l'ADN de la victime et de l'ADN du coupable présumé.

INTRODUCTION

The mission of the forensic DNA-scientist is to obtain a valid and highly informative DNA profile from a minimal amount of biological sample. STR analysis by multiplex PCR has put the forensic scientist in a position to derive DNA profiles from trace amounts of DNA. Using the commercially available AmpFlSTR SGM plus kit, reliable typing results can be obtained. The PCR reaction conditions for this multiplex system are consistent and robust under the conditions that are outlined in the manufacturers instructions. A complete and accurate STR profile can be obtained from 100 pg of DNA template (Cotton *et al.*, 2000). This amount of DNA is contained in the nuclei of no more than 18 diploid cells. By further increasing the sensitivity of the typing system DNA profiling from minute traces of evidence such as residues left in a fingerprint (Van Oorschot *et al.*, 1997) or palm mark on firearms or cartridges or the epithelial cells left by the assailant on a victim of strangulation (Wiegand *et al.*, 1997) comes within reach. Several methods have been described to increase the sensitivity of PCR amplification. Most methods use a preamplification step before locus specific amplification is initiated. Nested PCR (Taberlet *et al.*, 1996), whole genome amplification by degenerate oligonucleotide PCR (DOP-PCR) (Cheung *et al.*, 1996) and primer-extension preamplification (PEP) (Zhang *et al.*, 1992) utilise this principle.

An alternative and evident way to increase the sensitivity of PCR amplification is by simply extending the number of PCR cycles. By its simplicity this method is preferred to preamplification methods. An additional advantage in the forensic context is that this procedure does not depend on the transfer of the preamplified PCR products. This principle has already been applied on a range of different types of evidence that inherently contain low amounts of DNA. For example, successful DNA-typing has been reported on analysed epithelial cells transferred from an assailant to the victim after strangulation (Wiegand *et al.*, 1997). In addition, fingerprints from grips of tools (Van Hoofstat *et al.*, 1998) and hair shafts in the absence of the root (Barbaro *et al.*, 2000) were shown to be amenable to PCR-based STR-typing after hyperamplification. Increased sensitivity of the formerly heptaplex SGM typing system has been achieved by raising the number of PCR cycles from 28 to 34. Under these conditions DNA profiles could even be obtained from single (buccal) cells (Findlay *et al.*, 1998; Findlay *et al.*, 1997)

Increasing the number of cycles will unquestionably improve the PCR product yield but leads to a number of complications that are inherent to the amplification of low copy number (LCN) DNA samples; the most prominent complications are:

– Allele dropout (ADO) (Taberlet *et al.*, 1996; Gill *et al.*, 2000; Garvin *et al.*, 1998; Findlay *et al.*, 1998), a stochastic phenomenon due to the preferential amplification of one allele of a heterozygous pair during the first few PCR cycles; the other allele will be under-represented in the final PCR product and therefore undetectable. The generally observed imbalance in the peak height intensities of heterozygote loci is based on the same principle (Gill *et al.*, 2000).

– Locus dropout (LDO) occurs either when there is too little or degraded DNA or when the Taq polymerase becomes inhibited by substances that are co-extracted during DNA extraction. Unlike ADO, LDO is not a mere stochastic phenomenon but occurs predominantly at the loci with larger fragments (Sparkes *et al.*, 1996).

– Spurious alleles. LCN samples and single cells may generate DNA profiles with spurious alleles (Taberlet *et al.*, 1996; Gill *et al.*, 2000). Sporadic contamination of the DNA-extracts by cell debris containing fragments of single chromosomes or contamination of PCR reagents might lead to this phenomenon.

– Stutter may also generate artefacts. Slipped-strand mispairing during PCR in the first few cycles of STR loci results in the production of a fragment that is one repeat shorter than its template. This phenomenon is known as stutter (Meldgaard *et al.*, 1997; Walsh *et al.*, 1996; Hauge *et al.*, 1993) and under LCN conditions the stutter effect can be promoted (Gill *et al.*, 2000), thereby hampering the interpretation of the LCN DNA profile.

In earlier studies, it has already been reported that multiple testing of the same extracts increases the reliability of LCN typing. As most of the artefacts that are mentioned above are driven by stochastic fluctuation, it is not likely that these artefacts occur multiple times at the same locus in different PCR-reactions (Taberlet *et al.*, 1996; Gill *et al.*, 2000).

In this study we assessed the reliability of LCN methods and made a further inventory of the complications that adhere to these ultrasensitive DNA typing methods on forensic samples.

1. The sensitivity – with respect to the number of template DNA-molecules – of the 34 cycle PCR was assessed by typing single cells and highly diluted pristine DNA samples.

2. DNA-extracts obtained from 10-year old bones and teeth that produced no or only very partial profiles with the standard 28 cycles were subjected to 34 cycle PCR.

3. Stains from mixed bloodsamples – from a male and female donor – containing a declining amount of male blood in the presence of female blood were typed in order to assess if LCN typing leads to an increase in the success rate of typing the minor component in mixed samples. The ratio at which the male DNA could still be detected was assessed and compared with the typing results from the same experiment using 28 PCR cycles.

4. A variety of casework samples that failed to generate a (complete) profile under standard PCR conditions were subjected to 34 cycles.

5. Moreover, 28 cycle PCR-products of the same casework samples were subjected to a further 6 PCR cycles after AmpliTaq Gold® DNA Polymerase had been re-administered to the PCR reaction vessel. This approach might be relevant in those cases were too little original extract is left for (re-)analysis.

All these experiments were designed in order to validate ultrasensitive DNA typing technology and to introduce this new technology in actual forensic caseworks in The Netherlands.

MATERIALS AND METHODS

34 cycle PCR

In all our experiments, the Am*p*FlSTR® SGM Plus™ primer reagent mix (AmpFlSTR® SGM Plus™ User's manual (1999)) with AmpliTaq Gold® DNA Polymerase (Kebelmann-Betzing *et al.*, 1998) was used for PCR amplification. PCR-products were analysed on an ABI Prism 310 Genetic Analyzer (Wenz *et al.*, 1998). DNA from swabs was extracted using the Chelex extraction method (Walsh *et al.*, 1991). DNA from pulverised teeth and bones was decalcified and extracted using the phenol/chloroform method (Hochmeister *et al.*, 1991) DNA-quantification was carried out by dot blotting (Waye *et al.*, 1990).

The PCR reaction components and cycling conditions followed the manufacturers instructions (AmpFlSTR® SGM Plus™ User's manual (1999)) of the SGM plus kit. The thermal cycling conditions of the 34 cycle PCR are – except for the number of cycles (34 instead of 28) – identical to the instructions of the manufacturer. We maintained a sample volume of 10 µl in a PCR reaction volume of 25 µl.

Single cell PCR

Venous blood was obtained from a female laboratory worker and the white cell fraction was collected. The leukocytes were re-suspended in physiological saline. A small fraction of this suspension was mixed with a rich cell culture medium and placed under an inverted microscope. A single cell was picked out by micromanipulation and placed directly into a PCR-reaction tube already containing the PCR-reaction components. The negative single-cell controls underwent the same procedure with the exception that no cell was injected.

28 + 6 cycles PCR

2,5 U of AmpliTaq Gold® DNA Polymerase was added to the PCR product that had already been subjected to 28 PCR cycles. The cycling conditions for the "extra" six cycles were identical to the 28 cycles PCR. The polymerase was added in the post-PCR facility.

Mixed Stains

Mixed samples were produced by mixing liquid bloodsamples from two different donors, male and female in

varying (limiting dilution of the male component) proportions. From these mixtures stains were produced that were stored at room temperature. DNA from the mixed bloodstains was extracted by the Chelex method. 1 ng of extracted DNA was amplified under both standard (28 cycles) and LCN PCR conditions.

RESULTS

Characteristics of 34 cycle profiles from LCN samples

75 SGM plus profiles from mock case work DNA samples, which were derived from donors with a known SGM plus profile were analysed. Samples consisted of extracts from skin swabs and highly diluted DNA from buccal swabs. The extracts were subjected to 34 cycle PCR. Mixed profiles were excluded as well as profiles which contained flat top peaks. Partial profiles however were taken into account because they are frequently obtained from LCN DNA samples. The LCN profile data were subjected to three different analyses: heterozygote balance, between loci balance and stutter proportion. Additionally these data were compared with the data from Gill *et al.* (Gill *et al.*, 2000) showing the results of a collaborative exercise on the robustness and characteristics of a prototype of the SGM plus system under standard PCR conditions.

Heterozygote balance

The heterozygote balance (Hb) was obtained from: $Hb = \frac{\phi_{high}}{\phi_{low}}$ where ϕ_{low} and ϕ_{high} stand for the allelic peak areas of respectively the low and the high molecular weight alleles. Ideally the alleles of a heterozygote locus have the same allelic peak area, then the Hb would have a value of 1,0. Although there is a tendency for the low molecular weight allele to amplify preferentially, the Hb for STR loci is generally between 0.6 and 1.67 when amplified under standard PCR conditions according to the manufacturer's directives (Gill *et al.*, 2000). Table I shows the heterozygous balance of the SGM plus loci under both LCN conditions and standard PCR conditions. Under LCN conditions an increased tendency for peak asymmetry was observed. The FGA system was especially prone to heterozygote imbalance with the long FGA fragments tending to allele dropout.

Analysis of between loci balance

The between loci or inter-locus balance is obtained from the sum of the peak areas for the alleles of a specific locus (ϕL) divided by the sum total (ϕL_{tot}) of all peak areas. Ideally for each of the 10 polymorphic loci in the SGM plus system, the value should be 0.1 (Gill *et al.*, 2000).

The evaluation of the between loci balance (Table I) under LCN amplification conditions shows a major tendency for the short STR-loci to amplify preferentially. This effect appears to be inherent to the hyperamplification of LCN samples. Additionally DNA degradation and the presence of PCR inhibitors may add to the incidence of unbalanced profiles. Under standard PCR conditions and with pristine samples this phenomenon occurs to such a small extent that the effect is not manifest (data not shown).

Analysis of stutters

The proportion of stutter is taken from the peak area of the stutter band divided by the peak area of the allelic band. If a heterozygous locus showed two stutter peaks than the largest stutter proportion was taken into account. Under standard PCR conditions the median stutter proportion of the SGM plus loci is less than 8 % (Gill *et al.*, 2000). The increased number of PCR cycles had no dramatic effect on the incidence and proportion of stutter. All 10 STR loci showed median stutter values of 0.1 or less (Table I). The loci that displayed the largest amount of stutter were D3 (median stutter proportion 0.08 and maximum observed stutter 0.15) and D19 (median 0.10; maximum 0.18).

Single cell experiments

Seven different leukocytes from one (female) donor were subjected to 34 cycle PCR amplification. From the genomic input of 1 diploid cell, informative profiles could be obtained. Most loci in the SGM plus profiles exhibited peak heights between 500 and 4.000 RFU and flat top peaks were not observed. Table II shows the genotypes from the seven separately amplified and analysed cells. Both the phenomena of locus and allele dropout were clearly demonstrated. An example of a profile from a single cell demonstrating ADO is given in figure 1. Three out of the four blue loci displayed ADO. It should be noted that the single cells (cell 6 and 7) which showed the correct genotype displayed high allelic peaks (> 4000 RFU). Apart from the phenomena of ADO and LDO we observed three inconsistencies in the genotypes of the single cells (Table I). Contamination by personnel involved in the single cell typing could be ruled out based on their their genotypes. The first artefact (allele 15 of D19S433, cell # 3) is likely due to preferential amplification of the stutter of allele 16. The second artefact (cell # 4) was of special interest and is most likely due to a somatic mutation of the D8S1179 locus ($8 \Rightarrow 9$). The third artefact (cell # 5, allele 27 of D21S11) is due to spurious contamination and became evident after hampered amplification of this particular sample. According to the guidelines of Gill (Gill *et al.*, 2000) a consensus profile was retrieved from these results. The consensus profile matched the SGM plus profile from the cell donor (Table II).

The same single cell experiment was repeated with an amplification regime of 55 PCR cycles. The products of the 55 cycles PCR were in extreme unbalance with some loci producing flat top peaks interspersed with alleles that exhibited peak heights of less than 500 RFU. 55 cycles PCR had no clear effect on the occurrence of ADO and LDO (Table II). From these experiments it became evident that 34 cycles were sufficient for the

TABLE I. – Comparison of the heterozygous balance, interlocus balance and stutter proportion of the SGM plus loci under LCN and standard PCR conditions. For each characteristic, the table shows the first quartile, median and third quartile. The LCN characteristics are based on the results of 75 different samples that were analysed under LCN PCR conditions. The data relating to the standard PCR conditions of the SGM plus prototype kit were from Gill *et al.* [23] and refer to a collaborative exercise with the data of 21 forensic laboratories. Loci D3S1358 and D2S1338 were not part of this prototype.

Heterozygous balance	LCN PCR conditions (N = 75)			Standard PCR conditions		
locus	Quartile 1	Median	Quartile 3	Quartile 1	Median	Quartile 3
D3S1358	0.9	1.1	1.7	–	–	–
vWA	0.9	1.1	1.3	1.0	1.1	1.2
D16S539	0.8	1.3	1.9	1.0	1.05	1.2
D2S1338	0.8	1.4	1.9	–	–	–
D8S1179	0.9	1.2	1.6	1.0	1.05	1.2
D21S11	0.8	1.2	1.9	1.0	1.1	1.2
D18S51	1.1	1.4	1.8	1.0	1.05	1.1
D19S433	0.9	1.1	1.4	1.0	1.1	1.2
THO1	0.8	1.2	1.5	0.9	1.0	1.1
FGA	0.8	1.4	2.2	1.0	1.1	1.2
Interlocus balance						
D3S1358	0.08	0.12	0.20	–	–	–
vWA	0.11	0.16	0.18	0.10	0.12	0.15
D16S539	0.05	0.09	0.13	0.09	0.11	0.14
D2S1338	0.02	0.05	0.09	–	–	–
D8S1179	0.10	0.16	0.19	0.06	0.08	0.11
D21S11	0.06	0.10	0.12	0.08	0.09	0.10
D18S51	0.01	0.02	0.05	0.06	0.08	0.09
D19S433	0.09	0.14	0.21	0.07	0.09	0.11
THO1	0.05	0.09	0.11	0.05	0.06	0.07
FGA	0.04	0.05	0.07	0.09	0.10	0.11
Stutter						
D3S1358	0.06	0.08	0.11	–	–	–
vWA	0.06	0.07	0.09	0.05	0.06	0.06
D16S539	0.05	0.06	0.07			
D2S1338	0.07	0.08	0.09	–	–	–
D8S1179	0.05	0.06	0.06	0.04	0.05	0.06
D21S11	0.06	0.07	0.08	0.04	0.05	0.05
D18S51	0.06	0.09	0.10	0.05	0.06	0.07
D19S433	0.07	0.10	0.12	0.08	0.08	0.09
THO1	0.02	0.03	0.03	0.04	0.05	0.07
FGA	0.05	0.06	0.10	0.05	0.06	0.06

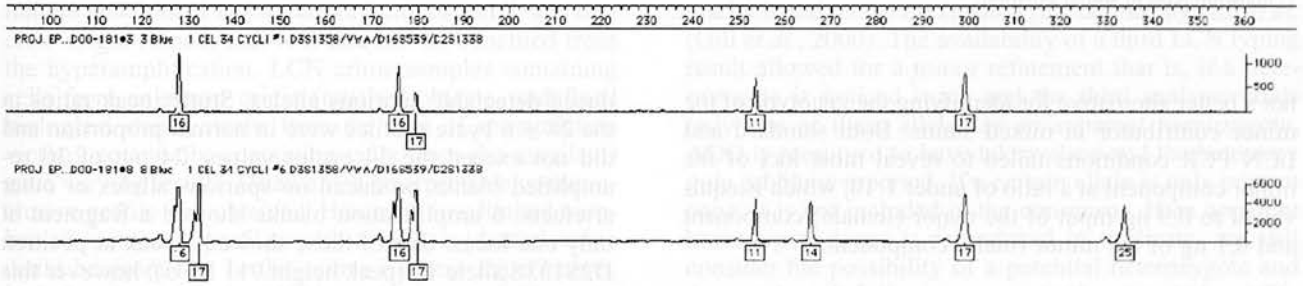

FIG. 1. – The electropherograms of the "blue" SGM plus loci (D3, vWA, D16 and D2) from two separate single cell experiments. The upper profile (cell 1, Table II) suffers from allele dropout for the loci D3, D16 and D2 and shows unbalanced peaks for the vWA locus. The second profile (cell 6) shows the correct genotype of the cell donor.

detection of the virtually minimal amount of DNA that can be encountered in forensic typing: the input which is equivalent to the quantity of DNA in one cell. A higher cycle number does not result in further sensitivity and only increases the possibility of further PCR artefacts such as high stutters, peak imbalance and the appearance of spurious alleles (Gill *et al.*, 2000).

The identification of the minor component in mixed stains using 34 cycle PCR

After genotyping the mixed bloodstains, the ratio was scored at which the minor (male) components could still be identified (Table III). From these experiments it became clear that PCR-typing under LCN conditions is

TABLE II. – SGM plus typing results from single cells.

34 PCR cycles	*D3S1358*	*vWA*	*D16S539*	*D2S1338*	*AMEL*	*D8S1179*	*D21S11*	*D18S51*	*D19S433*	*THO1*	*FGA*	
cell # 1	16	16 17	11	17	X	14	***	***	***	***	22	
cell # 2	17	16 17	11 14	***	X	8	29 33.2	17	13	9.3	***	
cell # 3	17	***	***	17	X	8	29 33.2	12 17	13 15 16	9.3	22	
cell # 4	16	16 17	14	17 25	X	9 14	29	12 17	13 16	9.3	22	
cell # 5	***	17 17	14	***	X	***	27	***	***	***	22	
cell # 6	16 17	16 17	11 14	17 25	X	8 14	29 33.2	12 17	13 16	9.3	22	
cell # 7	16 17	16 17	11 14	17 25	X	8 14	29 33.2	12 17	13 16	9.3	22	
Consensus	*16 17*	*16 17*	*11 14*	*17 25*	*XF*	*8 14*	*29 33.2*	*12 17*	*13 16*	*9.3F*	*22F*	
Reference (28 cycles)	16 17	16 17	11 14	17 25	XX	8 14	29 33.2	12 17	13 16	9.3 9.3	22 22	
34 PCR cycles												Total
ADO	4	1	3	2	0	3	1	1	1	0	0	16
LDO	1	1	1	2	0	1	2	2	2	2	1	15
55 PCR cycles												Total
ADO	4	2	0	2	0	2	2	5	5	0	0	22
LDO	0	0	2	1	2	1	1	1	0	1	1	10

Table II shows the SGM plus typing results from seven single leukocytes from the same donor that were successively amplified by 34 PCR cycles. It also shows the typing results from the reference sample and the rate of ADO and LDO under the 34 cycle and 55 (individual data not shown) cycle amplification regime.
In the consensus profile result, a heterozygote is reported in case both alleles are observed at least twice. If the different typing results hold one allele, an "F" designation is given in the consensus to denote the possibility of allele dropout.

TABLE III. – The inverse of the ratio at which the minor (male) component can still be reliably scored in a mixture of blood from a female and a male blood donor. Mixtures were tested in the following ratio's (1:1; 1:2; 1:3; 1:4; 1:6; 1:8; 1:10; 1:12; 1:16, 1:20; 1:50 and 1:100). 1 ng extracted DNA was added to the PCR mix. For example, the 1:10 mix contains 0.9 ng of the major (female) and 0.1 ng of the minor (male) component.

Locus	28 cycles	34 cycles	Locus	28 cycles	34 cycles	Locus	28 cycles	34 cycles
D3	6*	6*	Amel	12	10	D19	6	10
vWA	8	10	D8	8	8	THO	6	6
D16	10	8	D21	8	12	FGA	10	10
D2	8	8	D18	6	8			

*: male allele(s) in stutter position.

not a better alternative for identifying the genotype of the minor contributor in mixed stains. Both standard and LCN PCR conditions failed to reveal most loci of the minor component at a ratio of under 1:10, which is equivalent to 0,9 ng input of the major (female) component and 0,1 ng of the minor (male) component.

28 + 6 cycles

PCR products from 30 positive controls (1 ng DNA input) and 30 amplification blanks which were stored either at 4° C or – 2° C over a period ranging from one week to 6 months were subjected to a further 6 PCR cycles after the addition of 2.5 U AmpliTaq Gold. An improved PCR product yield was observed for all positive controls. Different storage time and temperature of the 28 cycle PCR-products had no visible effect on the quality of the profiles that were obtained after 6 extra PCR cycles. None of the positive control samples produced detectable spurious alleles. Stutter peak ratios in the 28 + 6 cycle profiles were in normal proportion and did not exceed the 34 cycles values. 24 out of 30 re-amplified blanks produced no spurious alleles or other artefacts. 6 amplification blanks showed a fragment at only one locus. One of these showed a peak at position D2S1338 allele 17 (peak height 911 RFU); however this allele had already been visible at 28 cycles (height 57 RFU). Three blanks showed spurious alleles in the amelogenin locus (X, heights 715, 527 and 230 RFU). One blank showed an allele in THO1 region (allele 8, height 808). One blank produced two peaks in the vWA region (allele 16, height 615 RFU and allele 18, height 1171 RFU).

The experiments showed that the profiles of 28 + 6 PCR cycles are consistent with the profiles of 34 cycles using the same extracts and DNA input. This means that if a certain DNA extract generates no profile after 28 + 6 cycles it will also fail to demonstrate a profile after

34 cycles. Moreover, if a partial profile is obtained after 28 + 6 cycles then a partial profile can likewise be expected after 34 cycles. However the loci which suffer from ADO and LDO may fluctuate.

Casework

The 34 respectively 28 + 6 cycle PCR protocols were applied to hundreds of actual casework samples of miscellaneous nature and the high sensitivity undoubtedly improved the success rates of DNA profiling on evidence types that previously showed unsatisfactory results using standard PCR conditions. One of the potentials of the LCN methods appeared once DNA profiles were generated from old human remains. Samples derived from bones or teeth often contain little DNA and/or much inhibiting substances. The standard PCR conditions applied to the SGM plus kit are not always able to generate (complete) DNA profiles from these types of evidence. In most cases both LCN protocols were able to generate complete DNA profiles despite the complications which adhere to hyperamplification. In order to obtain a complete profile, it sometimes appeared necessary to dilute the extract in order to gain a better ratio between the DNA and the inhibiting substances. In a major identification case 10-year old bones and teeth from victims that were retrieved from a mass grave had to be identified. Using standard PCR conditions SGM plus profiles could be generated in about half of the cases. The remaining cases were re-typed under LCN conditions, every single one resulting in profiles that could be used for comparison with the DNA profiles from putative first degree family members. The possibility of the occurrence of ADO in the profiles of the bone extracts was taken into account when comparisons were made with the data containing the profiles from the putative family members.

Likewise, the profiling of biological samples with minute amounts of DNA extract from a specific and discrete origin (blood, hair-root and saliva) benefited from the hyperamplification. LCN crime samples containing cells from unknown origin (touched objects, undefined biological stains, swabs from the skin of strangulation victims potentially containing cells from the assailant) gave varying results. Often a non-interpretable or inconclusive profile was obtained. However, in a limited number of instances, profiles with a high evidential value could be produced. In the course of these experiments, it was noticed that the template efficiency of the genomic template DNA could be enhanced through ultrafiltration of the DNA extract. In an actual crime case concerning the sexually related murder of a young girl and in which strangulation was the possible cause of death, a large (over 50) number of internal and external samples were retrieved from the body of the victim. All critical investigations were carried out before the autopsy at the scene of the crime by a team of forensic scientists and under stringent precautions that assured minimal risk of contamination by personnel attending the scene. All samples taken from the victim were Chelex extracted and subjected to both standard PCR and 34 cycle PCR. Of all samples that were subjected to DNA analysis only the samples that were retrieved from the neck of the victim were consequential. Under 34 cycle conditions it was revealed that this sample alone contained a complete but mixed profile containing all the alleles of the victim and the profile of an at that time unidentified male. In a later stage the authorities provided the laboratory with a reference sample of a suspect and it was shown that the mixed DNA sample from the neck of the victim was consistent with a mixture of DNA from both the victim and this suspect. In the course of further experiments we were able to obtain profiles from a large variety of exhibits such as spectacles, the brim of caps, strangulation cords, telogenic hairs, knife handles, tie wraps, envelopes, surgical gloves, butt-plates from (hand)guns, cartridges, etc.

LCN DNA-amplification was also applied to the reinvestigation of items that were related to unsolved crimes from the past ("cold cases"). In a number of cases we were able to retrieve DNA profiles from items that failed to produce a DNA profile during the earlier investigations.

During the evaluation of the profiling results of LCN case work samples, it became clear that the potential of a particular extract to generate a profile under LCN conditions depends mainly on trial and error and cannot be predicted from previous test results (DNA yield and standard 28 cycle PCR profile). Yet, the result of the 28 + 6 cycle PCR provides a clear indication on the outcome of the 34 cycle PCR, if the same amount of template is used. An additional advantage of the 28 + 6 strategy is that extra typing results are gained without unnecessary consumption of the precious extract. This extra typing result assists in the interpretation of the profiles and eases the derivation of the consensus profile from all LCN typing results (28 + 6 respectively 34 cycles in duplicate). The principles of our guidelines are primarily based on the duplication rule defined by Gill *et al.* (Gill *et al.*, 2000). The availability of a third LCN typing result allowed for a minor refinement that is, if a heterozygote is noticed twice and the third analysis holds only one of these alleles as an apparent homozygote, ADO is presumed to have taken place and the heterozygote profile is reported. If a certain allele is only present once, it is not included in the consensus. If an apparent homozygote locus is reproduced in triplicate, we still consider the possibility of a potential heterozygote and the match probability is conservatively estimated as $1/2f_a$, where f_a is frequency of the observed allele.

DISCUSSION

DNA typing from crime related biological samples that contain very few nucleated cells is becoming an important new application in the forensic field. In this study hyperamplification of LCN DNA samples has proven to be an efficient and reliable method when DNA profiles have to be generated from samples that contain

minute amounts of genomic DNA. Hyperamplification resulted in a limited increase in the proportion of the stutter bands when compared with genotyping under standard conditions and the stutter proportion boundary (in terms of peak height) had to be adjusted from a level of 10 % to a level of 15 % to minimise the risk of scoring stutters as true alleles. The interlocus balance under LCN conditions showed increased asymmetry with notable preferential amplification of the short STR loci and the long fragments tending to drop out. This effect becomes even more extreme when degraded samples are amplified under LCN conditions and partial profiles that contain flattop peaks in the low molecular weight region and complete drop out in the high molecular weight region are occasionally observed. The most striking feature and of great concern to the forensic application of LCN DNA typing is the phenomenon of ADO. Furthermore, this study has patently confirmed the occurrence of amplification artefacts when the genomes from single cells were subjected to hyperamplification. In the same experiment it was shown that the correct genotype of the cell donor could only be obtained when multiple cells are subjected to the same analysis. This multiple tube procedure still represents the most comprehensive and reliable approach for obtaining reliable typing results when single cells or forensic samples with minute DNA quantities have to be analysed. Gill *et al.* (Gill *et al.*, 2000) have provided the forensic community with an interpretation strategy which involves duplicate amplification of the forensic sample. A consensus profile is subsequently generated from the compiled results. This duplication rule was expanded by the incorporation of the results of one extra analysis: the 28 + 6 cycle genotype. This extra typing result which is obtained from the standard 28 cycle PCR product does not consume anymore of DNA-extract but facilitates the derivation of a correct consensus profile.

As a last resort the use of the 28 + 6 cycle analysis is worth considering when no original DNA-extract is available for LCN analysis and some 28 cycle PCR product is available. Interpretation however should be performed with great care and conservatism.

Only very recently Whitaker (Whitaker *et al.*, 2001) has shown that there is a correlation between the rate of ADO and the peak intensities of the observed alleles. The recorded incidence of allele dropout was zero when the peak area of the single allele of an apparent homozygote was > 10.000 RFU. Likewise, in the course of this study, a low incidence of ADO was observed when the peak intensity of the manifest homozygote allele was high. As an example the phenomenon was demonstrated in our single cell experiments (Fig. 1). However, occasionally LDO has been observed in the presence of high and even flattop peaks of the amplified fragments from the other loci. For this reason and for reasons of conservatory, care should be taken when peak intensities are included in the interpretation guidelines of LCN profiles.

Amplification of DNA under LCN conditions holds promising new applications in the forensic context. One foreseeable application is the typing of a small number of haploid sperm cells or diploid cells that have been separated out from suspended cells from mixed cell stains by the use of flow cytometry. This approach will ease the often cumbersome and labour intensive interpretation of profiles from mixtures. The LCN typing system has proved its merit in DNA identification of decomposed human remains. We have shown that reliable typing results were obtained from 10 year old bones. Until now, mt-DNA typing was considered to be the most sensitive system and the only possibility when the authorities demand the DNA identification of old bone samples. Due to the higher speed of analysis and the unsurpassed evidential value that is obtained through multiplex STR typing it can be foreseen that LCN typing systems will supersede mt-DNA typing and improve the identification of skeletal remains.

The new methodology requires a completely different approach when samples are collected from exhibits, scenes of crime or *corpora delicti*. Due to the diversity of evidential samples that have the potential to contain minute amounts of DNA evidence from which a profile can be generated, the forensic scientist and other scene of crime personnel must have a clear understanding of the unprecedented possibilities of this new technology. The strategy of the investigators at the crime scene must amalgamate the new possibilities of LCN profiling. Furthermore, the application of the LCN DNA technology can be of major importance when the authorities demand the review and reinvestigation of unsolved crime cases from the past.

Finally, due to the robust and conservative methods that allow for the derivation of the consensus profiles, the evidential value of LCN DNA-profiles is decreased compared to profiles that were obtained through conventional typing. However, a careful and conservative approach will prevent the scientist from mistypings or overestimation of the evidence.

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