Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics xxx (2008) xxx–xxx
www.elsevier.com/locate/fsig

Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ''low copy number'' (LCN) method for analysis of trace forensic DNA samples

Luke Forster, Jim Thomson *, Stefan Kutranov

LGC Forensics, Queens Road, Teddington, Middlesex TW11 0LY, UK

Received 1 February 2008; received in revised form 28 March 2008; accepted 9 April 2008

Abstract

The investigation of samples with low amounts of template DNA remains at the forefront of forensic DNA research and technology as it becomes increasingly important to gain DNA profile information from exceedingly trace levels of DNA. Previous studies have demonstrated that it is possible to obtain short tandem repeat (STR) profiles from <100 pg of template DNA by increasing the number of amplification cycles from 28 to 34, a modification often referred to as ''low copy number'' or LCN analysis. In this study, we have optimised post-PCR purification techniques applied after only 28 cycles of PCR, as well as using modified capillary electrophoresis injection conditions and have investigated the progressive application of these enhanced approaches. This paper reviews the characteristics of the profiles obtained by these methods compared with those obtained on the same samples after 34-cycle PCR. We observed comparable sensitivity to 34-cycle PCR in terms of the number of profiles with evidence of DNA and the number of allelic peaks per profile and we noted improved peak height and area magnitude with some sample types. Certain parameters reported to be adversely affected in 34-cycle LCN investigations, such as non-donor allele peaks and increased stutter peak ratio, were reduced by this approach. There are a number of advantages for trace samples in progressing from the standard 28-cycle process to the post-PCR processing method as compared to 34-cycle PCR method, including reduced sample consumption, reduced number of PCR amplifications required, and a staged approach to sample processing and profile interpretation.

Keywords: Short tandem repeats; STRs; Low copy number; LCN; Trace DNA; Capillary electrophoresis; Post-PCR purification

1. Introduction

This study looks at the effects that post-PCR modifications can have on short tandem repeat (STR) profile intensity, sensitivity and quality and, unlike other studies, compares these directly with the alternative 34-cycle LCN PCR method for trace DNA samples.

Low copy number (LCN) DNA testing using 34 PCR cycles is a common method of examining samples with less than 100 pg of template DNA [1–3], which often do not produce suitable profiles under standard 28-cycle PCR processing conditions (as recommended by Applied Biosystems for the AmpF*l*STR® SGM Plus® PCR amplification kit used in this study). Successful STR typing has been demonstrated using this technique [1,2] including successful STR results generated from a single buccal cell [4]. LCN 34-cycle PCR is reported to have been used over 21,000 times in forensic investigations within the UK [5]. Successful reported cases in the UK, where LCN typing has been used, included the capture of the Yorkshire Ripper hoaxer known as ''Wearside Jack'' [6] and the cold-case review of the 1983 murder of Jacqueline Poole [7]. However, the technique has come under close scrutiny following the failure to convict Sean Hoey on charges relating to the Omagh bombings [5,8].

LCN analysis is not without its drawbacks. These include larger stutter peaks, allele drop-out, heterozygote imbalance and the occurrence of unexpected allelic peaks. Methods for the processing of samples and approaches to interpretation of LCN profiles have been developed [1,2] to deal with these difficulties.

* Corresponding author. Tel.: +44 20 8943 7638; fax: +44 20 8943 7657.
E-mail address: jim.thomson@lgc.co.uk (J. Thomson).

1872-4973/$ – see front matter © 2008 LGC. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.fsigen.2008.04.005

Budowle et al. [9] suggested alternative approaches to 34-cycle LCN PCR for low template profiling. These were (1) reducing the PCR volume; (2) filtration of the amplicons to remove ions that compete with DNA when injected to the capillary; (3) use of formamide with a lower conductivity; (4) adding more amplified product to the denaturant formamide; and (5) increasing the injection time. These proposed modifications have been evaluated in various combinations, with varying degrees of success [10,11]. These studies did not report the increases in sensitivity demonstrated here, or include back-to-back comparisons of the results against those using 34-cycle PCR.

This paper examines the effects of combining steps (2) and (4) together, as well as (2), (4) and (5), as proposed by Budowle, and increasing the injection voltage. The parameters used within this paper were optimised within our laboratories and offered the best balance between concentration, sample consumption and consistent profile quality. These results are compared to both the standard 28-cycle PCR and 34-cycle PCR.

Specifically we have used the Qiagen MinElute® column to both clean-up and concentrate the AmpF*l*STR SGM Plus PCR product, which was amplified at the manufacturer's recommended volume of 50 μl, which is at least twice the volume used in other LCN studies [10,11], allowing for a subsequently greater concentration of product. An increased volume of the product was then added to the Hi-Di Formamide mix increasing the amount of product injected into the capillary. Lastly, the injection time and voltage was increased. All these factors together greatly increase the amount of amplified DNA injected into the capillary, compared to untreated post-PCR reaction mix.

This approach of using post-PCR enhancement methods to increase signal from trace DNA samples has a number of advantages over the use of additional PCR cycles. By using the same 28-cycle product there is reduced sample consumption compared with 34-cycle LCN analysis, as a new PCR reaction does not need to be performed (at least for the first of the two duplicate analyses described here). The staged approach to the method also means that it can be used on samples over a broader range of DNA content than 34-cycle LCN, and once a suitable profile is obtained the next stage does not need to be applied.

2. Materials and methods

2.1. Item and sample preparation

Sample types were chosen to represent the types of evidential material most commonly required to be processed through high sensitivity methods. The items were created in an attempt to closely simulate casework scenarios, in contrast to diluting concentrated, high quality DNA extracts. A total of 36 mock evidential items were prepared so as to mimic trace input DNA conditions. All donors were consenting volunteers with known SGM Plus STR profiles. The items were prepared as follows: **Touched Items (4 samples)**—donors washed their hands with soap then rubbed their hands together for 2 min to loosen epidermal cells. Each donor touched a clean, sterilised glass microscope slide (Mensal Glaser, Germany) with any two fingertips for 10 s. Cells were recovered from the glass surface by wiping the touched area with sterile 5 mm × 5 mm grade 1 11 μ filter paper (Whatman, UK) squares moistened with water followed by 5 mm × 5 mm grade 1 11 μ dry filter paper squares. **Trace Semen (8 samples)**—5 μl of fresh semen was pipetted onto a sterilised glass slide, creating a spot with ∼5 mm diameter. Each spot was dried separately on a hotplate. Once dry the slide was passed through a Bunsen flame to fix the stain, covered with Haemotoxylin (Fisher Scientific, UK) for 2 min and then rinsed with sterile distilled water. The semen spot was then covered with Eosin (VWR International Ltd., UK) for 30 s and washed with distilled water. Each slide was then dried by placing it back on the hotplate, and covered with Xam (BDH Laboratory Supplies, UK) and a cover slip and was examined at 400× magnification to score the number of spermatozoa observed and select samples likely to give low yields of DNA. The slides were then soaked in Xylene (Fisher Scientific, UK) for 24 h to remove the cover slips and the stain was then scraped into a 1.5 ml tube using a sterile scalpel. Xylene was then added to the scrapings, which was then allowed to evaporate off inside a laminar flow cabinet. No untreated semen was used. **Saliva (4 samples)**—One donor licked their lips and lightly kissed the back of a second donor's hand. Cells were recovered from the kissed areas by swabbing the kissed area with a dry swab (Sterilin, UK); **Worn Gloves (4 samples)**—donors washed their hands with soap then rubbed their hands together for 2 min to loosen epidermal cells. Donors then put on a new cotton glove (Fisher Scientific, UK) and opened and closed their fists 5 times before removing the glove. Cells were recovered from the inside of each glove by turning the gloves inside out and then repeatedly placing tape (Sellotape, UK) onto the glove. The recovered tape was cut into strips and placed into 1.5 ml tube; **Grabbed Clothing (4 samples)**—a donor grabbed the waist area of a newly laundered set of laboratory scrubs worn by another donor. Cells were recovered from the grabbed area by taping as above; **Weak Blood (4 samples)**—fresh blood spots of <0.5 mm diameter were dotted onto glass slides using the tip of a hypodermic needle. The spots were allowed to dry for 15 min and cells were recovered by wiping the blood spot with sterilised wet and dry 2.5 mm squares of filter paper (Whatman, UK); **Shed hairs (4 samples)**—shed telogen hairs with no visible follicle or sheath material were collected from donors. One hair (0.5 cm from the root end) was used per item; **Heat treated samples (4 samples)**—10 μl of fresh saliva was incubated at 36 °C for approximately 60 h, conditions likely to cause significant degradation of the DNA. 1 μl of saliva was then pipetted onto four pieces of clean cloth; an item was prepared from each of the areas containing the saliva.

All items were examined and prepared for DNA isolation by trained forensic examiners.

2.2. DNA isolation

DNA from all samples except semen samples was isolated using the Qiagen QIAamp® DNA Micro Extraction kit (Qiagen

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ''low copy number'' (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

Ltd., UK) in accordance with the manufacturer's instructions with a final volume of 105 μl. Semen samples were extracted by addition of Chelex® 100 resin (5%, w/v) (Bio-Rad Laboratories, USA), Proteinase K (10 mg/ml) (USB, USA) and DTT (15% w/v) (Aldrich, UK) to the recovered cells and incubating at 55 °C for 2 h; then at 100 °C for 8 min and then centrifuging at 12,000 rpm for 3 min. The extract was further purified using a Microcon® YM100 (Millipore, UK) Centrifugal Filter Unit the DNA was resuspended in 105 μl of Tissue Culture Water (Sigma, UK).

2.3. Quantification of DNA

Extracted DNA was quantified using the Quantifiler® Human DNA Quantification kit (Applied Biosystems, UK), according to the manufacturer's protocols on a 7500 Fast Real Time PCR System (Applied Biosystems, UK).

2.4. 28-cycle and 34-cycle SGM Plus PCR amplification

Extracted DNA was amplified using the AMPF*l*STR SGM Plus (SGM+) kit (Applied Biosystems, UK), which simultaneously amplifies ten tetranucleotide STR loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA) and the amelogenin locus. Each amplification was carried out in a 50 μl PCR volume (19.1 μl AMPF*l*STR PCR Reaction Mix, 10.0 μl AMPF*l*STR SGM+ Primer Set, 0.9 μl (4.5U) of AmpliTaq Gold DNA Polymerase, 20 ul DNA template). Cycling parameters were 95 °C for 11 min followed by either 28 cycles or 34 cycles (94 °C, 1 min; 59 °C, 1 min; 72 °C 1 min); 60 °C for 45 min. Each DNA extract was amplified four times, twice under the 28-cycle conditions (used for **conditions A, B and C**—see Section 2.5 and Table 1) and twice under the 34-cycle conditions (**condition D**, Table 1).

2.5. Electrophoresis of PCR product prior to post-PCR purification and concentration

Conditions A and D: Electrophoresis was performed on a 3130-XL Genetic Analyser (Applied Biosystems, UK) using POP-4 polymer (Applied Biosystems, UK) on a 36 cm capillary array. 1.0 μl of PCR product was mixed with 8.85 μl of Hi-Di Formamide (Applied Biosystems, UK) and 0.15 μl of GeneScan®-500ROX® (Applied Biosystems, UK). Samples were run according to the default run module, HIDFragmentAnalysis_POP4 set to dye set F (JOE, 5-FAM, NED, ROX). This incorporates an initial injection for 10 s at 3 kV.

2.6. Post-PCR purification and concentration of 28-cycle SGM+PCR product following initial electrophoresis

The remaining 49 μl of PCR product from each sample amplified under the 28-cycle conditions were subsequently purified and concentrated with the Qiagen MinElute PCR purification kit (Qiagen Ltd., UK) according to the manufacturer's instructions. Cleaned PCR product was eluted in 10 μl of the included Elution Buffer resulting in an approximate assumed 5× concentration of the PCR product.

2.7. Electrophoresis of PCR product following post-PCR purification and concentration

Following the post-PCR purification and concentration, each 28-cycle PCR was analysed under two different electrophoresis conditions.

Condition B: Samples were electrophoresed on the 3130-XL Genetic Analyser according to 2.5, except that 2.0 μl of PCR product was prepared with 7.85 μl of Hi-Di Formamide and 0.15 μl of GeneScan-500ROX. This is theoretically 10 times the amount of DNA added to the loading mix as in condition A. This is the equivalent of (2) and (4) proposed by Budowle et al. [9].

Condition C: As for condition B but with the injection conditions set to a 30 s injection time and a 4 kV injection voltage. This is the equivalent of (2), (4) and (5) proposed by Budowle et al. [9] as well as an increase in injection voltage. Table 1 lists a summary of conditions A, B, C and D.

2.8. Data analysis

Sample data from the 3130-XL Genetic Analyser was analysed using GeneMapper® ID Software v3.2 (Applied Biosystems, UK).

2.9. Interpretation

Interpretation of STR profiles was performed by trained forensic practitioners with no prior knowledge of the donor profiles. Donor and non-donor peaks were assigned following interpretation.

2.10. Prevention of contamination

During all pre-PCR steps of the sample processing (item preparation, item examination, DNA isolation, DNA quantification, and PCR set-up) stringent precautions against

Table 1
Shows a summary of the differences between conditions A, B, C and D

Condition	Number of PCR cycles	Volume of sample loaded onto 3130 (μl)	Post-PCR clean-up	Injection time (s)	Injection voltage (kV)
A	28	1	None	10	3
B	28	2	MinElute	10	3
C	28	2	MinElute	30	4
D	34	1	None	10	3

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

contamination of the sample by the analyst or other DNA sources were taken. Personal protective equipment comprising full scene suits, masks and elbow length gloves were worn. Two pairs of gloves were used during all processes. Outer gloves were wiped with 10% Microsol 3+ (Anachem, UK) followed by bactericidal, disinfectant alcohol wipes (Premier, UK) and were changed frequently.

Item examination areas were covered with Benchkote (Whatman, UK) and cleaned with 10% Microsol 3+ solution before and after the examination of each item. The lab areas and all lab equipment were cleaned with 10% Microsol 3+ solution. All consumables were autoclaved and/or UV treated.

Laboratory rooms for DNA isolation, quantitation, PCR and electrophoresis were strictly separated. To detect any possible contamination, ''environmental'' controls were prepared by swabbing the bench where the items were examined, the laminar flow DNA isolation cabinet and the laminar flow PCR set-up cabinet prior to use. Negative controls were included at DNA isolation, Quantitation, PCR and post-PCR clean-up. PCRs were set-up four times from each DNA extract; two duplicates being amplified at 28 and 34 cycles each. Each set of replicates were set-up in separate PCR batches separated by time to further reduce the chance of single contamination events affecting multiple replicates.

3. Results

3.1. Quantification data

Four samples had measured DNA concentrations indicating that >100 pg of DNA would be added to the PCR. The remaining 32 samples had DNA concentrations which would allow the addition of <100 pg of template DNA, confirming that these samples were suitable trace samples. The Internal Positive Control of the kit did not show any inhibition.

3.2. Profile improvements

Data from all peaks over 25 relative fluorescence units (rfu) in height were collected and compared for each of the conditions A, B, C and D tested. Four samples produced product that when run under conditions C and D produced profiles too strong for the 3130-XL Genetic Analyser detection system (and so exhibiting ''off-scale'' data) in at least one replicate under at least one condition. The sample types of these samples were hair (1) and grabbed clothing (3). Under condition A, the hair sample produced a full profile, while the grabbed clothing samples produced partial profiles. These samples also had the four highest measured DNA concentrations in the study (Table 2) which indicated that between 126 and 778 pg of template were added to the PCRs. On the basis that these did not, therefore, represent ''trace'' samples appropriate for these methods, together with the difficulty in quantitatively comparing ''off-scale'' peak data with the data from the other samples, these 4 samples were removed from the data set, leaving 32 samples carried through the full comparative analysis.

Table 3
Shows the number of sample profiles with ≥1 peak in the profile (n), with no evidence of DNA (NP), the mean number of peaks per profile for those profiles containing peaks and the mean peak heights and areas of the peaks present for conditions A, B, C and D

Condition	n	NP	Mean		
			No. of peaks	Peak height	Peak area
A	14	50	6	39	378
B	48	16	10	184	1906
C	52	12	11	768	8774
D	53	11	11	580	5946

Table 3 summarises the number and magnitude of allele peaks observed under the 4 conditions. Conditions B, C and D gave a higher proportion of sample profiles with at least one peak present, compared to condition A. A chi-square test for independence comparing all conditions gave a p-value not detectably different from zero. However, when conditions B, C and D only are considered, the p-value is 0.51 indicating that the difference is entirely with A.

Conditions B, C and D all had more peaks per profile compared to A (Table 3). Using a generalised linear model with Poisson distributed errors we found that conditions B, C and D produced significantly more peaks per profile than condition A (p-values of <2E-16). Conditions C and D were not significantly different to each other.

The variation in the number of peaks across loci was tested and compared for all the experimental conditions (data not shown). A chi-square test for independence produced a p-value of 0.0012 when all conditions were tested. When conditions B, C and D only were considered, the same test gave a p-value of 0.92. This shows that the difference in the number of peaks across loci is significant and is entirely with A.

Table 2
Samples that were too strongly amplified under conditions C and D (''OS''=off-scale, ''–''=PCR suitable for processing under the relevant conditions)

Sample	DNA concentration (ng/μl)	A		B		C		D	
		Replicate		Replicate		Replicate		Replicate	
		1	2	1	2	1	2	1	2
Hair 1	0.0371	–	–	–	–	OS	OS	OS	OS
Grabbed clothing 2	0.0063	–	–	–	–	OS	–	–	–
Grabbed clothing 3	0.0386	–	–	–	–	OS	OS	OS	OS
Grabbed clothing 4	0.0189	–	–	–	–	OS	–	–	–

The off-scale peaks for the grabbed clothing were from the ''grabber''.

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ''low copy number'' (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

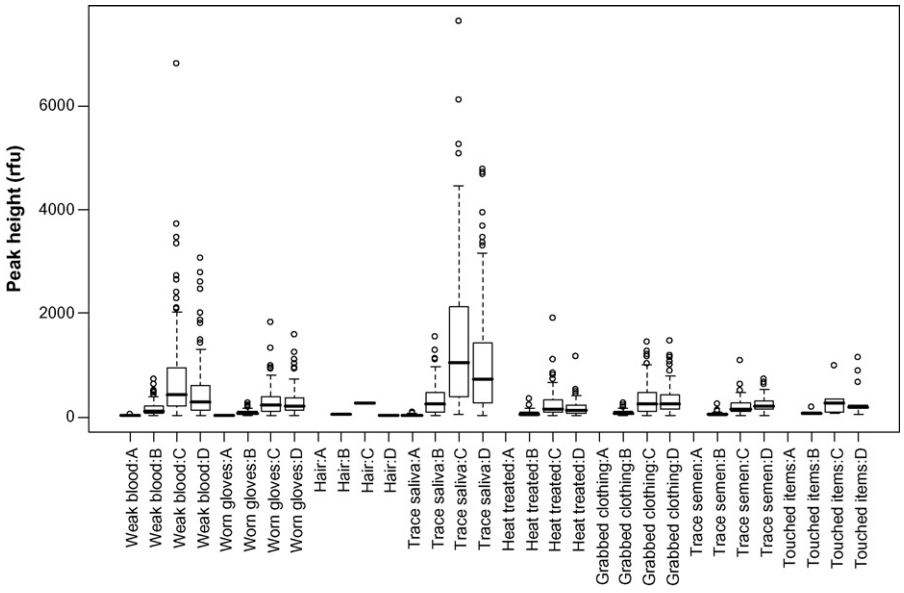

Fig. 1. Distribution of peak height for each sample type and condition. Key is as follows: Thick black line: Median, Box: interquartile range, Whiskers: contain remaining data if within 1.5 times the interquartile range, Circles: represent outliers.

Condition B, C and D all showed increases in peak height and area with C showing the greatest gains followed by D and then B (Table 3). Within the sample types there were very different distributions in peak height and area (Fig. 1 shows peak heights).

Comparing peak height and area for each sample type and locus for conditions C and D using a classical two-way ANOVA showed that the difference in peak height and area was significant for blood ($P_{height} = 0.000060$, $P_{area} = 0.00043$) and saliva ($P_{height} = 0.000018$, $P_{area} = 0.00019$) samples and not significant for other sample types, which is likely due to the lack of data for the other sample types.

Fig. 2 shows an example of the same blood sample processed through conditions A, B, C and D.

3.3. Increase in signal strength from post-PCR modifications

For peaks that are present in condition A, the increases in signal strength as measured by increases in peak height and area can be measured when the samples are processed through condition B and C. Direct comparisons for increases in signal strength only, could not be made to condition D as this condition requires the processing of a new and separate PCR which will have independent stochastic effects resulting in a profile with different peak proportions (condition D can be compared for other criteria as these involves total and mean scores). Table 4 shows these increases.

3.4. Allelic information loss between methods

Of the total of 90 peaks were observed under condition A, all of these were observed under conditions B and C; and all peaks seen under condition B were also observed under condition C. Condition D cannot be compared as a new PCR is generated.

3.5. Construction of "consensus" profiles

For each sample under each condition, "consensus" profiles were constructed which included only allele peaks that were present in both duplicate PCRs. This was done in line with published approaches to LCN interpretation [2,3] to reduce the chances of including spurious peaks caused by low level adventitious transfer of environmental DNA. Table 5 shows this data.

3.6. Peak area ratio data

Heterozygote balance was examined by calculating the peak area ratio (PAR) for each heterozygous locus with both allelic peaks present. PAR was calculated by dividing the peak area of the smaller area allele by the peak area of the larger area allele ($PAR = PA_{low}/PA_{high}$). The ratio is always ≤ 1 with 1 representing equally balanced peaks (note that for both peaks at a reported heterozygous locus to be loaded to the UK National DNA Database, the PAR must be >0.5). Table 6 shows the data obtained.

Comparison of C and D, and A, B, C and D showed there was no significant difference in PAR between the conditions (Kruskal–Wallis rank sum test). The distribution of PAR values of <0.5 between different loci was proportionate and no dependence on experimental conditions and sample type were found (by logistic regression).

3.7. Stutter peaks

Stutter peaks 4 bp smaller than the main allele peak were measured where they could be unambiguously assigned. Condition A had no stutter peaks >25 rfu present. Conditions B, C and D all had stutter peaks present with 85, 152 and 177 occurrences, respectively. The overall distribution across loci

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

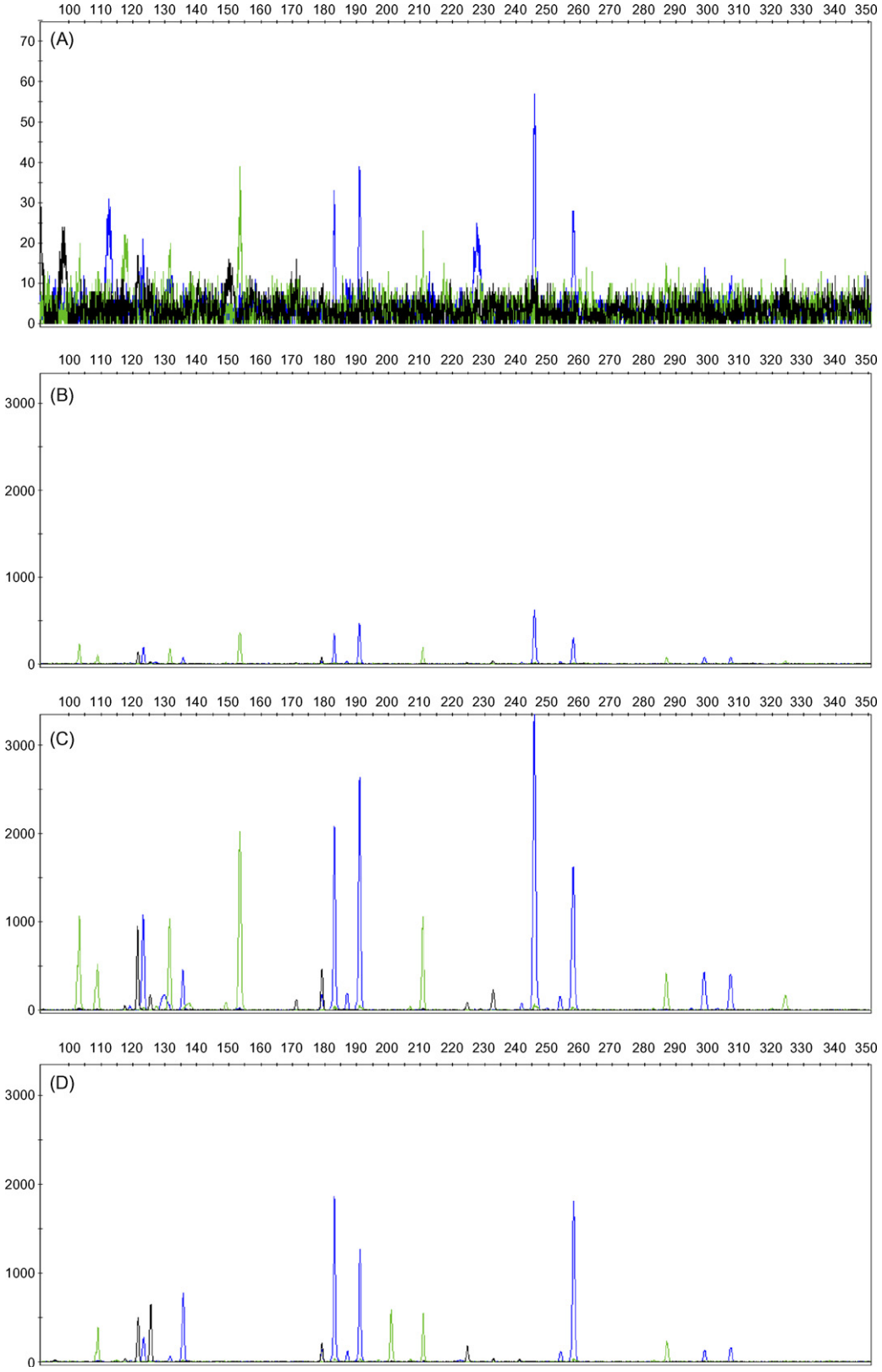

Fig. 2. Examples electropherograms of SGM Plus profiles for a sample processed though conditions A, B, C and D. The example is a blood sample.

was very similar for each condition with D18S51 exhibiting the largest mean stutter as a percentage of the main peak area and TH01 exhibiting the smallest. The mean stutter proportion for each locus was larger for condition D compared to C at all loci, and for condition D compared to B except for D16S539 (Fig. 3).

The peak area data was transformed into a form suitable for statistical modelling using classical assumptions. An analysis

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ‘‘low copy number’’ (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

Table 4
Mean peak heights and areas for conditions A–C, as well as the respective *n*-fold increase in each factor, for peaks present under condition A

Condition	Mean peak height (rfu)	*n*-Fold increase compared to A	Mean peak area (rfu)	*n*-Fold increase compared to A
A	39	–	379	–
B	536	14	5,408	14
C	2,638	67	30,294	80

Table 5
Shows the total number of possible donor peaks, the number of donor peaks duplicated between replicate PCRs, the number of non-donor peaks which were not duplicated between replicates PCRs and the number of missing donor peaks

Condition	Total possible number of donor peaks	Number of donor peaks duplicated between replicate PCRs	Number of donor peaks not duplicated between replicate PCRs	Number of donor peaks missing from either PCR	Percentage of observed donor peaks which are consistent
A	796	12	64	708	27
B	796	140	168	348	63
C	796	188	140	280	73
D	796	178	166	274	68

NB for donor peaks duplicated between PCR replicates a duplicated peak is counted once.

Table 6
Shows the average peak area ratio (PAR) for each condition and the number of these with PAR of <0.5

Condition	Number of heterozygote loci present	Average PAR	Number of PARs <0.5	% of heterozygous loci with PARS of <0.5
A	11	0.71	2	18
B	101	0.62	34	34
C	117	0.57	50	43
D	99	0.57	44	44

of variance and a linear model showed that there was little statistical difference between conditions B and C ($p = 0.057$), but, that condition D produced significantly larger stutter peaks ($p = 0.00064$).

3.8. Additional allelic peaks observed in negative controls

26 negative controls and environmental controls were processed through each condition. Per PCR batch these were 4 item examination environmental controls, 4 DNA isolation environmental controls, 4 DNA isolation negative controls and 1 PCR negative. No unexpected allele peaks were seen in negative controls in condition A but 2 were seen in separate negative controls in each of conditions B, C and D (Table 7).

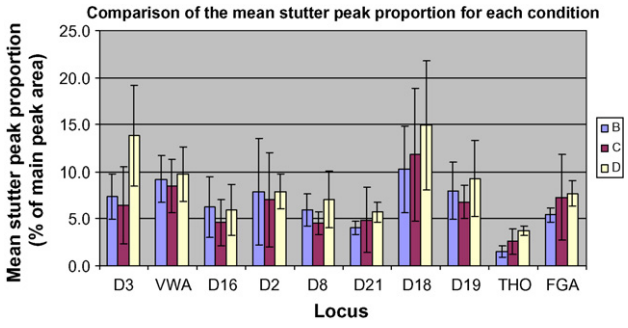

Fig. 3. The mean stutter for each locus for conditions B, C and D. Whisker bars represent ±1 standard deviation.

Importantly the additional peaks were not consistent between replicate amplifications. The rate of occurrence of additional peaks for each of the conditions B, C and D was 0.08 peaks per PCR (2 unexpected peaks/26 PCRs) or 0.007 peaks per locus.

3.9. Unexpected background allelic peaks observed in samples

Any observed peaks not matching the donor's known SGM+ profile were recorded as unexpected background allelic peaks. For condition A there were no such peaks. Conditions B, C and D all showed evidence of unexpected allelic peaks associated with some samples. Table 8 shows the non-donor peaks observed under each condition in the 16 samples where one or more additional peaks were observed.

The sample types that showed unexpected allelic peaks most frequently were the grabbed clothing and the worn gloves suggesting that there was a low level background DNA component in these items. The majority of the non-donor peaks were not consistent and did not match the profiles of the scientists involved in the project or of any persons on LGC's staff DNA database. Only the gloved sample (12) and the grabbed clothing sample (14) showed common peaks between conditions B and D and conditions C and D. The gloved sample (12) had 6 and 10 non-donor peaks, while the grabbed clothing sample (14) had 6 and 7 non-donor peaks observed in either PCR in conditions B and D, and conditions C and D.

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

Table 7
Lists the unexpected allele events associated with the negative controls

Condition	Control type	Surface swabbed	Locus	Peak height
A	–	–	–	–
B	Item examination environmental	Protective benchkote item examined on	D21	56
	Item examination environmental	Protective benchkote item examined on	D19	42
C	Item examination environmental	Protective benchkote item examined on	D21	309
	Item examination environmental	Protective benchkote item examined on	D19	202
D	DNA isolation environmental	Laminar flow cabinet	D19	205
	DNA isolation negative	Blank created with extraction batch	Amel	206

Table 8
Summary of the background contamination events both as individual events and as duplicated contamination events for each condition A, B, C and D

Sample	PCR replicate	Sample type	Number of additional peaks (number of additional peaks present in both replicates)			
			A	B	C	D
1	a	Touched slide	–	1	1	1
	b		–	1	1	1
2	a	Touched slide	–	–	–	–
	b		–	–	–	1
3	a	Blood	–	–	–	1
	b		–	–	–	1
4	a	Blood	–	–	–	1
	b		–	–	–	–
5	a	Mixture–kissed hand	–	–	–	2
	b		–	–	–	1
6	a	Mixture–kissed hand	–	–	–	3 (3)
	b		–	–	–	3 (3)
7	a	Mixture–kissed hand	–	–	–	3 (2)
	b		–	–	–	2 (2)
8	a	Mixture–kissed hand	–	–	–	2
	b		–	–	–	2
9	a	Worn glove	–	2	4	3
	b		–	–	1	–
10	a	Worn glove	–	–	–	1 (1)
	b		–	–	–	2 (1)
11	a	Worn glove	–	–	1	1
	b		–	4	6	2
12	a	Worn glove	–	6 (6)	9 (7)	9 (8)
	b		–	7 (6)	11 (7)	9 (8)
13	a	Hair	–	1	1	–
	b		–	–	–	–
14	a	Grabbed clothing	–	12	17	9 (1)
	b		–	4	8	15 (1)
15	a	Semen	–	1	1	–
	b		–	–	–	–
16	a	Semen	–	–	–	–
	b		–	1	1	–
Total duplicated peaks			0	12	14	30
Total unduplicated peaks			0	28	48	45
Total peaks			0	40	62	75

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ‘‘low copy number’’ (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

Table 9
Shows the summary data from the conditions examined

	No significant difference between conditions C and D					Criteria where condition C is superior to D			
	Number of samples with ≥1 allelic peak	Mean number of peaks	Average peak area ratio	% of heterozygous loci showing peak area ratios of <0.5	Allelic peaks in controls	Mean stutter peak ratio	Non-donor allelic peaks in samples	Mean peak height	Mean peak area
A	14	6	0.71[a]	18[a]	0	None	0	39	379
B	48[a]	10[a]	0.62[a]	34[a]	2	7.1[a]	40	184	1913
C	52[a]	11[a]	0.57[a]	43[a]	2	6.6[a]	63	766	8760
D	53[a]	11[a]	0.57[a]	44[a]	2	8.4	85	579	5946

[a] There is no significant difference between the asterisked conditions.

Examining total non-donor peaks using a generalised linear model with Poisson-distributed errors showed that the difference between condition B and C being very borderline significant ($p = 0.031$) and the difference between condition B and D being strongly significant ($p = 0.00078$), the difference between C and D is also strongly significant. These differences are caused by the increased low level non-donor contamination seen in condition D.

Examining the additional peaks in profiles under all conditions, the non-donor contamination seen in samples 12 (worn glove) and 14 (worn scrubs) were found to be strongly significant ($p = 7$, 1E-07 and 2.4E-0.8) for all conditions, indicating that these samples are different from the remaining samples, which had no significant difference to each other.

Although sample 14 had high levels of non-donor contamination within individual PCRs for each condition the number of duplicated peaks was very low. Comparing the 4 PCRs for this sample from condition C and D showed that 16/51 peaks were duplicated. This further demonstrates the stochastic nature of amplifying low levels of DNA and suggests that the contamination may be inherent in the sample but of extremely low level.

4. Discussion

The analysis of items of evidence that contain a minimal number of nucleated cells has become more frequent in past years. A widely studied method for such low template analysis is 34-cycle LCN PCR [1,2,12]. This adds an extra 6 PCR cycles to the standard 28-cycle method widely used within the forensic community, particularly for the commonly used SGMplus® and Identifiler® kits (Applied Biosystems, UK) [13]. With sufficient PCR reagents, 34-cycle PCR can result in a theoretical 64-fold increase in PCR product. This paper has examined and compared DNA from trace mock-evidential samples processed through 34-cycle LCN PCR (D) with the same DNA processed using 28-cycle PCR (A); concentrated by the use of the Qiagen MinElute column with increased sample loading (B); and further enhanced by increasing injection conditions (C). Clean-up, concentration, increased sample loading and injection conditions act to increase the amount of PCR product loaded onto a capillary during capillary electrophoresis.

This study demonstrates that gains in profile quality at least equivalent to those seen in 34-cycle PCR can be made by concentrating and increasing the loading of product from a 28-cycle PCR.

Both conditions C and D had similar numbers of total peaks and peaks which were duplicated between replicate PCRs. This number of duplicated peaks is important as interpretation of low copy number analysis commonly involves the creation of consensus profiles comprising those allelic peaks replicated in profiles from duplicate PCRs of the same sample [1,14]. Based on our data, the creation of a consensus profile will result in the loss of approximately one-third of the peaks obtained under either condition, but in doing so will significantly reduce the likelihood of reporting allele peaks attributable to spurious background DNA.

Condition C and D both had the same mean peak area ratio, with C having slightly less PARs below 0.5 (as a percentage of heterozygous loci). However, this difference was not significant. As with other observations, condition B generally followed the trends of C but was smaller in scale. Although the data appears to show that conditions B, C and D have a detrimental effect on PAR (with lower mean PAR values and greater numbers of PAR values <0.5 than condition A), this is most likely due of the lack of available data from heterozygote loci seen under condition A and is not statistically significant.

The size of stutter peaks is important in the interpretation of mixed profiles from multiple donors, where it is important to be able to distinguish a possible stutter peak from a genuine donor peak that forms part of the minor component [15]. The mean stutter for each locus for condition D was larger than for each locus of condition C, and for condition B, except at D16S539. These differences were statistically significant. Based on these criteria, data collected under conditions B and C will be more useful for interpretations of mixed samples than data from condition D due to smaller average stutter peaks.

All possible precautions were taken to minimise sample contamination. Despite these precautions, additional peaks were seen in some of the samples and negative controls under all conditions except condition A. The rate was low across each method and may be observed, and even expected, in any technique capable of detecting DNA originating from only a few cells. Any technique with increased sensitivity (compared to standard forensic techniques, which are already highly sensitive) is more likely to detect very low level background DNA which would not have been detected under normal conditions. DNA not from the perpetrator may be detected in

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ''low copy number'' (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

any forensic sample be it from the background environment [3,5] or secondary transfer [16,17]. Adventitious DNA could also be transferred from those collecting the evidence, those working in the laboratory and from within the laboratory itself [18]. However, in this study stringent steps were taken to minimise this.

By using duplicate amplifications and consensus assessment, single contamination events may still be observed but will not be reported and the risk of reporting very low level secondary DNA in the sample is much decreased [1,14]. Gill et al. [1] reported 21 of 30 negative controls in their study as showing evidence of contamination and state that under 34-cycle conditions, provided that the laboratory contamination is less than 0.3 per locus, then constructing consensus profiles by only including peaks seen in duplicate amplifications is statistically supported. The corresponding rate seen in this study was 0.007. This approach was supported in these experiments as any additional peaks seen in negative controls were not duplicated between replicate PCRs. In this study, condition D had the highest rates of detected adventitious transfer of DNA to a sample. These results were found to be statistically significant.

The results of this study, although similar to other publications [10,11] are not directly comparable due to different factors being examined and different DNA concentrations processed through different injection conditions. A distinct advantage to our technique is that it does not require an increase in the number of PCR cycles above 28, in part due to the larger concentration factor attainable by concentrating from a larger PCR volume of 50 μl compared to 25 μl or 12.5 μl used in other studies. Initial experiments carried out by us, comparing the use of a 50 μl PCR with a 25 μl PCR containing the same quantities of input DNA in each PCR showed that under condition A the 25 μl PCR resulted in profiles with more allelic peaks and peaks with greater peak heights. However, when processed through conditions B and C, the 50 μl PCRs produced STR profiles with more allelic peaks and peaks with greater peak heights.

Overall, this study showed that, by a combination of PCR product clean-up, concentration, increased sample loading and increasing injection parameters, STR profiles can be produced from 28-cycle PCRs with the same or better quality and sensitivity as those generated from 34-cycle PCR. The staged approach by which these conditions can be applied is also advantageous. By using condition B first it can be assessed whether the profile will yield a suitable level of information before deciding whether to continue to condition C. This also enables processing of samples which will contain too much DNA for conditions C and D. Hence, the use of a staged approach with conditions B and C allows the processing of a wider range of samples. To achieve an analogous position in the context of additional PCR cycles, a 30- or 32-cycle PCR could be used to obtain an intermediate result, but this has the clear disadvantage of increased sample consumption.

Interpretation of samples processed through conditions B, C and D were done using LCN interpretation guidelines, based on the creation of consensus profiles from duplicate PCRs. The main interpretation difficulties reported elsewhere for LCN analysis, and observed here in condition D, are due to stochastic effects inherent in PCRs when exceedingly low levels of template DNA are amplified. These stochastic effects are, specifically, allele drop-out, increased imbalance between heterozygote peaks and increased stutter. These problems are still anticipated, and indeed observed with condition B and C. While condition B and C are significantly better than condition D in some interpretation areas these differences are not large enough to warrant a different approach to interpretation. Table 9 shows a final summary of the result areas tested. This reinforces that for each result area condition C is as good as or better than condition D.

Amplifying samples with low levels of DNA template requires at least two PCR amplifications to produce consensus profiles. Given that 28-cycle PCR analysis will often be performed before 34-cycle PCRs, the use of the same purified and concentrated product from the 28-cycle PCR with enhanced capillary electrophoresis conditions will mean reduced consumption of DNA from precious samples, as only one further 28-cycle PCR, rather than two new 34-cycle PCRs will be necessary. The emphasis changes to achieving optimum data recovery from the 28-cycle product rather than reamplification of more of the primary DNA extract.

As we are reanalysing the same 28-cycle PCR product and effectively just magnifying the peak heights, the same peak proportions are kept and peaks seen under condition A, but which are of poor quality, can be confirmed under conditions B or C. In contrast, the generation of a new PCR via 34-cycle LCN analysis means that under a new set of random stochastic effects the peak proportions and presence of peaks are unlikely to be the same. The advantage of this in interpretation is that peaks seen in the standard 28 cycle condition A can be confirmed and can be given greater evidential strength than would have been the case had no further processing taken place.

Enhancing 28-cycle PCR processing therefore represents a comparable, but more flexible, approach to analysing low template samples than applying 34-cycle PCR processing.

Acknowledgements

We thank David Carrick-White, Vic Moore and Ana Pedrero-Llamas for technical laboratory assistance. We thank Nikki Peake, Helen Rose, Kerry Way, Gail Westenbrink for helpful comment and interpretation of the results. We thank Simon Cowen for carrying out the statistical testing and for helpful comments in the preparation of this paper. We thank Paul Debenham and Ric Treble for their input and discussion in writing this paper.

References

[1] P. Gill, J.P. Whitaker, C. Flaxman, N. Brown, J. Buckleton, An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA, Forensic Sci. Int. 112 (2000) 17–40.

[2] J.P. Whitaker, E.A. Cotton, P. Gill, A comparison of the characteristics of profiles produced with the AMPFlSTR® SGM Plus™ multiplex system for both standard and low copy number (LCN) STR DNA analysis, Forensic Sci. Int. 123 (2001) 215–223.

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ‘‘low copy number’’ (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005

[3] P. Gill, Application of low copy number DNA profiling, Croat. Med. J. 42 (2001) 229–232.
[4] I. Findlay, R. Frazier, A. Taylor, A. Urquhart, Single cell DNA fingerprinting for forensic applications, Nature 389 (1997) 555–556.
[5] BBC News Online, DNA test halted after Omagh case, BBC News Online, http://news.bbc.co.uk/1/hi/uk/7156051.stm, 23 December, 2007.
[6] J. Booth and agencies, Wearside Jack pleads guilty to Ripper hoax, The Times, March 20, 2006.
[7] Chris Summers, How science caught up with killer, BBC News online, http://news.bbc.co.uk/1/hi/uk/1459056.stm, 24 August, 2001.
[8] Crown Prosecution Service, Review of the use of Low Copy Number DNA analysis in current cases: CPS statement, http://www.cps.gov.uk/news/pressreleases/101_08.html, 14 January, 2008.
[9] B. Budowle, D.L. Hobson, J.B. Smerick, J.A.L. Smith, Low Copy Number—consideration and caution, in: Proceedings of the Twelfth International Symposium on Human Identification, Promega Corporation, Madison, Wisconsin, 2001, Available at: http://www.promega.com/geneticidproc/ussymp12proc/contents/budowle.pdf.
[10] M. Prinz, L. Schiffner, J.A. Sebestyen, E. Bajda, J. Tamariz, R.C. Shaler, H. Baum, T. Caragine, Maximization of STR DNA typing success for touched objects, in: International Congress Series, vol. 1228, 2006, 651–653.
[11] P.J. Smith, M.S. Ballantyne, J. Ballantyne, Simplified Low-Copy-Number DNA analysis by post-PCR purification, J. Forensic Sci. 52 (2007) 820–829.
[12] A.D. Kloosterman, P. Kersbergan, Efficacy of limits of genotyping low copy number DNA samples by multiplex PCR of STR loci, in: International Congress Series, vol. 1239, 2003, 795–798.
[13] E.A. Cotton, R. Allsop, J. Guest, R. Frazier, P. Koumi, I.P. Callow, A. Seager, R. Sparkes, Validation of AMPF*l*STR® SGM Plus™ system for use in forensic casework, Forensic Sci. Int. 112 (2000) 151–161.
[14] P. Taberlet, S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L.P. Waits, J. Bouvet, Reliable genotyping of samples with very low DNA quantities using PCR, Nucleic Acids Res. 24 (1996) 1389–3194.
[15] T.M. Clayton, J.P. Whitaker, R. Sparkes, P. Gill, Analysis and interpretation of mixed forensic stains using DNA STR profiling, Forensic Sci. Int. 91 (1998) 55–70.
[16] F. Alessandrini, M. Cecati, M. Pesaresi, C. Turchi, F. Carle, A. Tagliabracci, Fingerprints as evidence for a genetic profile: morphological study on fingerprints and analysis of exogenous and individual factors affecting DNA typing, J. Forensic Sci. 48 (2003) 586–592.
[17] A. Lowe, C. Murray, J. Whitaker, G. Tully, P. Gill, The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces, Forensic Sci. Int. 129 (2002) 25–34.
[18] A.L. Poy, R.A.H. van Oorschot, Trace DNA presence, origin and transfer within a forensic biology laboratory and its potential effect on casework, J. Forensics Identification 56 (2006) 558–576.

Please cite this article in press as: L. Forster, et al., Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle ''low copy number'' (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Gene. (2008), doi:10.1016/j.fsigen.2008.04.005