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Simplified Low-Copy-Number DNA Analysis by Post-PCR Purification

ABSTRACT: Frequently, evidentiary items contain an insufficient quantity of DNA to obtain complete or even partial DNA profiles using standard forensic gentotyping techniques. Such low-copy-number (LCN) samples are usually subjected to increased amplification cylces to obtain genetic data. In this study, a 28-cycle polymerase chain reaction (PCR) was used to evaluate various methods of post-PCR purification for their effects on the sensitivity of fluorophore-based allelic detection subsequent to capillary electrophoretic separation. The amplified product was purified using filtration, silica gel membrane, and enzyme mediated hydrolysis purification techniques and evaluated for their effect on fluorescent allelic signal intensity. A purification method was selected and its effect on fluorescent allelic signal intensity was compared with that of the unpurified PCR product. A method of post-PCR purification is described which increases the sensitivity of standard 28-cycle PCR such that profiles from LCN DNA templates (<100 pg DNA) can be obtained. Full DNA profiles were consistently obtained with as little as 20 pg template DNA without increased cycle number. In mock case type samples with dermal ridge fingerprints, genetic profiles were obtained by amplification with 28 cycles followed by post-PCR purification whereas no profiles were obtained without purification of the PCR product. Allele dropout, increased stutter, and sporadic contamination typical of LCN analysis were observed; however, no contamination was observed in negative amplification controls. Post-PCR purification of the PCR product can increase the sensitivity of capillary electrophoresis to such an extent that DNA profiles can be obtained from <100 pg of DNA using 28-cycle amplification.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, purification, low copy number, Identifiler, MinElute, Microcon, Montage PCR, ExoSAP-IT

Recently, a great deal of interest has been generated in obtaining a DNA profile from low template DNA samples including DNA transferred by casual contact, often referred to as trace DNA. A number of studies have demonstrated that profiles can be obtained from fingerprints and other objects that have been handled (1–5). However, success rates using standard forensic procedures are relatively low, ranging from 30% to 50% (5). Studies aimed at increasing sensitivity through extract concentration and reduced-volume polymerase chain reaction (PCR) have been performed with a measure of success (6,7). With a standard forensic procedure, the limit of detection for a DNA profile is anywhere from 100 pg to 500 pg, dependent upon the amplification multiplex and detection parameters utilized (8–11).

Samples containing <100 pg DNA fall into a category where specialized low-copy-number (LCN) techniques are employed. The most popular method of LCN analysis is to raise the number of amplification cycles from 28 to 30–34 cycles (1–3,12–16). Other strategies such as nested PCR and whole-genome amplification have also been described (12,17). These methods have proved highly successful in obtaining profiles from as little as 5 pg of DNA. However, LCN analysis is not without its drawbacks. Typical problems encountered include allele dropout and drop-in, higher stutter peaks, and sporadic contamination. Not withstanding these problems, strategies for dealing with these issues have been described (10,13,16,18) and LCN analysis sits at the forefront of forensic DNA inquiry.

An alternative method of LCN analysis through post-PCR purification is set forth in this study. When short tandem repeat (STR) PCR products are generated, they are separated by size using electrophoresis and the alleles detected using a variety of instrument platforms. Hutchison (19) purified microsatellite PCR products prior to separation on a slab gel and reported significantly reduced background as well as a 2–5 fold increase in the signal-to-noise ratio. However, in the forensic community STR PCR products are generated with a multiplex amplification procedure, and most often detected via capillary electrophoresis. Prior to capillary electrophoresis, the sample is electrokinetically injected into the capillary, typically for 5 sec. During electrokinetic injection a voltage is applied to the electrode, effectively drawing negatively charged molecules such as DNA into the capillary. The short injection time permits a limited amount of sample to be taken into the capillary. In this process the uptake of smaller components is favored; STR amplicons compete with primers, unincorporated deoxynucleoside triphosphates (dNTPs), salts and other negatively charged PCR reaction components. In theory, the removal of un-reacted amplification components should favor amplicon injection and lead to an increase in fluorescent signal intensity (20). This study explores the effects of increasing PCR sensitivity without increased amplification cycles by purifying the PCR product in an attempt to increase fluorescent allelic signal intensity.

Material and Methods

Isolation and Quantitation of DNA

DNA was isolated from two different, previously typed blood samples ('CTS'' and ''LH') on cotton cloth and FTA paper (Whatman, Florham Park, NJ) using a standard organic extraction

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method and quantified using the Quantiblot Kit (Applied Biosystems, Foster City, CA). DNA for the sensitivity studies was prepared from serial dilutions of the 1.25 ng/μL "CTS" and 0.625 ng/μL "LH" samples. Some samples (i.e., stutter studies and mock casework) were quantified with Quantifiler in accordance with the manufacturer's instructions. With the samples used, Quantifiler estimates were consistently 2–3 times larger than estimates by Quantiblot.

Amplification

The DNA was amplified using the AmpFℓSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems) in a reaction volume of 25 μL. The Identifiler™ PCR Amplification Kit amplifies 15 STR loci and the sexing locus Amelogenin. Amplification was performed in a GeneAmp® PCR 9700 thermal cycler (Applied Biosystems) for a 95°C 11 min incubation followed by 28 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, ending with a 60°C for 60 min extension and 25°C hold in accordance with the manufacturer's recommendations.

PCR Purification

Filtration—Amplified product was purified using the Microcon-50 and Montage PCR® (Millipore Corp., Danvers, MA) filter units by adding 375 μL TE^{-4} Buffer (10 mM Tris–HCL, 0.1 mM EDTA, pH 8) to the sample reservoir followed by 25 μL of the amplified product. The tubes were subjected to centrifugation (maximum speed for 12 min for the Microcon-50 and $1000 \times g$ for 15 min for the Montage PCR®). After discarding the eluate, 400 μL of TE buffer was added to the sample reservoir followed by a brief vortex and centrifugation as described above. This process was repeated for a total of four washes. The sample reservoir was placed in a clean collection tube and 10 μL of TE^{-4} buffer was added to the reservoir followed by a brief vortex, inversion of the sample reservoir and centrifugation ($1000 \times g$ for 3 min for the Microcon-50 and $1000 \times g$ for 2 min for the Montage PCR®).

Silica Gel Membrane—Purification with the Qiagen MinElute Kit (Qiagen, Valencia, CA) was performed by adding 125 μL high salt, low pH PB buffer to the column followed by 25 μL of amplified product and centrifugation at $\geq 10{,}000 \times g$ for 1 min. After discarding the eluate, 700 μL of ethanol containing PE buffer were added to the column to wash and centrifuged at $\geq 10{,}000 \times g$ for 1 min. The eluate was discarded and this step was repeated for a total of four washes followed by a dry centrifugation under the same conditions to clear the column. The amplified product was eluted with 10 μL low salt, high pH EB buffer into a clean 1.5 mL tube. The precise volume of eluate for the Microcon-50, Montage PCR® and MinElute purifications was measured to normalize for variability in volume recovery.

Enzyme-Mediated Hydrolysis of Reaction Components—ExoSAP-IT® (USB Corp., Cleveland, OH) reagent was added to amplified product in a ratio of 2 μL ExoSAP-IT® to 5 μL PCR product as recommended by the manufacturer. The entire 25 μL of amplified product was treated and incubated in a GenAmp® PCR 9700 thermal cycler (Applied Biosystems) at 37°C for 15 min followed by heat inactivation of the enzymes at 80°C for 15 min. The enzyme-treated amplified product was stored at −20°C until use.

Separation and Detection of STR Alleles

Samples were prepared for electrophoresis by adding 1.5 μL of unpurified PCR product, 0.5 μL GeneScan-500 LIZ Size Standard (Applied Biosystems) and 24.5 μL Hi-Di™ Formamide (Applied Biosystems) unless otherwise specified. Purified amplified product was prepared by mixing 1.5 μL PCR product, 0.1 μL GeneScan-500 LIZ Size Standard and 25 μL Hi-Di™ Formamide. When required, purified product (range 4–10 μL) was mixed with 0.2 μL GeneScan-500 LIZ Size Standard and 15 μL Hi-Di™ Formamide. The samples were heated to 95°C for 3 min and snap-cooled for at least 3 min. PCR products were separated and detected on the ABI Prism® 310 Genetic Analyzer (Applied Biosystems) using POP-4™ polymer (Applied Biosystems) with the GS STR POP4 (1 mL) G5 module. The data were analyzed with GeneMapper 3.2 NT software (Applied Biosystems) using a threshold of 100 RFU.

Comparison of Post-PCR Purification Methods

The four purification methods were evaluated by a comparison of profile integrity and relative fluorescent signal intensity. The 1.25 ng/μL DNA extract was diluted manually by serial dilution to permit the amplification of 156, 78, 39, and 20 pg total input template DNA. The diluted samples, negative amplification control, and reagent blank were amplified in quadruplicate. The samples were injected in triplicate to establish an average RFU for each allele before purification. Each input quantity of DNA along with the negative amplification control and reagent blank was subjected to a different PCR purification procedure and injected in triplicate. The purified products from the Microcon 50, Montage PCR®, and MinElute devices were eluted with a total of 10 μL of appropriate buffer. To account for variation in eluate volume, the average peak height (PH, measured in RFU) for each allele, after purification, was calculated and then normalized. The fold increase (FI) in signal intensity for each allele was calculated by $FI = PH_{purified}/PH_{unpurified}$. The average FI across all alleles for each sample was determined. PHs for unpurified samples were obtained by analysis at 20 RFU.

Efficacy of Post-PCR Purification Using Silica Gel Membrane (MinElute)

A direct comparison was made between unpurified and purified PCR product using the MinElute method. The 1.25 ng/μL and 0.625 ng/μL DNA extracts were manually diluted by serial dilution to permit the amplification of 625, 312, 156, 78, 39, 20, 10, and 5 pg DNA. The samples were amplified in duplicate using a 25 μL reaction volume. The unpurified amplified product (1.5 μL) was added to the formamide mix and injected under standard conditions. The samples were again amplified in duplicate and purified eluting into 25 μL. The purified product (1.5 μL) was added to the formamide mix with 0.1 μL LIZ size standard and injected. The negative amplification control and reagent blank were also purified and injected. The FI in signal intensity for each allele (allelic signal increase) was calculated by $FI = PH_{purified}/PH_{unpurified}$ and the average FI in signal intensity across the 15 loci for each input quantity of DNA was determined.

Use of Concentrated, Purified, Total PCR Product

The effects of injecting the entire MinElute purified product were investigated. Serial dilutions of the 1.25 ng/μL and

0.625 ng/μL extracts were prepared such that 5–625 pg DNA were amplified in duplicate and concentrated to 10 μL in a heat assisted rotary evaporator. The 10 μL of unpurified PCR product was added to 15 μL of formamide and 1.5 μL LIZ size standard and injected. The same samples were again amplified in duplicate with 5–78 pg DNA, post-PCR purified using the MinElute system and eluted into 10 μL of EB buffer. The 10 μL of purified PCR product were added to 15 μL formamide and 0.2 μL LIZ size standard and injected. The negative amplification control and reagent blank were similarly purified and injected.

A comparison of allelic signal increase between the injections of total purified PCR product and 1.5 μL of unpurified PCR product was made. The FI in signal intensity for each allele was calculated by $FI = PH_{purified}/PH_{unpurified}$ and the average FI in signal intensity across the 15 loci for each input quantity of DNA was determined.

Mock Casework Samples

To assess the effectiveness of PCR purification on casework type samples, two dermal ridge fingerprints (on paper and glass substrates) and a telogen hair root were collected from three subjects. The fingerprint on paper was collected on a sheet of commercial printing paper taken from the center of a ream. The subjects were asked to grip a 2 × 5 cm piece of paper between the thumb and forefinger for 1–2 sec before release. Microscope slides were removed from the center of a new package and cleansed with ethanol. The subjects were asked to grip the slide with the thumb on top for 1–2 sec before release. The slides were swabbed on the top with sterile water and the swabs were dried. The hairs were washed in sterile distilled water prior to extraction. The samples were extracted with the Qiagen mini blood extraction kit and concentrated to a volume of 25 μL. Quantification was performed with the Quantifiler quantification kit (Applied Biosystems). The samples were amplified in duplicate with the Identifiler™ kit (Applied Biosystems) under standard amplification conditions.

Results

Comparison of Post-PCR Purification Procedures

Initially, four post-PCR purification methods were evaluated by a comparison of profile integrity and relative fluorescent signal intensity. These included two filtration methods (using the Millipore Corporation Microcon-50 and Montage PCR® filters), binding to a silica gel membrane (using the Qiagen MinElute PCR Purification Kit) and enzymatic hydrolysis of primers and nucleotides (using ExoSAP-IT® from USB). DNA samples (156, 78, 39, and 20 pg) were amplified in quadruplicate. The samples were injected in triplicate to establish an average RFU for each allele before purification. Each sample containing a defined input quantity of DNA, along with the negative amplification control and reagent blank, was subjected to one of the four different PCR purification procedures and injected in triplicate. The elution volume for the Microcon 50, Montage PCR®, and MinElute purification methods was 10 μL whereas the entire 25 μL amplified product was used for enzyme hydrolysis.

Increase in allelic signal intensity compared with the standard non-post-PCR-purified product was observed with the Microcon-50 filter (3–6-fold), the Montage PCR® filter (6–8 fold), and with the MinElute silica column (4–6 fold) (Fig. 1). The ExoSAP-IT® results yielded poorer quality data, exhibiting a decrease in RFU, minus A, extraneous peaks and numerous 75–100 bp products. Subsequently, the ExoSAP-IT® treated samples were further purified using the MinElute column and injected. This eliminated most of the anomalies seen except for the minus A artifact. Nonspecific product resulting in off ladder calls was observed at D3S1358 using both filtration methods: off ladder alleles were called by the GeneMapper software in 11 out of 14 injections using the Microcon-50 and in 4 out of 14 injections with the Montage PCR® filter. Careful inspection of the electropherograms indicated that the selfsame off-ladder artifacts were present below threshold in most of the samples even though not called by the software. In contrast, the MinElute purified samples exhibited no off ladder calls at D3S1358 and practically no artifacts below threshold. Though the Montage PCR filter yielded a greater signal increase, the MinElute method was selected for subsequent studies because of the absence of artifacts and its speed (e.g., 1 min centrifugation vs. 15 min with the filters).

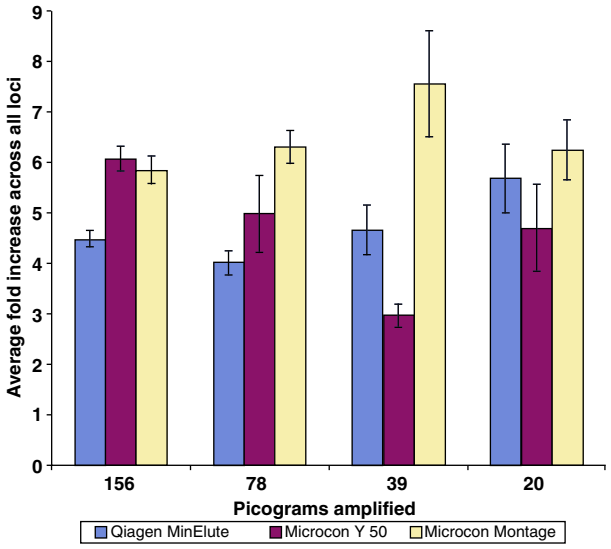

FIG. 1—*Comparison of Qiagen MinElute, Microcon Y50, and Microcon Montage PCR® purification products. Normalized data represents 1.5 μL of concentrated purified product (10 μL eluate) in formamide mix.*

Efficacy of Post-PCR Purification Using a Silica Gel Membrane (MinElute)

MinElute purification of the PCR products yielded full DNA profiles at 78 pg and partial DNA profiles with as little as 5–10 pg input DNA template (Table 1). The purified samples were eluted into 25 μL of EB buffer, and prepared for electrophoresis by adding 1.5 μL of the eluate to 25 μL of formamide and 0.1 μL LIZ size standard. In contrast, the standard method without PCR purification yielded full, albeit weak, profiles with 156 pg of DNA and partial profiles with 39 pg of DNA (Table 1). Allelic signal increases from 3.4 to 4.9 fold (mean = 3.9) were obtained by PCR purification compared with that by unpurified product

TABLE 1—*Increased sensitivity with post-PCR purification.*

PCR product	156 pg	78 pg	39 pg	20 pg	10 pg	5 pg
1.5 μL unpurified	30	15–25	5–9	0–1	0	0
1.5 μL purified	30	30	27–28	9–19	5–13	0–5
Entire purified product	N/D	30	30	30	22–28	12–27

PCR, polymerase chain reaction.

The number of alleles detected out of 30 possible alleles. Data indicate the range of alleles detected from four amplifications (two extractions amplified in duplicate). Complete profiles with or without purification were obtained for all samples amplified with 625–312 pg of DNA.

TABLE 2—*Fold increase in signal intensity injecting 1.5 μL purified product and the entire purified product.*

PCR product	625 pg	312 pg	156 pg	78 pg	39 pg	20 pg	10 pg
1.5 μL purified	4.9	3.5	3.4	3.8	3.7	4.2	4.3
Entire purified product	N/D	N/D	17.2	21.7	18.9	16.9	N/D

PCR, polymerase chain reaction.

(Table 2). There did not appear to be a relationship between the fold signal increase and the input quantity of DNA. As expected, a significant reduction in primer peak products was observed after post-PCR purification (Fig. 2*b*) compared with not having a purification step (Fig. 2*a*).

Use of Concentrated Purified, Total PCR Product

The effects of injecting the entire MinElute purified product were investigated. MinElute purified DNA (from 5–78 pg of input template DNA) was eluted into 10 μL of EB buffer, and all of it (i.e., 10 μL) added to 15 μL of formamide and 0.2 μL LIZ size standard and injected. For comparison, 5–625 pg DNA was amplified in duplicate and, without post-PCR clean up, concentrated to 10 μL in a heat assisted rotary evaporator. The 10 μL of resulting unpurified but concentrated PCR product was added to 15 μL of formamide and 1.5 μL LIZ size standard and injected.

Significantly, and reproducibly, complete DNA profiles were obtained from 20 pg of template DNA when injecting the entire PCR purified product (Fig. 3*b*), with partial profiles obtained with 5–10 pg of DNA (Table 1). Amplification of 20 pg of the same template DNA with a standard non-post-PCR purification method yielded no profile (Fig. 3*a*). Off scale data were produced with 78 pg of DNA when the entire purified product was added. Direct comparison with injection of the total, unpurified amplification product was not possible since this retarded migration of the alleles approximately 1.5–2 bp such that sample alleles did not align with the allelic ladder bins (data not shown). In addition, RFUs were decreased in the latter instance relative to the normal injection of 1.5 μL, possibly because of increased uptake of smaller reaction components, and spurious peaks were created. When compared with the injection of 1.5 μL of unpurified PCR product allelic signal increases from 16.9 to 21.7 fold (mean = 18.7) were obtained by injecting the total purified product (Table 2). This is a significant increase compared with standard methods, in which 1.5 μL of amplified product is injected without PCR purification. There did not appear to be a relationship between the fold signal increase and the input quantity of DNA

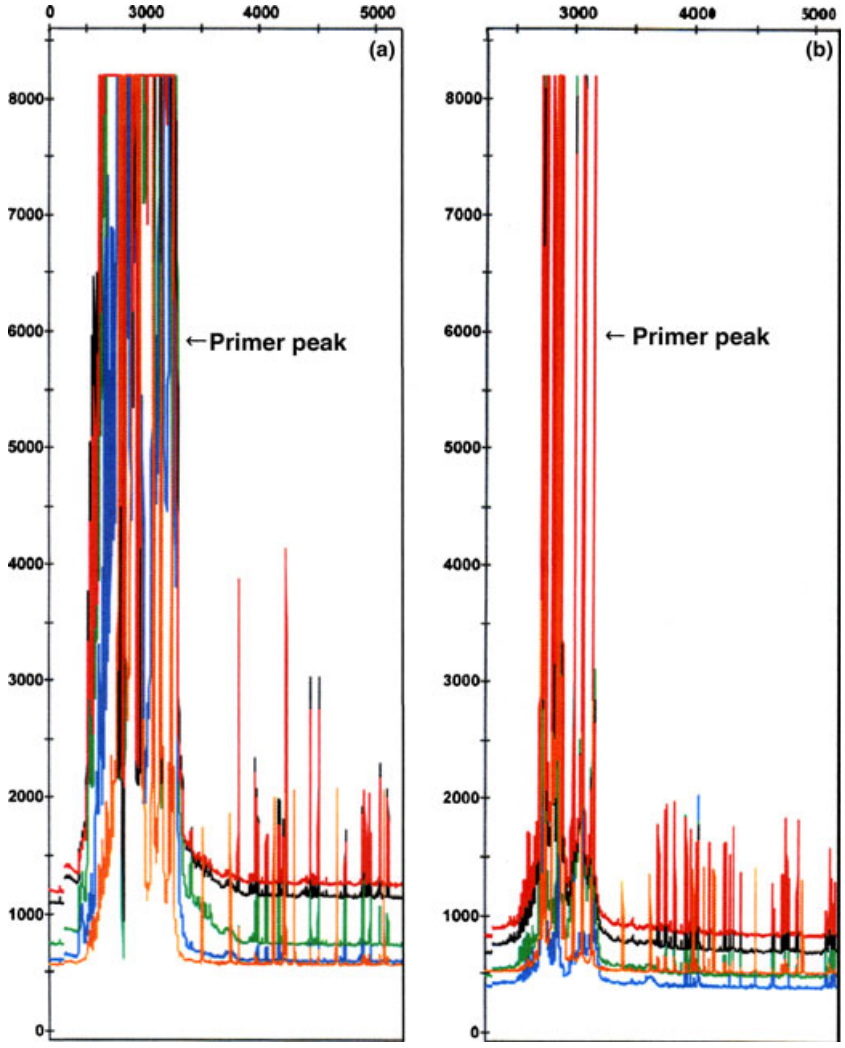

FIG 2—*Comparison of purified and unpurified primer peaks. Raw data from injecting 1.5 μL of unpurified product on the left (a). Raw data from injecting 1.5 μL of purified product eluted into 25 μL on the right (b).*

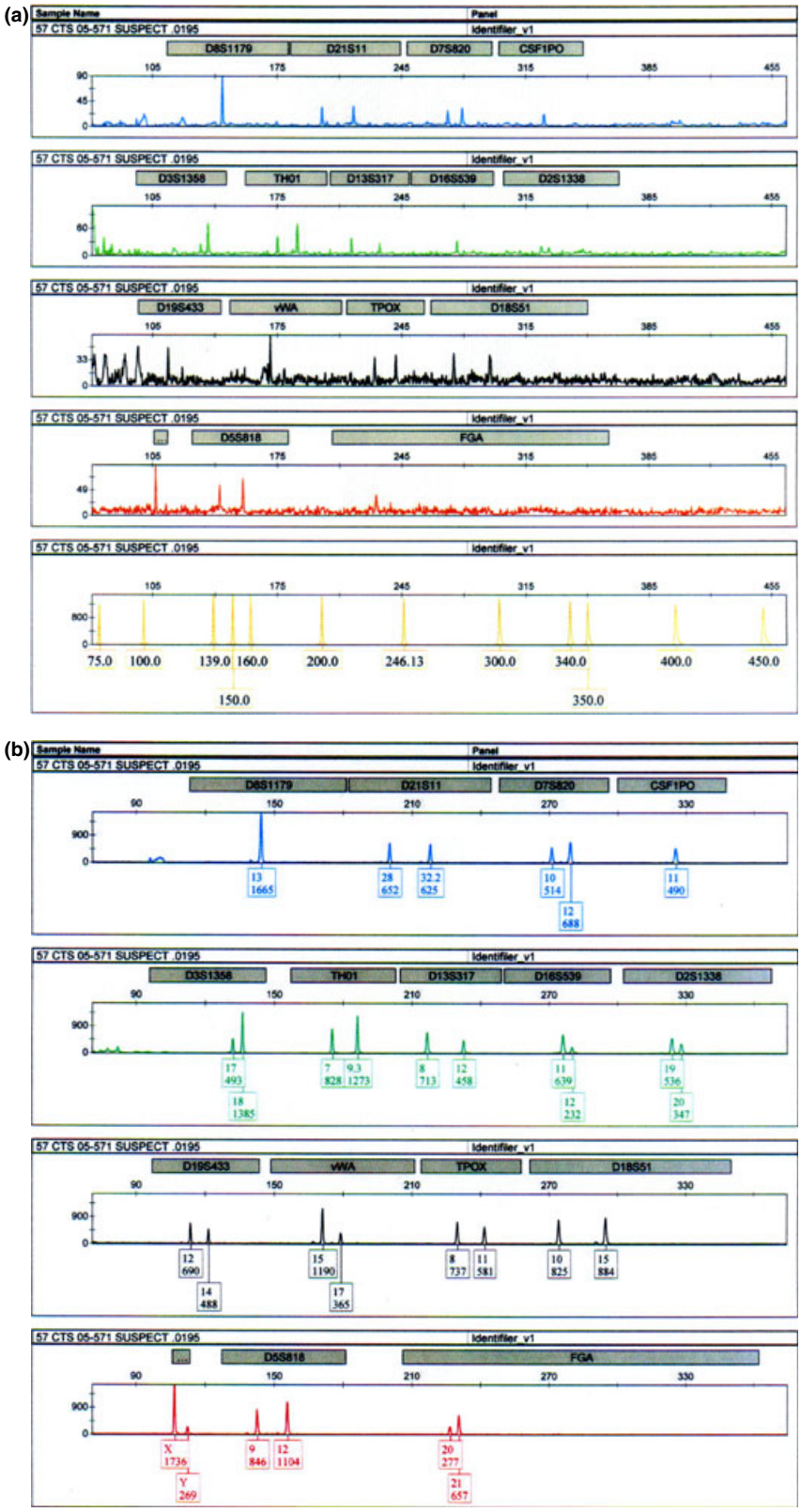

FIG. 3—*Short tandem repeat (STR) profile from 20 pg DNA without post-PCR purification (a). STR profile from 20 pg DNA (same sample as in (a)) after post-PCR purification using MinElute and injection of the entire concentrated purified product (b).*

Stochastic LCN-Like Artifacts

Stutter—Increased stutter was observed in post-PCR purified samples but the extent of this depended upon how much of the purified product was used. For example, when injecting 1.5 μL of the purified product (eluted into 25 μL of buffer) increased stutter was observed in only one of 900 (0.1%) of the allele calls (at the locus D5S818). However, increased stutter was observed in

approximately 25% of the samples (6/24) when injecting the entire concentrated purified product (10 μL), representing 0.8% of the allele calls (6/720). This increased stutter was often not seen when the same sample was reamplified and the total purified product injected, indicating the stochastic nature of the process.

A second, more comprehensive, stutter study was undertaken to compare stutter values between purified and unpurified product using 0.05–0.10 ng of input DNA for amplificaion. The shorter of the two alleles from heterozygotes that differed by one repeat unit were excluded from the stutter calculations. Fifty samples (from different individuals) of unpurified and 50 samples of concentrated purified PCR product (10 μL of eluate, injecting 4–10 μL) were analyzed for stutter and compared. At each locus, the mean stutter percentage was similar between the purified and unpurified PCR product, although PCR purification produced an increase in the variation of stutter at each locus (Fig. 4). In this study, 3.6% of the stutter values (37/1026) for the purified product exceeded Identifiler cut off values at the loci D5S818 (n = 9), D21S11 (n = 8), D8S1179 (n = 5), D19S433 (n = 4), D2S1338 (n = 3), vWA (n = 3), D7S820 (n = 2), CSF1PO (n = 1), TH01 (n = 1), and TPOX (n = 1). The unpurified product exceeded Identifiler cut off values 0.4% of the time (4/933) at the loci D5S818 (2) and D8S1179 (2). The highest stutter recorded for the purified product was 29.7% at vWA.

Stutter peaks are normally in the n−1 position, where n = the allele (in repeat units). In approximately 7% of the samples (7/105) an n + 1 stutter peak was observed when injecting concentrated purified product (range 1.5–10 μL). However these n + 1 peaks, although replicated upon reinjection, did not reappear in duplicate amplifications. No n + 1 stutter peaks were observed in the unpurified amplifications.

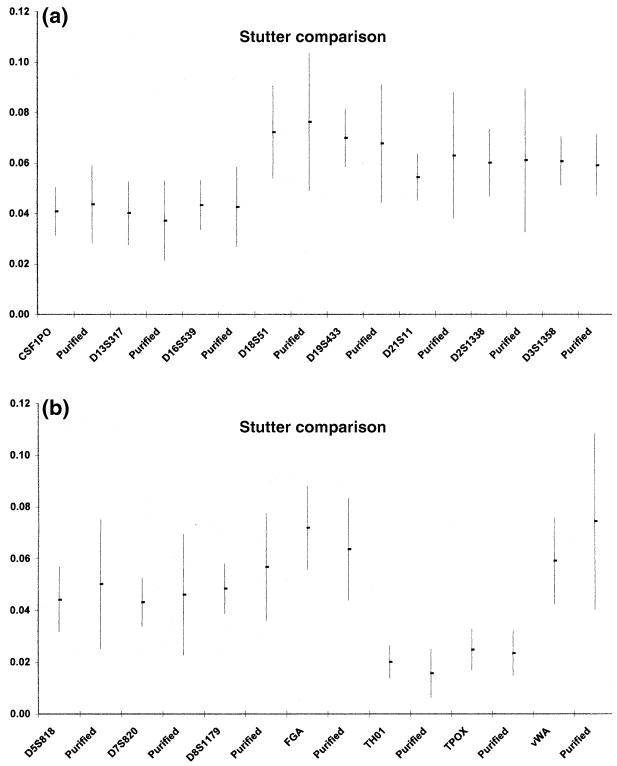

FIG. 4—*Comparison of stutter before and after purification for each locus. The average stutter percentage is indicated, with the length of the bar representing one standard deviation either side of the mean.*

In summary, the vast majority of the increased stutter (including the formation of n + 1 peaks) arises from the use of concentrated (i.e., 4–10 μL) purified product.

Allele Drop-in—In three of 60 amplifications in which 4–10 μL of concentrated purified product was injected, an unexpected allele was obtained at the locus D2S1338. No additional peaks below threshold were observed in these amplifications; moreover the negative amplification and reagent blank controls were amplicon-free. Importantly, the drop-in alleles were not reproducible upon re-amplification.

Heterozygote Peak Height Imbalance—Heterozygote PH imbalance in low template samples can be extreme (13,14,16). A comparison of heterozygous peak imbalance was made between unpurified product and purified product. The PH ratio (PHR) of heterozygous loci was calculated by dividing the PH of the lowest allele by the PH of the highest allele ($PHR = PH_{low}/PH_{high}$) such that the PHR is always ≤1, with 1 representing perfect PH balance. Heterozygous loci exhibiting allelic dropout were not included in the calculations. PHRs for purified product were obtained by injecting the entire purified product using input quantities of 78 pg (n = 56), 39 pg (n = 56), 20 pg (n = 56), 10 pg (n = 39), and 5 pg (n = 25). Four amplifications for each input quantity of DNA were analyzed. PHRs for unpurified product were obtained from 50 standard amplifications (samples from 50 individuals) using 1 ng DNA (564 heterozygous loci). The average PHR of non-PCR purified samples was 88% (Fig. 5). In contrast, post-PCR purified samples exhibited reduced PHR with the extent of the decrease being a function of initial input template quantity (Fig. 5). The mean PHR from postpurified PCR samples ranged from 77% (with 78 pg input DNA) to 52% (with 5 pg input DNA). The actual observed PHR ranged from 58–100% for unpurified samples to 15–99% for purified product, depending upon the DNA input (Table 3).

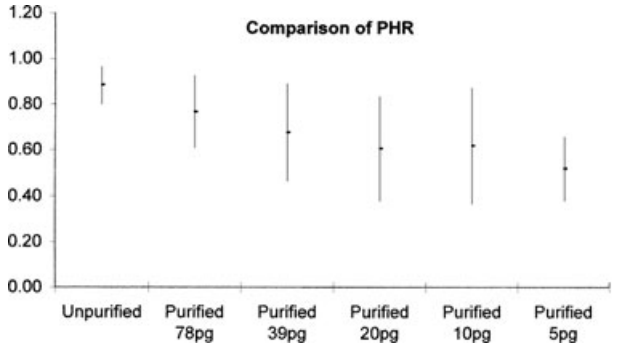

FIG. 5—*Comparison short tandem repeat (STR) heterozygote loci peak imbalance with purified and nonpurified PCR products. The average PHR is indicated, with the length of the bar representing one standard deviation either side of the mean. Unpurified* n = 564. 78, 39, and 20 pg, n = 56. 10 pg, n = 39. 5 pg, n = 25.

TABLE 3—*Range of heterozygote imbalance observed between amplifications of 1 ng unpurified PCR product and 78, 39, 20, 10, and 5 pg of purified PCR product.*

	Unpurified 1 ng	Purified 78 pg	Purified 39 pg	Purified 20 pg	Purified 10 pg	Purified 5 pg
PHR Range	58–100%	34–99%	22–99%	16–97%	15–99%	31–80%

PCR, polymerase chain reaction; PHR, peak height ratio.

Mock Casework Samples

The effectiveness of PCR purification on mock casework type samples was assessed using dermal ridge fingerprints (on paper and glass substrates) and a telogen hair root collected from three volunteer subjects. A significant improvement in the ability to obtain genetic signatures from the donor of dermal ridge fingerprints was obtained by post-PCR purification (for an example see Fig. 6).

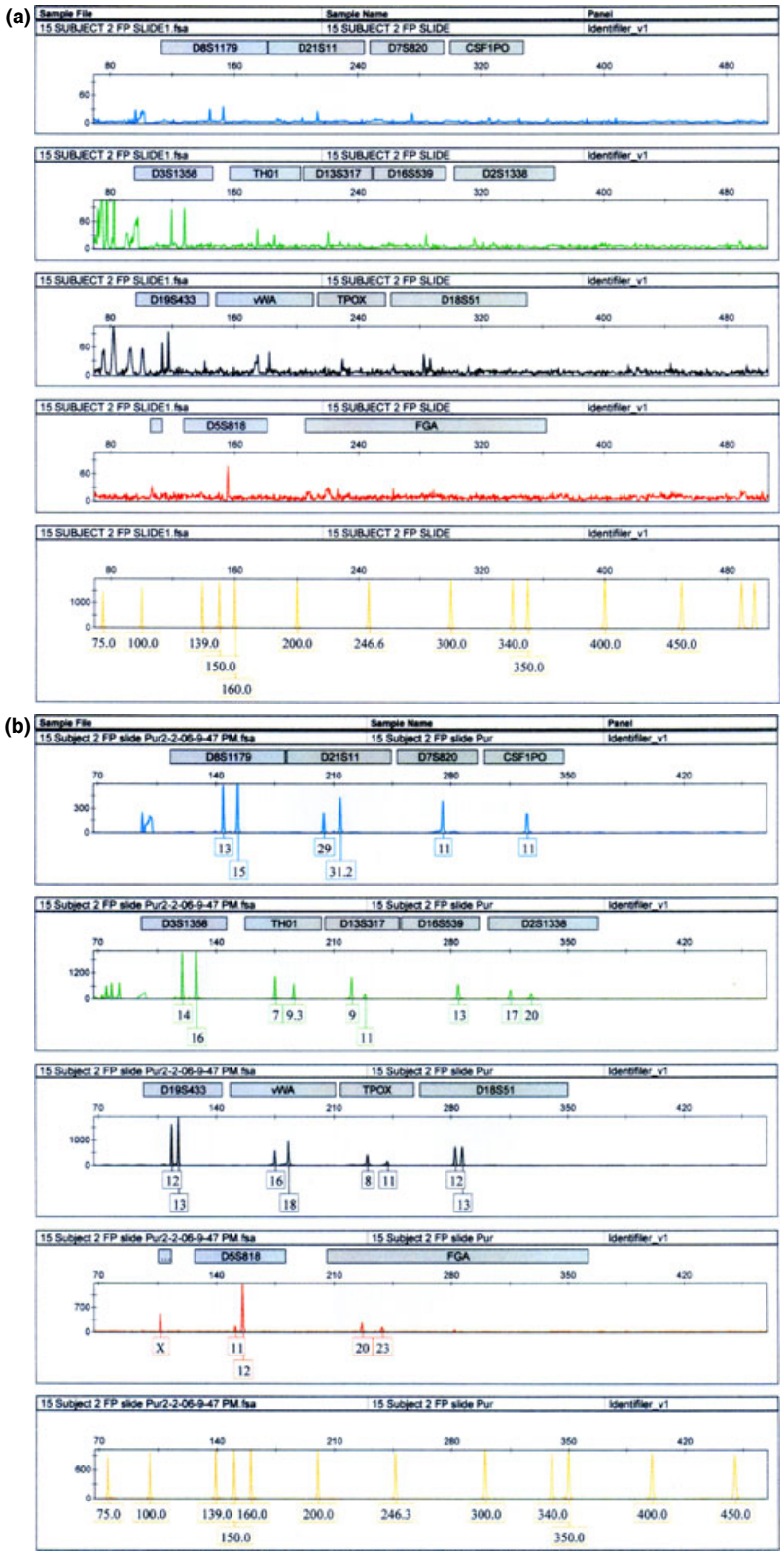

FIG. 6—*STR profile from dermal ridge fingerprint of subject 2 without post-PCR purification (a). Short tandem repeat (STR) profile from dermal ridge fingerprint of subject 2 after post-PCR purification using MinElute and injecting the entire concentrated purified product (b).*

TABLE 4—*Comparison of unpurified and purified PCR product in mock casework samples from subject 1.*

Subject 1	Subject 1 Profile	Fingerprint Paper		Fingerprint Glass	
		Unpurified	Purified	Unpurified	Purified
D8S1170	12, 14	*/*	12, 14/12, 14	*/*	12, 14/12, 14
D21S11	30, 31	*/*	30, 31/30, 31	*/*	30/29, 30, 31
D7S820	8, 11	*/*	11/8, 11	*/*	8/11
CSF1PO	10, 12	*/*	10, 12/10, 12	*/*	10, 12/*
D3S1358	16, 16	16/16	16/16	16/16	16/16
TH01	6, 8	8/*	6, 8/6, 8	*/*	8/6
D13S317	11, 11	11/*	11/11	*/*	11/11
D16S539	9, 13	*/*	9, 13/9, 13	*/*	9, 13/9
D2S1338	25, 25	*/*	25/25	*/*	25/*
D19S433	15, 15	15/15	15/15	*/*	15/14, 15
vWA	15, 17	*/15	15, 17/15, 17	*/*	15, 17/15, 17
TPOX	8, 11	*/*	8, 11/8, 11	*/*	11/ 11
D18S51	12, 12	*/*	12/12	*/*	12/*
AMEL	X Y	X/X	XY/XY	*/*	XY/XY
D5S818	11, 12	12,/ 11	11, 12/11, 12	*/*	11, 12/11, 12
FGA	21, 24	*/*	21, 24/21, 24	*/*	24/21, 24

PCR, polymerase chain reaction.
*Represents no data obtained. The results of each duplicate amplification are separated by a "/" in the columns.

Allele calls obtained from duplicate amplifications are reported in Tables 4–6. In these experiments the entire purified product was injected.

The purified fingerprint (glass) samples from subjects 2 and 3 exhibited elevated stutter at D5S818 and D2S1338, even upon duplicate amplification. This is consistent with the results of the previous stutter study of purified samples in which both D5S818 and D2S1338 exhibited elevated stutter. PHRs for the mock casework samples were calculated in the same manner as the heterozygote PH imbalance study ($PHR = PH_{low}/PH_{high}$). The average PHR ranged from 0.53 to 0.62 with standard deviations from 0.21 to 0.28. Equivalent PHRs and standard deviations were observed in purified samples with 5–20 pg of input DNA.

The subject 3 fingerprint (paper) results indicated the presence of a contaminating profile in the purified extracts (Table 6). Contamination was reflected in both amplifications and may have arisen from secondary transfer, a contaminant in the substrate or extraction tubes, or laboratory contamination. The contaminating alleles were not concordant with subjects 1 and 2 or with laboratory personnel. Two alleles were detected in a purified fingerprint reagent blank, but did not appear in the duplicate amplification. These alleles were not concordant with the contaminating profile.

No alleles were detected in the telogen root hair samples before purification. After purification, a total of eight alleles, concordant with the known profiles, were detected in the six amplifications (i.e., samples from three subjects, each with duplicate amplifications). However, no allele was reproduced in the duplicate amplification (data not shown). No contamination was detected in the purified negative amplification control or hair reagent blank.

Discussion

The intent of this study was to evaluate different post-PCR purification methods in an attempt to improve the analytical sensitivity of standard STR typing. Four different PCR purification methods were evaluated and, based upon the purity of the eluate obtained,

TABLE 5—*Comparison of unpurified and purified PCR product in mock casework samples from subject 2.*

Subject 2	Subject 2 Profile	Fingerprint Paper		Fingerprint Glass	
		Unpurified	Purified	Unpurified	Purified
D8S1170	13, 15	*/*	13, 15/13, 15	*/*	13, 15/13, 15
D21S11	29, 31.2	*/*	29, 31.2/31.2	*/*	29, 31.2/29, 31.2
D7S820	11, 11	*/*	*/11	*/*	11/ 11
CSF1PO	11, 11	*/*	11/11	*/*	11/ 11
D3S1358	14, 16	14/*	14, 16/14, 16	*/*	14, 16/14, 16
TH01	7, 9.3	*/*	7, 9.3/7, 9.3	*/*	7, 9.3/7, 9.3
D13S317	9, 11	*/*	9, 11/11	*/*	9, 11/9, 11
D16S539	13, 13	*/*	13/13	*/*	13/13
D2S1338	17, 20	*/*	17, 19, 20/20	*/*	17, 20/17, 20
D19S433	12, 13	13/*	12, 13/12, 13	*/*	12, 13/12, 13
vWA	16, 18	*/*	16, 18/16, 17, 18	*/*	16, 18/16, 18
TPOX	8, 11	*/*	8, 11/8	*/*	8, 11/8
D18S51	12, 13	*/*	12, 13/12, 13	*/*	12, 13/12, 13
AMEL	X Y	*/*	XY/Y	*/*	X/XY
D5S818	12, 12	*/12	11, 12/12	*/*	11, 12/11, 12
FGA	20, 23	*/*	20, 23/20, 23	*/*	20, 23/20

PCR, polymerase chain reaction
*Represents no data obtained. The results of each duplicate amplification are separated by a "/" in the columns.

TABLE 6—*Comparison of unpurified and purified PCR product in mock casework samples from subject 3.*

Subject 3	Subject 3 Profile	Fingerprint Paper		Fingerprint Glass	
		Unpurified	Purified	Unpurified	Purified
D8S1170	9, 15	*/*	9, 12	*/*	9, 14, 15/9, 15
D21S11	29, 29	*/*	29, 31	*/*	29/*
D7S820	8, 10	*/*	10	*/*	8, 10/8, 10
CSF1PO	12, 13	*/*	*	*/*	12/12, 13
D3S1358	15, 15	*/*	15, 16, 18	*/*	15/15
TH01	6, 9	*/*	6, 9, 9.3	*/*	*/6, 9
D13S317	12, 13	*/*	10, 13	*/*	12, 13/12, 13
D16S539	11, 12	*/*	10, 11, 12	*/*	11, 12/11
D2S1338	19, 20	*/*	19, 27	*/*	18, 19/18, 19, 20
D19S433	12, 16	*/*	13, 13.2, 16	*/*	16/12, 16
vWA	17, 18	*/*	17, 18, 19	*/*	18/17, 18
TPOX	8, 11	*/8	11	*/8	8/8, 11
D18S51	12, 15	*/*	*	*/*	12, 15/12, 15
AMEL	X X	*/*	X	*/*	X/XY
D5S818	11, 12	*/*	11, 12, 13	12/*	11, 12/*
FGA	19, 25	*/*	*	*/*	19, 25/*

PCR, polymerase chain reaction

*Represents no data obtained. The results of each duplicate amplification are separated by a ''/'' in the columns. Only one amplification is reflected in the fingerprint on paper purified column due to a sample migration issue.

effect on signal intensity, and ease of use, the Qiagen MinElute silica column was selected for detailed study. Purified PCR product using this method produced a fourfold increase in fluorescent signal intensity over unpurified product. Furthermore, by adding the entire concentrated purified PCR product, a 19-fold increase in signal intensity can be expected. Hutchinson (19) subjected microsatellite PCR products to Sephadex™ purification and briefly examined its effect using capillary electrophoresis. A 3.5-fold increase in signal intensity was reported, comparable with our observations of a fourfold increase using the unconcentrated purified product.

Post-PCR purification with the MinElute column can greatly enhance the sensitivity of the PCR process obtaining full profiles down to 20 pg input template DNA and generating significant data down to 5 pg without increasing amplification cycles. This purification method is simple, inexpensive, and can be accomplished in about 15 min. By adjusting the volume of eluate and the amount of purified product injected, the sensitivity of this technique can be controlled. Thus post-PCR purification fits easily into the flow of casework and can be used to enhance allelic signals beyond the threshold in a weak sample or as a technique for *bona fide* LCN analysis. Of course, as manipulation of PCR products is required, the method should be conducted in a room dedicated to post-PCR procedures.

The detection of a foreign profile in the mock casework samples implies the greatest risks for contamination that occurs in the collection and extraction process as opposed to amplification, purification, and electrophoresis setup. Thus, the need for strong contamination prevention guidelines is warranted. Incidents of contamination were not observed in the negative amplification controls as reported by some using increased amplification cycles (11,18). A single incident of contamination was detected in a mock casework sample reagent blank, but was not duplicated upon reamplification. No contamination was detected in any other reagent blank. Although great care was taken to observe good laboratory practices, these studies were carried out in a case-working laboratory without specialized precautions to prevent contamination. This suggests that there is less risk of detecting adventitious DNA with PCR purification using 28 cycles than with increased cycle amplifications.

The increased incidences of stutter, heterozygote imbalance and allelic drop-in are in accordance with the observations of Gill et al. (12–14,16,22) and support the necessity for having appropriate interpretation guidelines for LCN-generated profiles. It is possible that a combination of PCR purification and increased cycle number may permit complete profiles to be obtained from 5 pg templates with greater allele fidelity than simply increasing the cycle number alone.

Laboratory Guidelines for post-PCR purification using the MinElute column are provided in Table 7. As amplification efficiency can vary from sample to sample, these guidelines are based upon RFU observed rather than input quantities of DNA. Laboratories should establish at what point LCN procedures and interpretation guidelines should be applied, and perform validation studies before implementation on casework. After standard 28-cycle PCR amplification, samples should be injected prior to post-PCR purification. To minimize the risk of off scale data and maximize allelic information obtained, electropherograms should be examined to determine the highest above threshold and lowest below threshold PHs. A purification strategy based upon PHs (RFU) observed can be

TABLE 7—*Strategy for post-PCR purification of samples.*

Pre-Purification Peak Heights	≥50 and ≤1000 RFU	<600 RFU	<300 RFU
Volume of eluate	25 μL	10 μL	10 μL
Volume of formamide	25 μL	15 μL	15 μL
Volume of size standard	0.1 μL	0.1 μL	0.2 μL
Volume of purified PCR product	1.5 μL	1.5 μL	10 μL
Average increase in fluorescent signal	4-fold range (3–5)	* 6.5-fold range (5–8)	19-fold range (17–22)

PCR, polymerase chain reaction

*Data not normalized.

selected from Table 7. The efficiency of the purification can vary from sample to sample and the FI in fluorescent signal intensity can vary across alleles in a profile. If needed, fluorescent signal intensity can be optimized by increasing or decreasing the amount of purified product in the formamide mix. Up to a 50:50 mix of purified product and formamide have been injected in this study with good quality results. In addition, injection times may be altered to optimize results.

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