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TECHNICAL NOTE

Utility of amplification enhancers in low copy number DNA analysis

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Abstract One parameter that impacts the robustness and reliability of forensic DNA analyses is the amount of template DNA used in the polymerase chain reaction (PCR). With short tandem repeat (STR) typing, low copy number (LCN) DNA samples can present exaggerated stochastic effects during the PCR that result in heterozygote peak height imbalance, allele drop out, and increased stutter. Despite these effects, there has been little progress toward decreasing the formation of stutter products and heterozygote peak imbalance effects during PCR. In an attempt to develop a more robust system that is less refractory to stochastic effects, the PCR additives, betaine, DMSO, PEG, and PCRboost®, were investigated on low-quantity DNA samples. The effects of the additives were assessed by evaluating STR typing results. Of the four additives, the only positive effects were observed with betaine treatment. Betaine, at a final concentration of 1.25 mol/L, was found to improve the robustness of the amplification, specifically by decreasing stutter in a dual locus system. In contrast, the addition of 1.25 mol/L betaine to commercial STR amplification kits did not affect stutter ratios. However, the addition of betaine did lead to increased yield of PCR products in all commercial kits tested. The results support that betaine can improve amplification efficiency of LCN DNA samples.

Keywords PCR enhancer · Betaine · Stochastic effects · STR typing · LCN DNA

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Introduction

The examination of samples with low quantities of template DNA commonly referred to as "low copy number" (LCN) or low template DNA analysis has a major limitation: stochastic effects during the polymerase chain reaction (PCR) are exacerbated, causing heterozygote peak height imbalance, allele drop out, and increased stutter (i.e., artifacts due to slippage during the PCR). All these phenomena can complicate interpretation of LCN profiles. A potential approach to improve robustness of amplification of low template DNA is to modify the PCR by use of additives which effectively concentrate the target and enzyme (i.e., volume excluders), alleviate the paused extension of primer, stabilize the enzyme, and/or reduce instability of the template strand. Robustness of amplification can be measured by reduced stutter values, better heterozygote balance, and increased PCR product yield. A variety of PCR additives and enhancing agents have been the focus of efforts to improve amplification and stringency [1–15]. The most successful of the additives tested has been dimethyl sulfoxide (DMSO), glycerol, polyethylene glycol (PEG), betaine, and formamide. Weissersteiner et al. [16] first introduced the glycine betaine as a powerful PCR additive to counteract effects of NaCl and other high-salt inhibition of *Taq* polymerase. Since then, betaine has been used as a PCR facilitator, not only as a single compound, but also in combination with other additives [1–9, 11–13, 17–26]. Betaine is believed to facilitate PCR via strand separation, lowering melting temperature (T_m) and acting as an isostabilizing agent, equalizing the contribution of GC- and AT-base pairings to the stability of the DNA duplex [13, 21]. Furthermore, certain DNA sequences can cause the DNA polymerase to pause, a phenomenon that can be counteracted by betaine. It has been suggested that betaine disrupts the contorted DNA helix without perturbing the polymerase-DNA interaction [22]. In fact, betaine has been used to enhance formation of long PCR

products, in diagnostic PCR, on GC rich template, and in low-temperature PCRs [22]. Another advantage of using betaine is that it acts as an osmoprotectant, increasing the resistance of the polymerase to denaturation [1]. Betaine also allows the PCR to overcome some low level of contaminants that can co-purify with DNA, allowing for PCR of low-quality DNA samples [22].

Other potential PCR enhancers include PEG and DMSO. PEG is an additive which effectively concentrates the target and enzyme, acting as a volume excluder or molecular crowder [27–31]. DMSO is thought to assist in amplification by reducing secondary structure, facilitating strand separation by disrupting base pairing, which is particularly useful for GC rich templates [11, 18]. Recently, PCRboost® (Biomatrica, San Diego, CA, USA), a novel additive, became commercially available. Previous studies have shown that PCRboost® has the ability to enhance yield as much as five-fold, specificity, and consistency of the PCR [32].

There has been little research, however, on whether these additives can overcome some of the negative stochastic effects of LCN typing. Although these additives may have beneficial effects on some amplification systems, it is impossible to predict which agents will be useful in a particular context and therefore need to be tested. This paper investigated the effects on amplification of low-quantity DNA samples and STR products in the presence of betaine, DMSO, PEG, and PCRboost®.

Materials and methods

Buccal swabs

Buccal swabs of 100 individuals were obtained and stored at room temperature until extraction. All samples were collected with informed consent and were anonymized to ensure the privacy of the contributing subjects in accordance with the University of North Texas Health Science Center IRB.

DNA extraction

AutoMate Express™ Forensic DNA Extraction System (Life Technologies, Carlsbad, CA, USA) was performed according to manufacturer instructions. The DNA from the buccal swabs was extracted using the PrepFiler Express™ Forensic DNA Extraction Kit according to manufacturer instructions. The DNA extracts obtained were stored at 4 and −20 °C for short- and long-term storage, respectively.

PCR additives

Each additive was placed in the PCR at a final concentration as follows:

- Betaine (Sigma, St. Louis, MO, USA): 0, 0.5, 1.25, and 2 mol/L.
- Dimethyl sulfoxide (Sigma): 0, 1, 5, and 10 %.
- Mixtures of betaine and DMSO: 1.25 mol/L betaine and 5 % DMSO.
- Polyethylene glycol (PEG 8000) (Promega, Madison, WI, USA): 0, 1, 2.5, and 5 %.
- PCRboost® according to manufacturer's instructions (7.5 μl added to replace final water volumes in the amplification reaction mix) [33].

Primers

D18S51 and D21S11 primer information was provided kindly by Life Technologies (Life Technologies, Carlsbad, CA, USA), and primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). The forward primer for the D18S51 locus was fluorescently labeled with FAM. The forward primer for the D21S11 locus was fluorescently labeled with JOE. The reverse primers were not labeled. Primer concentrations were optimized to obtain comparable signal of D18S51 and D21S11 products, resulting in a final concentration of 0.25 μM for each primer.

DNA quantification

The quantity of extracted DNA was determined using a reduced volume protocol of the Applied Biosystems® Quantifiler™ Human DNA Quantification Kit (Life Technologies, Foster City, CA, USA) on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies). Negative (no template DNA) and reagent blank controls were included on each assay plate. Samples then were normalized to 25 and 100 pg/μL (amounts routinely considered low copy number).

DNA amplification

For amplifications of the D18S51 and D21S11 duplex, thermal cycling was performed on a GeneAmp® PCR System 9700 (Life Technologies) as follows: initial denaturation at 95 °C for 11 min; 34 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min; hold at 60 °C for 60 min; and an indefinite hold at 4 °C. Amplifications for commercially available STR kits were performed according to the manufacturer's instructions but with six additional PCR cycles. For the AmpFℓSTR® Identifiler® kit (Life Technologies), thermal cycling was performed on a GeneAmp® PCR System 9700 (Life Technologies) as follows: initial denaturation at 95 °C for 11 min; 34 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min; hold at 60 °C for 60 min; and an indefinite hold at 4 °C. For the AmpFℓSTR® Identifiler® Plus kit (Life Technologies), thermal cycling was performed on a

GeneAmp® PCR System 9700 (Life Technologies) as follows: initial denaturation at 95 °C for 11 min; 34 cycles of 94 °C for 20 s, 59 °C for 3 min; hold at 60 °C for 10 min; and an indefinite hold at 4 °C. For the PowerPlex® ESI 17 Pro System (Promega Corporation, Madison, WI, USA), thermal cycling was performed on a GeneAmp® PCR System 9700 as follows: initial denaturation at 96 °C for 2 min; 36 cycles of 94 °C for 30 s, 59 °C for 2 min, and 72 °C for 90 s; hold at 60 °C for 45 min; and an indefinite hold at 4 °C. Positive (9947A), negative (no template DNA), and reagent blank controls also were included on each assay plate.

Capillary electrophoresis

Capillary electrophoresis was performed on an Applied Biosystems® 3130xl Genetic Analyzer (Life Technologies) using POP-4™ polymer (Life Technologies), and data were analyzed using Applied Biosystems® GeneMapper® ID v3.2 software (Life Technologies), according to the manufacturer's recommended protocol. For this study, the analytical threshold was set at 25 relative fluorescence unit (RFU) to capture as many stutter peaks as possible.

Data analysis

PCR product yield (based on RFU), peak height ratio (PHR) for heterozygous loci, and proportion of stutter and variance of these ratios were evaluated and compared with controls using an in-house program designed using Microsoft® Excel. Intra-locus PHRs were calculated for a given locus by dividing the peak height of an allele with a lower RFU value by the peak height of an allele with a higher RFU value and then multiplying this value by 100 to express the PHR as a percentage. Stutter percentages were calculated by dividing the peak height of the stutter allele (generally $n-4$ position) by the peak height of the true allele and then multiplying this value by 100 to express the stutter as a percentage.

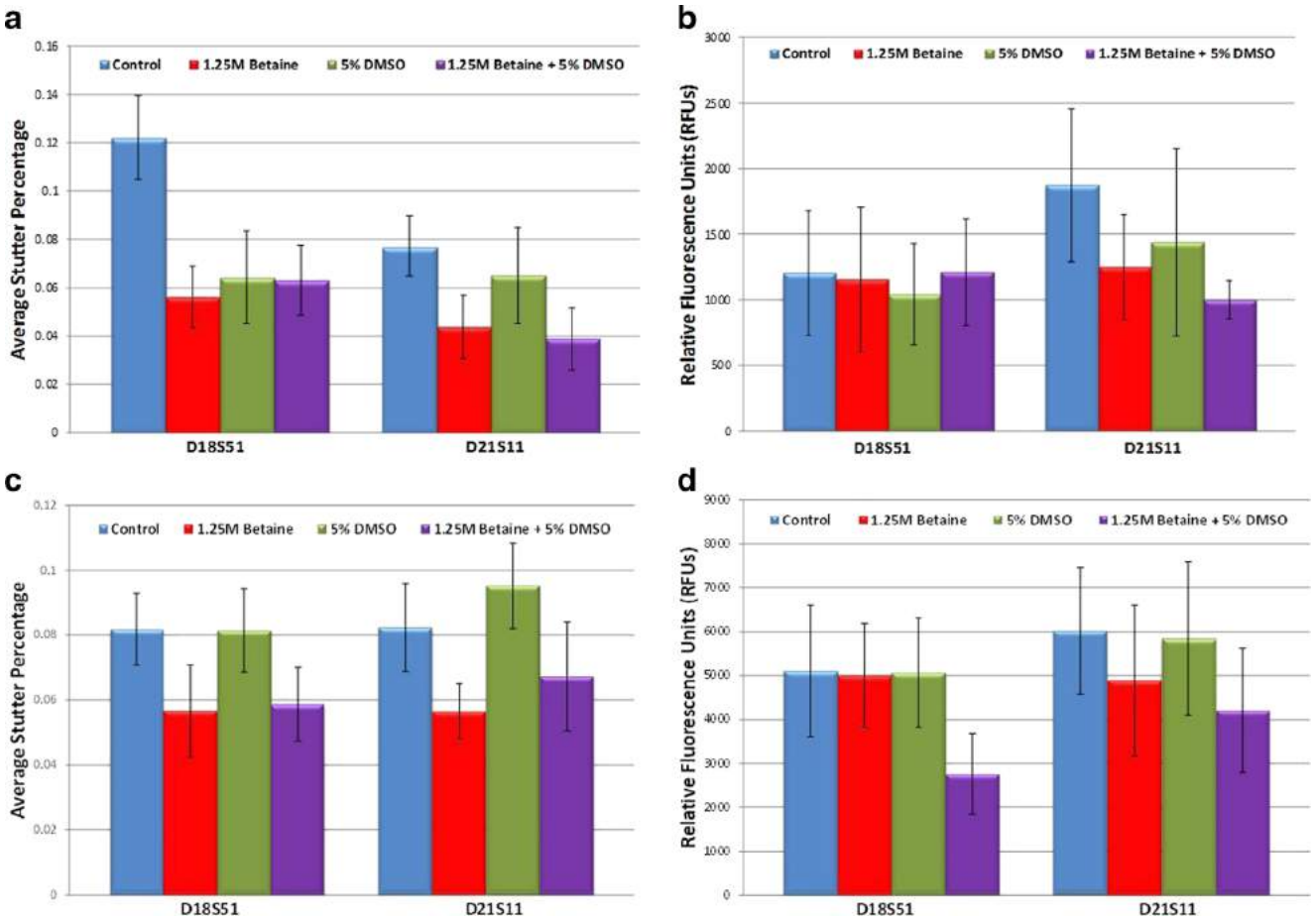

Fig. 1 DNA from five different individuals at 25- or 100-pg total DNA were added to custom duplex reaction mixes containing the following: control—no PCR enhancer, 1.25 mol/L betaine, 5 % DMSO, or a mixture of 1.25 mol/L betaine and 5 % DMSO. Samples were amplified in triplicate. Average stutter percentages and RFUs of alleles were calculated. **a** Average stutter percentage—25 pg; **b** average peak height—25 pg; **c** average stutter percentage—100 pg; and **d** average peak height—100 pg

Table 1 Average stutter percentage for control and betaine treated samples

	D18S51 betaine stutter	D18S51 betaine true allele	D18S51 control stutter	D18S51 control true allele	D21S11 betaine stutter	D21S11 betaine true allele	D21S11 control stutter	D21S11 control true allele
25 pg								
Average RFUs	63	720	90	889	70	1,024	89	1,054
STDEV	48	534	70	637	64	864	71	833
Stutter (%)	0.06		0.1		0.06		0.08	
STDEV	0.03		0.05		0.02		0.03	
100 pg								
Average RFUs	116	1,735	177	1,757	152	2,347	214	2,421
STDEV	87	1,395	127	1,249	133	1,721	169	1,788
Stutter (%)	0.05		0.09		0.07		0.08	
STDEV	0.02		0.05		0.09		0.03	

RFUs relative fluorescence units, *STDEV* standard deviation

Results and discussion

Effect of DMSO and betaine on a two-locus multiplex

To determine the potential for reduction of stochastic effects in LCN DNA samples, the effects of betaine and DMSO were evaluated separately and in combination. These reagents initially were tested with a limited sample size of five to establish concentration and reagent combination parameters which would then be explored in a larger evaluation study. A two-locus multiplex (D18S51 and D21S11) was developed to test the impact of the additives. These loci were selected because

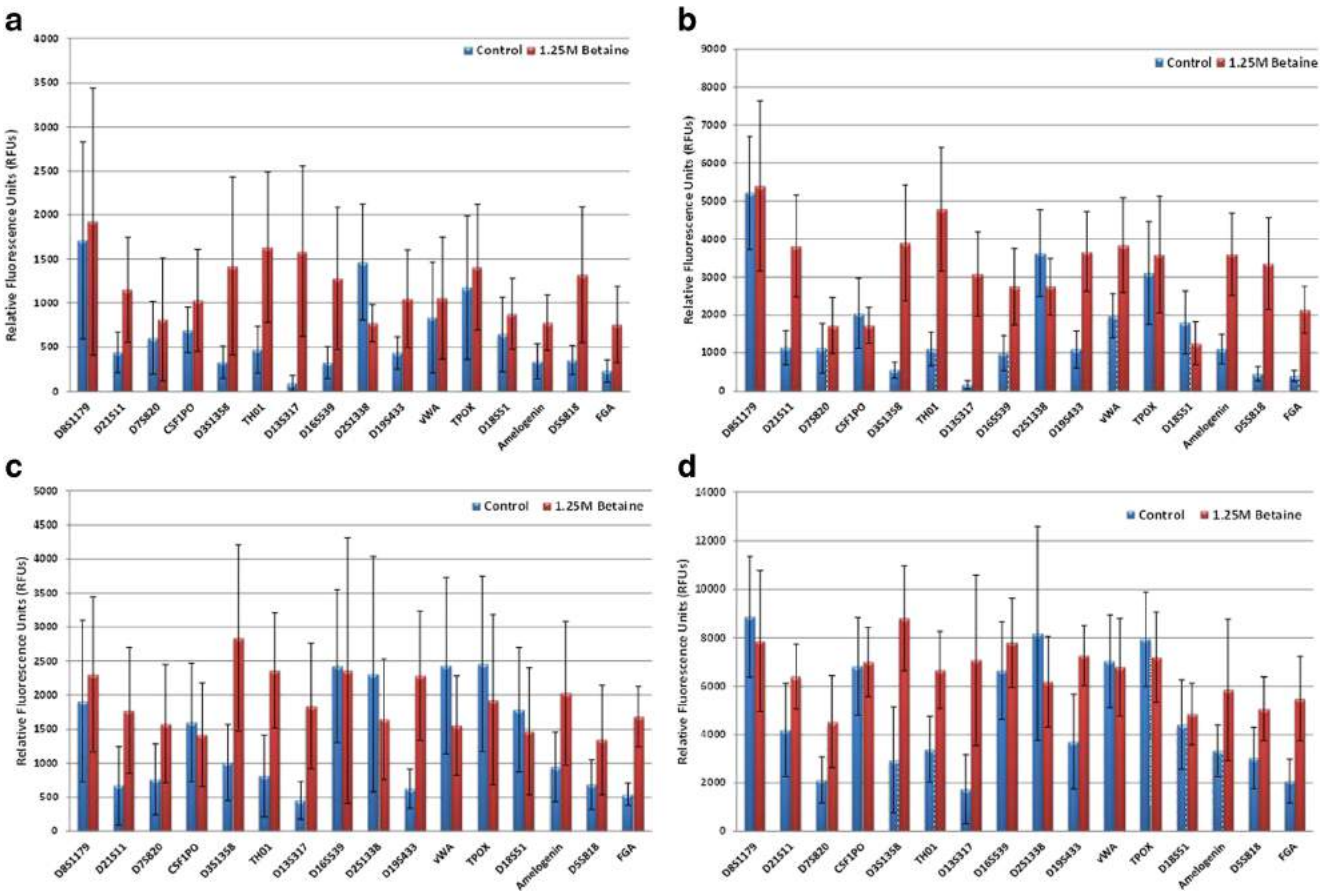

Fig. 2 DNA from ten different individuals at 25- and 100-pg total DNA were added to Identifiler® or Identifiler® Plus amplification mix containing the following: control—no PCR enhancer or 1.25 mol/L betaine. Samples were amplified in triplicate. Average RFUs were calculated. **a** Identifiler®—25 pg; **b** Identifiler®—100 pg; **c** Identifiler® Plus—25 pg; and **d** Identifiler® Plus—100 pg

they tend to display higher amounts of stutter than other loci [34]. Preliminary testing was carried out using 0.5, 1.25, and 2 mol/L of betaine and 1, 5, and 10 % of DMSO to determine the best concentrations for further investigation. The concentration of 1.25 mol/L betaine and 5 % DMSO were found to have the most positive effects on stutter and thus were pursued with further testing (data not shown). Higher concentrations of betaine (2 mol/L) and DMSO (10 %) had no observable differences on STR typing results. In fact, in high amounts, DMSO can reduce *Taq* polymerase activity by up to 50 % [21].

DNA template amounts of 25 or 100 pg were amplified in reactions with and without additives: control—no PCR additive, 1.25 mol/L betaine, 5 % DMSO, and a mixture of 1.25 mol/L betaine and 5 % DMSO. The 1.25 mol/L betaine treatment (based on error bar distribution) significantly reduced stutter by approximately 50 % or more at both loci in the 25-pg samples and by 25 % in 100-pg samples (Fig. 1a and 1c, respectively). DMSO treatment of 5 % reduced stutter by 50 % and 16 % of the mean stutter value at the loci D18S51 and D21S11, respectively, in the 25-pg samples but showed no effect in reducing stutter at the D18S51 locus in the 100-pg samples (Fig. 1a and 1c, respectively). In fact, treatment with DMSO increased stutter at the D21S11 locus in the 100-pg samples (Fig. 1c). A mixture of 1.25 mol/L betaine and 5 % DMSO did not provide any observable difference than that observed with the betaine treatment alone, suggesting no benefit. While treatment with betaine reduced stutter, no differences were observed on average allele peak height among the treatment groups in either 25- or 100-pg samples at the D18S51 locus, but a reduction in signal was observed at the D21S11 locus (Fig. 1b, d).

Based on these results, only betaine was selected for further study. DNA from 81 individuals at two known quantities (25 and 100 pg/μL) were amplified for the same two-locus multiplex in the presence of 1.25 mol/L betaine and compared with a no betaine control. Although not significant, reduction in stutter was observed for betaine-treated 25- and 100-pg samples at both loci, with the effect of betaine greater at the D18S51 locus (Table 1).

Effects of betaine on Identifiler and Identifiler Plus

The effect of betaine on two commercially available forensic identification kits, AmpFℓSTR® Identifiler® and AmpFℓSTR® Identifiler® Plus (Life Technologies) was assessed on a limited sample size of ten individuals.

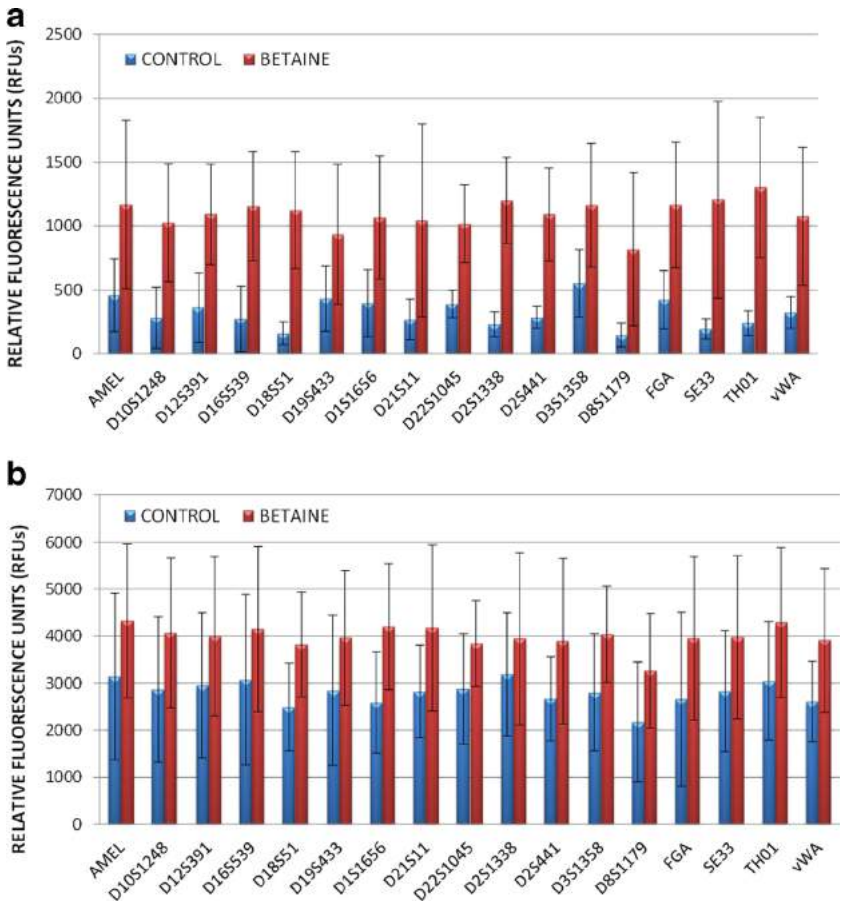

Fig. 3 DNA from 81 individuals at 25 and 100 pg were added to PowerPlex® ESI 17 Pro System amplification mix containing the following: control—no PCR enhancer or 1.25 mol/L betaine, and amplified. Samples were amplified in triplicate. Average RFUs were calculated. **a** 25 pg and **b** 100 pg

Amplification reaction mixes were prepared for control (no betaine) and a final concentration of 1.25 mol/L betaine test groups on either 25- or 100-pg total template DNA. The effect of betaine treatment on peak height of 25- and 100-pg samples for Identifiler® and Identifiler® Plus amplifications are shown in Fig. 2a–d. A trend of higher RFU values was observed for most loci with both 25- and 100-pg total DNA samples following betaine treatment using the Identifiler® kit (Fig. 2a and 2b, respectively) and Identifiler® Plus amplification kit (Fig. 2c and 2d, respectively) compared with controls. The effect of betaine treatment on RFU values appeared to be greater for several loci (D5S818, TH01, D13S317, D21S11, and D3S1358) in both the Identifiler® and Identifiler® Plus amplification kits.

No reduction on stutter was observed following betaine treatment for either the 25- or 100-pg total DNA samples using the Identifiler® and Identifiler® Plus amplification kits (data not shown). This observation is not consistent with the duplex data. It may be that the Identifiler® and Identifiler® Plus kits already contain some additives (proprietary information not available to us) that maximize the benefits of stutter reduction.

Regarding allele recovery, the total number of observed alleles (combined for ten individuals) was compared with the total number of actual alleles (combined for ten individuals). At 25-pg total input DNA, betaine treatment yielded 83 % allele recovery, while approximately 82 % of alleles were recovered in the control group using the Identifiler® amplification kit (data not shown). With Identifiler® Plus, betaine treatment showed a total allele recovery of 82 % compared with 75 % in the control group at 25-pg template DNA. At 100-pg total input DNA, betaine treatment yielded 100 % allele recovery using both the Identifiler® and Identifiler® Plus amplification kits, while the control group had an average percentage of total allele recovery of 99 % for both amplifications (data not shown).

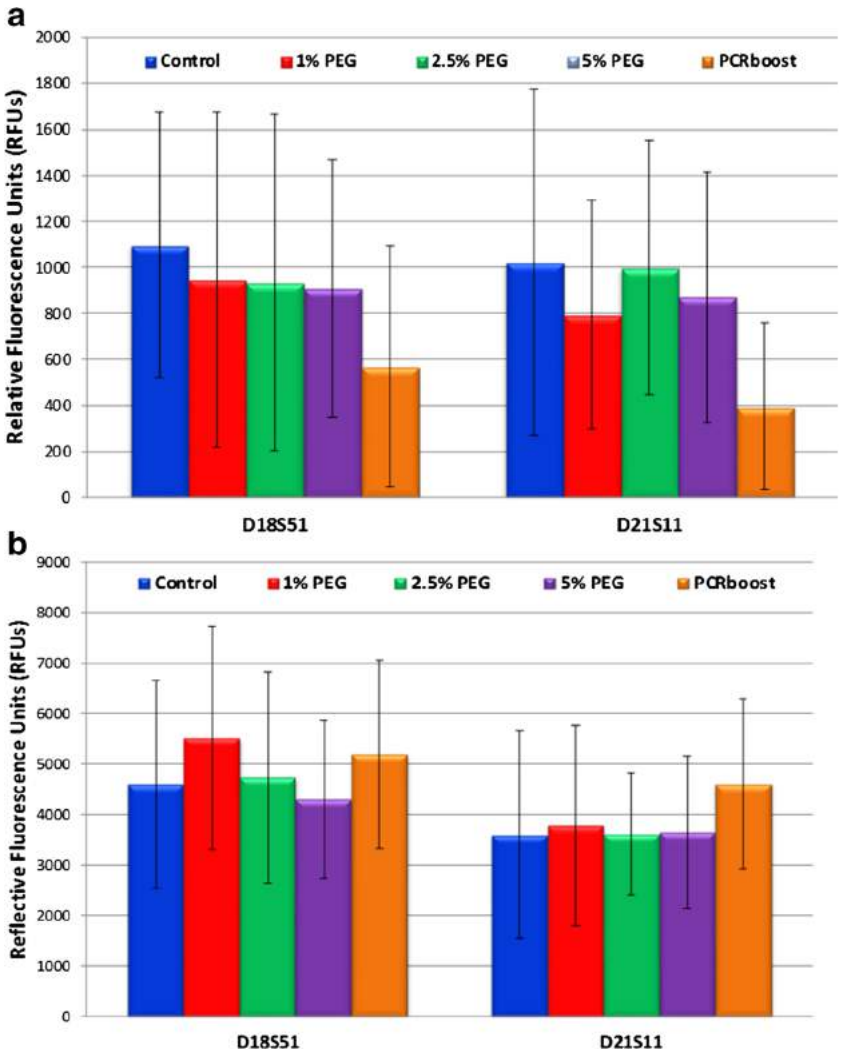

Fig. 4 DNA from five different individuals at 25- or 100-pg total DNA were added to custom duplex reaction mixes containing the following: control—no PCR enhancer, 1 % PEG, 2.5 % PEG, 5 % PEG, and PCRboost®, and amplified. Samples were amplified in triplicate. Average RFUs were calculated. **a** 25 pg and **b** 100 pg

Effect of betaine on PowerPlex® ESI 17 Pro System—larger evaluation study

A larger study then was performed using 25- and 100-pg DNA from buccal samples from 81 individuals using a different commercial kit, i.e., PowerPlex® ESI 17 Pro System. No differences in stutter percentages were observed following betaine treatment for either the 25- or 100-pg total DNA samples (data not shown). However, betaine treated samples displayed increased RFU values for the 25- and 100-pg samples (Fig. 3a, b). Betaine treatment of 25-pg samples yielded higher total alleles recovered and hence more complete profiles compared with no treatment. For the 100-pg samples, betaine treatment yielded 73 (of 81) complete profiles, while the control group only yielded 23 complete profiles. The data described above suggested that betaine treatment enhances PCR product yield based on increased allele peak heights and increased number of complete profiles observed when compared with no treatment.

Effect of PEG and PCRboost®

The effectiveness of PEG and PCRboost® on the PCR of LCN DNA samples with the D18S51 and D21S11 duplex also was tested. There were no improvements following treatment with either PEG or PCRboost® on the amplification of LCN DNA samples. For the 25-pg samples, PEG and especially PCRboost® treatment reduced peak height values (Fig. 4a). For the 100-pg samples, 1 % PEG and PCRboost® treatment yielded slightly higher average peak height values, while no changes were observed at higher concentrations of PEG (Fig. 4b). No positive improvements were observed in PHR values at either the 25- or 100-pg samples. No reductions in stutter were observed with either PEG or PCRboost® treatment compared with controls (data not shown). In fact, in low concentrations, PEG slightly increased the stutter at the D18S51 locus in samples at the lower DNA quantity of 25 pg.

The effect of PEG and PCRboost® on a commercially available forensic identification kit, AmpFℓSTR® Identifiler® Plus (Life Technologies), was performed on a limited sample

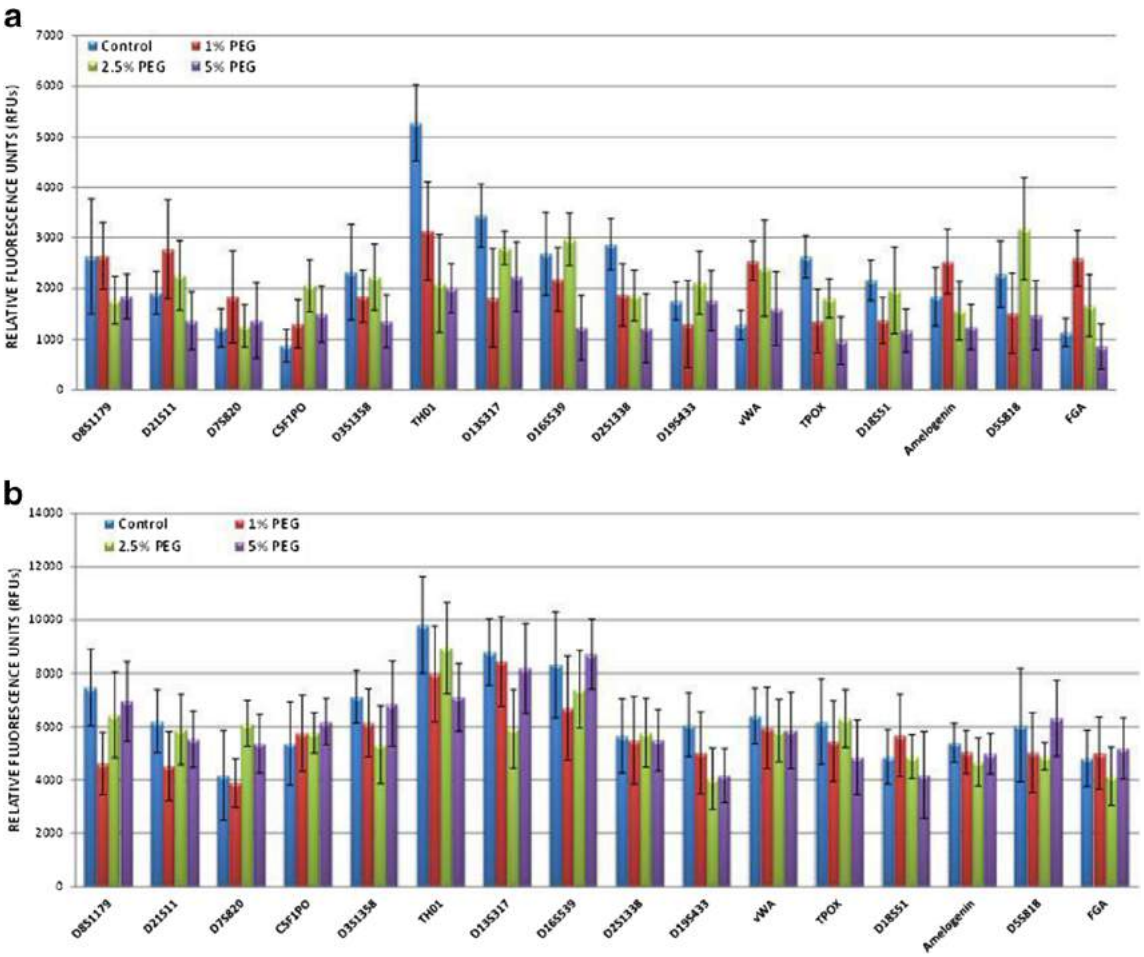

Fig. 5 DNA from five different individuals at 25- or 100-pg total DNA were added to Identifiler® Plus amplification mix containing the following: control—no PCR enhancer, 1 % PEG, 2.5 % PEG, and 5 % PEG, and amplified. Samples were amplified in triplicate. Average RFUs of alleles were calculated. **a** 25 pg and **b** 100 pg

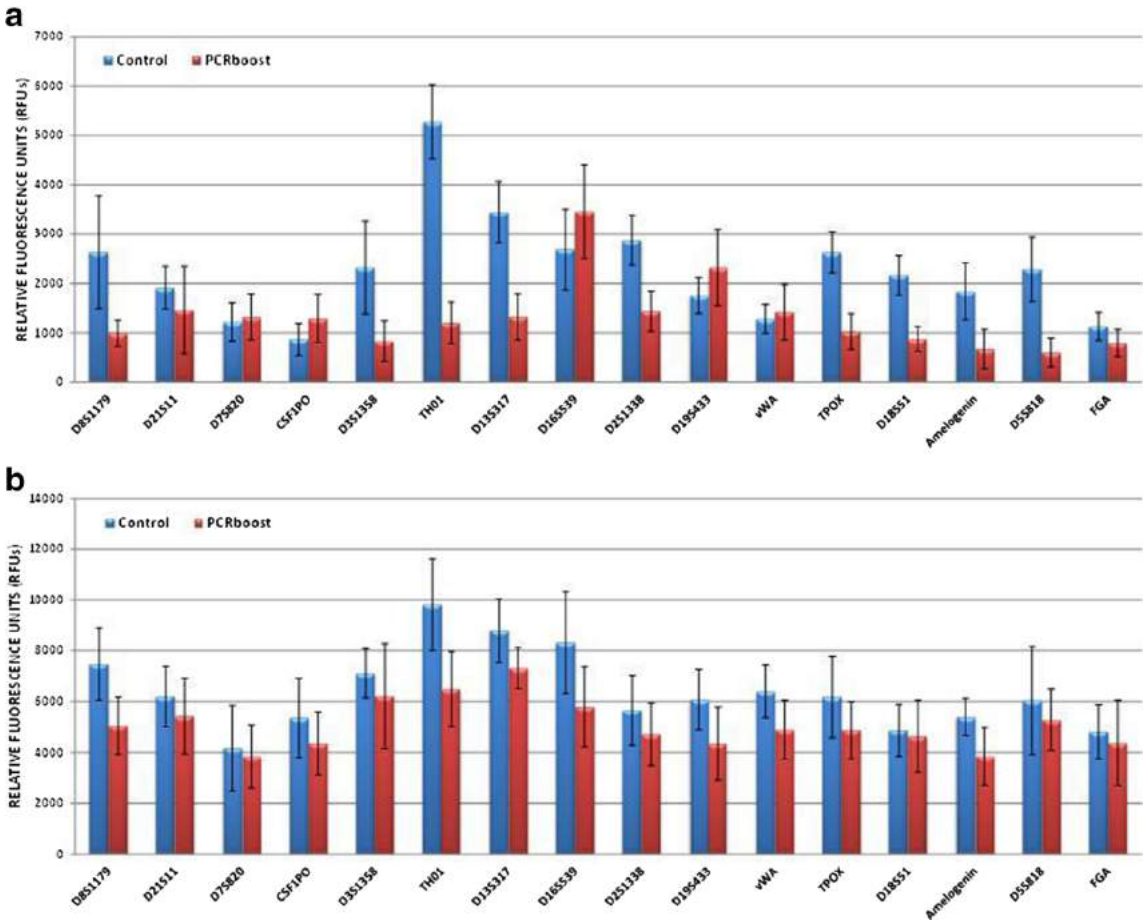

Fig. 6 DNA from five different individuals at 25- or 100-pg total DNA were added to Identifiler® Plus amplification mix containing the following: control—no PCR enhancer or PCRboost® and amplified. Samples were amplified in triplicate. Average RFUs of alleles were calculated. **a** 25 pg and **b** 100 pg

size of ten individuals. The effect of PEG and PCRboost® treatment on average allele peak height of 25- and 100-pg samples is shown in Figs. 5 and 6, respectively. No improvements in peak height were observed with either PEG or PCRboost® treatment. In fact, PCRboost® treatment decreased peak heights on average compared with the control, notably at some loci (Fig. 6). Additionally, stutter was not reduced and in some instances, increased, for 25- and 100-pg samples (data not shown). The results indicated that in our hands, neither PEG nor PCRboost® improved the amplification of LCN DNA samples.

Conclusions

This study showed that addition of betaine can increase the yield of PCR products in LCN samples in the two-locus multiplex and all commercial kits tested. Contrary to previous studies, the other additives had no impact on PCR product yield [27, 33], and in fact, PCRboost® had a negative impact. While betaine treatment showed an initial promise with decreasing stutter with the duplex, this effect was not observed in the commercial kits. In fact, significant reductions in stutter were not observed with any PCR additive tested. Seo et al. [35] demonstrated some reduction in stutter peaks by lowering the annealing/extension temperature to 56 °C. To date, this is the only method that has shown any potential in the reduction of stutter peaks while maintaining number of detected alleles and peak heights. Addition of betaine may be able to reduce the number of PCR cycles used; however, overall imbalance in peak height ratio will likely still persist. The data herein are consistent with other studies that it is quite difficult to reduce stutter or improve heterozygote peak height imbalance [36–39]. However, similar to other approaches, betaine treatment increased PCR product yield, resulting in reduced allele dropout and better representation of the true DNA profile [37, 40–43]. Therefore, betaine may be another consideration for enhancing allele detection of the PCR of LCN DNA samples.

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The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice.

Conflict of interest The authors PLM, JLK, and BB declare that they have no conflict of interest.

Ethical standards The work described above was performed in accordance with all laws (both Federal and State) that apply to research, researcher conduct, and the protection of human test subjects. We also operate under the guidance of and in accordance with the policies of the UNTHSC Institutional Review Board.

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