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TECHNICAL NOTE

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Improved specificity of Y-STR typing in DNA mixture samples

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Abstract Y-STR loci are beneficial for the analysis of forensic samples especially in sexual assault cases or other situations where mixtures of male and female cells are present. However, the amplification of Y-chromosomal STRs is also known to result in the formation of artefactual amplification products, mainly due to insufficient PCR specificity. This is a major drawback of the method, as the sensitivity as well as the correct Y-STR interpretation are affected. In our study, the addition of a PCR enhancer to the reaction master-mix resulted in a significant increase of specificity of Y-STR typing. This was clearly demonstrated by a loss of artefactual signal with increasing enhancer concentration, while the peak heights of the Y-STR alleles were not significantly affected by the enhancer. Mixtures of up to 1:500 (200 pg male and 100 ng female DNA) gave correct Y-STR profiles when the PCR enhancer was added to the reaction, while artefactual amplification succeeded over Y-specific amplification when no PCR enhancer was present.

Keywords Y-chromosomal STRs · PCR artefacts · PCR enhancer · Mixtures · Forensic

Introduction

The analysis of Y-chromosomal short tandem repeats (Y-STRs) has become a useful technique in forensic casework particularly for the investigation of sexual assault cases and in kinship testing [1, 2]. Sexual assault cases often involve mixture samples from one or more male contributors, which are to be identified against the background of a victim's component, which is female in the majority of cases. Mixtures of cells which cannot be separated by differential lysis [3] prior to DNA extraction are particularly problematic when only a minor male component is present [4]. As a rule of thumb, autosomal STRs only allow reliable identification of minor components in a mixture for ratios up to approximately 1 in 20. It has been shown that this ratio can be drastically increased with the use of Y-chromosomal STRs [5, 6, 7, 8, 9, 10, 11]. The authors described that they were able to detect male contributions in various male/female DNA mixtures even in the order of up to 1 in 4000. Some of these studies also report the occurrence of artefactual PCR products as a result of the amplification of X-chromosomal and/or autosomal DNA. Artefacts have also been observed in other Y-STR related publications not dealing with mixture samples, especially for multiplex PCR applications [12, 13, 14, 15, 16]. The formation of artefactual products has a negative impact on Y-STR analysis in two ways: firstly, they lead to problems for the correct interpretation of the Y-STR results when the fragment lengths overlap with those of actual alleles, secondly, their formation reduces the amplification efficiency of the correct PCR product leading to decreased PCR yield. The formation of artefactual amplification products can be suppressed by raising the specificity of the PCR conditions, e.g. by adding a PCR enhancer to the reaction master-mix. In the current study, we were interested whether or not the addition of a PCR enhancer to an established Y-STRs multiplex protocol [17] would help decrease artefactual amplification in male/female mixture samples.

Material and methods

The four Y-chromosomal STR loci, DYS19, DYS385, DYS392 and DYS393 were co-amplified in a multiplex PCR [17]. The total reaction volume was 25 µl including 1×PCR buffer II, 2 mM $MgCl_2$, 200 µM each dNTP, 2 U Amplitaq Gold polymerase (Applied Biosystems , Foster City, Calif.), and 0.5 µM each primer for DYS19, 0.1 µM each primer for DYS385, 0.5 µM each primer for DYS392 and 0.15 µM each primer for DYS393 (primer sequences according to [1]). A series of PCR master-mixes were produced containing different concentrations (0×, 0.25×, 0.5×, 0.75×, and 1×) of the PCRx Enhancer (Invitrogen, Life Technologies, Carlsbad, Calif.).

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The AmpF/STR control DNA 007 and control DNA CEPH 1347-10 were used as DNA sources known to originate from a male individual as well as control DNA CEPH 1347-02, known to originate from a female person (all Applied Biosystems). In the text these are referred to as "male DNA" and "female DNA". Mixtures of these DNA samples were prepared in ratios of 1:20, 1:100 and 1:500, combining 200 pg and 1 ng male DNA with 20 ng and 100 ng female DNA, respectively. The 1:100 ratio was produced using 2 different male DNA concentrations, 200 pg male DNA combined with 20 ng female DNA and 1 ng male DNA combined with 100 ng female DNA, respectively.

PCR was performed on a Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, Conn.) comprising 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min following initial denaturation at 95°C for 11 min and final incubation at 72°C for 30 min. Aliquots of 2 µl of the amplification products were combined with 20 µl deionized formamide including 1 µl internal lane standard (Genescan-500 ROX, ABI), heat-denatured at 95°C for 2 min, snap-cooled on ice, and subjected to electrophoresis on an ABI PRISM 310 Genetic Analyzer using POP 4 and default conditions. Data were analyzed using GeneScan Analysis (Version 2.1) and Genotyper (Version 2.5).

The correlation between the arithmetic means of the artefacts peak heights were calculated using Pearson's correlation coefficient (SPSS, Windows, Version 10.0.7). The correlation coefficient was considered to be significantly different from 0 when the significance level p_α was less than 0.05 (double-sided).

Results and discussion

A variety of additives are available which are known to increase the specificity and/or yield of PCR such as betaine [18], dimethyl sulfoxide (DMSO [19]) or tetramethyl ammonium chloride (TMAC, [20]). In a pilot study (data not shown) we tested DMSO and diverse commercially available PCR enhancing chemicals for their effectiveness to improve amplification and specificity of a Y-STR multiplex. What we aimed at was an adequate yield of the target DNA accompanying a minimization of undesired non-specific PCR products. These preliminary experiments identified the PCRx Enhancer as a promising option.

The PCRx Enhancer system is a PCR cosolvent that offers higher primer specificity, broader magnesium concentration optima, broader annealing temperature optima and improved thermostabilization of Taq DNA polymerase, as described by the manufacturers. It is also reported to improve multiplex PCR performance. In the current study, we tested the effects of the PCRx Enhancer on a multiplex PCR consisting of four Y-STR loci. Male and female DNA samples were amplified in different mixture ratios in order to test sensitivity and specificity of the reaction. Five different enhancer concentrations (0×, 0.25×, 0.5×, 0.75× and 1×) were used in order to reveal potential inhibitory effects of the PCRx Enhancer itself. The manufacturer recommends titration series up to 4.0×. In an initial experiment we observed strong reduction of Y-STR peak heights for the 2.0× enhancer concentration. This is why further experiments were carried out using an enhancer concentration range from 0× to 1.0×. All experiments were carried out at least in duplicate, the majority in triplicate format.

In order to test the sensitivity of the Y-STR multiplex 50 pg–2 ng male DNA were amplified. 200 pg male DNA

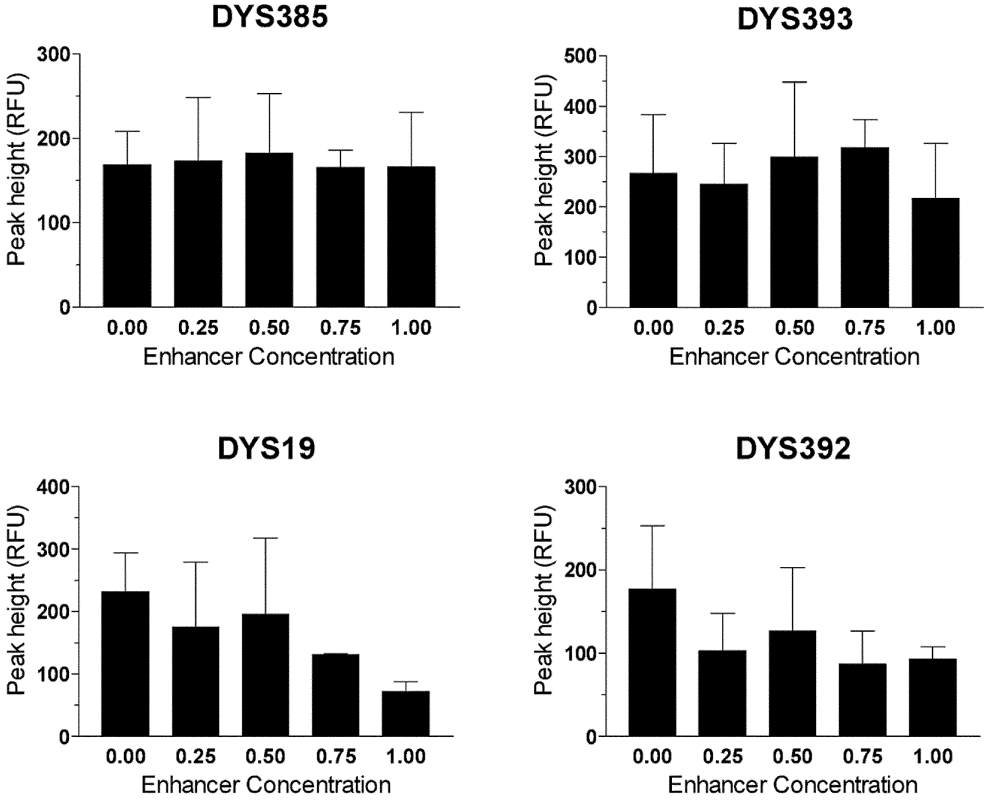

Fig. 1 Sensitivity of the Y-STR multiplex as a function of the PCRx Enhancer concentration. There was no significant correlation between the enhancer concentration and the peak height of the Y-STR alleles for the loci DYS385 and DYS393. DYS19 and DYS392 showed a reduction of peak height with increasing enhancer concentration up to about a third of the peak height obtained without PCRx enhancer

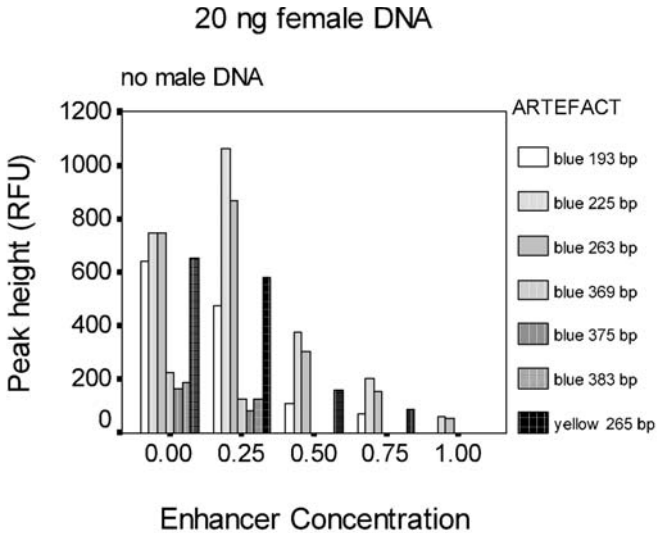

Fig. 2 Amplification of 20 ng female DNA using the Y-STR multiplex. Artefacts significantly decrease with increasing enhancer concentration [Correlation coefficient (Pearson) –0.648; p_{∞}(double-sided) 0.000; *N*=20]

brought full profiles and peak heights well above 100 RFUs. Using this DNA concentration, we found no significant correlation between the peak heights of the Y-STR alleles and the PCRx Enhancer concentration for the Y-STR loci DYS385 and DYS393, whereas DYS19 and DYS392 showed a reduction of peak height up to a third of the peak height obtained without enhancer (Fig. 1). Consistent results were also obtained for male DNA concentrations below 200 pg (100 pg and 50 pg). However, under the PCR and electrophoresis conditions described above, amplification of these DNA concentrations resulted in peak heights below 100 RFUs and some Y-STR loci tended to show drop-out. As a consequence, for further mixture studies the lower concentration limit for male DNA was set to 200 pg.

The Y-STR multiplex was also tested on pure female DNA in order to study the formation of artefacts. The amplification of 20 ng female DNA resulted in a pattern of artefactual peaks when no PCRx Enhancer was added to the PCR master-mix. However, with increasing enhancer concentration the artefact peaks drastically decreased in height (Figs. 2 and 3). In the female artefact peak pattern we were particularly concerned about the 265 bp yellow panel peak as well as the 369 bp, 375 bp and 383 bp blue panel peaks, as they were all located in the category ranges of the Y-STR loci DYS392 and DYS385 (Fig. 3). In the following mixture studies, these peaks as well as the blue panel peaks at 193 bp, 225 bp and 263 bp were closely observed, as their formation constituted a dominant portion of all artefactual peaks observed in the female DNA sample.

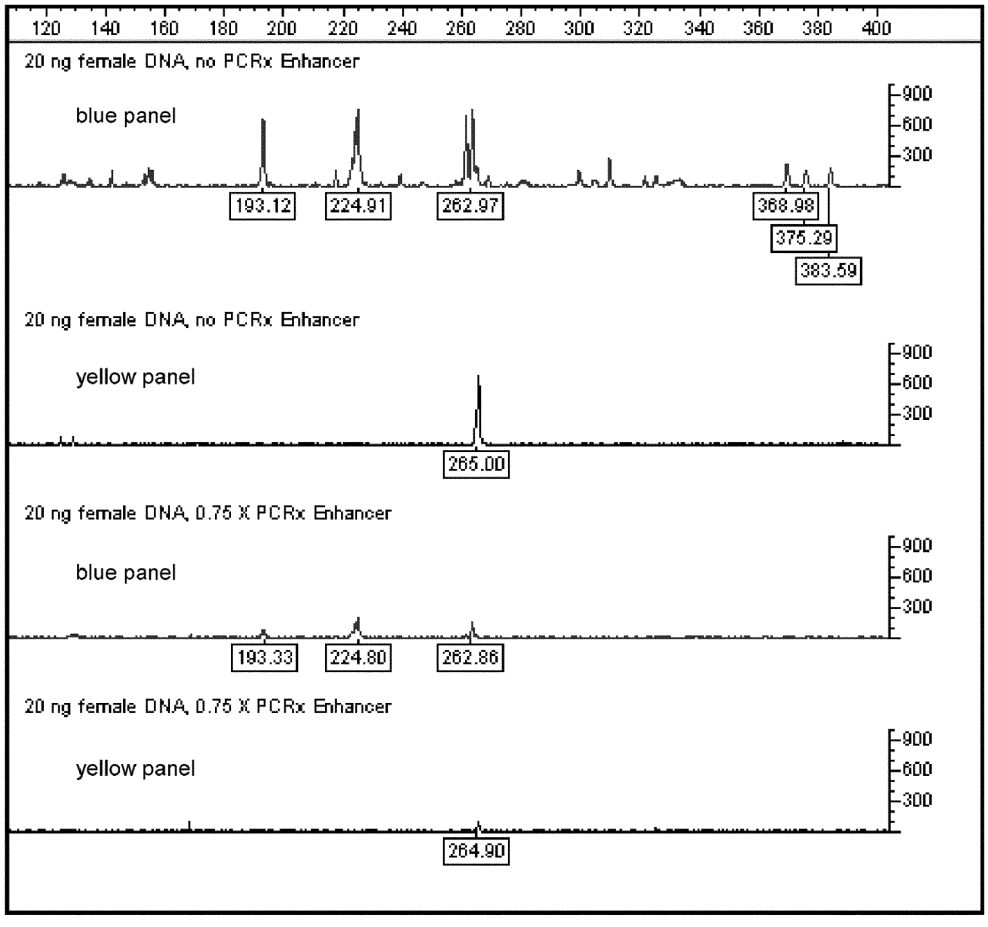

Fig. 3 Electopherogrammes of the amplification of 20 ng female DNA using the Y-STR multiplex with and without 0.75× PCRx Enhancer concentration. Among the artefactual peak pattern, predominant peaks were found within the category range of the STR loci DYS385 and DYS392 (blue panel: 369 bp, 375 bp, 383 bp; yellow panel: 265 bp). These, as well as artefacts outside the category ranges (blue panel: 193 bp, 225 bp, 263 bp) were studied with respect to their peak heights in the mixture experiments

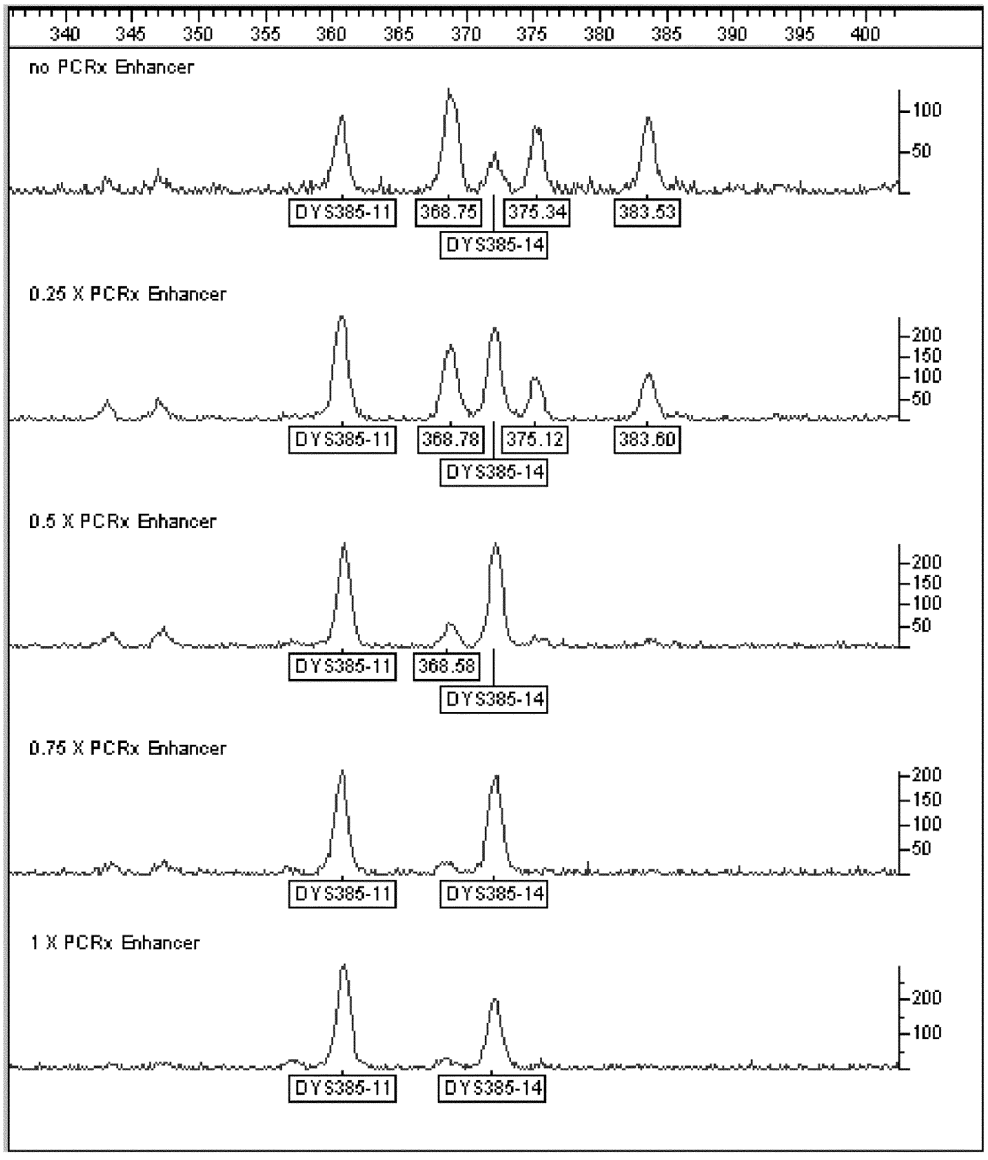

Fig. 4 Electopherogram showing the amplification of DYS385 in the 1:100 DNA mixture (200 pg male DNA/ 20 ng female DNA). The alleles (11, 14) cannot be identified correctly when no PCR enhancer is present but artefacts decrease with increasing enhancer concentration

The mixture studies were carried out with DNA ratios consisting of 1:20 (1 ng male DNA), 1:100 (200 pg as well as 1 ng male DNA) and 1:500 (200 pg male DNA). When no PCRx Enhancer was used, the amplification led to the formation of the artefactual peak pattern observed in the female DNA samples for all mixture examples. In the 1:500 mixture sample this led to the complete suppression of the Y-STR profile. In all other mixtures, male Y-STR profiles may have been present, but unambiguous allele calling was hampered due to overlapping of artefact peaks and actual alleles (for example see Fig. 4).

With the increase of enhancer concentration a significant reduction of the artefactual peaks was observed again (Fig. 4, Table 1), allowing the male Y-STR profile to be clearly identified and making correct allele calling feasible. Generally, peak heights of the Y-STR profile (sum of all alleles) were not severely affected by the enhancer (Fig. 5). Regarding the peak heights of the individual Y-STR loci, only slight differences were observed. The locus DYS385 tended to display little positive correlation between the enhancer concentration and the peak heights of the alleles, whereas peak heights in DYS393 and DYS19 tended to be slightly negatively correlated. Interestingly, the 0.75× enhancer concentration gave the highest Y-STR allele peaks for the mixture ratios 1:500 and 1:100 (using 1 ng male DNA), whereas the 0.25× enhancer concentration brought the highest Y-STR peaks for the mixtures 1:20 and 1:100 (using 200 pg male DNA). However, the 0.25× enhancer concentration still exhibited substantial artefactual signals, rendering allele calling problematic. In all four DNA mixtures, a slight (but not significant) decrease of Y-STR peak heights was observed between 0.75× and 1× PCR enhancer concentrations (Fig. 5).

The PCRx Enhancer is known to potentially decrease PCR yield. According to the results obtained in our study (PCRx enhancer concentrations up to 1.0×) this effect only played a minor role (Fig. 1) and was compensated by far by the increased specificity of the reaction (reduction

Table 1 Summary of the correlation between PCRx Enhancer concentration and artefactual peak height

Peak	Correlation	
Artefact blue 369 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=25	–0.925 0.000
Artefact blue 375 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=15	–0.905 0.000
Artefact blue 383 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=25	–0.908 0.000
Artefact blue 193 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=25	–0.911 0.000
Artefact blue 225 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=25	–0.761 0.000
Artefact blue 263 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=25	–0.768 0.000
Artefact yellow 265 bp	Correlation coefficient (Pearson) p_{∞} (double-sided) *N*=25	–0.819 0.000

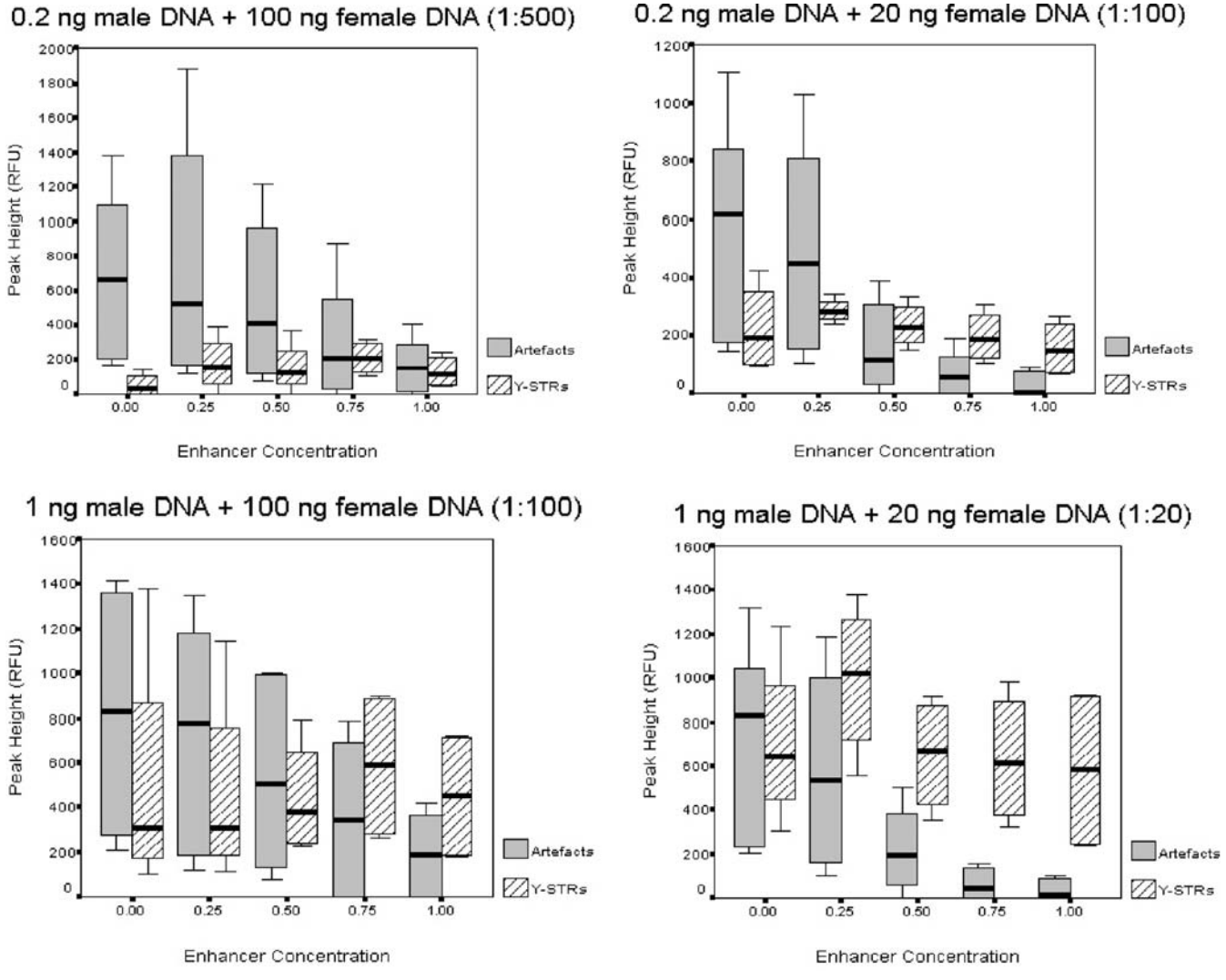

Fig. 5 Histograms of the Y-STR amplification of male/female DNA mixtures (see Materials and methods for setup of DNA mixtures) comparing peak heights of Y-STR alleles with peak heights of artefactual peaks. The increase of the PCRx Enhancer concentration added to the master-mix resulted in a significant reduction of artefactual peak heights in all examples (see also Table 1)

of the artefacts) and the increased ability to call a Y-STR profile, which would not have been interpretable without the PCR enhancer. As a consequence, the results of our study suggest that a concentration of 0.75× seems to be a good compromise between the specificity and yield-decreasing effects of the PCRx Enhancer. Nevertheless, it should be pointed out that this value should be considered as a recommended starting point to be adapted on an individual basis, but not as an absolute value for all settings and situations.

In a casework example published previously the addition of 0.75× PCR enhancer led to the identification of a suspect's Y-STR profile on a lip swab sample from the victim, where prior analysis using genomic STR markers in combination with the gender-specific Amelogenin marker failed to give a male-specific profile [21].

Conclusions

The results of this study demonstrate very clearly that the addition of the PCRx Enhancer to the reaction mix increases the specificity of Y-STR typing of male/female mixture samples. Although in some instances a slight PCR yield-decreasing effect of the PCRx Enhancer was observed, the positive effect of the increased specificity compensated for this. Male/female DNA mixtures up to a ratio of 1:500 gave the correct Y-STR haplotype and made the identification of a male contribution possible, which would not have been recognised using autosomal STRs. This method may be a useful tool for the analysis of mixture samples even when the gender-specific Amelogenin marker shows no indication of a male contribution.

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