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# Variability of mixed DNA profiles separated on a 3130 and 3500 capillary electrophoresis instrument

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In this paper, the variability of peak heights for mixed DNA profiles separated on two different models of capillary electrophoresis instrument is examined. The Applied Biosystems 3500xl instrument produces larger peaks than the 3130xl instrument. If the relative difference in peak heights between the two instruments was a constant factor then all relative heights should be preserved. However, if that factor differed, say, for small versus large peaks then relative heights would change. The effect of peak height and dye on the relative difference in peak height between injections of the same amplicon on a 3500xl and 3130xl instrument for a series of mixed DNA profiles using the Promega PowerPlex® 21 multiplex is described. The ratio of peak heights between instrument models resulted in values up to fourfold higher on the 3500 compared to the 3130. The magnitude of this difference was shown to be dependent on the dye but not on the peak heights themselves. Relative parameters stutter, heterozygote balance, and mixture proportion were very similar between the two instrument models indicating that the interpretation guidelines developed on one machine are likely to be transportable to different capillary electrophoresis instrument models and different machines of the same model.

**Keywords:** DNA interpretation; capillary electrophoresis; mixed DNA profiles; heterozygote balance; 3500; validation

## Introduction

The forensic analysis of DNA is most often undertaken by the amplification of short tandemly repeated lengths of DNA (STRs) by the polymerase chain reaction (PCR). In the PCR process, primers flanking the region of interest are annealed and then extended by the enzyme DNA polymerase to produce a copy of the target sequence [1]. Fluorescently labelled dyes on the primers are used to colour code the allelic products. Capillary electrophoresis (CE) is the primary method for separating and detecting the labelled alleles. A review of forensic DNA literature shows that the Applied Biosystems' series of capillary electrophoresis instruments (models 310, 3100, 3130 and the 3500) dominate the market. During CE, alleles are separated through a capillary with an applied electric field to allow separation of the differing lengths of STRs. As the different lengths of amplified DNA travel through the capillaries of the electrophoresis instrument, a laser excites the primer dyes which emit a fluorescent signal. The signal emissions are recorded with a charge coupled device (CCD) camera that can be used to determine what dye is present as they pass the detection window [2].

Each of the different dyes emits its maximum fluorescence at a different wavelength. The emitted light is collected at specific wavelengths to capture the signal from each dye colour. The instrument detector and data collection software are calibrated to differentiate between the different dyes. A spectral calibration is undertaken by testing a standard set of DNA fragments labelled with the individual dyes. Spectral calibrations are specific to each instrument, a factor of the instrument's individual laser and detector [3].

The fluorescent signals are visualised as peaks. The size of the peaks in base pair (bp) can be assigned by comparison with known size fragments from an internal size standard run simultaneously with the amplified sample. Barring artefacts, each distinct peak at a locus may correspond with an allele. The height of the peaks is a measure of amplified product and measured in relative fluorescent units (rfu). Peak height is approximately proportional to the initial amount of DNA template and the relationship between amplified product and height of the response peak is linear over a certain range [2]. The DNA fragment sizes are then compared to a look-up table, or 'ladder', containing all common STR lengths, and peaks are assigned allelic values. The plot of peaks against size is known as an electropherogram (EPG).

It is a requirement of many quality standards to conduct internal validation experiments when introducing new technology to a forensic DNA laboratory. The introduction of new instrumentation such as a 3500 to a laboratory would be subject to such requirements and several such validation studies have been reported previously [4, 5]. Internal validation includes the determination of forensic DNA profile interpretation guidelines, including those for mixture interpretation [6].

The Applied Biosystems 3500 Genetic Analyser is a relatively new capillary electrophoresis instrument on the market. The 3500*xl* is a 24 capillary instrument with a 505 nm solid state diode laser [3]. The 3130*xl* is a 16 capillary instrument with an argon ion laser with a wavelength of 488 nm for fluorescence excitation. Applied Biosystems report a three- to four-fold increase in rfu scale with the 3500 models over the older Applied Biosystems 3100 and 3130 instruments [7].

The effort to determine interpretation guidelines for profiles when implementing an additional capillary electrophoresis instrument of the same model is expected to be minimal. A check to ensure there is no significant difference in performance between machines would suffice. More significant work would be required to implement a different model of instrument.

The development of interpretation guidelines for mixed DNA profiles is independent of the type of match probability applied. A good understanding of the variability of standard phenomena such as stutter ratio, heterozygote balance, and mixture proportion over a profile is required [8]. These statistics are functions of peaks at a locus and are determined during the amplification process. Assuming peak heights between different instruments are related by a constant *scale factor* $(\delta)$, stutter ratio, heterozygote balance, and mixture proportion would remain constant between profiles generated by different injections of the same amplicon on different instruments. The scale factor describes the relative difference in peak height measured in rfu between the two instruments:

$$\delta = \frac{O_{3500}}{O_{3130}}$$

where $O_{3500}$ and $O_{3130}$ are observed heights of peaks in rfu run on the 3500 and 3130, respectively.

In this paper we investigate the effect of peak height and dye on the scale factor, the relative difference in peak height measured in rfu between a 3130 and 3500 capillary electrophoresis instrument. Injections of the same amplicons were made on the two instruments for a series of mixed DNA profiles using the Promega PowerPlex® 21 multiplex. We also examine directly the variability in heterozygote balance, stutter ratio, and mixture proportion. We have previously demonstrated that pristine DNA is a suitable substitute for 'real' casework samples when determining interpretation guidelines [9].

If there was a different scale factor for small and large peaks then the relative heights of peaks at a locus would change. A systematic effect of height on the scale factor would therefore result in a change in the behaviour of the stutter ratio, heterozygote balance, and mixture proportion. We investigate this by regression analysis using height as an explanatory variable. If there is no effect on the scale factor due to height, then we expect no difference in the statistics and any variability observed must be accounted for by differences in the respective instrument's injection and/or data generation, collection and analysis parameters.

**Method**

Twenty-eight two contributor mixed DNA profiles were prepared with the approximate mixture proportions of 1:1, 1:2, 1:5 and 1:10 by amplifying DNA extracted using Applied Biostsem's PrepFiler® (Life Technologies, Carlsbad CA) from known sources. In addition, 24 three contributor mixed DNA profiles were prepared with the approximate mixture proportions of 1:5:10 in varying contributor orders using extracted DNA from known sources. An estimate of the DNA quantity was made using Applied Biosystem's Quantifiler™ real time PCR quantitation kit (Life Technologies, Carlsbad, CA). DNA from the three different donors were diluted to 0.1 ng/μL and each target mixture proportion was amplified in duplicate at 1.0 ng and 0.5 ng total DNA using Promega's PowerPlex® 21 (Promega, Madison, WI) multiplex (30 cycles) in an Applied Biosystem 9700. The same amplicons were separated on both the Applied Biosystem 3500*xl* and 3130*xl* (injection 1.2kV for 24 sec) and 3130*xl* (injection 3kV for 10 sec) genetic analysers. Each duplicate amplification was subsequently considered independently as described below.

Profiles were analysed using Applied Biosystems' GeneMapper™ ID-X (version 1.2). The 3500 data was analysed using an analytical threshold of 80 rfu and the 3130 data 30 rfu. Analytical thresholds had been determined previously using the method of Gilder et al. [10], with modifications for 3500 file format . The analytical thresholds were lower than those used in routine casework analysis in order to increase the number of peaks detected, whilst ensuring no increase in baseline noise.

Within both datasets, the height of alleles that have not been detected above the analytical threshold have been replaced with a value half the threshold; 40 and 15 rfu for the 3500 and 3130 data, respectively.

The heights for each allele and stutter peak between different injections of the same amplicon were compared directly. In addition, heterozygote balance and mixture proportion for appropriate loci were calculated as described. Heterozygote balance (*Hb*) was calculated as:

$$Hb = \frac{O_{HMW}}{O_{LMW}}$$

where $O_{HMW}$ refers to the observed height of the high molecular weight allele and $O_{LMW}$ the height of the low molecular weight allele.

Since the genotypes are known, the mixture proportion $\left(M_x^{\ell}\right)$ may be calculated for each sample at each locus, $\ell$. Mixture proportions and average peak heights (*APH*) for the two person mixtures were estimated according to the equations in Table 1, as per Bright et al. [11]. The average mixture proportion $\left(M_x\right)$ across loci is treated as the estimate for that sample. The variability of the mixture proportion for each locus across a profile can be determined by calculating the difference, *D*, of the estimate at each locus from the average mixture proportion estimate:

$$D = M_x^{\ell} - M_x$$

For three person mixtures, $M_x^\ell$ can only be estimated for a subset of all possible locus genotype combinations. These are loci where the two alleles of an individual contributor are not shared, where the individual contributor of interest is a homozygote and this allele is not shared and loci where one allele of the individual contributor of interest is unshared. The equations are summarised in Table 2. Other locus combinations may be obtained by subtraction. A proxy *APH* was calculated by the sum of all peak heights at the locus multiplied by $M_x^\ell$.

Table 1: The form of the equations for estimation of the mixture proportion $\left(M_x^\ell\right)$ and average peak height (*APH*) for two person mixtures at individual loci styles, where $O_A$, $O_B$, $O_C$, and $O_D$ represent the observed heights of alleles A, B, C and D, respectively. Locus style is given as contributor 1:contributor 2.

| Genotype combination | $M_x^\ell$ | *APH* |
|---|---|---|
| AB:AA | $\frac{2O_B}{O_A+O_B}$ | $\frac{O_A+O_B}{4}$ |
| AA:AB | $\frac{O_A-O_B}{O_A+O_B}$ | $\frac{O_A+O_B}{4}$ |
| AB:AC | $\frac{O_B}{O_B+O_C}$ | $\frac{O_B+O_C}{2}$ |
| AA:BB | $\frac{O_A}{O_A+O_B}$ | $\frac{O_A+O_B}{4}$ |
| AA:BC | $\frac{O_A}{O_A+O_B+O_C}$ | $\frac{O_A+O_B+O_C}{4}$ |
| AB:CD | $\frac{O_A+O_B}{O_A+O_B+O_C+O_D}$ | $\frac{O_A+O_B+O_C+O_D}{4}$ |

Table 2: The form of the equations for estimation of the mixture proportion $\left(M_x^{\ell}\right)$ and a proxy average peak height (*APH*) for three person mixtures at individual loci styles, where $O_A$ and $O_B$, represent the heights of observed alleles A and B, respectively. Locus style is given as contributor 1:contributor 2: contributor 3.

| Locus style examples | $M_x^{\ell}$ | *APH* |
|---|---|---|
| AB:CD:EF<br>AB:CD:CD<br>AB:CC:CC | $\frac{(O_A+O_B)}{\sum \text{All peaks}}$ | $\sum All\ peaks\ \times M_x^l \times 2$ |
| AA:BC:DE<br>AA:BB:CC<br>AA:BB:BB | $\frac{O_A}{\sum \text{All peaks}}$ | $\sum All\ peaks\ \times M_x^l \times 2$ |
| AB:BC:DE<br>AB:BB:CD<br>AB:BB:BB | $\frac{2O_A}{\sum \text{All peaks}}$ | $\sum All\ peaks\ \times M_x^l$ |

Statistical analysis was undertaken using R [12] and MS EXCEL™.

## Results

### *Comparison of peaks heights*

An investigation into the effect of peak height and dye on the scale factor was undertaken for a selection of the amplicons separated on the 3130 and 3500. Allele height and dye colour were investigated as explanatory variables to explain the logarithm of the scale factor in a regression analysis. A summary of the difference in heights between different dyes on the 3500 and 3130 and their *p*-value are in Table 3.

Table 3: Summary of regression analysis investigating dye effect on peak height

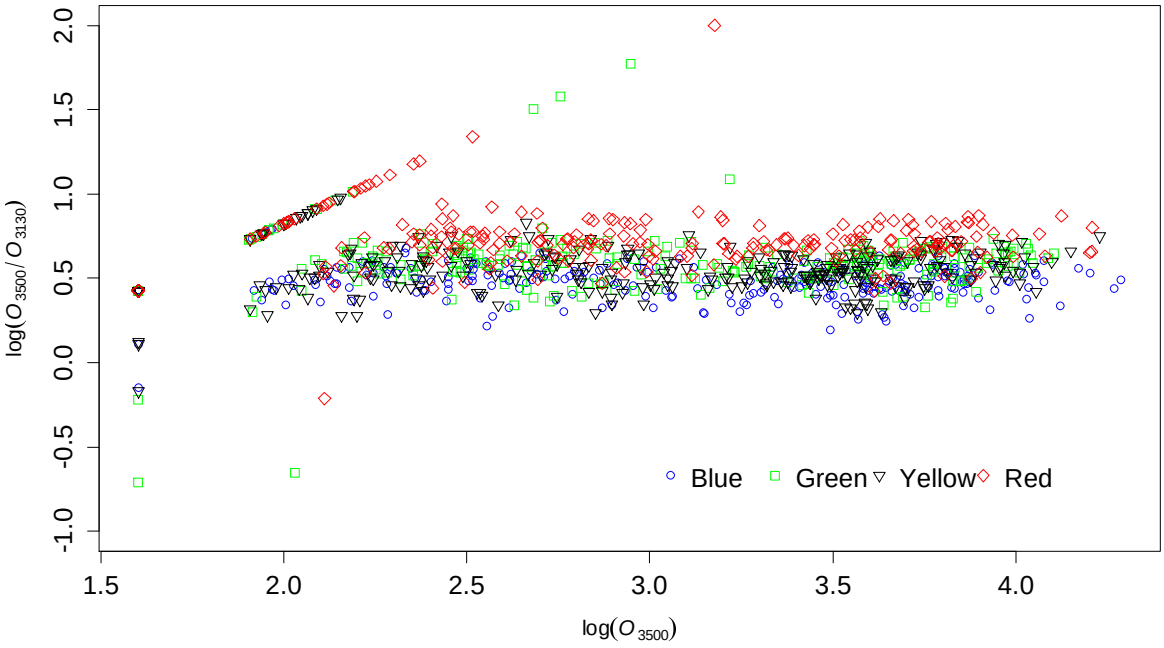
| Dye | Colour | $\frac{O_{3500}}{O_{3130}}$ | *p*-value |
|---|---|---|---|
| Fluorescein | Blue | 2.58 | <0.001 |
| JOE | Green | 3.22 | <0.001 |
| TMR-ET | Yellow | 3.00 | <0.001 |
| CXR-ET | Red | 4.44 | <0.001 |

There was a small effect due to height ($p$ = 0.02), however as the coefficient was very small $\left(8.09\times10^{-6}\right)$, this will have little effect on the scale factor. This indicates that ratios of heights at a locus (for example, stutter ratio, heterozygote balance, and mixture proportion) will not differ between the machine types. Dye is a significant explanatory variable. This indicates that different dyes have different effects on the scale factor (Table 3) for this dataset. Large differences in the laser between CE instrument models and smaller differences due to spectral values within the same CE instrument model are expected to have an effect on the heights of loci at different dyes.

In Figure 1, $\log(O_{3500}/O_{3130})$ is plotted against $\log(O_{3500})$. Inspection of Figure 1 shows that (with the exception of a few peaks) peaks from amplicons run on the 3500 are larger in rfu than peaks for the same amplicons run on the 3130. In addition, peaks within markers labelled with CXR-ET dye (red) are larger compared to the other dyes (also refer Table 3). The CXR-ET labelled peaks separated on a 3500 were on average 4.44 times higher than the equivalent peaks run on a 3130. In comparison, fluorescein labelled peaks run on a 3500 were only 2.58 times their 3130 separated counterparts.

The pattern of data points in Figure 1 at approximately 50° are the alleles that appear in the 3500 data but have fallen below the analytical threshold in the 3130 data. The smaller pattern of vertical data points at approximately 1.6 on the *x*-axis are peaks that appear in the 3130 data but are below the analytical threshold in the 3500 data. This data replacement was described in the methods and results in the observed patterns.

Figure 1: $\log\left(O_{3500}/O_{3100}\right)$ versus $\log\left(O_{3500}\right)$ for a selection of data points where $O_{3500}$ and $O_{3130}$ are the observed height of the peaks run on a 3500 and 3130, respectively. Peaks labelled with the Fluorescein (blue) dye are represented by circles, JOE (green) as squares, TMR-ET (yellow) as black triangles and CXR-ET (red) as diamonds.




***Heterozygote balance and mixture proportion***

A summary of log(*Hb*) versus *APH* and *D* versus *APH* for the two and three person mixtures for both 3130 and 3500 data is in Figure 2.

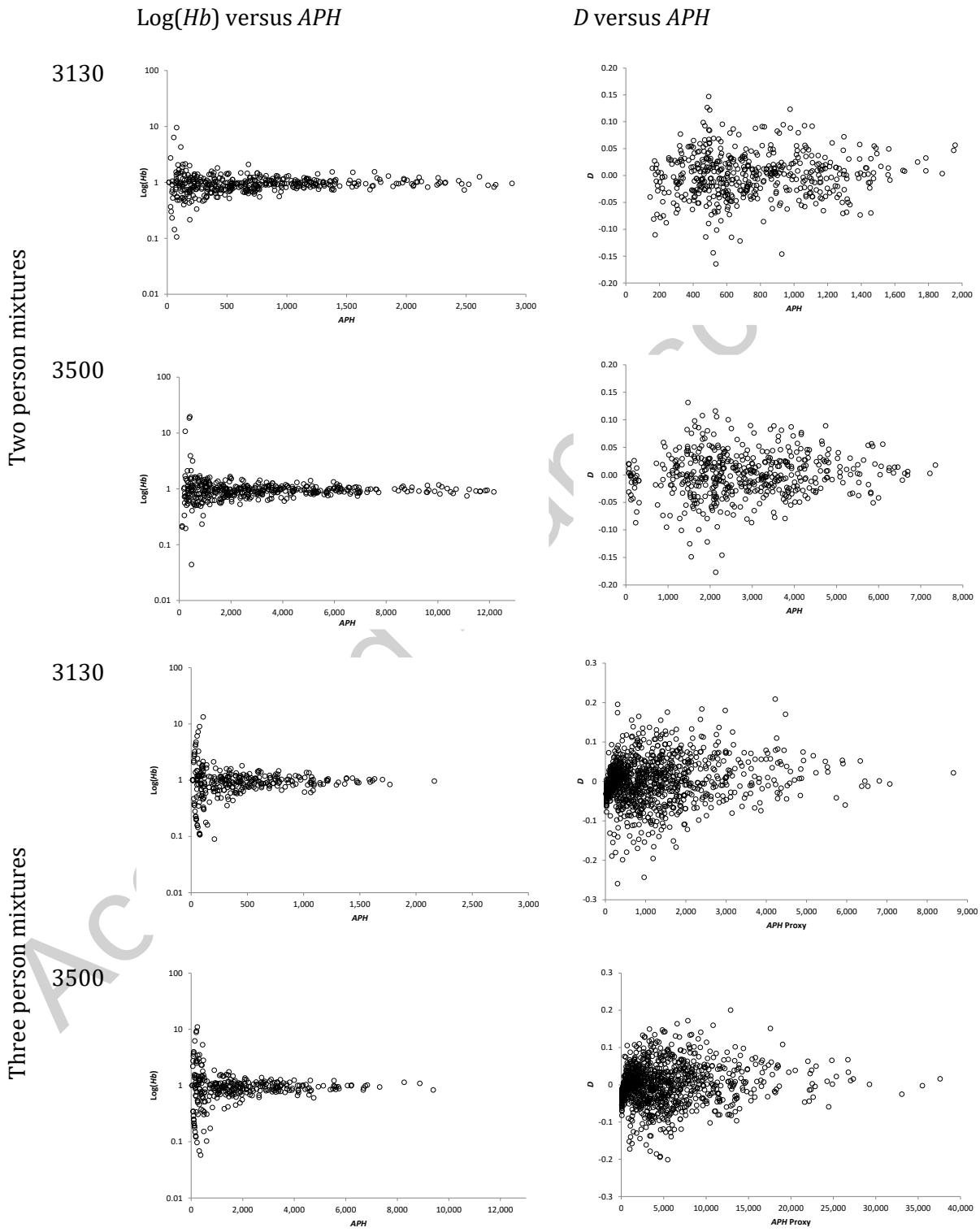
The arc pattern of data points visible in the plots in Figure 2 for log(*Hb*) versus *APH* represents data where one of the alleles was not present above the analytical threshold.

The y-axes of the plots in Figure 2 have been kept the same to allow easier comparison of the data. Inspection of the plots shows no apparent difference in the variation of log(*Hb*) and *D* versus *APH* between the different capillary electrophoresis instruments. The three person mixtures appear to be more variable than the two person mixtures. This may be due to the *APH* approximation.

The ratio of the logarithms of the stutter ratios for unambiguous alleles and stutters were calculated as in Equation 1. This is the logarithm of the scale factor for stutter ratio. Unambiguous alleles are where the difference in allele repeat numbers is greater than 1. Fifteen of the 20 loci resulted in data for analysis.

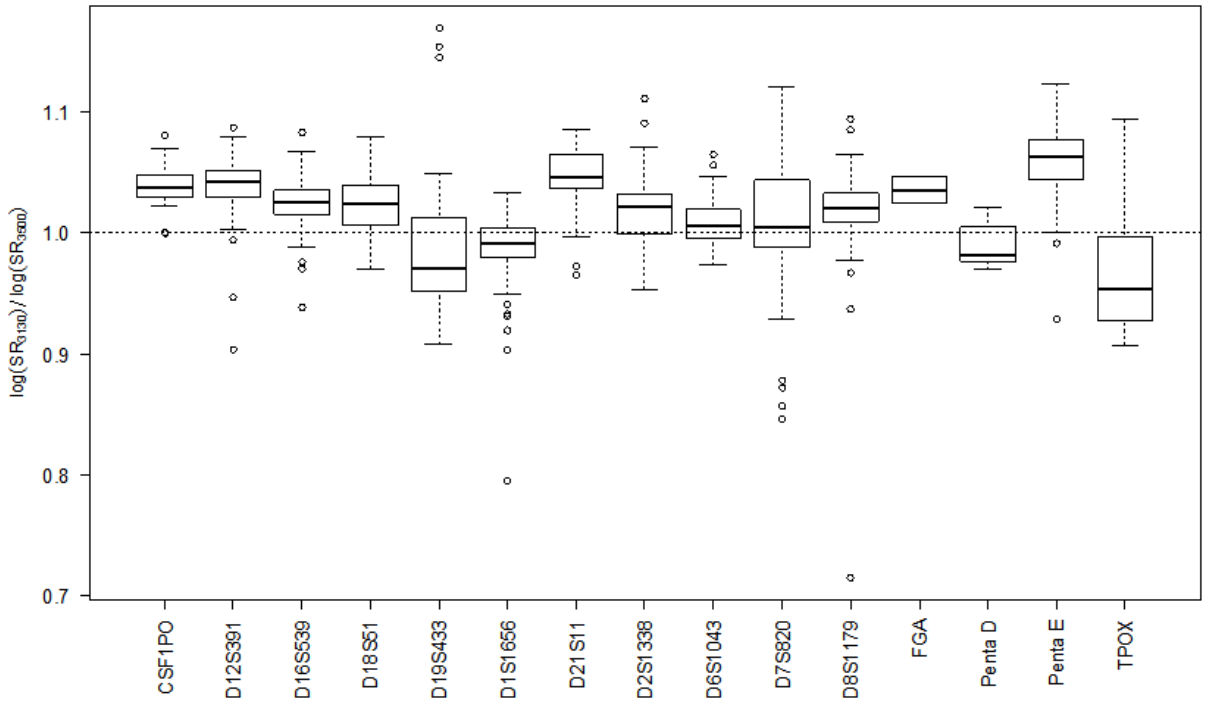
$$\frac{\log SR_{3130}}{\log SR_{3500}} = \frac{\log\left(O_{a-1}^{3130}/O_{a}^{3130}\right)}{\log\left(O_{a-1}^{3500}/O_{a}^{3500}\right)} \qquad \text{Equation 1}$$

Figure 2: Log(*Hb*) versus *APH* and *D* versus *APH* for two and three person mixtures analysed on the 3130 and 3500. Log(*Hb*) is the logarithm of the heterozygote balance, *APH* the average peak height and *D* the difference of the estimate at each locus from the average mixture proportion estimate.



If there is no effect of peak height on the logarithm of the stutter ratio specific scale ratio, then the expected ratio is one. The average of Equation 1 is 1.015 suggesting the stutter ratio of 3130 data is larger than the corresponding 3500 data. This can also be observed in Figure 3, a plot of the spread of values for Equation 1 for loci with unambiguous alleles. This effect could be due to minor non-linearity of the peaks. Only data where the stutter allele was above the analytical threshold in both the 3130 and 3500 profiles were used for Figure 3 and in Equation 1.

Figure 3: Boxplot of $\frac{\log SR_{3130}}{\log SR_{3500}}$ for each loci with unmasked alleles




## Discussion

Peak heights were observed to be up to fourfold higher for the same amplicons when run on a 3500 versus a 3130 (Table 3). The magnitude of this difference was shown to be dependent on the dye. This dye effect is likely to be caused by both the instrument's laser and camera. We have observed significant difference in the relative dyes between machines of different CE types and expect smaller differences within the same type.

The ratios studied (stutter, heterozygote balance and mixture proportion) respond very similarly in the two machines. The knowledge and guidelines developed on one machine are likely to be transportable to different CE instruments and different machines of the same instrument type. This finding will reduce the validation costs when implementing new instrumentation into a laboratory and will remove the requirement for the different analysis thresholds per machine. The assessment of analytical thresholds is still an important requirement for CE instrumentation validation.

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**References**

[1] K. Mullis, F. Faloona, Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction, Methods in Enzymology. 155 (1987) 335 - 350.

[2] J. M. Butler, E. Buel, F. Crivellente, B. R. McCord, Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis, Electrophoresis. 25(10-11) (2004) 1397-1412.

[3] J. M. Butler. Advanced Topics in Forensic DNA Typing: Methodology: Academic Press; 2011.

[4] E. L. R. Butts, M. C. Kline, D. L. Duewer, C. R. Hill, J. M. Butler, P. M. Vallone, NIST validation studies on the 3500 Genetic Analyzer Forensic Science International: Genetics Supplement Series. 3(1) (2011) e184-e185.

[5] A. Kirkham, J. Haley, Y. Haile, A. Grout, C. Kimpton, A. Al-Marzouqi, et al., High-throughput analysis using AmpFlSTR® Identifiler ® with the Applied Biosystems 3500xl Genetic Analyser, Forensic Science International: Genetics. 7(1) (2013) 92-97.

[6] DNA Advisory Board, Quality Assurance Standards for Forensic DNA Testing Laboratories and for Convicted Offender DNA Databasing Laboratories, Forensic Science Communication. 2(3) (2000).

[7] Applied Biosystems. User Bulletin Applied Biosystems® 3500/3500xL Genetic Analyzer. Foster City, CA; 2011.

[8] P. Gill, C. H. Brenner, J. S. Buckleton, A. Carracedo, M. Krawczak, W. R. Mayr, et al., DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures, Forensic Science International. 160 (2006) 90-101.

[9] J.-A. Bright, K. McManus, S. Harbison, P. Gill, J. Buckleton, A comparison of stochastic variation in mixed and unmixed casework and synthetic samples, Forensic Science International: Genetics. 6(2) (2012) 180-184.

[10] J. R. Gilder, T. E. Doom, K. Inman, M. Crim, D. E. Krane, Run-Specific Limits of Detection and Quantitation for STR-Based DNA Typing. , Journal of the Forensic Sciences. 52(1) (2006) 97-101.

[11] J.-A. Bright, J. Turkington, J. Buckleton, Examination of the variability in mixed DNA profile parameters for the Identifiler™ multiplex, Forensic Science International: Genetics. 4(2) (2009) 111-114.

[12] R Development Core team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2004.