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Title: STR profiling of epithelial cells identified by X/Y-FISH labelling and Laser Microdissection using standard and elevated PCR conditions

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1 Highlights

2 • 28 cycle Identifiler analysis can be undertaken on X/Y-FISH epithelial cells
3 • 75 or more LMD X/Y-FISH epithelial cells, is optimal for a 28 cycle Identifiler test
4 • For 30 or less LMD X/Y-FISH epithelial cells, 34 cycle SGM Plus is recommended
5 • Hb was not improved for Identifiler profiles from LMD X/Y-FISH epithelial cells.
6
7

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7 **Title**: STR profiling of epithelial cells identified by X/Y-FISH labelling and Laser
8 Microdissection using standard and elevated PCR conditions
9
10 **Authors**: Laura Lynch[1], Amelia Gamblin[1], Sue Vintiner[1*], Joanne Simons[1,2]
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24
25 **Abstract**: During the investigation of allegations of sexual assault, samples are
26 frequently encountered that contain DNA from a female and a male donor. These may
27 represent contributions of DNA from the complainant and potentially, the offender.
28 Many semen stained samples successfully undergo DNA analysis and interpretation using
29 a differential extraction method that separates sperm from the epithelial cells present in
30 the stain. However, for those mixed cell samples that contain only epithelial cells,
31 separation of any male cells from female cells is problematic. This paper describes the
32 application of fluorescent in situ hybridisation (FISH) for the gender identification of
33 epithelial cells and subsequent recovery of target cells using laser microdissection
34 (LMD). The profiling results obtained from samples of known cell numbers using the
35 Identifiler™ multiplex at standard 28-cycle PCR conditions and, when cell numbers are
36 low, the SGM Plus™ multiplex at elevated 34-cycle PCR conditions (also known as Low
37 Copy Number DNA analysis (LCN)) are described.
38
39
40
41 **Keywords**: Forensic DNA, Epithelial cells, laser microdissection, fluorescent in situ
42 hybridisation, Identifiler, Low Copy Number, SGM Plus
43
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44 **Introduction**

45

46 The examination of samples associated with an allegation of sexual assault frequently
47 involves the analysis of samples that comprise mixtures of cells. Separating cell mixtures
48 prior to undertaking DNA testing simplifies downstream DNA profile interpretation as
49 profiling results are more likely to originate from single contributors. Furthermore, more
50 complete profiles are likely to be obtained from a minor DNA contributor through the
51 removal of the masking effect of shared DNA results with a major DNA contributor.
52 Methods, such as preferential extraction, have focussed on the separation of sperm from
53 epithelial cells based on physical differences in cell structure [1]. However, it may be
54 necessary to separate cells for DNA profiling when sperm are not present in a cell mixture,
55 such as semen stained genital swabs containing azoospermic semen. Laser microdissection
56 (LMD) technology, which involves microlaser ablation to collect target cells from cellular
57 samples deposited on slides, has been utilised by the forensic community over recent years to
58 isolate sperm from cell mixtures [2,3], foetal cells from maternal tissue [4], nucleated cells
59 from hair follicles [5] and to isolate blood cells from cell mixtures [6,7].

60

61 A method which distinguishes between morphologically similar cells, such as epithelial cells,
62 of male and female origin, is fluorescent in situ hybridisation (FISH). In order to
63 differentiate cells based on gender, different coloured fluorescent probes to the X and Y sex
64 determining chromosomes are applied to samples of cells, which are usually fixed onto
65 microscope slides. This X/Y-FISH labelling method has a particular application for those
66 forensic samples where cells of one gender are mixed with a large number of cells from the
67 other gender, such as may occur with azoospermic semen mixed with vaginal epithelial cells
68 [8,9], female cells on post coital penile swabs [10] or condoms [11]. Cells of interest can be

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69 identified by X/Y-FISH labelling and then separated, by LMD, from other cells in the
70 sample. The recovered cells are then be subjected to DNA profiling analysis.
71
72 A viable DNA extraction method has been developed which allows for the release of DNA
73 from recovered cells coupled with denaturation of cellular proteins and endogenous nucleases
74 [12]. This method enables DNA extraction and PCR to be performed in the same tube,
75 providing time benefits and improved sensitivity. It is also hypothesized that a further benefit
76 would be reduction of the stochastic effects in DNA profiling brought about by unequal
77 sampling of alleles from a DNA extract as, in a one-tube test, all of the DNA from the
78 recovered cells is progressed to PCR.
79
80 Production of DNA profiles from X/Y-FISH LMD cells has, so far, to our knowledge, been
81 limited to ultra-sensitive methods of DNA analysis, such as the Low Copy Number (LCN)
82 technique using 34-cycles of the PCR versus the manufacturer's recommended 28 cycles
83 [13]. Given the relatively small number of forensic laboratories employing an ultra-sensitive
84 DNA profiling method, this has likely limited the application of X/Y-FISH in forensic
85 analysis. This research combines the use of a one-tube extraction and amplification method to
86 samples of known numbers of laser microdissected X/Y-FISH labelled cells to obtain DNA
87 profiles using either the Identifiler™ multiplex at standard 28-cycle PCR conditions or the
88 SGM Plus™ multiplex using 34-cycle PCR conditions. We present data of the profiling
89 success rates using these two protocols and the observed variation in heterozygote balance in
90 these profiles. The theorised effect on stochastic variation from sampling prior to PCR was
91 investigated.
92
93 **Materials and Methods**

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94

95 Sample collection

96 Epithelial cells were collected from consenting male (n=3) and female (n=3) participants,
97 with known Identifler™ DNA profiles, between the ages of approximately 20 and 50 years
98 old. Buccal epithelial cells were self-collected by participants, by rubbing the insides of their
99 cheeks and gums with sterile swabs for 20 seconds. Swabs were placed back into the swab
100 casings, which were cut, and placed in a laminar flow hood to dry. Once dry, the samples
101 were placed into a paper envelope and stored at room temperature until sample processing
102 commenced.

103

104 Cell recovery and slide preparation

105 Cells were recovered from swab heads by agitation in 500 µL of Tris Extraction buffer (10
106 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 8.0) and collected by centrifugation at 10,000
107 rpm for 10 minutes. Cells were chemically fixed using either 30 µL of Carnoy's fixative (3:1
108 Methanol: Acetic Acid) or 1:1 Methanol: Acetone and re-suspended single source cell pellets
109 were placed onto Polyethylene Terephthalate (PET) membrane slides (Leica Microsystems,
110 Germany). Slides were stored at room temperature in a laminar flow hood to dry completely
111 and left, at least overnight, prior to X/Y-FISH labelling or Christmas Tree staining.

112

113 X/Y-FISH Labelling

114 X/Y-FISH was performed using the CEP® X SpectrumOrange™ Y SpectrumGreen™ DNA
115 Probe Kit (Vysis, Des Palines, IL, USA) following the manufacturer's instructions. The slides
116 were immersed in a denaturing solution (70% Formamide in 2 x SSC pH 7.0-8.0) within a
117 Coplin jar in a water bath at 73 ^{o}C±1 ^{o}C for five minutes. The slides were dehydrated in an
118 ethanol series by soaking for one minute in each of 70%, 85% and 100% ethanol then placed

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119 on a 42 ^{o}C hot block to dry for two minutes. Ten μL of probe solution was added to the
120 sample area on each slide. A cover slip was applied and sealed with rubber solution. Slides
121 were incubated in a humidified chamber overnight at 42 ^{o}C. Following hybridisation the
122 coverslips and rubber solution were removed and the slides were washed in 0.4x SCC at 73
123 ^{o}C for 2 minutes and 2x SCC/0.1% NP-40 at room temperature for 1 minute. Slides were air
124 dried in the dark, before 10 μL of DAPI II counterstain and then coverslips were applied.
125
126 Christmas Tree Staining
127 For comparison, additional slides were stained with CTS using reduced times for nuclear fast
128 red and picroindigocarmine staining, so as to minimise any deleterious effect of the chemicals
129 but still providing effective visualisation of cells, as described in Meredith et al. [12].
130
131 Laser Microdissection
132 The slides were examined on a Leica LMD6000 laser microdissector (Leica Microsystems,
133 Wetzlar, Germany) at 25x and 40x lens magnification using appropriate DAPI/Green/Orange
134 filters for the detection of fluorescent signals. Male cells were confirmed by the presence of
135 one orange and one green signal within the DAPI II stained nucleus, while female cells were
136 defined as having two orange signals within the nucleus.
137
138 Samples of X/Y-FISH labelled cells were collected by laser microdissection, with the number
139 of cells in each sample ranging from 2 to 150. These cell sets were collected into the caps of
140 Axygen 0.2mL flat top, long hinged, microcentrifuge collection tubes (Raylab, New Zealand)
141 containing an extraction solution, as described below. Following collection of the selected
142 cells, the tubes were centrifuged at 13,000rpm for 1 minute to move samples from the cap
143 into the main body of the tube.

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144

145 One-tube extraction and amplification

146 DNA extraction and amplification was carried out according to the method of Meredith et al.
147 [12]. Epithelial cells were recovered into the caps of tubes containing a solution consisting of
148 Tris Extraction buffer, Tween 20 at 0.2% v/v and 0.1 mg/mL Proteinase K (PK). Different
149 quantities of reagents were used depending on the DNA profiling kit. The two profiling kits
150 selected for use in this study are ones that have been validated for casework analysis in the
151 authors' laboratory, at the cycle numbers described below. Cells intended for amplification
152 with the AmpF*l*STR Identifiler^{TM} multiplex (Applied Biosystems, Life Technologies™,
153 Carlsbad, CA) were extracted in a final volume of 10 µL, and cells amplified by LCN
154 AmpF*l*STR SGM Plus^{TM} (Applied Biosystems, Life Technologies™, Carlsbad, CA) were
155 extracted in a final volume of 20 µL. Samples were incubated in a thermal cycler for 1 hr at
156 56 ^{o}C and inactivation of the PK was achieved by heating the sample at 95 ^{o}C for 10 min
157 before cooling to 4 ^{o}C. Samples were stored at 4 ^{o}C prior to amplification of the DNA.

158

159 For the Identifiler^{TM} amplification reactions, the whole 10 µL extract was used and the
160 reaction was undertaken in the same tube as DNA extraction. The DNA, in a total volume of
161 25 µL, was amplified at 28 cycles in a silver block 9700 thermal cycler (Applied Biosystems,
162 Life Technologies™, Carlsbad, CA), according to the manufacturer's instructions. For the
163 LCN SGM Plus^{TM} reactions, half of the 20 µL extract was transferred to a new tube and two
164 replicate amplifications were each carried out in a total volume of 50 µL, in a silver block
165 9700 thermal cycler, according to the manufacturer's instructions, but at 34 cycles.

166

167 A total of 30 samples, comprising six replicates each of 2, 4 10, 20 and 30 cells, were profiled
168 using LCN SGM Plus ^{TM}. Seventy three (73) samples were profiled using the Identifiler ^{TM}

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169 multiplex. These comprised sets of 15 cells (n=10), 25 cells (n=8), 30 cells (n=10), 40 cells
170 (n=10), 50 cells (n=22), 75 cells (n=8) and 100 cells (n=3).
171
172 Data Analysis
173 Amplified products were separated on a 3130*xl* Genetic Analyser (Applied Biosystems, Life
174 Technologies™, Carlsbad, CA) and analysis of DNA profiles was undertaken using the
175 GeneMapper™ *ID* version 3.2.1 (Applied Biosystems, Life Technologies™, Carlsbad, CA)
176 software. A peak detection threshold of 50 RFU was applied to all profiles. A stochastic
177 threshold of 4500RFU was applied for the LCN SGM Plus™ profiles and 400 RFU for the
178 Identifiler™ profiles.
179
180 Heterozygote peak balance *(Hb)* was calculated in this study using the following formula:
181
$$Hb = \frac{O_{HMW}}{O_{LMW}}$$
182 where O_{HMW} is the height of the high molecular weight peak and O_{LMW} the height of the lower
183 molecuar weight peak. Prior to interpretation, all alleles known to have dropped out were
184 returned to the dataset at half the peak detection threshold (25 RFU). When determining the
185 parameters of a disturbution such as *Hb* ignoring censored data can bias the estimate [14].
186 Substitution is a simple way of handling missing data and is sustainable if the proportion of
187 missing alleles is small, as is this dataset [15]. Alleles were only included in the LCN SGM
188 Plus™ profiles if they were present in the profile from both reactions, in accordance with the
189 consensus model [13, 16]. Stutter peaks were assigned using a profile wide threshold of 15%.
190 To avoid outlier data affecting conclusions, work has been undertaken using the central 0.95
191 quantile of data. All data analysis was conducted in MX Excel.
192
193 **Results and Discussion**

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194

195 After X/Y-FISH labelling of epithelial cells, within the DAPI II stained nucleii, female cells
196 displayed two orange fluorescent signals and male cells displayed one green and one orange
197 fluorescent signal.

198

199 Samples, comprising between 15 and 150 dissected cells were profiled using Identifiler™ at
200 28-cycle PCR conditions and samples comprising between 2 and 30 dissected cells, were
201 profiled using LCN SGM Plus™ PCR conditions. All profiling results obtained from
202 samples were found to correspond to donors' profiles. From the DNA profiling results
203 obtained, the percentage of alleles detected in the profiles from the different samples and the
204 average peak heights across profiles were determined (Table 1). For the LCN SGM Plus™
205 profiles, extracts were halved to enable duplicate amplifications and reporting of consensus
206 profiles.

207

208

	Total number of cells collected	Average number of alleles observed[α] ±SEM	Range of possible alleles observed (%)	Average peak height (RFU) ±SEM
Identifiler™ by 28-cycle PCR	15 (n=10)	14.9 (± 2.1)	18.8 – 81.3	70.2 (± 3.5)
	25 (n=8)	20.8 (± 1.9)	50.0 – 96.9	112.7 (± 5.4)
	30 (n=10)	21.4 (± 2.9)	21.9-93.8	130.5 (± 6.0)
	40 (n=10)	29.6 (± 1.1)	71.9-100	249.1 (± 10.2)
	50 (n=22)	29.5 (± 0.5)	81.3-100	277.1 (± 10.4)
	75 (n=8)	30.0 (± 0.9)	78.1-100	307.4 (± 11.22)
	100 (n=3)	30.7 (± 1.3)	87.5-100	453.3 (± 35.6)
	150 (n=2)	32.0	100	1018.8 (± 49.9)
SGM Plus™ by 34-cycle PCR[β]	2 (n=6)	3.3 (±1.1)	0-36.4	264.6 (±67.4)
	4 (n=6)	3.2 (±1.2)	0-36.4	403.1 (±83.1)
	10 (n=6)	9.2 (±1.3)	27.3-63.6	653.0 (±177.2)
	20 (n=6)	12.8 (±1.9)	22.7-81.8	801.2 (±302.6)
	30 (n=6)	17.3 (±0.7)	72.7-95.4	1183.4 (±396.7)

209 [α] Total number of STR alleles and Amelogenin alleles (32 for Identifiler™ and 22 for SGM
210 Plus™). [β] Extracts halved for duplicate amplification by 34-cycle SGM Plus™ analysis.

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211
212 Table 1. Comparison of Identifiler™ and LCN SGM Plus™ profiling results for different
213 numbers of FISH-labelled epithelial cells collected by LMD. Data presented includes the
214 average number of alleles observed and the average peak height for an allele, ± the standard
215 error of the mean (SEM), in the profiles.
216
217 From the results obtained after Identifiler™ analysis, the two samples tested that comprised
218 150 X/Y-FISH labelled cells each produced full DNA profiles. When the cell number was
219 reduced to 75 cells, the average total number of alleles observed was approximately 30, out
220 of a possible total of 32. Where results were missing in the 75 cell sample profiles, on every
221 occasion, the height of two peaks failed to meet the stochastic threshold. As expected,
222 average peak height (APH) was reduced from approximately 1000 RFU, in the 150 cell
223 samples, to 300R FU in the 75 cell samples. Although the 50 and 40 cell samples also
224 produced profiles comprising an average of approximately 30 alleles, the APH was further
225 reduced to approximately 270 RFU and 250 RFU respectively.
226
227 The Identifiler™ profiling success for the X/Y-FISH dissected cell samples was compared to
228 data obtained from the Identifiler™ analysis of dissected epithelial cell samples treated with
229 reduced Christmas Tree stain, as reported in the study by Meredith et al. [12]. In this study
230 they report that full DNA profiles were obtained from four 50 dissected cell samples, with an
231 APH of 281 RFU (±SEM=21.5). This is comparable to the results obtained in this study
232 where the 50 X/Y-FISH dissected cell samples (n=22) produced between 81% to 100% of the
233 profile, with an APH of 277 RFU (±SEM=10.4). Further comparison between the APH from
234 the Identifiler™ profiles of 25 dissected cell samples (n=9) and 25 X/Y-FISH dissected cell
235 samples (n=8), showed that the APH was reduced from 153RFU (±SEM=9.0) to 112RFU
236 (±SEM=5.4) respectively. The slight reduction in peak height in the 25 X/Y-FISH cell
237 samples is indicative of an effect that could be due to the FISH process on the smaller

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238 amounts of DNA within these samples. A similar effect on APH was also observed after LCN
239 SGM Plus™ analysis of X/Y-FISH dissected cells. APHs of Christmas Tree stained epithelial
240 cells after LMD DNA testing have been reported [17] to be approximately twice that of the
241 X/Y-FISH dissected cells (Table 1).
242
243 Informative DNA profiles can be obtained from X/Y-FISH dissected cells when using the
244 Identifiler™ multiplex at 28-cycle PCR conditions, with samples containing 40 or more
245 dissected cells producing near complete DNA profiles. When the cell number was reduced to
246 15 X/Y-FISH dissected cells, the average number of alleles observed reduced to
247 approximately 50%. Although not tested in this study, it is anticipated that samples
248 containing less than 15 cells would produce Identifiler™ profiles with even fewer results
249 along with a corresponding reduction in APH. As this study has been undertaken using fresh
250 and pristine cell samples, the quality of the DNA recovered from samples is likely to be
251 superior to that obtained in casework [18]. Therefore, a minimum optimal number of X/Y-
252 FISH dissected epithelial cells required for Identifiler™ analysis has been set at 75, coupled
253 with a recommendation to collect up to 150 cells, if present, to compensate for any
254 degradation of the target DNA.
255
256 The LCN test requires replicate testing to be undertaken to produce a consensus profile, in
257 accordance with the method advocated by Gill et al. [13]. The consequence of this model is
258 that an extract must be split into at least two fractions, to enable replicate amplifications, and
259 therefore some loss in profiling information may occur. This may be ameliorated, in some
260 instances, by splitting the original sample into three fractions, so that if one DNA result of a
261 heterozygote pair is missing in the first replicate amplification, and the second result in the
262 pair is missing in the second replicate amplification, the third amplification may provide a

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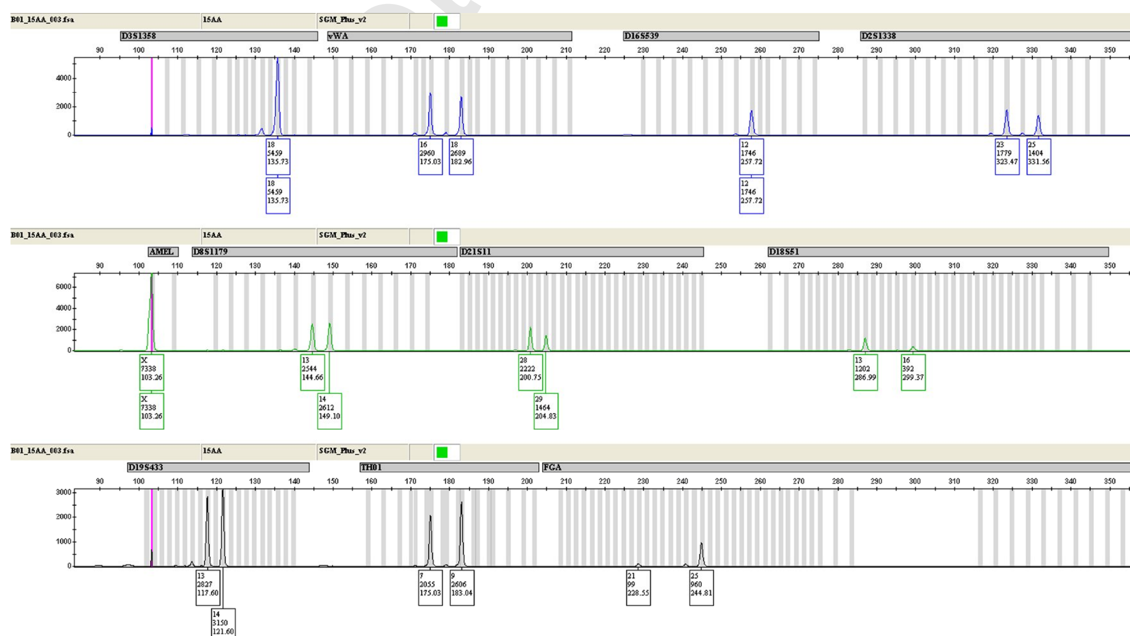
263 duplicate to one or both results. However at very low cell numbers, splitting the sample into
264 three fractions may be counterproductive and the sample may be best split two ways to
265 maximise duplication of results.
266
267 A comparison between the profiling results obtained from 30 X/Y-FISH dissected epithelial
268 cells after Identifiler™ analysis and LCN SGM Plus™ analysis, indicate that at this cell
269 number the methods are approximately equal with an average of 21 and 17 alleles detected in
270 the respective profiles. The average of 17 alleles reported from LCN SGM Plus™ analysis of
271 30 cell samples is from consensus results, with individual profiles containing at least this
272 number of results. Representative 30 cell profiles are shown in Figure 1. The results from
273 this study indicate that when there are less than 30 X/Y-FISH labelled epithelial cells, LCN
274 DNA analysis should be undertaken as the increased sensitivity of this test will likely
275 compensate for the lower amounts of potentially poor quality DNA. The recommeded lower
276 limit for Identifiler™ DNA analysis of X/Y-FISH labelled epithelial cells is 30 cells.
277



278

279 SGM Plus™ profile from half a 30 cell extract, at 34-cycle PCR.

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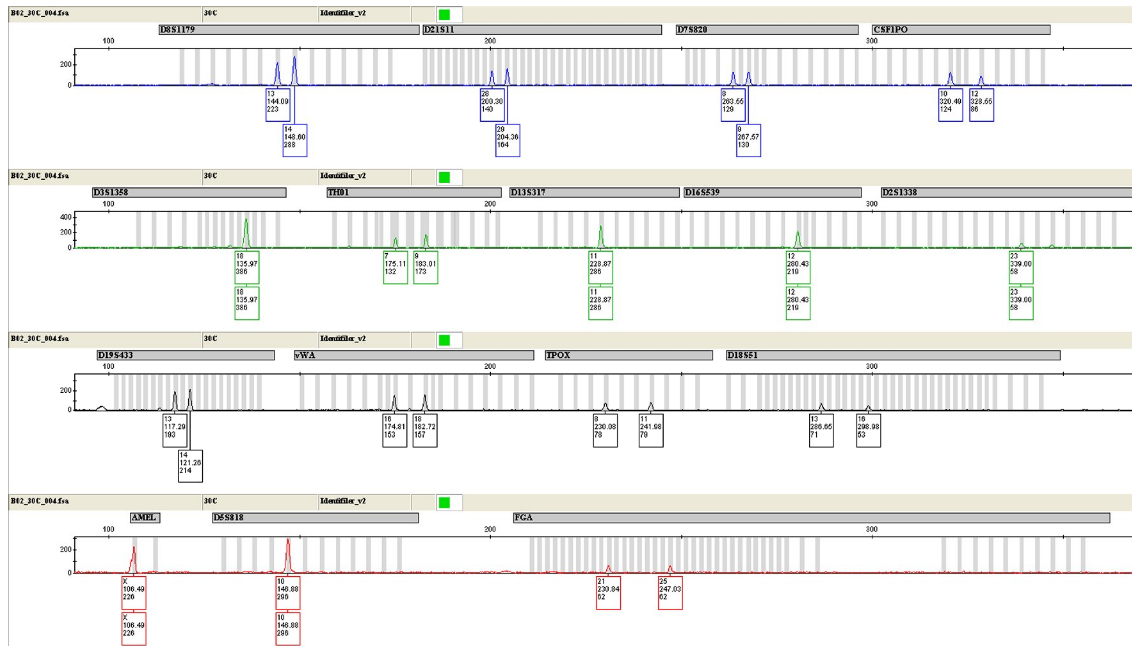

Identifiler™ profile of 30 cells, at 28-cycle PCR.

Figure 1. Profiling results obtained after SGM Plus™ analysis and Identifiler™ analysis of 30 cell samples. To enable duplicate amplification by 34-cycle SGM Plus™, the 30 cell extract was halved; one of two replicate profiles shown.

Lucy et al. [19] suggest that a DNA extract from theoretically 8 intact and non-degraded haploid cells is required for a 90% chance that there is at least one copy of all alleles in the profile from 10 heterozygote loci using 34-cycle PCR. This theoretically equates to the DNA content from 4 diploid cells and to fulfil duplicate profiling, 8 diploid cells would be required. This probability does not reflect the effect of factors such as DNA quality, extraction and the PCR processes. As observed in our data, the 10 cell samples tested at 34-cycle PCR contained on average 9 alleles, equivalent to approximately half of a SGM Plus™ profile.

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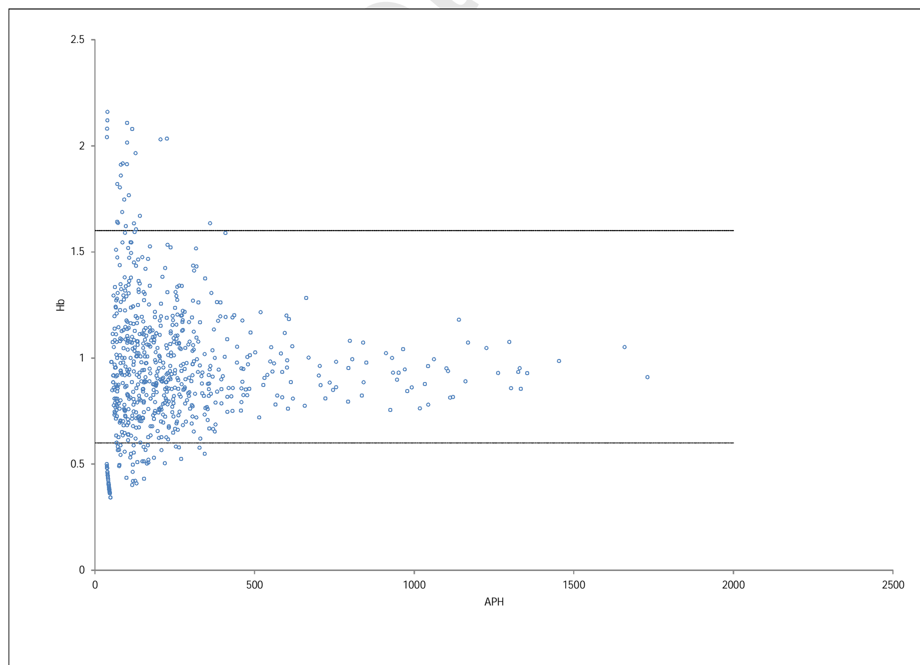
300 As LMD technology enables cells to be individually collected into a tube and the DNA
301 extracted from the cells can be directly amplified within the same tube, a direct method of
302 extraction and PCR should, theoretically, reduce any stochastic effects introduced by the
303 random sampling of alleles prior to amplification. That is, as the entire DNA extract is
304 available for amplification, the alleles at each locus should be equally, or more equally,
305 represented than what is observed when an aliquot of an extracted DNA sample is amplified.
306 Therefore, it was hypothesised that this testing would result in minimal differences in peak
307 heights between heterozygote alleles at a locus in the Identifiler™ profiles of X/Y-FISH
308 dissected cell samples.
309
310 A commonly used bound on *Hb* in 28-cycle PCR is $0.6 < Hb < 1.66$ [20, 21]. Examination of
311 the plot, *Hb* vs. APH of heterozygous peaks within the Identifiler™ profiling data, indicates
312 that *Hb* was more variable at low APH, with the bound found to hold when the APH was
313 above 361 RFU (Figure 2).
314



315

316 Figure 2. A plot of *Hb* vs. APH of pairs of peaks at the heterozygous loci of Identifiler™
317 profiles of X/Y-FISH LMD cells. The dashed lines represent the *Hb* bounds of 0.6 and 1.66.
318

319 A study conducted by Bright et al. (2011) of 131 single source casework sample Identifiler™
320 profiles, of varying sample type and profile quality, showed that the bound on *Hb* was met
321 above an APH of 267 RFU for non-stutter affected peak heights and 265 RFU for stutter
322 affected peak heights [21]. Therefore, no improvements in peak height balance were found in
323 the profiles from the X/Y-FISH dissected cell samples. The one-tube extraction and PCR of
324 the entire DNA extract should, in theory, minimise allele imbalance introduced through
325 stochastic effects from sampling. These results suggest that other factors such as the PCR
326 process, and possibly also any associated DNA degradation or PCR inhibitory effects
327 introduced through the FISH labelling process, could have a greater effect on peak balance.
328

329 To further investigate the effect, if any, of the FISH labelling process on peak balance, *Hb* vs.
330 APH of heterozygous peaks within the Identifiler™ profiling data obtained from CTS stained
331 cells was compared to data obtained from this study for 50 cells (n=6 per treatment) and 25
332 cells (n=3 per treatment).
333

334 A plot, *Hb* vs. APH of heterozygous peaks within the Identifiler™ profiling data for X/Y
335 FISH labelled cells and CTS stained cells, shows that for these cell numbers the *Hb* bound
336 was met at an APH of 209 RFU for X/Y-FISH labelled samples and 144 RFU for CTS
337 stained samples (Figure 3) . These results indicate that the X/Y-FISH process itself has a
338 contributory negative impact on peak balance.

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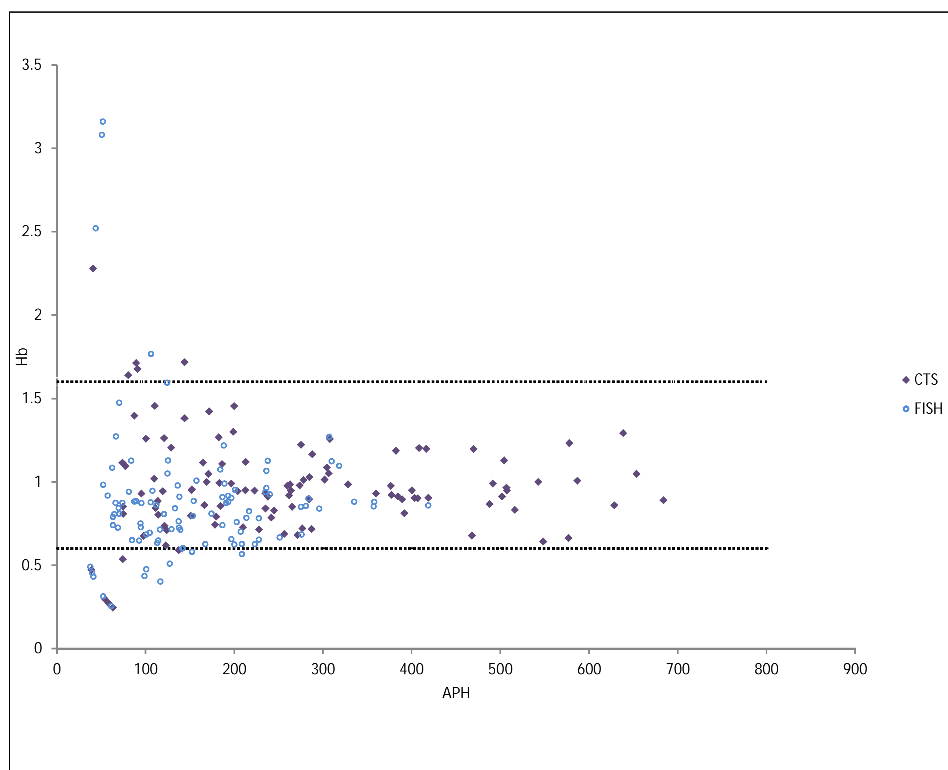

339

340 Figure 3. A plot of *Hb* vs. APH of pairs of peaks at the heterozygous loci of Identifiler™
341 profiles of X/Y- FISH labelled epithelial cells and CTS stained epithelial cells, after 6
342 amplifications of 50 cells and 3 amplifications of 25 cells for each staining method. The
343 X/Y- FISH labelled data was randomly selected from the larger data set that was available at
344 these cell numbers. The bound of 0.6 <*Hb*< 1.66 is displayed on the graph as dashed lines.
345

346 Heterozygote balance of LCN SGM Plus™ profiles from X/Y-FISH dissected cell samples
347 was also investigated. A plot of log *Hb* vs. APH of pairs of peaks at the heterozygous loci of
348 the LCN SGM Plus™ profiles is provided in Figure 4. Log values were taken as these
349 provided a better visual representation of the observed data.
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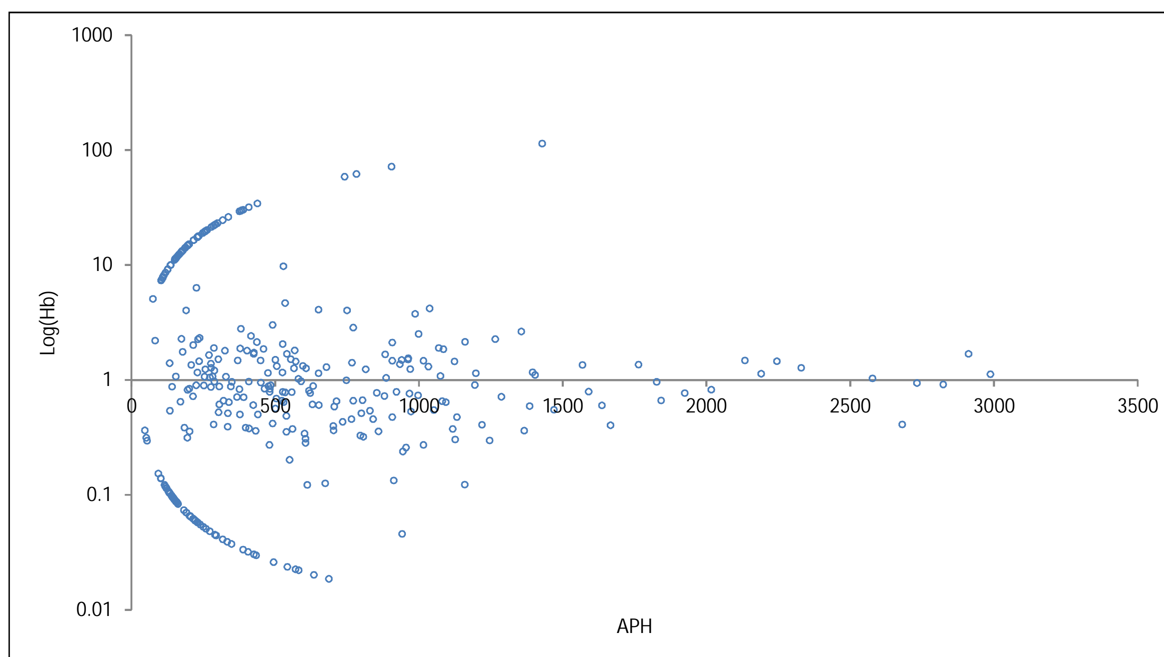

351
352 Figure 4. A plot of log [*Hb*] vs. APH of pairs of peaks at the heterozygous loci of the SGM
353 Plus™ profiles from X/Y-FISH LMD cells. The hammer-head effect for many of the low
354 APHs is due to the occurrence of allelic drop–out, where missing alleles were added to the
355 dataset at 25 RFU, half the peak detection threshold.
356
357
358 The peak balance of heterozygous loci in LCN SGM Plus™ profiles of X/Y-FISH dissected
359 epithelial cells was expected to be more variable than what had been observed in the
360 Identifiler™ profiles, due to the stochastic effects introduced by removing aliquots of DNA
361 from the extract for duplicate amplification, potentially resulting in unequal amounts of
362 template DNA in the starting reactions. The sampling effect was expected to be further
363 exacerbated by the limited amount of starting material and natural variations in the PCR, as
364 described in Buckleton et al. [22]. These effects were observed in the LCN profiling data as
365 indicated by the more variable *Hb,* although *Hb* did generally approach 1 with higher APH.
366 The hammer-head effect observed for many of the low APHs is a consequence of allelic
367 drop-out, where missing alleles were re-inserted in the data at half the peak detection
368 threshold.

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369 As shown in Figure 5, peak balance is improved in the SGM Plus™ profiles of the higher cell
370 number samples, as seen by the trend of increased clustering around one for the 10 to 30 cell
371 samples. For samples of 2 and 4 cells, where there is lower starting DNA template, there is
372 less clustering around one as drop-out is more pronounced. A similar trend is also seen with
373 the Identifiler™ profiles, with a trend of increased clustering around one for the 15 to the 150
374 cell samples (Figure 6).

375

376 Figure 5 here

377

378 Figure 5. A plot of log [*Hb*] vs. cell numbers for SGM Plus™ profiles.

379

380 Figure 6 here

381 Figure 6. A plot of log [*Hb*] vs. cell numbers for Identifiler™ profiles.

382

383 **Conclusions**

384

385 Examination of the DNA profiling results for 28-cycle Identifiler™ analysis and 34-cycle
386 SGM Plus™ analysis of X/Y-FISH LMD cells indicate that results, suitable for comparison
387 purposes in a forensic investigation, can be obtained from the analysis of epithelial cell
388 samples identified using X/Y-FISH labelling and recovery by LMD using either profiling
389 method. For this dataset, the recommended number of epithelial cells for 28-cycle
390 Identifiler™ analysis has been set at an optimal minimum of 75 and the limit of detection for
391 this profiling system set at approximtely 30 epithelial cells. When the epithelial cell numbers
392 available for testing reduce below 30 cells, DNA analysis using a more sensitive method,
393 such as LCN DNA analysis, is recommended.

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395 An investigation of *Hb* and APH for Identifiler™ profiles indicates that a stochastic threshold
396 of 400 RFU holds for the anaysis of X/Y-FISH LMD epithelial cells. The expected
397 improvement to peak balance for pairs of peaks in the profiles of the X/Y-FISH labelled
398 LMD epithelial cells was not observed, with our data suggesting that the X/Y-FISH labelling
399 process itself has some influence on DNA profiling.

400

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