

A proof of principal study on the use of direct PCR of semen and spermatozoa and development of a differential isolation protocol for use in cases of alleged sexual assault

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Abstract

Sexual assault samples are some of the most common samples encountered in forensic analysis. These samples can require a significant time investment due to differential extraction processes. We report on the first record of successful direct amplification of semen for STR analysis. Neat seminal fluid, dilutions ranging from 1:5 to 1:160 and GEDNAP samples were successfully amplified using a direct method. A mild differential isolation technique to enrich spermatozoa was developed and successfully implemented to separate and directly amplify a mixture of semen and female epithelial cells. Aliquots of samples subjected to the differential isolation protocol were stained with Haemotoxylin and Eosin for sperm scoring. Samples stained after PCR showed a complete lack of intact spermatozoa demonstrating that the cells are lysed during the PCR process. This paper demonstrates the potential to incorporate direct PCR in cases of sexual assault to more rapidly obtain results and achieve a higher sensitivity. (150)

Keywords

Direct PCR; Sexual Assault; Semen; Seminal Fluid; Differential Isolation; spermatozoa

1. Introduction

Amplification without prior DNA extraction, known as direct PCR, has gained increased interest in forensic science due to the reduced time to DNA profile and increased sensitivity of the technique. Although its application to forensic science is relatively recent, the technique itself has been used since the 1990s [1-3].

Benefits of direct PCR include reduced time and expenses compared to standard DNA extraction prior to amplification. In direct PCR, manual processing of the sample as well as the amount of buffers and consumables needed are limited to a minimum which helps to avoid the introduction of contamination [4, 5]. Furthermore, DNA extraction results in significant loss of DNA from the samples due in part to multiple tube changes and also due to sometimes low DNA recovery rates [6]. This is especially concerning in samples originally containing very low amounts of DNA.

Direct PCR has already been tested successfully for various sample types, including touch DNA (epithelial cells and cell free DNA) [4]; blood [1, 2, 7-10]; buccal swabs/cells [8-10]; hair [2, 11]; fingernails [12]; tissues [3], and plant material [13]. Other highly significant forensic sample types are those relating to sexual assault allegations, specifically semen and spermatozoa; however, to our knowledge, investigation for the use of direct PCR in these types of crimes has not yet been undertaken. Direct PCR has been used to identify pathogens in semen (e.g. [14, 15]), but never for the identification of the semen donor, as required in forensic investigation. Sexual assault allegations can make up a large proportion of casework samples and can also be more time intensive due to specific aspects such as differential extraction [16] and, microscopy and sperm scoring [17].

Presumptive identification of suspected semen is well documented and mainly consists of the use of Acid Phosphatase (AP) [18, 19] and Prostate Specific Antigen (P30/PSA) [20, 21], however these presumptive tests are known to cross react with a variety of substances [22-26]. Direct observation of spermatozoa is therefore needed in some regions as conclusive proof of ejaculation so microscopy and sperm scoring may still be required, however direct PCR would still offer major benefits including: minimal amount of sample destruction; faster analysis; greater sensitivity; decreased cost, and; reduced risk of contamination. Semen can be found on intimate body swabs (often as a mixture), on skin, clothing and bedding items, all of which are routinely submitted for forensic examination [27, 28]. Dependant on if the semen is neat and expected as a single source, as it may be with bedding and clothes, or a mixture is expected, as with intimate swabs, will determine the downstream analysis method. Mixed samples from intimate swabs are processed using a two-step differential extraction [16] to separate the sperm fraction from the epithelial fraction.

As the samples are not purified through an extraction process, direct PCR incorporates all elements of a sample in to the analysis. This includes the total DNA of a sample, but also cellular components and other potential inhibitors, which can have an adverse effect on the PCR process. Semen has been demonstrated to have between 15 and 200 million spermatozoa per mL for males with normal sperm counts [29] and up to 5 million other non-spermatozoa cells per mL, mainly constituting leucocytes and round cells (debris and immature sperm) [30], but which will still contain the donors DNA. Heme, a component of blood, has been shown to act as a PCR inhibitor by blocking the active site of the polymerase [31, 32]. Other cellular or tissue based inhibitory substances that can be commonly encountered in forensic cases include: polysaccharides and humic acid, found in soils and plants [33-35]; urea, found in urine [36], and calcium, found in bones [37]. Other inhibitory

78 substances such as indigo dye, found in blue jeans, have also been demonstrated to pose problems
79 with the amplification process [38]. The overall inhibitory effect of different substances can be
80 reduced based on the particular polymerase used in the PCR [39, 40], but to date various sample
81 types have been successfully typed using direct PCR without problem.

82 In this study, we investigated the potential for direct amplification of spermatozoa and seminal fluid
83 in order to determine the donor. We subjected stains containing seminal fluid (pure and mixtures) to
84 the technique of direct amplification and investigated a differential isolation method prior to direct
85 amplification.

86 **2. Materials and Methods**

87 All samples were collected and obtained in accordance with ethical requirements as set out by the
88 University of Strathclyde.

89 **2.1 Semen**

90 Semen was obtained from a fertility clinic and anonymous donors. All samples contained normal
91 sperm counts between 15 and 200 million spermatozoa/mL [29], which equates to between 15,000
92 and 200,000 spermatozoa/ μ L and between 48.7 and 649.3 ng/ μ L DNA.

93 **2.2 Samples in cases of sexual assault**

94 Semen dilutions (1:5, 1:10, 1:20, 1:40, 1:80 and 1:160) were spotted on sterile cotton, air dried in a
95 safety cabinet and subjected to direct PCR. Dilutions were performed in sterile distilled water to a
96 final volume of 1 mL. Two repetitions at each dilution were performed. Four stains from previous
97 proficiency tests of the German DNA profiling (GEDNAP) group were subjected to direct PCR: stain D
98 of GEDNAP 38 (semen on toilet paper), stain 4 of GEDNAP 43 (blood-semen-mixture on tissue), stain
99 2 of GEDNAP 45 (semen on condom, stain was transferred to a swab), and stain 2 of GEDNAP 46
100 (semen on toilet paper). GEDNAP stains were each processed twice to ensure consistency. Neat
101 semen (10 μ L) and blood (approximately 10 μ L) were deposited on to the various substrates and
102 allowed to air dry in a safety cabinet to avoid contamination. This was done in duplicate for each
103 fluid and each substrate.

104 **2.3 Differential lysis**

105 Body fluid mixtures often encountered in sexual assault allegations were prepared to test a short
106 differential enrichment protocol. Mixtures of semen and saliva (1:10), to a final volume of 100 μ L,
107 were prepared and kept in liquid form and also spotted onto sterile cotton. Saliva was donated by a
108 female volunteer to more closely simulate expected male/female mixtures. Several short fibres, for
109 dried stains, and 5 μ L, for liquid samples, were submerged in 18 μ L Swab solution (Promega).
110 Proteinase K (2 μ L, 20mg/mL) was added and samples were incubated for 15, 30 and 60 min at 70°C.
111 After centrifugation 2 μ L of the supernatant was transferred for direct PCR while the pellet was
112 washed twice with 100 μ L swab solution and then transferred for direct PCR.

113 Five μ L of the resuspended sperm fraction (SF) and non-sperm fraction (NSF) were slide mounted
114 and stained using Haemotoxylin and Eosin (HE). Slides were assessed for spermatozoa using a Leica
115 DM 2500 Microscope (Wetzlar, Germany) with magnification of 500x (ocular 10x, objective 50x).

116 **2.4 Direct PCR**

117 Samples deposited on fabric, including toilet paper and tissue, were collected for direct PCR by
118 taking two to three fibres, approximately 2-3mm in length, from the substrate and adding directly to
119 the PCR reagents. NSF samples were added at 8 µL to the PCR and the total pellet from the SF was
120 added, minus the sample removed for sperm scoring.

121 All samples were amplified without extraction using PowerPlex ESX 16 and 17 Kits (Promega Corp.).
122 All samples were processed according to manufacturer's specifications; reaction volumes were
123 reduced to 12.5 µL for samples processed with ESX 17. Samples treated with the differential
124 enrichment protocol additionally had 1 x Amp Solution (Promega) added to the mastermix. For solid
125 substrate samples (fibres, tissue and toilet paper) amplification-grade water was added up to the
126 final volume required by the PCR kit.

127 Amplification took place in a 2720 Thermal Cycler (Life Technologies) and all batches were processed
128 with both a negative and positive controls. All PCR products were visualised on a 3130 Genetic
129 Analyser (Life Technologies, UK). Data were analysed using Genemapper ID v.3.2.1 software (Life
130 Technologies, UK) with a threshold of 50 rfu used as the limit of detection, and 150 rfu as the limit
131 for a homozygote. Local and global balance were calculated according to [41].

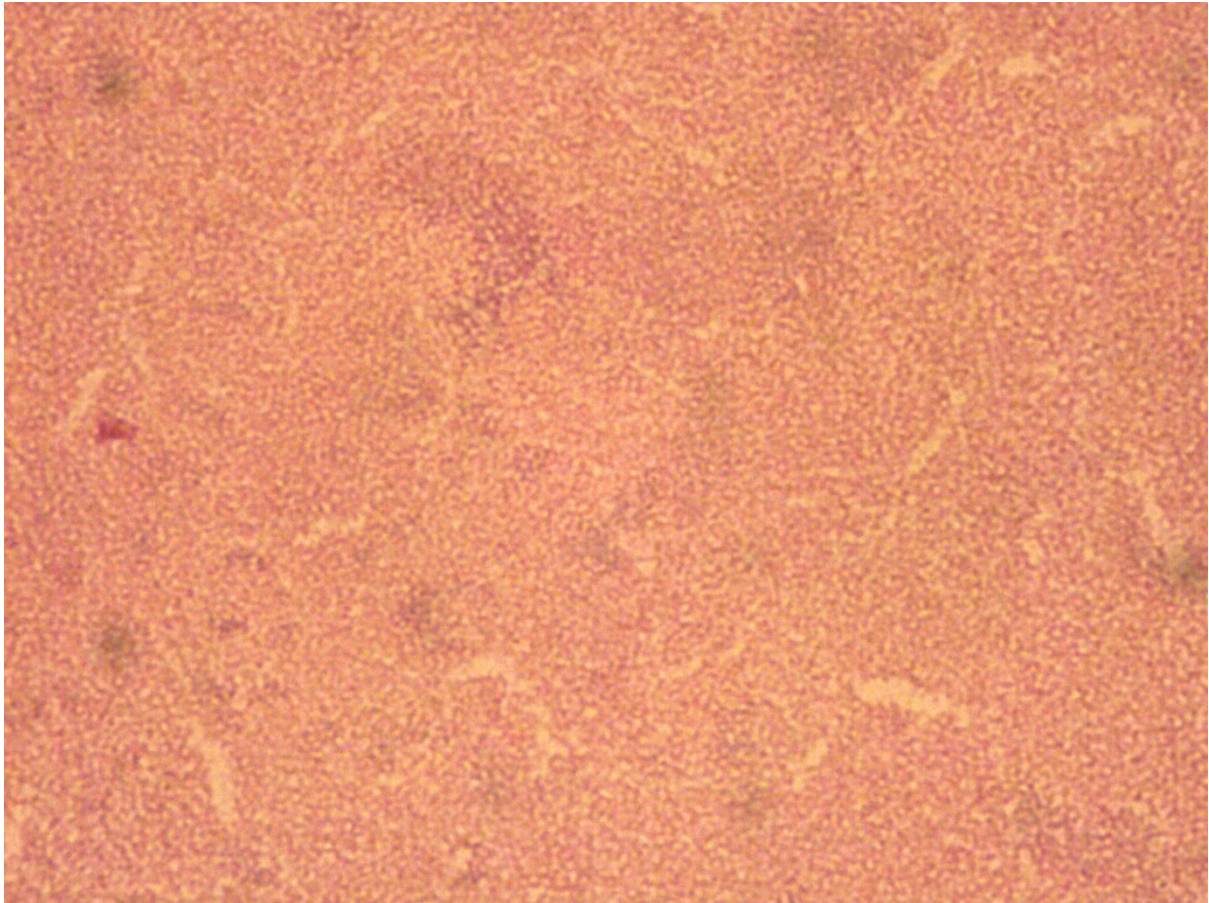
132 **2.5 Lysis of spermatozoa**

133 To determine if the spermatozoa were being lysed during the PCR process, samples were centrifuged
134 post-PCR and 10 µL was removed from the bottom of the PCR tube (where a pellet would have
135 formed) and then slide mounted, HE stained and examined as previously described.

136 **3. Results and Discussion**

137 **3.1 Lysis of spermatozoa**

138 Samples HE stained post-PCR showed a complete lack of intact spermatozoa (Figure 1). This is the
139 first instance that the lysis of spermatozoa during PCR has been demonstrated and supports the use
140 of direct PCR in cases of sexual assault for SF samples. Complete lysis of the spermatozoa indicates
141 that direct PCR can be used for sexual assault samples and that despite the protein coat, samples
142 can be fully analysed without the risk of missing potentially vital genetic material, important with
143 low level samples. In this way case samples that may previously have yielded insufficient levels of
144 DNA, due to minimal levels of DNA combined with low extraction efficiency, can now be analysed
145 with the total DNA compliment offering resolution of difficult, low level, case samples.

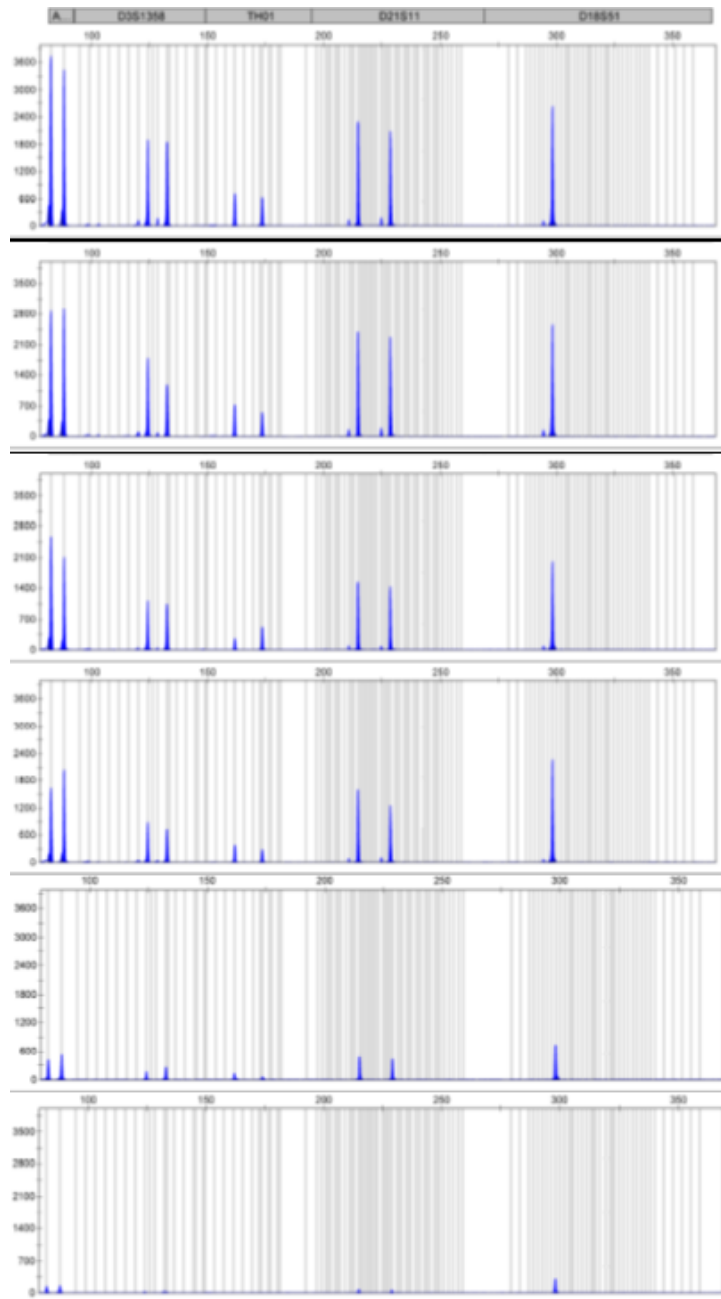


146

147 **Figure 1: Post-PCR sample (1:1), centrifuged and pellet area mounted and HE stained; magnified at 500x demonstrating**
148 **the lack of intact spermatozoa.**

149 ***3.2 Dilution of semen and GEDNAP samples***

150 The dilution series of seminal fluid produced full profiles at all dilutions to 1:80 demonstrating the
151 potential for direct PCR in cases of sexual assault. Global balance for full profiles ranged from 0.78
152 (one 1:40 dilution) to 0.91 (one 1:10 dilution) with an average of 0.84. Local balance showed a
153 minimum range from 0.4 (SE33 in one 1:20 dilution) to 0.72 (D19 in one 1:10 dilution) and an
154 average of 0.6. One sample at 1:80 produced a full profile, while the other sample showed one locus
155 (D19) and one allele (THO1) dropping out. Both samples at 1:160 showed higher levels of dropout,
156 with one sample containing 50 % of the expected profile and the other 47 %. Peak heights reduced
157 as the dilution increased (Figure 2). Stains demonstrating a weak presumptive positive, or which are
158 known to be diluted as in the case of washing, may therefore benefit from the addition of more
159 fibres or concentration via a differential isolation protocol as described below.

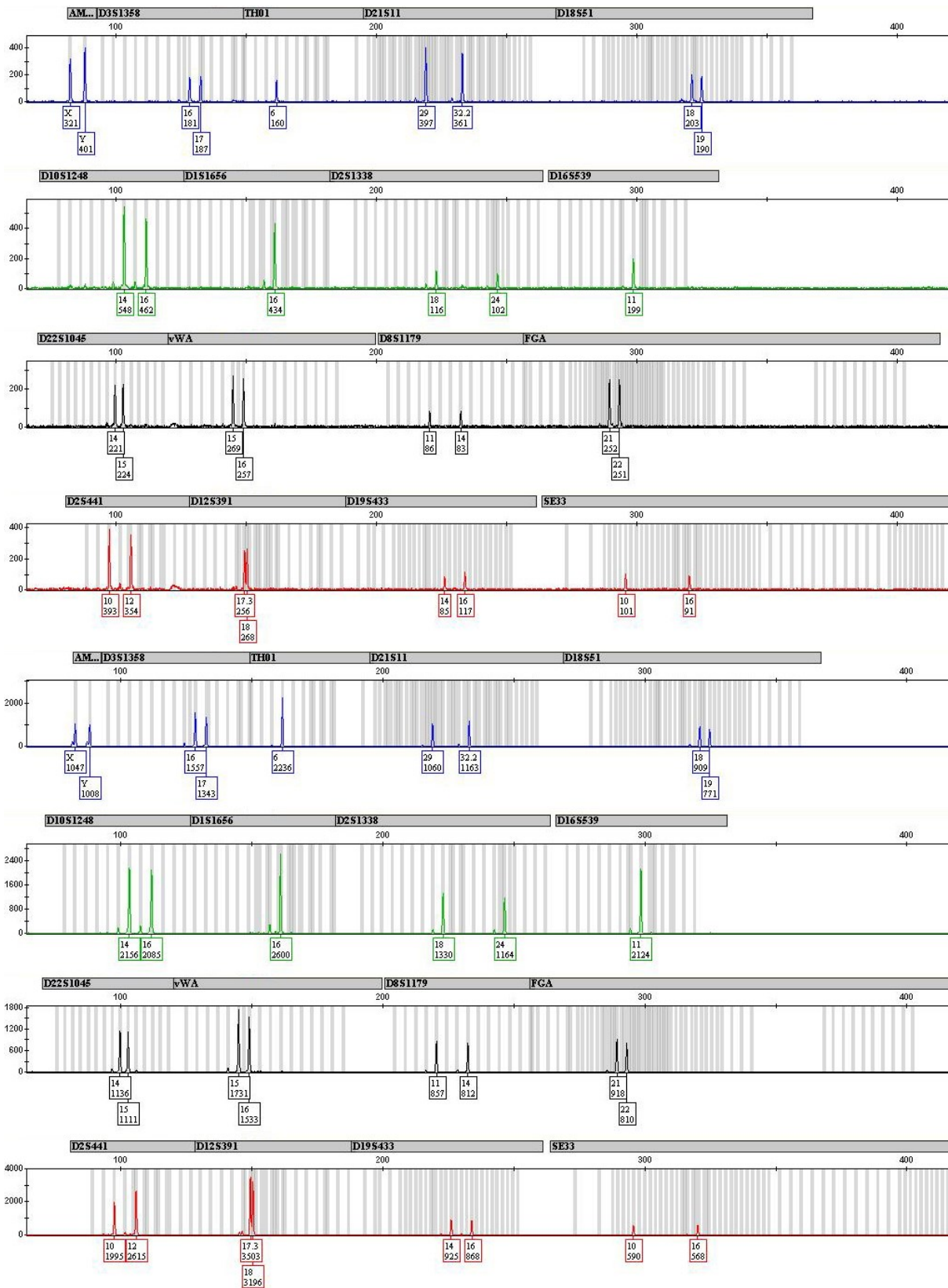


160

161 **Figure 2: Blue dye channel of ESX17 showing direct PCR result from dilutions of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 (top**
 162 **to bottom).**

163 All GEDNAP-stains produced low-level, but full and reproducible profiles (Figure 3) containing the
 164 same alleles as when analysed using a traditional differential extraction technique. Total peak height
 165 and average peak height were 7,570 and 222.65 ± 123.68 for the direct PCR sample, and 52,829 and
 166 1553.79 ± 464.67 for the traditionally extracted sample. The traditionally extracted and amplified
 167 sample showed a local allelic balance between 0.79 and 1, with a global balance of 0.928, whereas
 168 the direct PCR sample showed a local allelic balance between 0.73 and 1, with a global balance of
 169 0.925. While the average peak height for the direct PCR sample (Figure 3 top) is 14.3 % the height of
 170 the traditionally extracted sample (Figure 3 bottom), it is still complete and could be fully called
 171 using the Genemapper ID v.3.2.1 software. The local and global balances are similar, with only a 0.32
 172 % difference between the global balances, indicating that the direct PCR approach amplified

173 efficiently and as would be expected from a traditionally extracted sample. Considering that only
174 two 2 mm fibres were sampled for direct PCR relative to a sample of approximately 2 mm² section
175 for the traditional extraction sample, the direct amplification of seminal fluid and stains is a less
176 destructive process, leaving valuable evidence behind for subsequent analysis or re-evaluation.
177 GENAP samples represent simulated case samples that are used for proficiency testing relating to
178 presumptive testing and STR analysis. The ability to amplify these samples using direct PCR therefore
179 demonstrates the robustness and applicability of direct amplification of semen to forensic casework.



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Figure 3: Top - Full profile obtained by direct PCR from 2 fibres (2mm each) of stain 2 of GEDNAP 46 (semen on toilet paper). Bottom – Full profile obtained by traditional differential extraction of 2 of GEDNAP 46.

184 ***3.4 Differential isolation of spermatozoa in male/female mixtures***

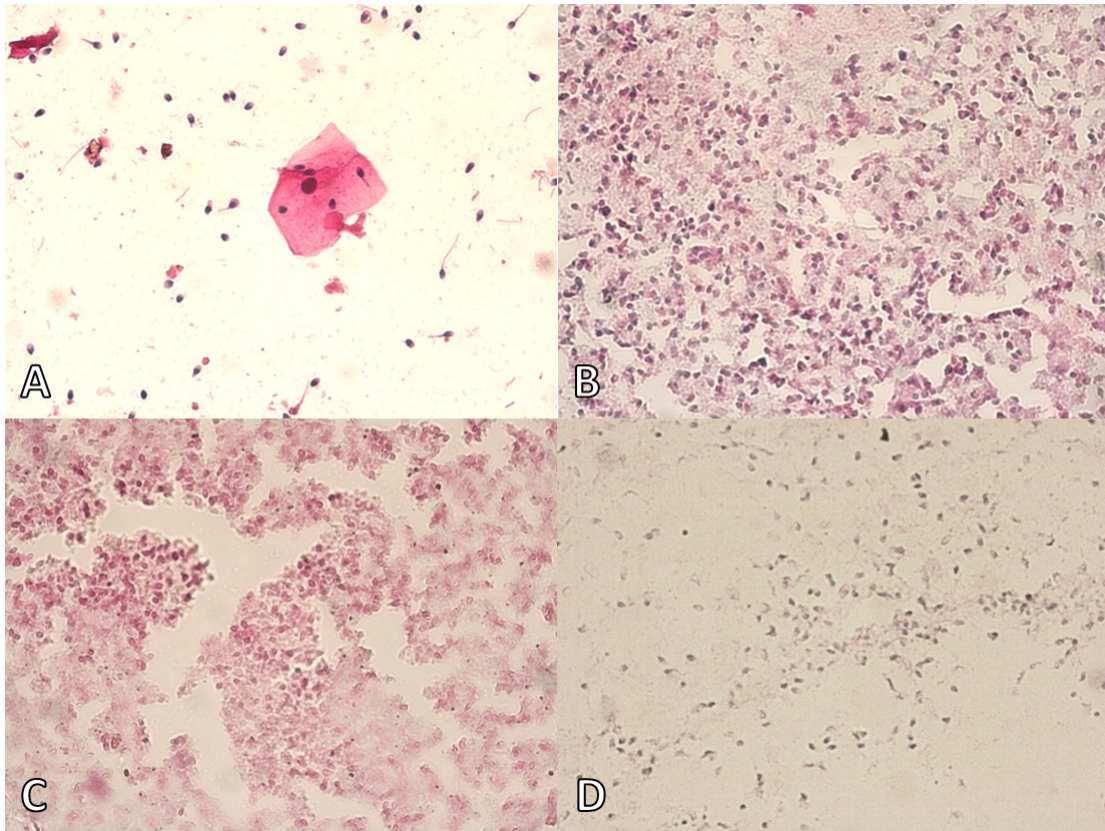
185 The ability to distinguish between male and female fractions of a mixed sample in cases of sexual
186 assault is of primary importance to forensic investigators. This has been customarily accomplished
187 using a differential extraction protocol [16] or through other techniques such as laser
188 microdissection of spermatozoa or epithelial cells [42]. These techniques can add several hours to
189 the analysis of sexual assault evidence and contribute to backlogs in the processing of sexual assault
190 casework. A more rapid method for differentiating cells from the complainant (female) and accused
191 (male) in allegations of sexual assault would therefore offer obvious benefits in reduction of time for
192 analysis and, in conjunction with a direct amplification approach, greater sensitivity.

193 The mild differential isolation technique used to enrich spermatozoa and remove the female fraction
194 was successful as demonstrated by the HE stained samples (Figure 4). Most non-sperm cellular
195 components were digested after 15 minutes (Figure 4b) and after 60 minutes of incubation the non-
196 sperm cells were completely digested (Figure 4d). Our results indicated that the optimal incubation
197 time was 60 minutes (data not shown). This incubation time showed the best separation between
198 the male and female STR profiles, with the female profile component reducing in intensity as the
199 incubation time increased.

200 Profiles resulting from the differentially isolated samples show high levels of amplification with good
201 balance across all loci. Global balance ranged from 0.78 to 0.87 for liquid mixtures and 0.78 to 0.9 for
202 dried mixtures on cotton.

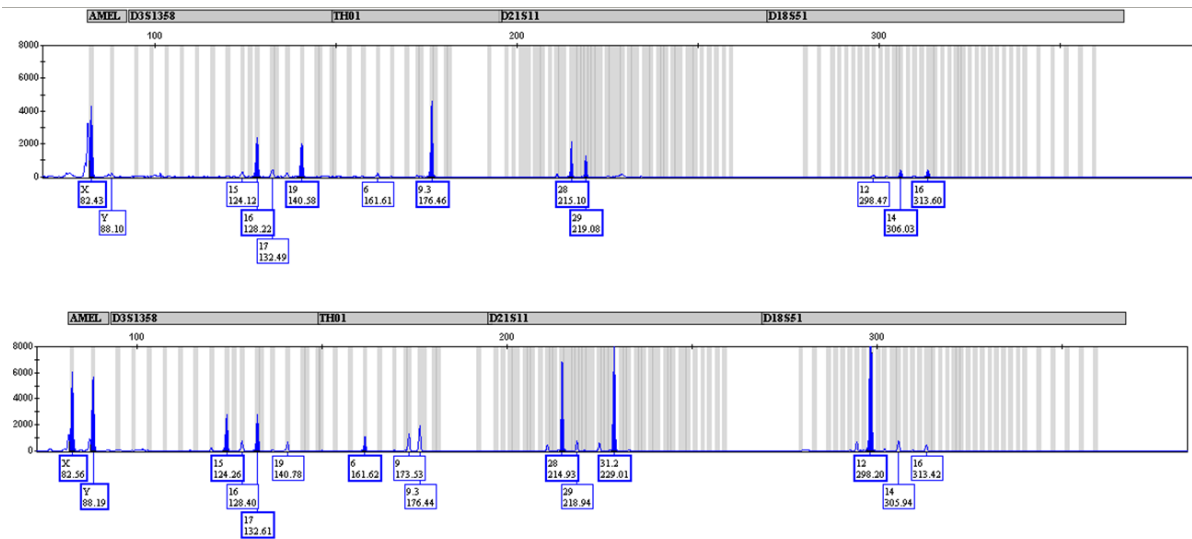
203 While the differential isolation method adds a small amount of time to the direct amplification
204 process, it resulted in strongly enhanced male profiles. Even though the female fraction was still
205 present at low levels after enrichment, the male profile was clearly distinguishable (Figure 5). The
206 female fraction, derived from the supernatant, similarly showed a mixture with the female fraction
207 accounting for the major contributor (Figure 5).

208 We conclude that even in samples with a massive excess of female cells, direct PCR can produce
209 male profiles with surprisingly strong peak heights if combined with the described differential
210 isolation protocol. Using the whole cell pellet directly in the PCR means that little or no genetic
211 material is lost during the DNA extraction processes. This ability to use the whole sample makes
212 direct amplification a powerful technique for forensic analysis.



213

214 **Figure 4: Differentially isolated samples stained using HE at various incubation times, magnified 500x. a) no incubation;**
 215 **b) 15 min incubation; c) 30 min incubation, and; d) 60 min incubation.**



216

217 **Figure 5: Direct PCR after differential enrichment of a semen-saliva mixture (1:10). The blue channel of the ESX**
 218 **amplification is shown exemplarily, containing the markers amelogenin, D3S1358, TH01, D21S11, and D18S51. The male**
 219 **DNA profile obtained from the cell pellet after differential lysis (top) reveals surprisingly high peaks, while the female**
 220 **fraction in the supernatant (bottom) is also clearly distinguishable from the male fraction.**

221

222 4. Conclusion

223 We have demonstrated that even though spermatozoa are enclosed in a firm cell wall that they can
224 be analysed by direct PCR, without the need of labour and time intensive extraction methods. While
225 the DNA in the sample may in part be derived from the non-spermatozoa cells of the semen donor,
226 our results from the post-PCR HE stained samples demonstrate that the spermatozoa are being lysed
227 during the PCR process and are directly contributing to the resultant profile.

228 Simulated case samples and GEDNAP proficiency testing samples were all successfully typed offering
229 significant improvements in time and sensitivity in the analysis of sexual assault evidence. For the
230 first time the successful direct amplification of semen and spermatozoa on various substrates and in
231 simulated sexual assault samples has been achieved.

232 Without the need for extraction, up to 5 hours (if using differential extraction) can be saved and the
233 chance of success is increased. Standard extraction techniques can lose greater than 70 % of the
234 genetic material, therefore direct PCR of semen can allow amplification of samples which may have
235 previously failed to yield results. The use of standard equipment and reagents means that this
236 technique can be implemented rapidly into most forensic laboratories with simple validation testing.
237 The successful use of direct PCR with semen samples will allow rapid and more sensitive analyses in
238 sexual assault casework samples.

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243 References

- 244 1. Mercier B, Gaucher C, Feugeas O, Mazurier C (1990) Direct PCR from whole blood, without
245 DNA extraction. *Nucleic acids research* 18: 5908.
- 246 2. Ohhara M, Kurosu Y, Esumi M (1994) Direct PCR of whole blood and hair shafts by
247 microwave treatment. *Biotechniques* 17: 726, 8-, 8.
- 248 3. Panaccio M, Georgesz M, Hollywell C, Lew A (1993) Direct PCR from solid tissues without
249 DNA extraction. *Nucleic acids research* 21: 4656-.
- 250 4. Linacre A, Pekarek V, Swaran YC, Tobe SS (2010) Generation of DNA profiles from fabrics
251 without DNA extraction. *Forensic science international: genetics* 4: 137-41.
- 252 5. Vallone PM, Hill CR, Butler JM (2008) Demonstration of rapid multiplex PCR amplification
253 involving 16 genetic loci. *Forensic Science International: Genetics* 3: 42-5.
- 254 6. Butts ELR, Kline MC, Almeida JL, Vallone PM. (2010) DNA extraction efficiency: is it what you
255 thought? *Mid-Atlantic Association of Forensic Scientists State College, PA*
- 256 7. Aki K, Kawazoe K, Izumi A, Tada T, Minakuchi K, Hosoi E (2014) Direct determination of ABO
257 blood group genotypes from whole blood using PCR-amplification of specific alleles method.
258 *American Journal of BioScience* 2: 49-55.
- 259 8. Park SJ, Kim JY, Yang YG, Lee SH (2008) Direct STR Amplification from Whole Blood and
260 Blood-or Saliva-Spotted FTA® without DNA Purification*. *Journal of forensic sciences* 53: 335-41.
- 261 9. Wang DY, Chang C-W, Oldroyd NJ, Hennessy LK (2009) Direct amplification of STRs from
262 blood or buccal cell samples. *Forensic Science International: Genetics Supplement Series* 2: 113-4.
- 263 10. Vandewoestyne M, Van Hoofstat D, Franssen A, Van Nieuwerburgh F, Deforce D (2013)
264 Presence and potential of cell free DNA in different types of forensic samples. *Forensic Science*
265 *International: Genetics* 7: 316-20.

- 266 11. Ottens R, Taylor D, Abarno D, Linacre A (2013) Successful direct amplification of nuclear
267 markers from a single hair follicle. *Forensic science, medicine, and pathology* 9: 238-43.
- 268 12. Tie J, Uchigasaki S (2014) Detection of short tandem repeat polymorphisms from human
269 nails using direct polymerase chain reaction method. *ELECTROPHORESIS*: n/a-n/a. doi:
270 10.1002/elps.201400061
- 271 13. Gausterer C, Stein C, Stimpfl T (2012) Application of direct PCR in a forensic case of yew
272 poisoning. *Int J Legal Med* 126: 315-9. doi: 10.1007/s00414-011-0607-0
- 273 14. Amin AS, Hamdy MER, Ibrahim AK (2001) Detection of *Brucella melitensis* in semen using the
274 polymerase chain reaction assay. *Veterinary Microbiology* 83: 37-44. doi:
275 [http://dx.doi.org/10.1016/S0378-1135\(01\)00401-1](http://dx.doi.org/10.1016/S0378-1135(01)00401-1)
- 276 15. Van den Brule A, Hemrika D, Walboomers J et al (1993) Detection of *Chlamydia trachomatis*
277 in semen of artificial insemination donors by the polymerase chain reaction. *Fertility and sterility* 59:
278 1098-104.
- 279 16. Yoshida K, Sekiguchi K, Mizuno N et al (1995) The modified method of two-step differential
280 extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. *Forensic*
281 *Science International* 72: 25-33. doi: [http://dx.doi.org/10.1016/0379-0738\(94\)01668-U](http://dx.doi.org/10.1016/0379-0738(94)01668-U)
- 282 17. Tobe SS, Dennany L, Vennemann M (2015) An assessment of the subjectivity of sperm
283 scoring. *Forensic Science International* 251: 83-6.
- 284 18. Sivaram S, Bami HL (1971) Identification of Seminal Stains by the Inhibition of Acid
285 Phosphatase by L(+) Tartrate. *Journal of the Forensic Science Society* 11: 187-94. doi:
286 [http://dx.doi.org/10.1016/S0015-7368\(71\)70652-5](http://dx.doi.org/10.1016/S0015-7368(71)70652-5)
- 287 19. Sivaram S (1970) A modified azo-dye method for identification of seminal stains. *Journal of*
288 *forensic sciences* 15: 120.
- 289 20. Balk SP, Ko Y-J, Bubley GJ (2003) Biology of prostate-specific antigen. *Journal of Clinical*
290 *Oncology* 21: 383-91.
- 291 21. Hochmeister MN, Budowle B, Rudin O et al (1999) Evaluation of prostate-specific antigen
292 (PSA) membrane test assays for the forensic identification of seminal fluid. *Journal of forensic*
293 *sciences* 44: 1057-60.
- 294 22. Hooft PJ, van de Voorde HP (1994) Interference of body products, food and products from
295 daily life with the modified zinc test and the acid phosphatase test. *Forensic Science International*
296 66: 187-96. doi: [http://dx.doi.org/10.1016/0379-0738\(94\)90343-3](http://dx.doi.org/10.1016/0379-0738(94)90343-3)
- 297 23. Vennemann M, Scott G, Curran L, Bittner F, Tobe S (2014) Sensitivity and specificity of
298 presumptive tests for blood, saliva and semen. *Forensic Science, Medicine, and Pathology* 10: 69-75.
299 doi: 10.1007/s12024-013-9515-6
- 300 24. Tirimanna ASL (1972) Acid phosphatases in the tea leaf. *Experientia* 28: 633-. doi:
301 10.1007/BF01944944
- 302 25. Papotti M, Paties C, Peveri V, Moscuza L, Bussolati G (1988) Immunocytochemical detection
303 of prostate-specific antigen (PSA) in skin adnexal and breast tissues and tumors. *Basic and applied*
304 *histochemistry* 33: 25-9.
- 305 26. Yu H, Diamandis EP (1995) Prostate-specific antigen in milk of lactating women. *Clinical*
306 *chemistry* 41: 54-8.
- 307 27. Allard JE, Baird A, Davidson G et al (2007) A comparison of methods used in the UK and
308 Ireland for the extraction and detection of semen on swabs and cloth samples. *Science & Justice* 47:
309 160-7. doi: <http://dx.doi.org/10.1016/j.scijus.2007.09.010>
- 310 28. Nittis M (2013) Evidence Collection in Cases of Sexual Assault. *Legal and Forensic Medicine*:
311 1335-58.
- 312 29. Cooper TG, Noonan E, von Eckardstein S et al (2010) World Health Organization reference
313 values for human semen characteristics. *Human Reproduction Update* 16: 231-45. doi:
314 10.1093/humupd/dmp048

- 315 30. Bellabarba GAD, I. Tortolero VV, Molina CZ, Bellabarba C, Velazquez E (2000) Nonsperm Cells
316 In Human Semen And Their Relationship With Semen Parameters. *Systems Biology in Reproductive*
317 *Medicine* 45: 131-6. doi: doi:10.1080/01485010050193896
- 318 31. Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K (1994) Identification of the heme
319 compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of
320 polymerase chain reaction (PCR) amplification. *Journal of forensic sciences* 39: 362-.
- 321 32. Al-Soud WA, Rådström P (2001) Purification and Characterization of PCR-Inhibitory
322 Components in Blood Cells. *Journal of Clinical Microbiology* 39: 485-93. doi: 10.1128/jcm.39.2.485-
323 493.2001
- 324 33. Tsai YL, Olson BH (1992) Rapid method for separation of bacterial DNA from humic
325 substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology* 58:
326 2292-5.
- 327 34. Watson RJ, Blackwell B (2000) Purification and characterization of a common soil component
328 which inhibits the polymerase chain reaction. *Canadian Journal of Microbiology* 46: 633-42. doi:
329 10.1139/w00-043
- 330 35. Demeke T, Adams R (1992) The effects of plant polysaccharides and buffer additives on PCR.
331 *Biotechniques* 12: 332-4.
- 332 36. Khan G, Kangro HO, Coates PJ, Heath RB (1991) Inhibitory effects of urine on the polymerase
333 chain reaction for cytomegalovirus DNA. *Journal of Clinical Pathology* 44: 360-5. doi:
334 10.1136/jcp.44.5.360
- 335 37. Powell HA, Gooding CM, Garrett SD, Lund BM, McKee RA (1994) Proteinase inhibition of the
336 detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. *Letters in Applied*
337 *Microbiology* 18: 59-61. doi: 10.1111/j.1472-765X.1994.tb00802.x
- 338 38. Shutler GG, Gagnon P, Verret G et al (1999) Removal of a PCR inhibitor and resolution of
339 DNA STR types in mixed human-canine stains from a five year old case. *Journal of forensic sciences*
340 44: 623-6.
- 341 39. Al-Soud WA, Rådström P (1998) Capacity of nine thermostable DNA polymerases to mediate
342 DNA amplification in the presence of PCR-inhibiting samples. *Applied and environmental*
343 *microbiology* 64: 3748-53.
- 344 40. Hedman J, Nordgaard A, Rasmusson B, Ansell R, Rådström P (2009) Improved forensic DNA
345 analysis through the use of alternative DNA polymerases and statistical modeling of DNA profiles.
346 *Biotechniques* 47: 951-8.
- 347 41. Hedman J, Ansell R, Nordgaard A (2010) A ranking index for quality assessment of forensic
348 DNA profiles. *BMC research notes* 3: 290.
- 349 42. Di Martino D, Giuffre G, Staiti N, Simone A, Le Donne M, Saravo L (2004) Single sperm cell
350 isolation by laser microdissection. *Forensic science international* 146: S151-S3.