Comparison of the QIAcube® to manual differential separation:
Man versus Machine

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Abstract

Sexual assault is a serious public safety concern worldwide, with the resulting caseload backlog posing significant challenges for forensic laboratories. Each sexual assault kit is likely to contain a number of samples with female-male mixtures on which a differential extraction must be performed. Differential extraction is the process of separating sexual assault victim epithelial cells from the perpetrator sperm cells in order to obtain an assailant profile. Unfortunately, differential extraction is a lengthy process, requiring repeated pipetting and centrifugation. Furthermore, due to factors such as experience, the quality and consistency of separation may be variable between individuals. Because of the reagent cost, time, and manual work involved in working with these cases, sexual assault backlogs have unfortunately become commonplace. In an effort to identify ways to reduce these backlogs and benefit a scientist's workflow, it is worth evaluating the qualities of automated processes. This study focused on determining the utility of the QIAGEN® (Hilden, Germany) QIAcube® for differential separation of samples, and compared it to the current manual method. The QIAcube® was originally designed to extract nucleic acids and proteins, and it is capable of centrifuging, vortexing, pipetting reagents, and extracting a supernatant from a pellet. This study evaluated the QIAcube®'s abilities, using a custom protocol, to perform differential separations on up to 12 mock sexual assault samples at a time. Experiments included a cross-contamination study using mixed female blood and semen; a sensitivity study based on a 1:3 serial dilution of semen, with and without female epithelial cells present; a reproducibility study, utilizing mixed female epithelial cells and semen; as well as a matrix or mock evidence study, consisting of a mixture of female epithelial cells and semen pipetted onto different fabric types and swabs. All studies were performed by a novice student using the QIAcube®. For comparison, the sensitivity and reproducibility studies were also

performed by one or more experienced analysts, using a validated manual separation and wash procedure. Each method was evaluated with respect to cost-effectiveness, time efficiency, reproducibility, and sensitivity. The QIAcube® did prove to be a very efficient way to perform differential separations, with excellent sensitivity, and superior reproducibility. There was no sign of cross-contamination between samples. Conversely, more reagents were wasted with the automated method. Furthermore, loading the instrument proved to be precarious at first; but the ease of training a novice on the instrument had great potential, especially in comparison to differential extraction training. The instrument may not add hours of hands-free time, with the need to prepare the reagents and set up the instrument – but it is quite nice to push "Go" and walk away for about half an hour while the instrument does all the centrifuging and pipetting. Lastly, the factor of general human error – for example, bumping a tube and having to re-pellet sperm cells – is subtracted from the extraction process. In conclusion, the use of the QIAcube® has the potential to help a scientist work more efficiently simply by freeing an analyst or technician from repetitious pipetting and centrifuging.

Introduction

A sexual assault is committed every two minutes in the United States, and approximately 46% of these crimes are subsequently reported to the police (RAINN 2009). As a result, there are a large number of sexual assault cases to be analyzed, which requires lengthy, work-intensive processing procedures. In fact, there are so many sexual assault cases that a backlog of rape kits are held up in evidence lockers, waiting for analysis. In an effort to handle the sheer volume of evidence received by forensic laboratories, scientists at the Marshall University Forensic Science Center,

in cooperation with Dr. Mark Guilliano from QIAGEN®, have evaluated a relatively new instrument and its ability to perform differential separation.

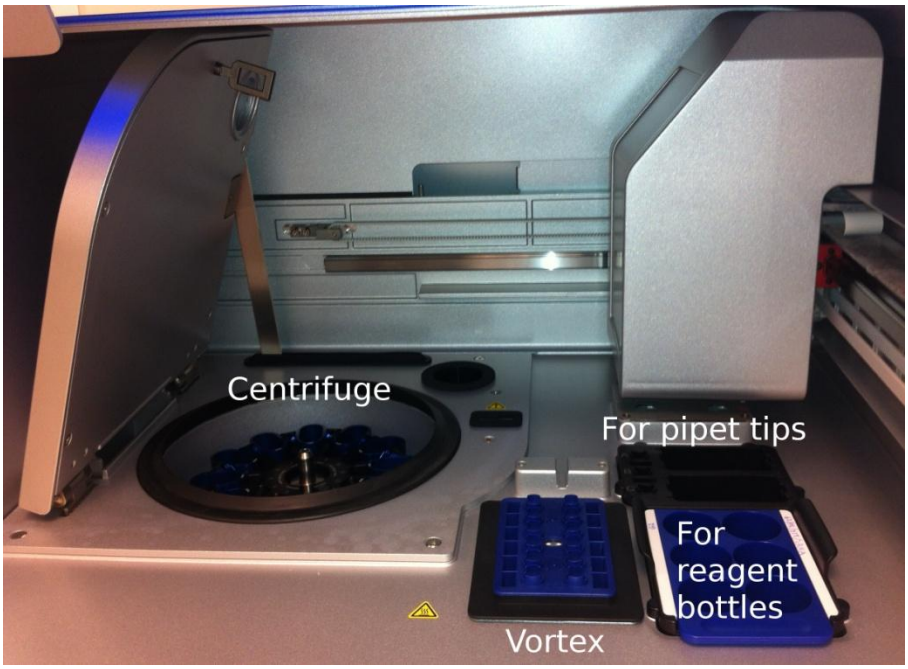
Differential extraction is the process of separating cell types based on their ease of lysis, and then extracting the DNA to obtain cell-type specific profiles. Sperm cells from the perpetrator are more difficult to lyse than the epithelial cells of the victim due to the presence of many disulfide bonds across the structure of the sperm cell membrane. They are also denser. In order to separate the sample based on cell type, one can lyse the epithelial cells, centrifuge the sperm cells into a pellet, remove the supernatant containing epithelial material, wash the sperm pellet, and finally lyse the sperm cells. Ideally, the sperm fraction would yield a perpetrator-only profile. For many years, this process, referred to as "differential extraction," is what has been performed in forensic laboratories to separate the victim's DNA from that of the perpetrator.

Unfortunately, differential extraction is a lengthy, process involving intensive manual labor. Due to the nature of manual techniques, error and inconsistencies between laboratories, analysts, and individual samples may occur. This delay in processing the backlog, and the potential for variability, provide an opportunity to explore a way to better serve the justice system, victims of sexual assault, and wrongly accused individuals. At a roundtable meeting in Washington, DC, led by the U.S. Department of Justice's Office on Violence Against Women, it was determined that "more research on how to perform DNA analyses faster, better, and at a lower cost" was necessary to facilitate a victim-centered approach to sexual assault cases (2010). One way of accomplishing this goal may be to investigate using automation. This study investigates the

abilities of the QIAcube® to help assist technology to move forward in the field of differential extraction and sexual assault kit processing.

In 2007, QIAGEN® released the QIAcube® (QIAGEN 2012), an instrument designed for the lysis of cells and DNA extraction. It is capable of performing a range of functions, including pipetting, centrifuging, vortexing, and the separation of a supernatant from pelleted material (Figure 1).

Figure 1: Interior of the QIAcube®



Photograph by Joshua Stewart, MSFS

While the QIAcube® does come with several programs readily available for download, it is also possible to create custom protocols to fit the diverse needs of many types of laboratories and studies. In this study, two custom protocols were used to perform the differential separation of sperm cells from epithelial cells – a process which would take a human hours to perform by hand. This study aims to investigate the degree to which the QIAcube® may aid forensic DNA

analysts in working more efficiently; as well as to evaluate any other possible advantages or disadvantages to using automation to process these types of samples.

Previously, studies have been performed using the QIAcube® for medical research; for determining time since deposition in bloodstains (Hanson et al. 2011); and in forensic validation studies, comparing the QIAcube® to other instruments or methods of extraction (Lee et al. 2010; Leon, Guilliano, and Della Manna; Phillips et al. 2010). One study compared the QIAcube® to two other automated systems, the MagNA Pure LC (Roche Diagnostics, Mannheim, Germany) and the Magtration System 12GC (Precision Science Co., Ltd., Chiba, Japan), in processing human whole blood (Lee et al. 2010). They concluded that all three instruments performed comparably, leaving it up to laboratories to decide which is best for their purposes. A second validation study was performed comparing the QIAcube® to two manual methods for extracting nucleic acids from buccal swabs and blood samples on filter paper (Phillips et al. 2010). They concluded that the QIAcube®'s results were comparable to manual methods, but that the amount of time and effort was reduced with the use of the automated procedure. Finally, a study was performed to determine the feasibility of using the QIAcube® to perform differential separation of mixed samples. The results were promising, generating profiles comparable to manual separation with no cross-contamination between samples (Leon, Guilliano, and Della Manna).

Materials and Methods

160 µL Buffer G2, 10 µL Proteinase K, and 40 µL 1M DTT were added to to 10 µL of raw semen sample. The mixture was vortexed vigorously for 10 seconds, then incubated at 70 °C for

10 minutes at 900 rpm on a thermomixer. After the incubation, the sample was vortexed for another 10 seconds and purified on the QIAGEN® EZ1®. Finally, it was quantitated using Promega™ Plexor® HY.

After this initial quantitation, a semen dilution was prepared with 1X Phosphate Buffered Saline, pH 7.2 (PBS). This dilution was used for the reproducibility study and as the starting concentration for a 1:3 dilution series for the sensitivity studies. An approximate 1:2 dilution with female saliva was also prepared using PBS, which was used in the sensitivity, reproducibility, and matrix studies. For the cross-contamination study, female blood was mixed with semen for the positive samples.

Automated Separation

Before placing them on the QIAcube®, the samples prepared for automated differential separation and lysis each (except for the cross-contamination study) received 210 µL of a lysis mixture, which was a ratio of 200 µL Buffer G2 to 10 µL Proteinase K to 1 µL carrier RNA. Samples were then vortexed, incubated at 56°C on a heat block for 1.5 to 2 hours, vortexed again, and placed on the QIAcube® for centrifugation and separation using the first custom protocol designed by QIAGEN®. After the first protocol had finished its run, the student would cap the epithelial cell fractions and run the QIAcube®'s second custom protocol, which would wash the pellet with Buffer G2 and lyse the sperm cells using a mixture of 160 µL Buffer G2 to 10 µL Proteinase K to 40 µL DTT to 1 µL carrier RNA. After removing the sperm fractions from the QIAcube®, they were vortexed for ten seconds vigorously and placed on a thermomixer

for 10 minutes at 70°C and 900 rpm. All samples were then vortexed again for ten seconds and placed on the QIAGEN® EZ1® for DNA purification.

Manual Separation

Manual samples underwent the validated procedure for differential extraction of casework samples. 200-250 µL Buffer G2 and 10 µL Proteinase K were added to the samples, which were incubated for 1-2 hours at 56°C. The tubes were centrifuged at 13,200 rpm for 5 minutes, and the supernatant (the epithelial cell fraction) was transferred to an EZ1® sample tube. 1 µL of carrier RNA was added to the epithelial cell fraction, which was then ready for EZ1® DNA purification. The sperm fraction was washed at least three times with 500 µL Buffer G2. After washing, 160 µL Buffer G2, 10 µL Proteinase K, 40 µL 1M DTT, and 1 µL carrier RNA were added to the sperm fraction. It was vortexed vigorously for 10 seconds, incubated at 70°C for 10 minutes at 900 rpm, and placed on the EZ1® for DNA purification.

A cost analysis was performed by summing the initial cost of reagents and other supplies required to perform a differential separation on one sample. The automated and manual methods were compared.

All samples, manual and automated, were quantitated using Promega™ (Madison, WI) Plexor® HY kits and placed on Applied Biosystems™ (Grand Island, NY) 7500 Real-Time PCR Systems. Amplification was performed using Promega™ PowerPlex® 16 HS, and capillary electrophoresis was performed on the Applied Biosystems™ 3130 or 3130xl. Electropherograms were analyzed using GeneMapper™ ID version 1.2.3 with a 50 RFU analysis

threshold and a marker specific stutter ratio filter. The QIAcube® was lent to the MUFSC by QIAGEN®.

Cross-contamination study

Two runs were performed on the QIAcube® for the purpose of determining the risk of cross-contamination between samples. One run placed positive samples in odd positions and negative samples in even positions (Figure 2). The other run was the opposite, with positives in the even positions and negatives in the odd positions.

Positive samples contained 20 µL of female blood, 2 µL of semen, 250 µL of G2 buffer, and 10 µL of Proteinase K. Negative samples contained 250 µL of G2 buffer and 10 µL of Proteinase K. After the separation, sperm fractions were digested as previously described.

Figure 2: The QIAcube® centrifuge with 12 rotor positions

Photograph by Joshua Stewart, MSFS

Sensitivity & linearity study

Part A: Semen only

A 1:3 semen dilution series was prepared (Table 1), and 50µL of each semen dilution was differentially extracted.

Table 1: Semen dilution series (per Mark Guilliano from QIAGEN®)

Dilution Name	Semen Serial Dilution (ul)		PBS to 720	Calculated ng/50ul dilution	Calculated ul semen	ng/ul in eluate
A	720	ul of A	0ul	45.91	0.308	1.148
B	240	ul of A	480	15.30	0.103	0.383
C	240	ul of B	480	5.10	0.034	0.128
D	240	ul of C	480	1.70	0.011	0.043
E	240	ul of D	480	0.57	0.004	0.014
F	240	ul of E	480	0.19	0.001	0.005

The set of dilutions A-F was created in duplicate to test the QIAcube®'s sensitivity to decreasing amounts of semen. A second duplicated set of dilutions A-F were separated manually for comparison.

Part B: Semen dilution series with female saliva

A quadruplicate set of dilutions A-D (Table 1) was added to 50µL of an approximate 1:2 dilution of female saliva. One duplicate set of dilutions was separated manually, while the second duplicate set was separated using the QIAcube®.

Reproducibility study

Samples were prepared using 50 µL of semen dilution A (Table 1) and 50 µL of the 1:2 saliva dilution. Six sets of three samples were separated manually by different members of the Marshall University Forensic Science Center DNA laboratory. The rest of the samples were

separated using the QIAcube® in three sets of six (minus one lost sample), for a total of 35 samples tested.

Matrix study

This study may also be referred to as a "mock evidence" type study, using several different prepared cloth types (towel, jeans, blue sock, brown shirt, and white shirt) and a swab. The matrices were digested at 56°C for about 1.5 hours, in a spin basket, with the same 200 µL Buffer G2 to 10 µL Proteinase K to 1 µL carrier RNA mixture. They were then centrifuged for 5 minutes at 13,000 rpm to draw the liquid off the substrate. The baskets and substrates were removed, and extraction continued on the QIAcube® as usual.

Results

Cross-Contamination Study

Two representative positive samples were amplified and typed. Both samples yielded the expected profiles (Figures 3 and 4). Three of the control negative samples contained small peaks that aligned with three allele bins: bin 30 of D21S11, bin Y of Amelogenin, and bin 17 of vWA (Figure 5). However, neither the D21 nor the vWA suspected alleles listed match those of the control male or female samples. No peaks were called by GeneMapper ID. The remaining 21 negative controls showed no evidence of contamination.

Sensitivity Study

Semen-only samples

The samples from the QIAcube® showed full profiles in the sperm fraction through dilution D and for one of the two E dilutions. For the other E and dilution F, dropout was noted. The manual samples displayed dropout starting at dilution D, and seemed to have a higher level of dropout than the automated samples. When the peak heights from capillary electrophoresis were totaled for each dilution, the QIAcube® appeared to be more sensitive than the manual method with overall higher peak heights (Figure 6). Both the manual and automated methods seemed to follow the expected threefold decrease in slope.

Semen with saliva

Quantitation data for both the automated and manual methods showed the expected threefold decrease in concentration for the sperm fractions (Figure 7). The epithelial fraction seemed to show that more DNA may have been extracted with the use of the QIAcube®. Some dropout was noted in some of the epithelial fractions for both the manual and automated methods.

Reproducibility Study

The quantitation of samples from the QIAcube® displayed a higher level of consistency than the manual samples for both the epithelial and the sperm fractions (Figures 8 and 9). Both the manual and automated methods gave the expected DNA profiles.

Matrix or Mock Evidence Study

Quantitation of the sperm and epithelial fractions both showed lower values than for the previous liquid-only studies. Upon amplification and capillary electrophoresis of the samples, dropout was noted in most of them, especially the sperm fractions. However, the observed dropout was not consistent, even among the same fabric types (Figure 10).

Cost Analysis (per Season Seferyn, MSFS)

The instrument itself costs $17,802. In addition, it costs about $24.79 to set up the QIAcube® (Table 2), as a certain level of reagent is required in the reagent bottles, and specialized plastics are also needed. After the initial expense, it costs about $17.78 to separate and extract one sample. In comparison, the manual method costs about $18.58.

Table 2: QIAcube® Set-Up and EZ1® Purification Cost (per Season Seferyn, MSFS)

Item	Amount per set-up	Number needed	Total
EZ1® expense (cartridge, G2, Pro K, etc.)	*8.104166667*	*2*	*16.20833*
Tips - 1000uL QIAGEN, wide-bore	*0.077734375*	*10*	*0.777344*
Rotor Adapter and Elution Tube	*0.150833333*	*1*	*0.150833*
Surplus Proteinase K	*0.0267*	*140*	*3.738*
DTT (35000uL)	*0.003371429*	*640*	*2.157714*
Buffer G2 (260mL) - 6900uL surplus needed	*1.756846154*	*1*	*1.756846*
Total for e-cell & sperm cell	**-**	**-**	**24.78907**

Discussion

Although some small peaks in bins were noted during the cross-contamination study, these were not determined to necessarily be true alleles. If a Y allele was truly present, it could have come from either the sperm sample or any of the three men working on the QIAcube® that day.

Furthermore, since the other suspect peaks registered at very small height values, and since they did not match the known profiles, the instrument was deemed not to be responsible for any cross-contamination.

The results of both the sensitivity and reproducibility tests showed that the QIAcube®'s performance at least equaled that of an experienced analyst. It may have even proved to be more sensitive in generating a profile from a sperm pellet. Also, there was a marked improvement in consistency with the use of the QIAcube®, which had been pitted against the combined experience of 30 years of manual technique.

The matrix study presents many questions which could be addressed with further study. Firstly, during incubation, the spin basket sits relatively high in the tube. It may be worth investigating whether the sample is reaching the proper temperature during incubation. It would also be worth comparing the results of the matrix study to a liquid-only control, as well as a manual comparison. Lastly, it may be worth following QIAGEN's protocol exactly, rather than making an attempt to mirror the other methods performed during this study.

Although there is an initial set-up cost for the instrument, the amount per sample is almost a dollar less for the automated process than the manual one. If running more than one sample, and more than one run of the instrument per day, the set-up expenses would most certainly be reduced. Therefore, except for the initial cost of the instrument, the expense of running the QIAcube® is roughly equivalent to manual differential separation.

Conclusions

One important way to better serve the justice system and victims of sexual assault is to "Develop enhanced training for new DNA analysts, since training is currently a resource-heavy, time-consuming process [and e]xplore ways to streamline training for criminalists" (US DOJ 2010). The QIAcube® addressed this issue mainly by being easy to understand and use, as well as allowing some hands-free time to work more efficiently. A novice could be trained very quickly on the instrument, which could drastically reduce training time for new analysts, and allow a new worker to progress to handling backlogged cases more quickly. The instrument introduced no risk of contamination and general human error was eliminated. The QIAcube® is a viable alternative to manual differential separation, reducing repetitive motions and providing equal or greater consistency and sensitivity.

Acknowledgements

The authors would like to express their gratitude toward the Marshall University Forensic Science Center DNA Laboratory for hosting this project and providing laboratory personnel and equipment. Heather Harrah-Lea, MS, and Season Seferyn, MSFS, have the thanks of the authors for performing the manual portions of the sensitivity study and for helping with cost analysis, respectively. The authors would also like to acknowledge QIAGEN for lending the QIAcube® to the laboratory; and the National Institute of Justice, whose generous grant made these experiments possible.

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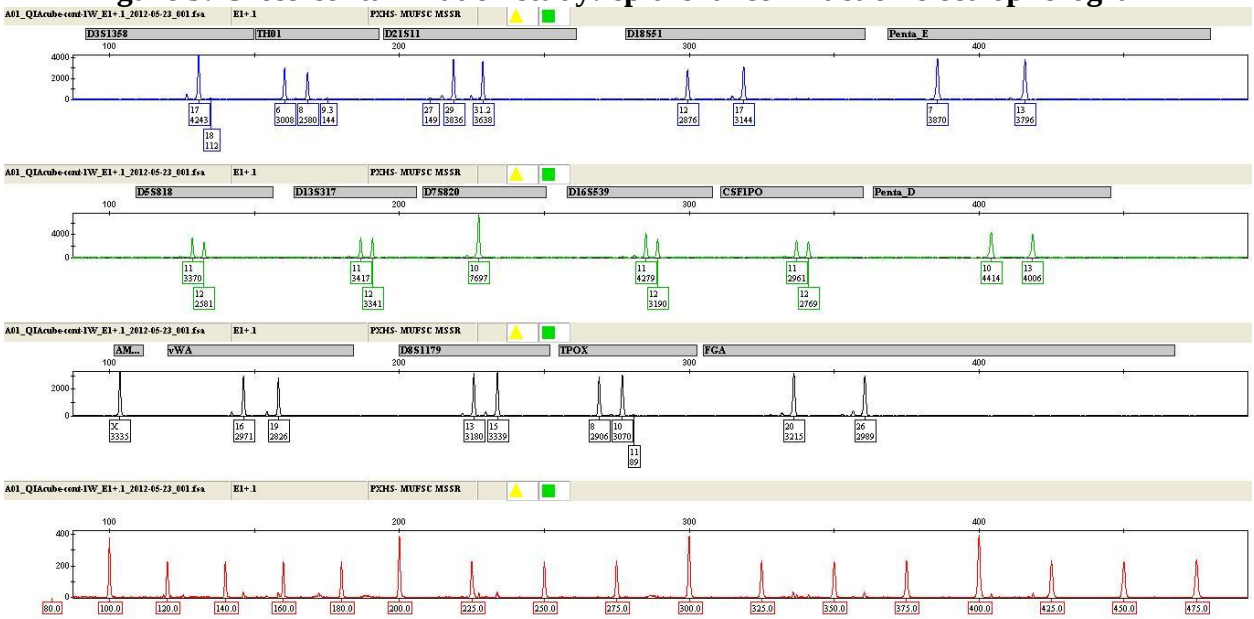
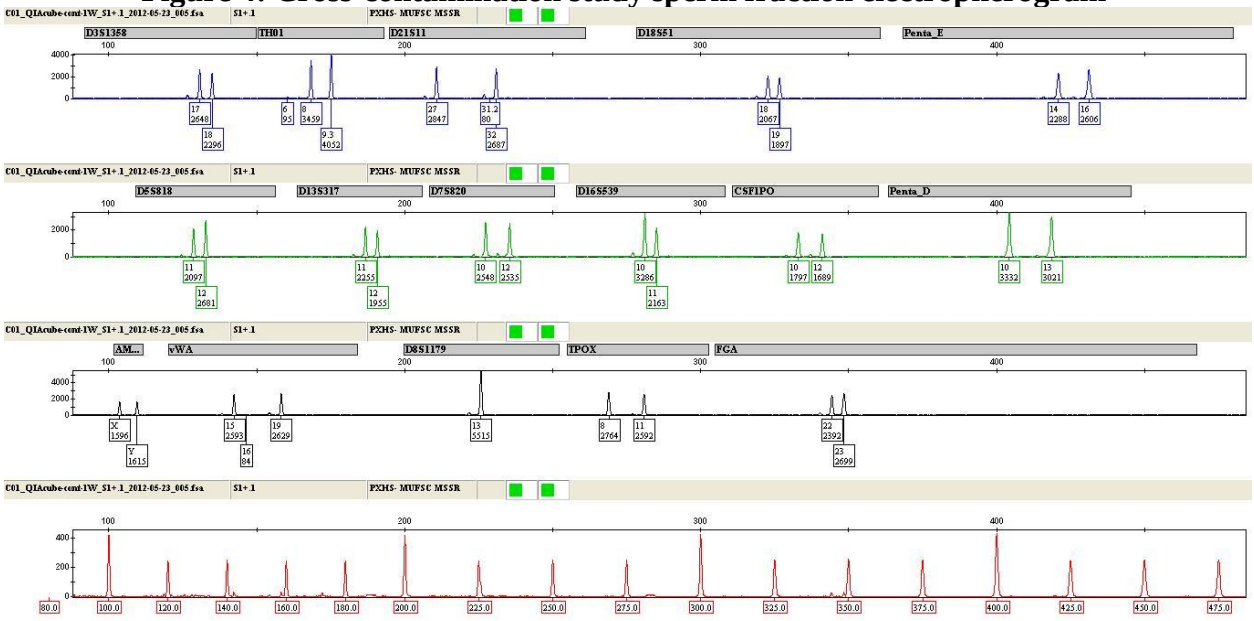
Figures 3-10

Figure 3: Cross-contamination study: epithelial cell fraction electropherogram



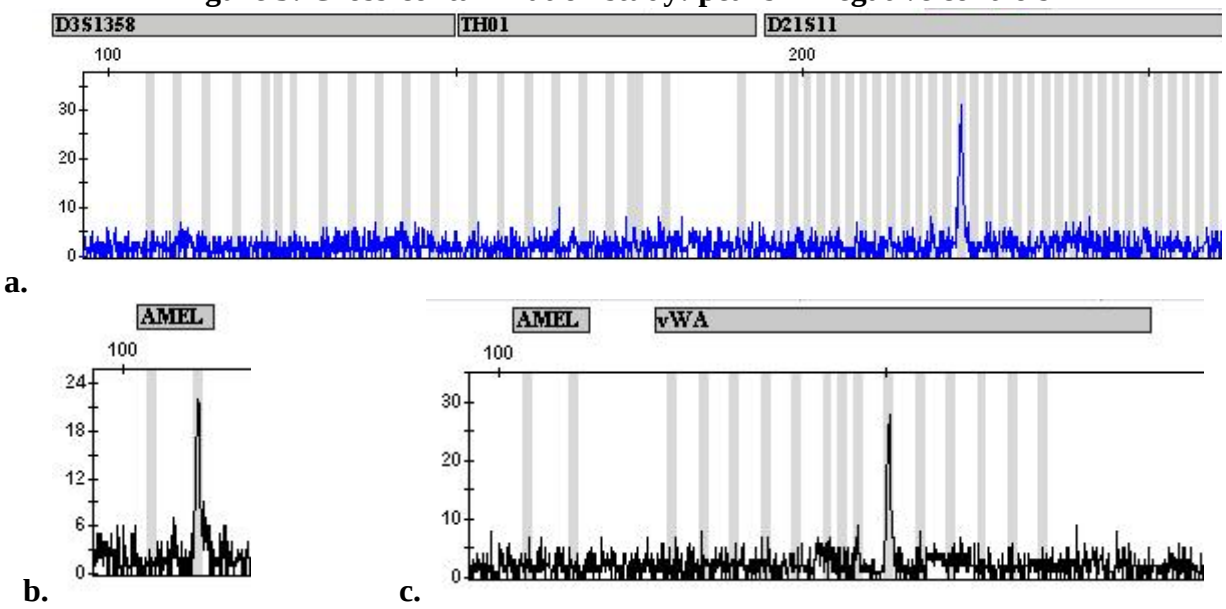
An example electropherogram from one of the positive controls of the cross-contamination study. The epithelial fraction of the sample yielded the expected profiles.

Figure 4: Cross-contamination study sperm fraction electropherogram



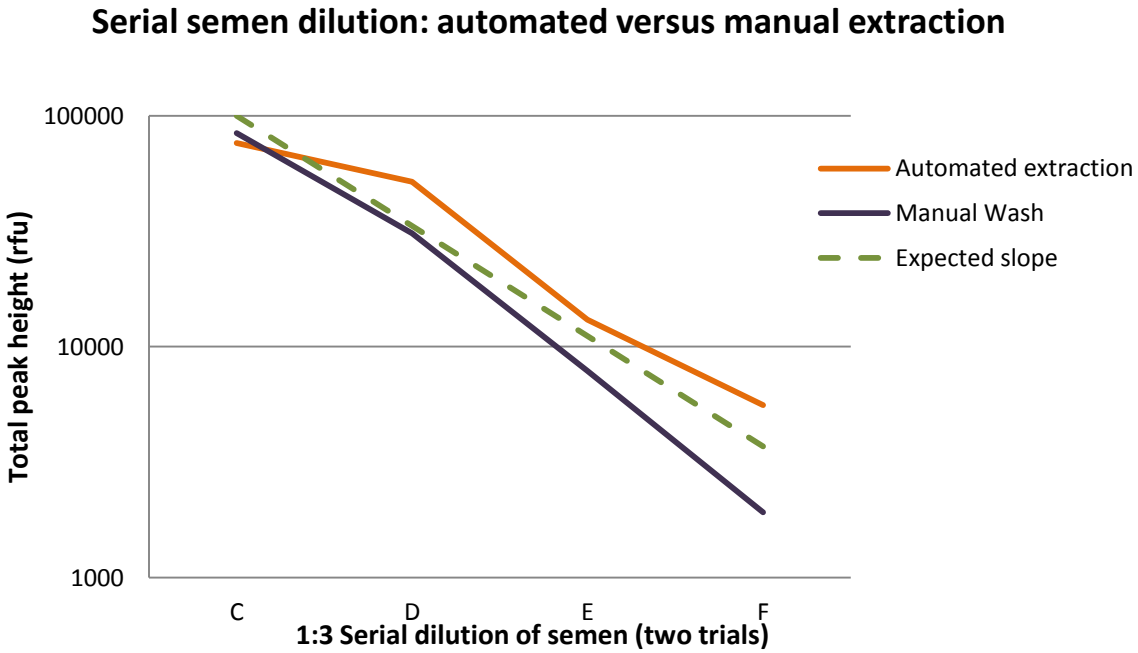
An example electropherogram from the same positive control's sperm fraction. The expected profiles were generated.

Figure 5: Cross-contamination study: peaks in negative controls



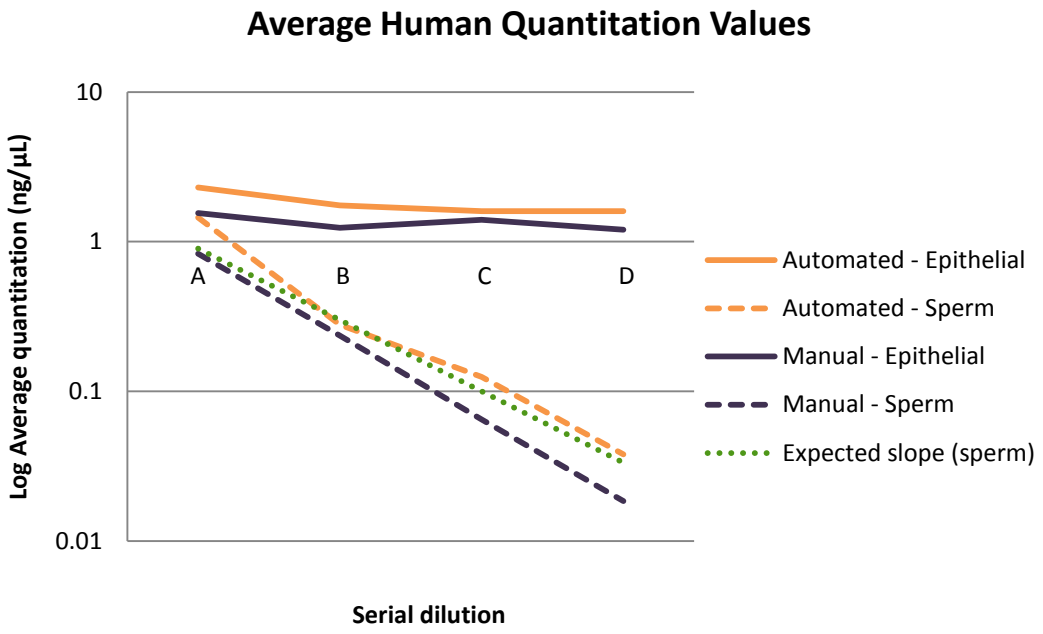
*A few very low peaks were noticed in three negative controls of the cross-contamination study. All three peaks were below the 50 RFU analysis threshold. Two of the bins (**a.** and **c.**) did not match the known alleles from the blood or semen donors. It was determined that, if these were true alleles rather than "noise," they were not due to the performance of the QIAcube®.*

Figure 6: Sensitivity study



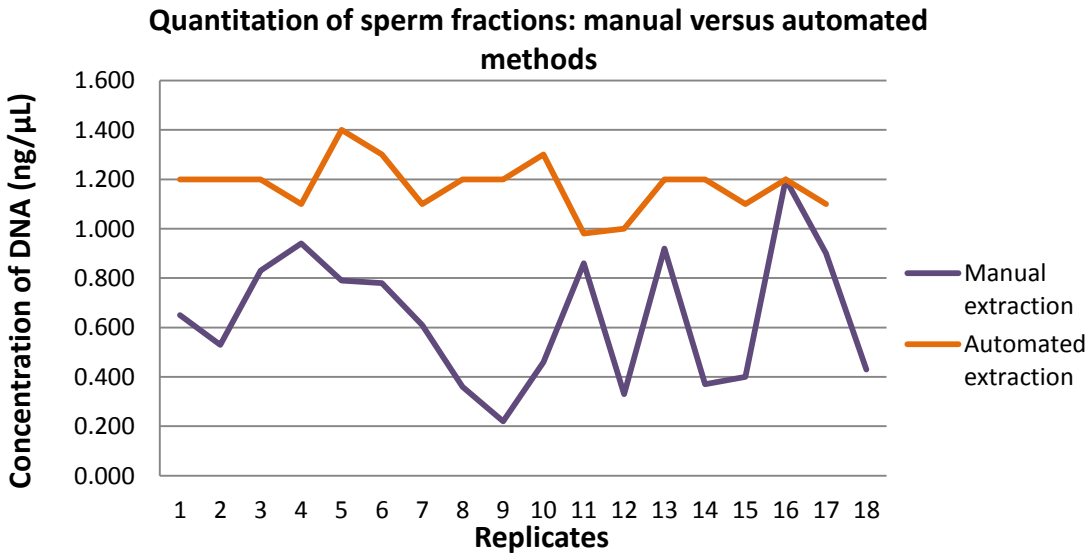
Total peak height of two trials per test (manual and automated) was summed after capillary electrophoresis and compared to a slope that decreased threefold. Dilutions A and B are not shown because they were normalized before amplification. The automated extraction shows a slightly higher peak height than the manual extraction.

Figure 7: Sensitivity study



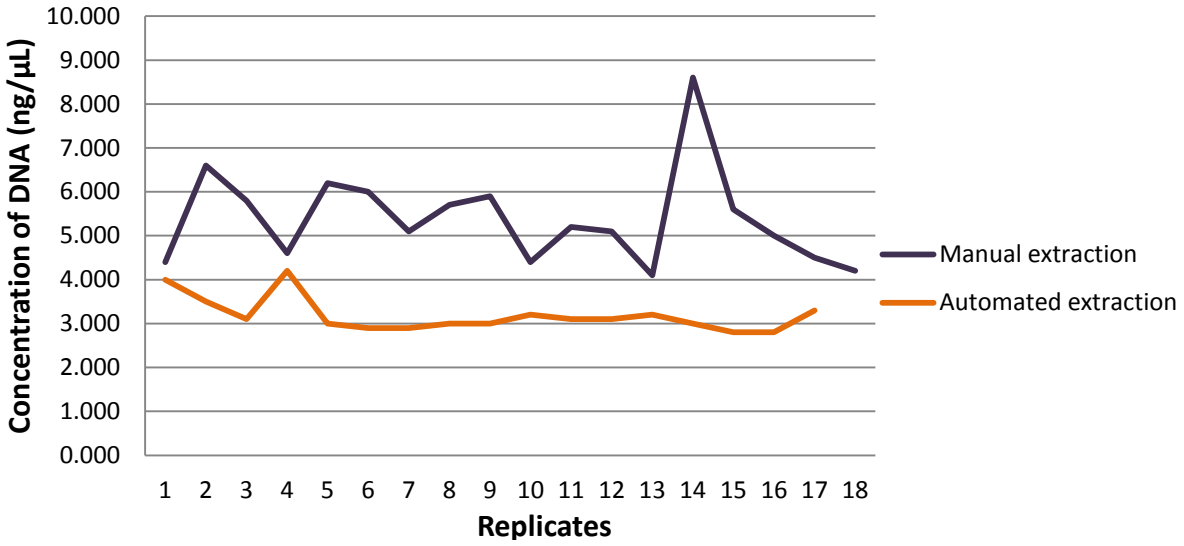
Quantitation values of the sensitivity study (semen with saliva) showed that the sperm fractions of both the manual and automated extraction methods followed the expected decreasing trend. The automated method yielded slightly higher quantitation results in both fractions than the manual method.

Figure 8: Reproducibility study



Quantitation values of the sperm fractions for the reproducibility study. All samples contained approximately the same amount of sperm and saliva dilution. The quantitation values of the automated extraction show more consistent and slightly higher quantitation results than those of the manual extraction method. CV for manual extraction was 43%; CV for automated extraction was 9%.

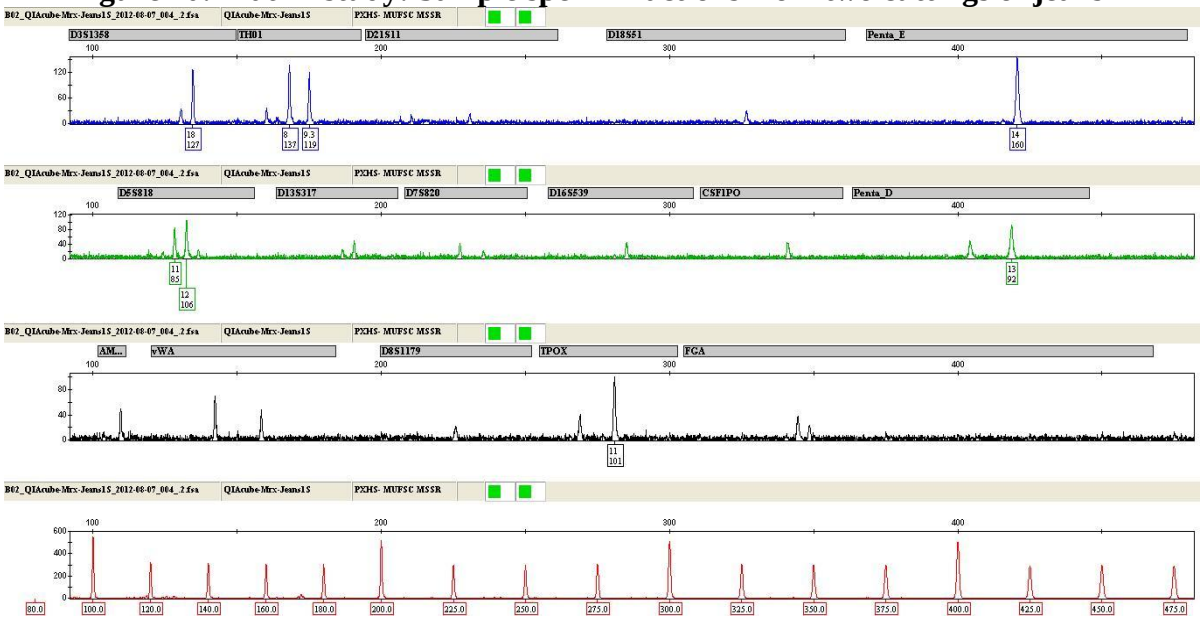
Figure 9: Reproducibility study
Quantitation of epithelial fractions: manual versus automated methods

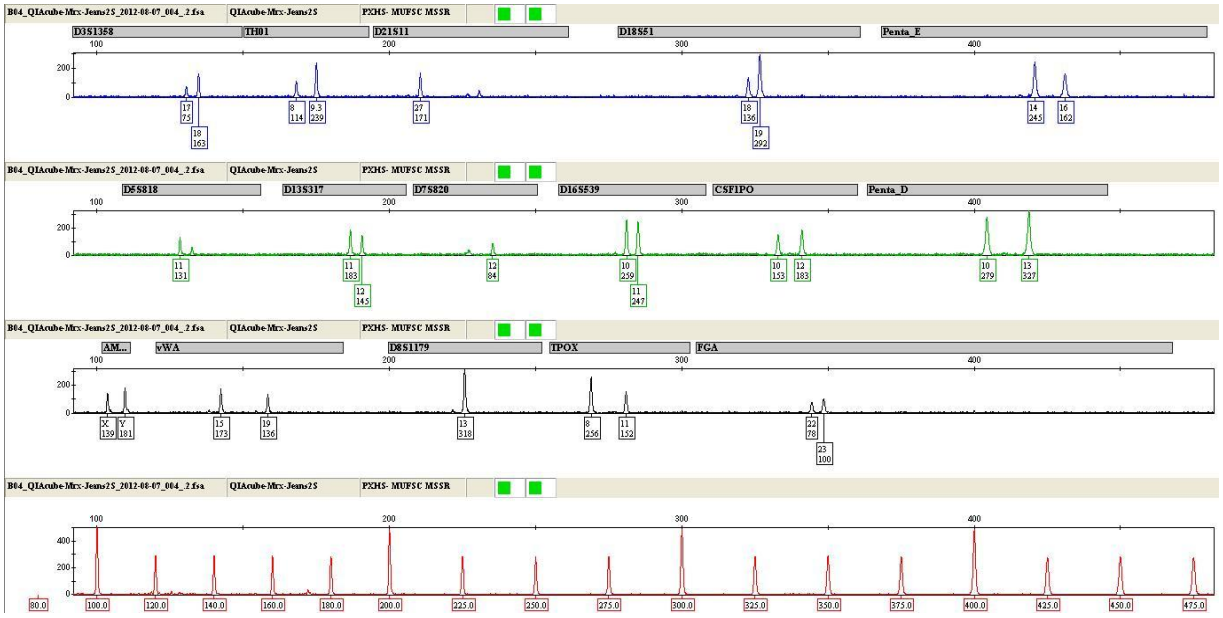


Reproducibility study: Much like the sperm fractions, the epithelial fractions showed greater consistency with the automated method than with the manual method, although the quantitation values are lower on average. CV for manual extraction was 20%; CV for automated extraction was 12%.

Figure 10: Matrix study: Sample sperm fractions from two cuttings of jeans

a.



b. 

Dropout was noted with the sperm fractions of the matrix study samples. This dropout was not necessarily consistent within the same fabric type. This example of blue jean fabric shows that the two samples had entirely different levels of dropout in their sperm fractions.