

Evaluation of manual differential separation in comparison to QIAcube[®] automation

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Abstract

Sexual assault is a concern to communities around the world and throughout the United States, as it continues to be a prevalent issue. While the U.S. rate of forcible rapes has decreased by 3.2% since 2010, the state of Arizona has seen a 2.0% increase in this rate (1). The high volume of sexual assault cases received in forensic laboratories, and the time needed to process these cases, has led to a backlog of cases in labs across the country. The Phoenix Police Department (PPD) Crime Laboratory is a high throughput lab that receives hundreds of sexual assault cases a year, in addition to the hundreds of non-sexual assault cases received annually. Samples obtained from sexual assaults often contain a mixture of sperm and epithelial cells, which are ideally separated to yield DNA profiles without mixtures. To complete this separation a differential extraction procedure is employed thereby reducing cross-contamination of cell types in the newly separated fractions. The differential extraction procedure currently in use at the PPD Crime Lab involves manual washing of the sperm pellet prior to lysis which is a time-consuming, labor-intensive process requiring many steps that may yield variation in results due to differences in skill and experience of the analyst. The QIAGEN[®] (Hilden, Germany) QIAcube[®] is a robotic workstation capable of automating separation of sperm and epithelial cells for up to 12 samples via a customized protocol. This offers the potential to streamline the analysis of sexual assault samples by improving analyst efficiency and eliminating the potential for human error. This study assessed the possibility of using the QIAcube[®] for separating sperm and epithelial cells by comparing it to the manual method currently validated and in use at the PPD Crime Lab. Experiments included a buffer study comparing the efficacy of Buffer ATL versus Buffer G2, a sensitivity and linearity study using a 1:2 serial dilution of semen, a reproducibility study using a 1:1 mixture of a 1:2 semen dilution with a 1:2 saliva dilution, and a

cross-contamination study. Results were evaluated with respect to the sensitivity, reproducibility, and time required for the QIAcube[®] in comparison with the currently validated manual method. The QIAcube[®] demonstrated sensitivity and reproducibility comparable to the current manual method and showed no cross-contamination. Conversely, the QIAcube[®] method used more reagents and consumables and required more time to process samples than the manual method. To make the QIAcube[®] differential extraction procedure more suitable for use in the PPD Crime Lab, it would be worthwhile to perform additional studies regarding the recovery of sperm cells from a matrix, to determine the minimum volume required for the sperm lysis buffer, and to investigate the possibility of customizing the protocols further so that sperm lysis buffer is added by the analyst instead of by the QIAcube[®].

Introduction

There were 1,725 rapes reported in Arizona in 2012, and there has been an increasing rate in the number of rapes that occur annually in the state (1, 2). As a result, there has been an increase in the number of sexual assault cases to be analyzed, which leads to a backlog of evidence to be processed. According to the PPD Forensic Biology section, 679 sexual assault kits were processed in 2013, with 946 samples being processed using differential extraction during DNA analysis. Differential extraction is the process of separating sperm cells from epithelial cells in an effort to obtain a DNA profile of the perpetrator of the sexual assault.

Differential extraction is used for the separation of sperm cells from non-sperm cells, and is utilized whenever there is a mixture of sperm and epithelial cells from different donors in a single sample source, such as in the case of a sexual assault. By using this procedure, the DNA profile of the victim of a sexual assault and that of the perpetrator can potentially be obtained,

ideally with little or no cross-contamination. Differential extraction works by exploiting the higher resistance that sperm cells have over epithelial cells when exposed to certain chemicals, and the fact that sperm cells are denser than epithelial cells. Disulfide bonds in spermatozoa allow sperm cells to stay intact while epithelial cells are lysed, and centrifugation pellets the sperm cells so that the lysed epithelial cells can be removed. Ultimately, separating out the different cell types before purification can help alleviate, or even prevent complications in later downstream analysis by eliminating cross-contamination and providing DNA profiles without mixtures.

The challenge to this type of extraction is that it is a time-consuming, labor-intensive process requiring many steps that may yield variable results based solely on the experience, skill, and natural difference between individuals performing the procedure. In an attempt to save time and obtain more consistent results, regardless of the individual performing the separation, it is worth considering automated methods for this procedure. There are many methods available to extract the DNA from each cell fraction once they have been separated, with the separation of the two fractions often being done by hand. The QIAcube[®] is a robotic workstation designed to automate the purification of proteins and nucleic acids for up to twelve samples. Custom protocols can be obtained to use the QIAcube[®] for other procedures, such as the separation of sperm and epithelial cells. The PPD Crime Lab currently has two QIAcube[®] instruments for DNA extractions and sample purification. They have purchased customized differential wash protocols which require validation prior to implementation into casework. Efficacy and reliability of the QIAcube[®] using the custom protocols was compared to that of the currently validated manual method in use at the PPD Crime Lab. Currently, the validated method involves manually separating the sperm and

epithelial cells, and manually washing the sperm pellet, which requires many steps and a lot of time to complete. This study focused on comparing the manual wash portion of differential extraction to the automated version on the QIAcube[®].

Previous studies on differential extraction demonstrated that variation occurs between labs using this separation technique (3). Since there is not a standard method to separate sperm and epithelial cells, the amount of DNA recovered from sperm and epithelial fractions is highly variably. In a study comparing various purification methods for the extraction of viral nucleic acid, it was concluded that the QIAcube[®] had good sensitivity and specificity for detecting various viruses (4). In another study researchers evaluated two manual DNA extraction methods, as well as extraction on the QIAcube[®] and they found that while all the procedures yielded similar results, the QIAcube[®] provided an efficient option that reduces the potential for contamination (5). Considering the QIAcube[®] has demonstrated to yield reliable results, it is worthwhile to investigate the automation of the differential extraction process on the QIAcube[®].

Methods and Materials

Sperm ratings, based on sperm searches, were given to semen dilutions and stains made with semen dilutions. In order for a sperm search to be performed, either 2 μ L of the liquid semen was spotted onto individual slide wells, or a cutting approximately 1 mm x 1 mm in size was taken from a stain and fixed to a slide. The ratings given ranged from 4+ to negative for sperm, and were used to determine which samples would be most representative of samples received in casework. Forensic biology screeners determine these ratings before evidence items are received by DNA analysts and processed for DNA. Typically, only samples with low sperm ratings go on to DNA analysis within PPD Crime Lab, while samples with high sperm ratings are sent to an external lab for analysis. Due to the fact that DNA analysts usually receive evidence items with a

low sperm rating, the samples with a low sperm rating in this study provided the best representation of samples that are most often seen in casework. Ratings were given by an experienced forensic screener and were based on the number of spermatozoa found in each field of view on a slide at 400X magnification (Table 1).

Table 1: Sperm ratings (at 400X magnification)

Rating	Sperm seen on slide
Negative	No spermatozoa found
Few	Less than 5 spermatozoa found
+	Hard to find
++	Some in some fields, easy to find
+++	Many or some in most fields
++++	Many in every field, 4 or more per field

A rating of 4+ was indicative of the presence of 4 or more spermatozoa per field of view on a slide; a rating of 2+/3+ was indicative of the presence of 2 – 3 spermatozoa per field of view on a slide; a rating of few/1+ was indicative of the presence of 1 spermatozoa per field of view on a slide or few spermatozoa on the entire slide; and a rating of negative was indicative of no spermatozoa present on the slide.

10 μ L of raw semen sample was digested using 400 μ L Buffer ATL, 20 μ L Proteinase K, and 20 μ L DTT, then incubated at 56 °C for 15 minutes on a thermomixer set at 900 rpm. The sample was manually purified using the QIAGEN[®] QIAamp[®] DNA Investigator Kit, and subsequently quantitated using the Promega[™] (Madison, WI) Plexor[®] HY System. A 1:2 semen dilution series was made with TE⁻⁴ buffer based on the value obtained from quantitation. This dilution series was used in the sensitivity, reproducibility, and contamination studies.

A 1:2 saliva dilution was prepared with TE⁻⁴ buffer using female saliva, and a 1:2 saliva dilution was prepared with TE⁻⁴ buffer using male saliva; these dilutions were used in the reproducibility and contamination studies.

Automated Separation

Prior to being loaded on the QIAcube[®], the samples were prepared for automated differential separation. Samples that were to be purified manually or on the QIAcube[®] received 520 μ L of an epithelial cell lysis mixture, containing 500 μ L Buffer ATL and 20 μ L Proteinase K. Samples that were to be purified utilizing the QIAGEN[®] QIASymphony[®] received 500 μ L of an epithelial cell lysis mixture, containing 475 μ L Buffer ATL and 25 μ L Proteinase K. The samples were then vortexed, and incubated on a thermomixer with shaking at 900 rpm at 56 °C for approximately 15 minutes. The samples were briefly centrifuged to remove condensation from the lid and then loaded on to the QIAcube[®] for separation of the sperm and epithelial cells using the custom protocols designed by QIAGEN[®]. Once samples were on the QIAcube[®], the samples were centrifuged to pellet the sperm, before epithelial fractions were drawn off by the instrument and placed into a new tube. The sperm pellet was then washed four times with Buffer ATL, except during the buffer study when samples were washed with either Buffer ATL or Buffer G2. Finally, the QIAcube[®] added sperm lysis buffer containing 75% Buffer ATL, 20% DTT, and 5% Proteinase K to the sperm fractions. After the sperm fractions were removed from the QIAcube[®], the sperm cells were lysed by incubation at 56 °C for 15 minutes on a thermomixer set 900 rpm.

The protocol provided by QIAGEN[®] utilizes Buffer G2 for the wash buffer because of a concern that foaming may occur with Buffer ATL. The PPD wanted to investigate the possibility of using Buffer ATL as the wash buffer since it is the buffer currently in use at the lab, and since another lab reported success with it. Therefore, an initial study was run on the QIAcube[®] comparing Buffer ATL and Buffer G2. Favorable results were obtained for samples processed with Buffer ATL so this buffer was used for the remaining studies.

Each study performed on one QIAcube[®] was replicated on a second QIAcube[®] as a performance check to coincide with the validation process.

Manual Separation

Samples undergoing manual separation of the sperm and epithelial fractions were processed using the method that is currently validated and in use for differential extraction of casework samples at the PPD Crime Lab.

Samples received an epithelial cell lysis buffer and were incubated using the same procedure used for samples undergoing automated separation. The samples were centrifuged at 5 minutes at maximum speed to pellet any sperm present. Approximately 500 μ L of the lysate was transferred to a new tube; this was the epithelial fraction that was set aside for later extraction. The sperm pellet was re-suspended in 500 μ L of TE⁻⁴ buffer, vortexed, and centrifuged again for 5 minutes at maximum speed. A total of three washes were performed on the sperm pellet before sperm lysis buffer was added and the samples were incubated.

Samples that were to be purified manually or on the QIAcube[®] received 300 μ L of sperm lysis buffer, containing 280 μ L Buffer ATL, 10 μ L Proteinase K, and 10 μ L DTT. For samples that were to be purified on the QIASymphony[®], 500 μ L of sperm lysis buffer, containing 400 μ L Buffer ATL, 50 μ L Proteinase K, and 50 μ L DTT. Before purification the samples were incubated on a thermomixer with shaking at 900 rpm at 56 °C for approximately 15 minutes.

Samples purified manually or on the QIAcube[®] were purified using the QIAGEN[®] QIAamp[®] DNA Investigator Kit. Samples purified on the QIASymphony[®] were purified using the QIASymphony[®] DNA Investigator Kit. Prior to being loaded onto the QIASymphony[®], sperm fractions that were separated on the QIAcube[®] were brought up to a volume of 500 μ L by adding 250 μ L of Buffer ATL or sperm lysis buffer and transferred to a screw-capped tube. All samples were quantitated using the Promega[™] Plexor[®] HY System and the Applied Biosystems[™] (Grand Island, NY) 7500 Real-Time PCR System. Quantitation data was analyzed with the Plexor[®] Analysis Software. Amplification was performed using the Applied Biosystems[™] AmpFISTR[®] Identifiler[™] Plus Amplification Kit. Capillary electrophoresis was performed on the Applied Biosystems[™] 3500xL Genetic Analyzer, using a 24 second injection, and data was analyzed using Applied Biosystems[™] GeneMapper[™] ID-X software.

Buffer study

A 1:4 semen dilution series was prepared, six replicates were prepared for differential extraction, and sperm ratings were given to each dilution, ranging from 4+ to sperm negative. 20 μ L of each dilution was spotted onto a cotton t-shirt and the entire stain was used for analysis. One replicate was washed and lysed on each QIAcube[®] using Buffer ATL. The second replicate was washed and lysed using Buffer G2. Two manual replicates were washed using PPD's current protocol with TE⁻⁴ buffer as the wash buffer.

Sensitivity and linearity study

A 1:2 semen dilution series (dilutions A-F) was prepared (Table 2), and six 50 μ L replicates were prepared for differential extraction. Two replicates were differentially washed on one

QIAcube[®], two replicates were differentially washed on the second QIAcube[®], and two replicates were differentially washed using the manual method currently validated and in use for casework samples.

Table 2: 1:2 Semen dilution series

Dilution	Volume of semen dilution	Volume of TE ⁻⁴ buffer	Calculated human [DNA] (ng/ μ L) in 50 μ L eluate
A	300 μ L	0 μ L	4.93
B	100 μ L of A	200 μ L	1.64
C	100 μ L of B	200 μ L	.548
D	100 μ L of C	200 μ L	.183
E	100 μ L of D	200 μ L	.0609
F	100 μ L of E	200 μ L	.0203

Semen dilution A was prepared by mixing 100 μ L of semen with 200 μ L of TE⁻⁴ buffer. A sperm search was performed for each dilution and sperm ratings were subsequently given (Table 3).

Table 3: Sperm ratings for semen dilutions

Dilution	Sperm Rating
A	1+
B	Few
C	1+/few
D	Negative
E	Negative
F	Negative

Reproducibility study

Samples were prepared by using dilutions C – E from the sensitivity study since the quantitation values and sperm ratings for these dilutions were most representative of the casework samples typically received for DNA analysis at the PPD Crime Lab. Samples were prepared by adding 50 μ L of the 1:2 saliva dilution to 50 μ L of dilutions C – E. One set of samples consisted of

dilutions C – E in duplicate, with the first duplicate being mixed with female saliva and the second duplicate being mixed with male saliva. Two sets were differentially washed manually by two different analysts, two sets were differentially washed using the first QIAcube[®], and two sets were differentially washed using the second QIAcube[®].

Cross-contamination study

The risk of cross-contamination between samples was assessed using the same sample sets used in the reproducibility study, with the two studies being run simultaneously. Two sets were run on the first QIAcube[®] and two sets were run on the second QIAcube[®]. The first set run on each QIAcube[®] was run with samples in odd rotor positions in the centrifuge and reagent blanks in even positions, and the second set run on each QIAcube[®] was run with samples in even positions and reagent blanks in odd positions. Reagent blanks contained 475 μ L of Buffer ATL and 25 μ L of Proteinase K.

Results

Buffer study

The profiles obtained from samples processed with Buffer ATL and from samples processed with Buffer G2 were similar, showing no substantial difference in the results from quantitation and capillary electrophoresis. Although both buffers performed equally well, Buffer ATL was used for the remaining studies because it is the buffer currently in use at the PPD crime lab.

Sensitivity and linearity study

The results of the sensitivity study showed that the QIAcube[®] performed similarly to the currently validated method. Full profiles were obtained for the epithelial fractions for dilutions A

– D, with the profiles becoming uninterpretable at dilution E, for all samples. In the first set of samples on the second QIAcube®, dilution D exhibited alleles below the laboratory's stochastic threshold, however, the profile was still interpretable according to the laboratory's guidelines. Samples from the second QIAcube® also showed lower quantitation values for dilution E. Full profiles from the sperm fractions were obtained for dilutions A through E, with the profiles becoming uninterpretable at dilution F, for the samples run on the first QIAcube® and the samples processed manually. On the second QIAcube®, full profiles were obtained for the sperm fractions for dilutions A-D, while dilution E exhibited dropout, and no profile was obtained for dilution F. Overall, results were consistent between the automated and manual separation methods (Figures 1 and 2).

Reproducibility study

The expected profiles were obtained from samples processed manually and samples processed on each QIAcube®, and similar trends with mixtures were seen across replicates in both methods. In addition, quantitation results were consistent across replicates for both sperm and non-sperm fractions.

Cross-contamination study

The expected profiles were seen both for samples sets processed manually and sample sets processed on the QIAcube®. Reagent blanks run in both even and odd positions on each QIAcube® yielded a clean profile with no peaks, and all 48 samples were free of contamination.

Discussion

When running more than six samples on the QIAcube[®] there an initial problem in getting the protocol to run. The sperm lysis buffer was prepared according to the protocol provided by QIAGEN[®] but an error occurred with the instrument that indicated that there was not enough buffer and that the process could not continue. A technician from QIAGEN[®] determined that each instrument was in proper working order, but due to tolerance in the liquid volume detection by the system, the minimum amount required for operation is higher than the protocol states. This required troubleshooting to determine what volume of sperm lysis buffer was needed for a run above six samples, and that could work on both instruments. One instrument required slightly more volume than the other so it was best to find one volume to work for both. An excess amount of sperm lysis buffer was required which became a concern of reagent waste, however since the volume of the sperm fractions had to be brought up before being put on the QIASymphony[®], it was determined that the excess buffer could be used to do this to yield less waste. Due to variations with the volume detection by the system, the minimum required volume could not be determined. Further studies would be essential to find the minimum volume of sperm lysis buffer required.

There is a lot of time required to prepare the samples when working with the QIAcube[®]. When the DNA screeners receive evidence, a portion is placed in a 2.0 mL Dolphin tube and sent forward for DNA analysis. Samples to be processed using differential extraction would then have to be transferred to an 1.5 mL elution tube compatible with the QIAcube[®], and then transferred to a 2.0 mL screw-capped tube (or a 2.0 mL Dolphin tube) for extraction on the QIASymphony[®]. This requires a lot of tube changes, which makes the process of automation labor-intensive. It

also increases the chance of contamination or sample mix-ups. If the QIAcube[®] became an integral part of the PPD crime lab, it would be possible to modify protocols for DNA screeners to put items for differential extraction into the 1.5 mL elution tube needed for the QIAcube[®]. While this is a good option for samples received after the QIAcube[®] is in use, it would not help eliminate the numerous tube changes for the items of backlog evidence that have already been processed and are still awaiting DNA analysis.

When running more than six samples, the differential wash process must be completed in two different protocols on the QIAcube[®] which means the analyst must be available when one protocol ends to refill tips, replenish the buffer supply, and begin the next protocol. Although the QIAcube[®] allows for downtime during the two processes, there is a lot of work that goes into preparing samples to be used on the cube, and taking supplies on and off the cube.

Dropout seemed to occur earlier on the second QIAcube[®], which could possibly be attributed to factors such as variation in the preparation of dilutions; however, it would be necessary to assess the efficiency of the second QIAcube[®] with further studies. Due to the time spent troubleshooting the required volume of the sperm lysis buffer, matrix studies were not performed. Further studies on the ability of QIAcube[®] to recover sperm cells from a matrix would be worth performing. It would also be worthwhile in future studies to consider further customizing the procedure to have the sperm lysis buffer added by an analyst after removing the sperm fraction, instead of having it added by the QIAcube[®] since there was so much trouble getting the procedure to run on the QIAcube[®], and since the final volume of the sperm fractions has to be brought up before being placed on the QIASymphony[®]. Additionally, the PPD plans to

evaluate a material modification to use the same sperm lysis buffer ratios as the current PPD method since the QIAcube[®] uses a much higher concentration of DTT per sample.

Conclusion

Differential extractions are useful in resolving mixtures of sperm and epithelial cells, which helps alleviate downstream complications due to mixed profiles. The QIAcube[®] has the potential to standardize the differential wash procedure while giving analysts free time to take care of other tasks while the instrument runs. Due to the extra amount of processing that must be done to samples, the QIAcube[®] did not prove to be a faster method for the separation of sperm and epithelial cells, however it does reduce the potential for human error, and it did prove to provide results with equal sensitivity and reproducibility to manual processing. More studies must be performed to determine if the QIAcube[®] differential extraction procedure can fit in with the current workflow of the PPD Crime Lab.

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Figures 1 – 2

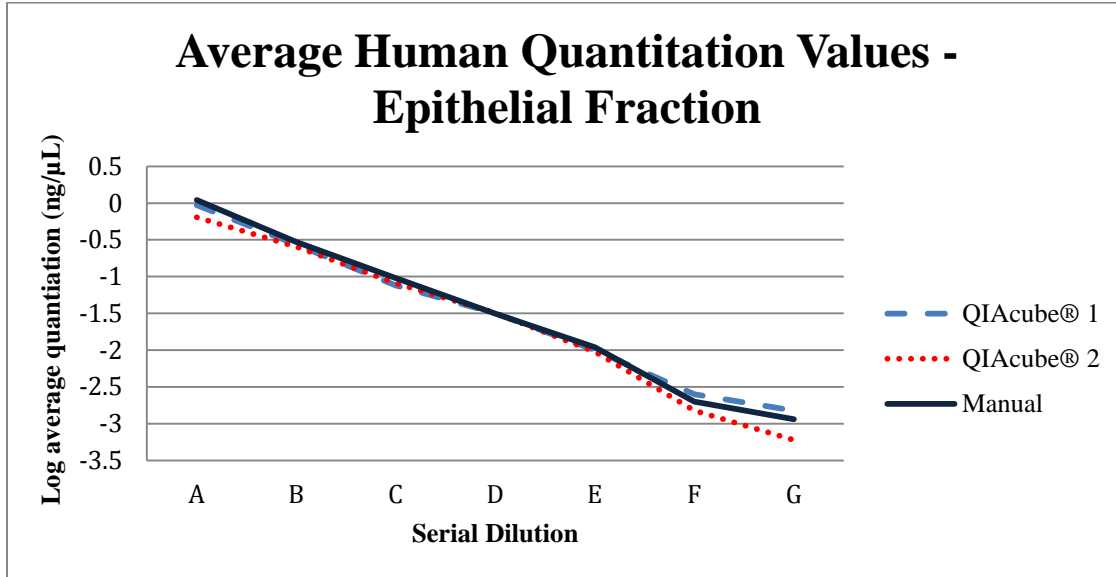


Fig. 1 Quantitation values of the sensitivity study showed that the epithelial fractions of the automated and manual extraction methods showed a decreasing trend as expected, and that results were consistent between methods.

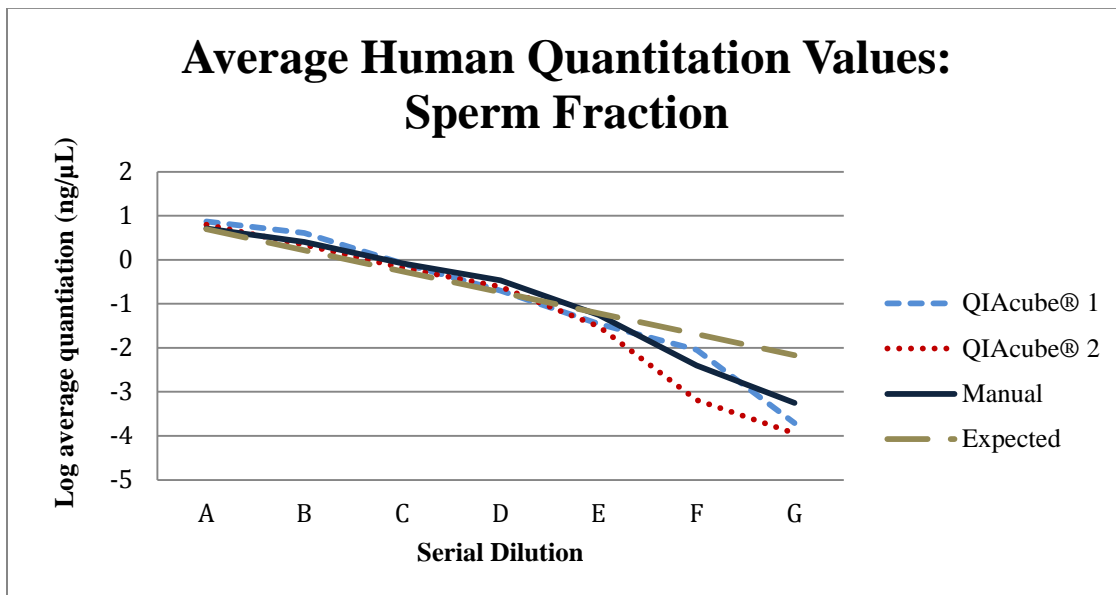


Fig. 2 Quantitation values of the sensitivity study showed that the sperm fractions of the automated and manual extraction methods showed a decreasing trend as expected, and that results were consistent between methods.