Optimization of the QIAGEN® QIAcube® for Differential Separation of Forensic Casework Samples

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Abstract
After reading this article, readers will have a better understanding of the QIAGEN® QIAcube® with respect to the processing of sexual assault kits and whether this automated technique is appropriate for integration into the workflow of certain crime laboratories. This article will impact the forensic science community by showing how to streamline the processing of sexual assault kits to reduce the national backlog.

Sexual assault is a national ongoing problem. The occurrence of sexual assaults continues at a rate that makes it difficult for crime laboratories to keep pace with the backlog of sexual assault evidence. Most sexual assault evidence is collected in "kits" containing several samples that may consist of biological mixtures of the victim and the actor. In the case of sexual assault, most victims are female while most perpetrators are male. A differential extraction is typically performed to separate male and female fractions consisting of the female epithelial cell fraction and the male sperm fraction, if present. The QIAGEN® QIAcube® is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications where a protocol for differential separation has been developed. Designed to efficiently manage 12 or fewer samples, the QIAcube® can centrifuge, vortex, and pipette reagents and the supernatant to accomplish separation of the epithelial fraction from the sperm pellet. The QIAcube® is an automated instrument allowing analysts to place samples on the instrument and walk away to perform other tasks. This study focuses on the optimization and implementation of this differential separation method for forensic casework samples. The studies performed on the QIAcube® included buffer optimization, sensitivity, reproducibility, cross-contamination, and mock-case work. These studies served to evaluate the various challenges of differential separation since sexual assault evidence can be collected hours or even days after the assault. For the buffer optimization experiment, buffer G2 and diluted ATL buffers were compared for the primary incubation step where it was found that the diluted ATL buffer performed better than Buffer G2. Thus, the remaining studies incorporated diluted ATL buffer in lieu of Buffer G2. The sensitivity studies were based on a 1:2 and 1:10 dilution series of semen, performed in duplicate. Each series was performed once with an epithelial and a seminal fluid mixture and then with a seminal fluid dilution. The reproducibility and cross-contamination studies were performed on the QIAcube® using contrived samples compromised of a 1:512 semen dilution deposited on a buccal swab. The mock casework samples were post-coital samples obtained from three different sets of anonymous volunteers at various time intervals. These mock casework samples were compared to the Differex™ method validated previously by the Allegheny County Office of the Medical Examiner. All samples were quantified using Plexor® HY and amplified with PowerPlex® 16. Upon completion of the validation, a QIAGEN® protocol was developed to fit the needs of the Allegheny County Office of the Medical Examiner.

Future studies for this validation involve quantitating swipe test samples obtained throughout the validation in order to determine if the cleaning methods are appropriate in between runs. The second QIAcube will be performance checked against the validation data. It is recommended that a more thorough comparison between Differex™ and the QIAcube® will more samples.

Introduction

The sexual assault kit backlog is a national problem and at the end of 2015 Pennsylvania had reported 1,850 kits in the backlog (1). At the Allegheny County Medical Examiner's Office (ACOME) in 2015, 52 sexual assault kits were processed from 13 different medical facilities. In November 2015, Allegheny County Forensic Biology streamlined the way kits are processed by removing redundant serological testing. As of July 29, 2016, 52 sexual assault kits have been processed which represents the same number of sexual assaults kits processed for the entire year in 2015. The ACOME currently has 91 unworked sexual assault kits in their backlog.

A differential separation is performed when a evidence sample is suspected to be a mixture of female and male, such as epithelial cell and sperm cell, fractions. This procedure is particularly useful in sexual assault kits where mixed DNA profiles may be difficult to interpret.

The goal of differential extraction is to separate the two cell types found in a single sample. As the typical sexual assault sample is collected from a female, the epithelial cell fraction will typically overwhelm the sperm fraction unless these fractions are not first separated. The scientific principle behind the differential extraction exploits the disulfide bonds in the sperm head that allows spermatozoa to resistance chemical digestion to which the epithelial cells are susceptible. Initial separation of the male and female fractions can facilitate a clear-cut single source or a simple mixture determination for a more straightforward interpretation of profiles. By including differential separations in sexual assault casework analysis, the potential exists that full profiles from the epithelial cell contributor and sperm cell contributor will result.

The sexual assault kit backlog has sparked labs across the country to identify and optimize automated methods for differential separation that is faster than the manual method. The QIAcube® (QIAGEN®, Hilden, Germany) is one such method that provides automated pipetting, centrifuging, and vortexing of samples. The QIAcube® can be used for many different types of protocols but forensic casework focuses on the two differential separation protocols. The QIAcube® spins the samples down and generates a sperm pellet, pipetting off the epithelial fraction within the first five minutes of processing. The sperm fraction then goes through a series of wash steps. Unlike an open, 96-well plate, platform, this instrument allows the analyst to completely walk away and perform another task. This study aims optimize and implement a QIAcube® method that is comparable or superior to the current Differex™ (Promega ®, Madison, WI) with DNA IQ® (Promega®, Madison WI) method of differential separation used at the Allegheny County Office of the Medical Examiner.

Traditional differential separation can be a time-consuming process where one study found that interlaboratory variability was quite common and calling for the commercialization of differential separation techniques, thus the development of the QIAcube® (2). Since there is no universal separation technique, all labs must validate and optimize their own technique, creating high variability in methods employed in the recovery of sperm cells from casework samples. A developmental validation by the manufacturer was performed using the QIAmp® DNA Investigator® Kit (QIAGEN®, Hilden, Germany) on the QIAcube® (extracts samples, then amplifies them), showing that the QIAcube® had good sensitivity and reproducibility with low contamination in the recovery of DNA from FTA cards and saliva when compared to a manual extraction method (3). In a comparison study of three different automated extraction methods,

the QIAcube® was found to be just as robust and efficient as the other two automated extractions tested (4). The QIAcube® is an easy to use automated instrument using consumables that can be incorporated with little effort into the extraction process, especially when coupled with the QIAGEN® EZ1 or EZ1 Advanced XL (QIAGEN®, Hilden, Germany) (5). Based on the results from these articles, the QIAcube® is a repeatable, robust instrument with low levels of contamination, which is appropriate for forensic casework.

Materials and Methods

For this validation, swabs were prepared for each of the following studies: buffer, sensitivity, reproducibility, cross-contamination, and mock casework. The buffer study samples were 1:512 semen dilutions and a female buccal swab. The sensitivity study occurred in two parts. The first part was a 1:2 and 1:10 seminal fluid dilution series with a female buccal swab. The second was the same 1:2 and 1:10 seminal fluid dilution series without the female buccal. Reproducibility and cross-contamination were performed together with six swabs of 1:512 semen dilution and six reagent blank samples. This study was performed in triplicate; samples in the even well and blanks in the odd, vice versa, and random placement of blanks and samples. Combining the studies allowed us to save time and resources. The mock casework samples were post-coital swabs at various time intervals. Twelve swabs were tested in duplicate on the QIAcube® using a draft protocol that the ACOME would eventually utilize.

Optimization Buffer study

Samples were placed into 1.5 mL tubes; either 480 µL of diluted ATL buffer (QIAGEN®, Hilden, Germany) or Buffer G2 (QIAGEN®, Hilden, Germany) and 20 µL of Proteinase K were added to each sample. Six samples were run with each buffer type, in duplicate. The samples incubated in a thermomixer at 56.0˚C at 900 rpm for 1.5 hours. The substrates were transferred to spin baskets and centrifuged for 5 minutes to dry out the substrate. The substrate was then discarded and the samples were loaded onto the QIAcube® using the protocol "Separation and Lysis 12A." Following the 12A protocol, the epithelial fractions were removed and loaded onto the QIAGEN® EZ1 for extraction. A Sperm Lysis master mix of Buffer G2, Pro K, and DTT was made and placed on the instrument; the "Separation and Lysis 12B" protocol was performed to wash the sperm fraction. Upon completion of protocol 12B, the sperm fraction was incubated for 10 minutes at 70˚C in the thermomixer. The samples were then loaded onto the EZ1 for extraction.

All subsequent samples to the optimization study were incubated in a diluted ATL buffer and enriched with the above-mentioned protocols before being placed in the EZ1 Advanced XL for extraction utilizing the following protocols:

Non-Sperm Fraction on EZ1: large volume protocol, elute in TE at 100 uL
Sperm Fraction on EZ1: trace protocol, elute in TE at 100 uL.

Sensitivity Study

Seminal fluid dilution series of 1:2 and 1:10 were created and 18 buccal swabs from the same female individual were obtained. 70 µL of each dilution was pipetted onto its respective swab, either a swab that contained epithelial cells or a plain swab, and allowed to dry overnight. These swab sets were created in duplicate: 1:2 with epithelial, 1:2 without epithelial, 1:10 with epithelial, and 1:10 without epithelial.

Reproducibility
A 1:512 seminal fluid dilution was prepared and pipetted onto 18 female buccal swabs. The samples were run in triplicate with 6 reagent blanks and 6 samples. The first round was separated on the QIAcube® with samples in the odd number rotor buckets and the reagent blanks in the even number rotor buckets. Round 2 was the opposite, with samples placed in the even number rotor buckets and reagent blanks in the odd. The third round was a randomly assigned set up, to ensure that the QIAcube® accurately identified the locations of samples.

Contamination
The potential for cross contamination between samples was studied in tandem with the reproducibility study, to expedite the validation process, and constituted the 6 reagent blanks run in triplicate in various locations in the QIAcube®. Optimal decontamination intervals were also studied to avoid contamination from the instrument. The instrument was cleaned with alcohol and deionized water after each use and the removable parts, shaker adapter, buffer holder, and shaker plugs, were soaked in Decon-Quat®, per the manufacturer suggested protocols. To measure the success of the decontamination, swabs were collected from the following locations before each run (after cleaning) and after each run (before cleaning): the rotor buckets, centrifuge lid, and tip dispenser. The wet-dry method, which is a wet swab moistened with sterile water applied to the area followed a dry swab to collect any residual fluid, was utilized to collect these samples. These samples were extracted on the EZ1 and eventually quantitated with Plexor® HY (Promega® Madison, WI).

Mock Casework
Since spermatozoa levels vary due to a number of factors, such as sperm count of the actor as well as the actions of the victims after deposit, post coital samples were obtained from three different couples at various intervals. The interval ranged from as little as 8 hours to 53 hours post coital. The varying time intervals mimic that of sexual assault kits in casework, due to variability in time intervals the victims could present at the hospital. The 12 samples were extracted twice using the methods from the previous studies.

All samples, unless otherwise noted, were quantitated with Promega® Plexor® HY using a Biomek 3000 for the plate set-up. The samples were tested on the Applied Biosystems™ 7500 Sequence Detection System (Applied Biosystems™, Foster City, CA) and analyzed using the Plexor® Analysis software (Promega®, Madison, WI). The samples were normalized to a 1 ng target template and then amplified with Promega® PowerPlex® 16 (Promega®, Madison, WI), then run on the Applied Biosystems™ Genetic Analyzer 3130 (Applied Biosystem™, Foster City, CA). Based on the template levels detected in the quantitation step, injection times were chosen for capillary electrophoresis appropriately, which spanned from 3 seconds to 15 seconds. The samples were analyzed on GeneMapper® ID software using an analytical threshold of 75 relative fluorescent units (RFU) and a stochastic threshold of 150 relative fluorescence units (RFU).

Results

Optimization Buffer Study

The buffer study samples were processed only up to quantitation. Although the Buffer G2 was more consistent across the board (Figure 1), the diluted ATL buffer had higher quantitation values over all. The hypothesis for the t test was as follows "there will be no statistically significant difference between the average quantitation values for buffer G2 and diluted ATL buffer." The quantitation values were assessed with a statistical two tailed t test. The test calculated a t_{calc} of -2.51. The t_{crit} from known table was assessed under a 95% confidence interval at 19.675. The t_{calc} was less than the t_{crit} determining there is no statistical difference between the two buffers.

Sensitivity Study

Semen deposited on buccal swabs

The quantitation data showed an expected decrease in both dilution series 1:2 and 1:10 respectively (Figure 2). Variability in data could be due to the affinity of spermatozoa for the cotton swab heads. In the 1:2 dilution series, the separation was sufficient in that major and minor peaks were observed, resulting in a possible resolution of the male fraction up to the 1:512 dilutions. At the lower end of the series, 1:1024, 1:2048, and 1:4096, the results varied but the profiles correlated with what the quantitation results showed. The 1:10 dilution series was also in agreement with the quantitation results with some variability between samples.

Semen only

Quantitation data showed the dilution decrease that was expected. There were a few no call results in the epithelial cell fraction, showing that optimal separation occurred. No call refers to the melt curve displaying the expected target temperature but there is no enough amplified product for the melt curve to cross the melt threshold. The 1:2 and 1:10 series showed optimal separation of epithelial and sperm fractions. Most epithelial cell samples presented with few to no allele calls. The 1:2 series exhibited full profiles at the 1:512 dilution and partial profiles at lower dilutions. The 1:10 series showed full male profiles in the sperm fraction at 1:1000.

Reproducibility and Cross-contamination

The epithelial cell and sperm fractions were completely separated in the majority of the samples. The peak height ratios were even and the relative fluorescence units (RFU) were consistently the same for all samples.

Contamination

All but four of the reagent blanks had no amplification product. Only 1 allele in each contaminated reagent blank was called, each at a different loci. The contaminated reagent blanks showed no quantitation values indicating contamination at that point No results were obtained from the QIAcube® swipe tests since they were not quantitated at this time due to quantitation issues throughout the validation and supplies.

Mock Casework

The quantitation data presented as expected, with an abundance of autosomal DNA and varying values of male DNA in the sperm fraction that correlated to the time interval of the post coital swab. The samples were performed twice to examine the variability between sampling as well as

donors. The sperm fraction samples were classified into five categories: single full male profile, single male profile + trace female, partial male profile, mixtures, and no male DNA present. There were 24 sperm fraction samples. 25% were full single male profiles, 21% were single male profile + trace female, 29% mixtures, 8% partial male profile, and 17% had no male DNA present. In the 17% that showed no male DNA amplified, one sample was an "N/A" for quantitation while the others were at a quantitation value of 10^{-4} ng/µL or lower, correlating with the sensitivity samples that showed no male DNA at those values. All of the samples that resulted in full single source male profiles quantitated at 1 ng or higher.

Discussion

Optimization Buffer Study

The goal of the optimization study was to determine whether one buffer functioned better than the other in the recovery of DNA. These buffers, ATL or G2, are used in the 1.5-hour incubation step. The data shown in Table 1 in the results section reported the ATL buffer recovering male DNA unsurpassed by G2. A student's t test was calculated to determine whether these two data sets were statistically different. Equal variances were used in the calculation since the two buffers in theory should be recovering DNA similarly. The t test determined t calculated was less than the t critical. Although statistically there was no significance to the difference of the buffers, the ATL buffer produced higher quant values than the G2 and was used throughout the remainder of the study.

Sensitivity Study

With both the buccal swab-semen samples and the semen only samples, optimal separation occurred. In the buccal swab-semen samples, the epithelial fraction exhibited no drop out as expected, since it was a high template buccal swab. The sperm fraction displayed drop out beginning at the 1:1024 dilution and less alleles were observed as the dilution factor increased. The peak height ratios were more uneven as the dilution factor increased which we attributed to lower template number. When compared to the quantitation data, the samples that quantitated with 10^{-4} ng/µL or less showed partial profiles or profiles unsuitable for comparison. In the 1:10 series based on buccal swab-semen samples, the samples that resulted in "N/A" on the quantitation data across the board may have been removed from the tray, as nothing was shown when these samples were amplified and run. The 1:10,000 dilution showed partial profiles and 1:1000 to 1:10 dilutions showed full profiles or mixtures suitable for deduction of the male fraction.

When the semen-only samples were analyzed, there was a clear decrease in quant values as the dilution series suggested. As the dilution factor increased, the lower template samples showed partial or unsuitable electropherograms for comparison. alleles were called in multiple samples that had "no call" or "N/A" in the epithelial fraction. Again, full male profiles were detected up to the 1:512 and 1:1000 dilutions. All dilutions following 1:512 or 1:1000 were partial profiles and drop out was observed. The quantitation concentration of 10^{-4} ng/µL or less theme continued in these samples, with partial or negligible profiles. Some high template samples exhibited drop out and poor peak height ratios which we attributed to the nonhomogeneous nature of semen itself.

Reproducibility
The epithelial fraction was consistent throughout every sample and the peak height ratios were in balance. As expected, no epithelial fraction dropout was displayed since the epithelial fraction was in abundance. The sperm fraction showed more variability where we attributed this to the nature of the biological fluid itself. There was still good separation. although some samples had drop out in the larger loci. In all the samples, there were only trace amounts of female epithelial cell even when there was drop out of the male fraction.

Contamination
The four reagent blanks that were contaminated at the 15 second injection time with one allele call were looked at in detail. There were two reagent blanks extracted on the same day RB_1S and RB_8S. The other two RB_9E and RB_13E were extracted on two completely however, both RB_13E and RB_9E shared the same position in the Shaker position 5. This seems to be a coincidence as the other samples in that position were reviewed and no contamination was perceived. The samples on either side of shaker positon 5 were also reviewed and it appears there is no contamination in those. The contamination in the RB_9E appears to be sporadic as the allele call did not match the two individuals used to create the sample or the person that extracted the samples. In the RB_13E sample the allele is common with the individual that processed the samples, however, because it is only one allele there is no way to truly tell whether the individual contaminated the sample or it was sporadic. RB_1S showed an X call at amelogenin; because amelogenin is a gender based chromosome, there is no way to tell where this DNA came from since everyone involved with processing and the sample make up has an x chromosome. The RB_8S allele contamination was associated with the individual processing the samples, however, it cannot be known whether it was true contamination or sporadic. Based on the analysis of the reagent blanks it is safe to assume that the QIAcube® set up will not contaminate the samples. The 15 second injection was not used for interpretation of the majority of the samples, but the contamination was still documented.

Mock casework
The mock casework post-coital samples provided optimal separation as well. Of the 24 sperm fractions 75% of the electropherograms obtained allowed for the recovery of a male profile, a profile suitable for major/minor resolution, or a profile suitable for intimate deduction. The other 25% were either over 48 hours post-coital or were variable and only recovered a partial profile. The partial male profiles can be attributed to the variability from male to male and the longevity of that male's sperm. As said previously, seminal fluid is not homogenous and the sperm holds onto cotton swabs more readily than other surfaces. In the full single male profile, single male + trace female, and mixtures, the peak height ratios were mostly even. The mixture samples were analyzed using the current ACOME math model to calculate average mixing ratio. Over half of the mixtures, 57%, obtained were determined to be resolvable based on the average mixing ratio calculation. Of the mixtures that were not resolvable based on the average mixing ratio calculation, an intimate deduction could be made provided there was a victim reference, which in most if not all casework samples, are available. These results are exceptionally better than the previous method of Differex™, with the validation data showing that only 10% of sperm fraction samples had resolvable mixtures. Only 17% of the sperm fraction samples had no male DNA, and these were at time intervals of 24, 52, and 53 hours. It is interesting to note that the samples

are unpredictable from donor to donor. A sexual assault or post-coital sample that is 8 hours from one person could appear the same as a 36 hours sample from another person.

In general, there were significant issues associated with the quantitation step. The y intercept frequently fell out of range, in addition to the slopes that fell outside the range. Considering that a robot with little variability was setting up the standards this could be an issue in the future and ACOME will be pursuing the routes necessary to fix this problem. Every quantitation run for this validation was performed in duplicate.

Conclusions and future needs

Upon completion of the optimization buffer study, the diluted ATL buffer was found to yield higher concentrations of DNA and will be used in the incubation step per ACOME standard operating procedure.

The QIAcube® showed optimal separation in the majority of samples and allowed for the deductions of profiles even at low templates. Separation occurred at the high and the lower ends of the both dilution series in both scenarios: buccal swab-semen and semen only. The 1:512 dilutions are most comparable to the sexual assault kits that are submitted into evidence, so obtaining a full profile from the sperm fraction or a major minor contributor would be helpful in making an intimate deduction. The separation occurred optimally and consistently in the reproducibility study and contamination of the reagent blanks in the cross-contamination study so it is safe to assume that the robot knows how and where to pipette appropriately and will not contaminate the samples. There was some sporadic contamination in the reagent blanks of the contamination study, however, this was the only place throughout the entirety of the validation contamination was seen. The peaks in the samples correlated to who was supposed to be in them and there were no added alleles. If the procedure is performed properly, then contamination can be minimized. Using the sperm fraction for comparison, the casework like samples produced better results than previously acquired in the Differex™ validation study. The results obtained from the various studies allowed for the successful implementation of this method in the ACOME laboratory.

In the future, analysts at ACOME will quantitate the swipe test samples in order to detect if the QIAcube® cleaning method is appropriate. Since there were two QIAcubes® purchased, a performance check on the QIAcube® not included in this study will need to be performed.

References

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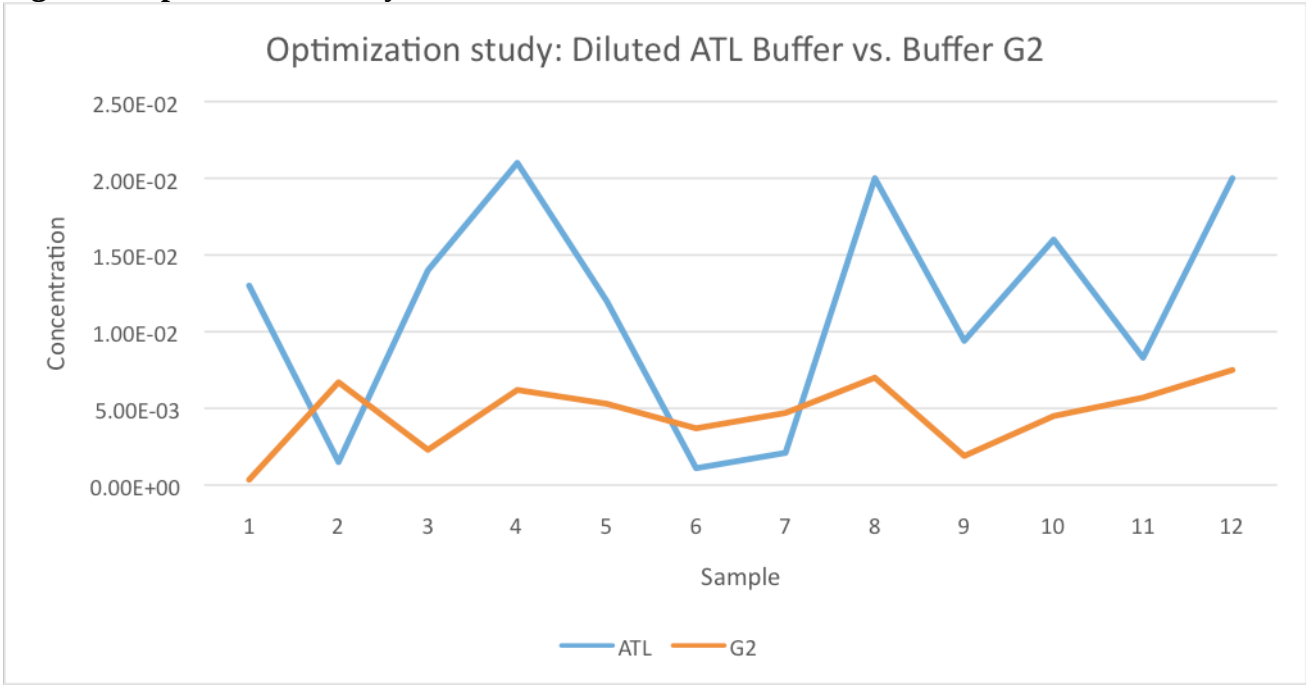
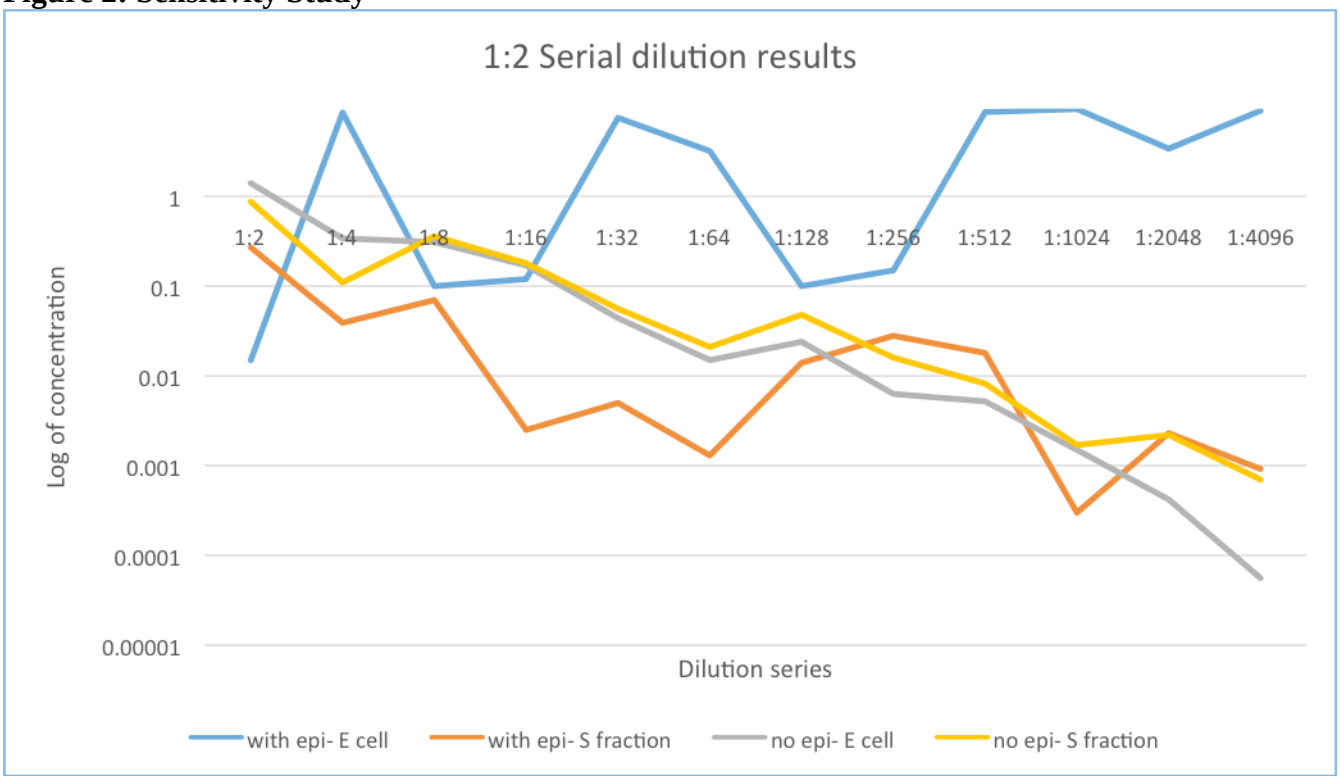
Figure 1: Optimization study



Quantitation values for the incubation using diluted ATL buffer and Buffer G2 are shown in the chart above. The diluted ATL buffer removed the sperm from the cotton swab better than the Buffer G2, although the buffer G2 was more consistent.

Figure 2: Sensitivity Study



Quantitation values for the 1:2 dilution series are shown in the chart above. The serial dilution quantitated as predicted.