Internal Validation of the QIAGEN® Investigator® Lyse&Spin Basket Kit and the PTC Erase Sperm Isolation Kit

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Abstract

The use of automated DNA extraction methods for DNA purification for forensic samples have been instrumental in streamlining the DNA extraction process, improving laboratory efficiency, and reducing the opportunity for analyst-introduced contamination [4]. However, many of these automated purification methods still require manual pre-processing steps. Two commercial products, the QIAGEN® Investigator® Lyse&Spin Basket Kit (QIAGEN®, Hilden, Germany), which combines sample lysis and lysate/substrate separation to reduce sample handling, and the Paternity Testing Corporation (PTC) Erase Sperm Isolation Kit (PTC, Columbia, MO), which eliminates laborious sperm washing steps during differential extractions by utilizing nuclease activity to reduce female carry-over to the sperm cell fraction and minimize manual sample handling are currently available solutions to potentially reduce the required pre-processing of DNA samples prior to extraction [1, 5, 8]. The use of the QIAGEN® Investigator® Lyse&Spin Basket Kit or the PTC Erase Sperm Isolation Kit in conjunction with an automated DNA extraction method could potentially decrease the amount of manual sample pre-processing and reduce the opportunity for sample loss and contamination.

The purpose of this study was to conduct an internal validation of the QIAGEN® Investigator® Lyse&Spin Basket Kit and the PTC Erase Sperm Isolation Kit for use in extractions on the QIAGEN® EZ1® Advanced XL BioRobot (QIAGEN®, Hilden, Germany) and compare the results obtained to the current extraction methods utilized by Palm Beach County Sheriff's Office (PBSO) Forensic Biology Unit. As part of the validation, sensitivity, stochastic threshold, repeatability, reproducibility, precision, accuracy, concordance/known, mixture, and contamination studies were completed as per the FBI Quality Assurance Standards to verify that both of the kits would produce reliable and reproducible results [9]. Overall, these validations

demonstrated that both the QIAGEN® Investigator® Lyse&Spin Basket Kit and the PTC Erase Sperm Isolation Kit would produce consistent and reliable results comparable to the extraction methods currently used by the PBSO. Future studies could be conducted to further evaluate these kits by testing vaginal swabs with the Erase Kit and investigating the use of longer injection times with the Lyse&Spin Baskets.

Introduction

Forensic DNA analysis is a multi-step process that begins with the extraction of DNA from evidentiary samples and ends with the potential generation of a DNA profile. The extraction step has the most potential for sample loss and contamination, and there must be laboratory protocols in place to protect the evidence [4]. PBSO commonly encounters evidentiary samples referred to as touch samples, which generally contain very low amounts of DNA. Sexual assault samples are also commonly submitted and often contain a biological mixture of male and female cellular material [2]. The quality of a DNA extraction process directly affects the recovery of DNA typing results from samples. As a result, the evaluation of all reagents, consumables, and methods used in the DNA process is critical. PBSO currently employs the QIAGEN® EZ1® DNA Investigator® Kit (QIAGEN®, Hilden, Germany) in conjunction with the QIAGEN® EZ1® Advanced XL BioRobot for extraction. This method utilizes an automated purification step, but still requires the use of manual sample pre-processing steps. In order to minimize the potential for sample loss and/or contamination, it is important to eliminate as many laborious, time-consuming, manual intervention steps as possible while still maintaining optimum DNA recovery. For this reason, alternative pre-processing methods to reduce sample handling, analyst time, while increasing sample quality and yield were investigated. The QIAGEN® Investigator® Lyse&Spin Basket Kit and the Paternity Testing

Corporation (PTC) Erase Sperm Isolation Kit are possible alternatives to the current sample pre-processing.

The QIAGEN® Investigator® Lyse&Spin Basket Kit is used for pretreatment of forensic samples in combination with a manual and automated extraction kit such as the QIAGEN® EZ1® DNA Investigator® Kit. The Lyse&Spin baskets allow for sample lysis and solid substrate separation in one simple procedure and work with a variety of substrates such as swabs, fabric, paper, cigarette butts, and gum. The Lyse&Spin baskets work by retaining the lysis buffer during the lysis step of the extraction procedure. Due to the preassembled configuration, there is no need for tedious manual handling steps during sample transfer. Upon centrifugation, holes in the bottom of the spin basket open and allow the sample lysate to pass through while retaining the sample substrate [5].

Sexual assault evidence frequently consists of a biological mixture of male and female cellular material. When processing these samples, an often time-consuming and laborious differential extraction is performed in an attempt to separate the male sperm DNA from the female DNA. Therefore, the most important steps in the differential methodology are the sperm cell pellet washes [1]. However, despite efforts to isolate the cell types prior to DNA extraction and purification, it is not uncommon to produce mixed DNA profiles due to an incomplete separation process [2]. A DNA mixture may make interpretation and statistical evaluation difficult and time-consuming. The Erase Sperm Isolation Kit is a differential extraction kit that utilizes nuclease activity to destroy epithelial cells in solution leaving the sperm cells unaffected and intact. This method does not include sperm washes, yet presumably provides for a single source male DNA profile from the sperm fraction [8].

Following pre-processing with the Lyse&Spin basket protocol or the Erase protocol, the samples can then be purified using the QIAGEN® EZ1® Advanced XL in conjunction with the QIAGEN® EZ1® DNA Investigator® Kit. An internal validation was performed at PBSO to evaluate if the Lyse&Spin baskets and the Erase Sperm Isolation Kit would provide a more efficient method for sample pre-processing as well as, quality and yield of DNA that was equal to or better than the current method. Sample preparation, quantification and amplification methods, injection time, and thresholds were determined based on PBSO's existing protocols [3]. Sensitivity/stochastic, repeatability, reproducibility, precision, accuracy, concordance/known samples, mixture, and contamination studies were completed as part of the validation. The overall quality and quantity of the DNA obtained was evaluated. The results were compared to the results obtained from a side-by-side comparison of the current in-house method and the original EZ1® validation where applicable.

Materials and Methods

Validation Studies

Sensitivity and Stochastic Studies

Lyse&Spin Baskets

A serial dilution was prepared using liquid blood and sterile water. The ratios included neat, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024. Each liquid dilution (200 µL) was spotted onto Whatman® (GE Healthcare Life Sciences, Pittsburgh, PA) filter paper and allowed to dry. The samples (~2 mm x 2 mm cuttings) were extracted in duplicate with the exception of the neat sample, which was included as a quality control measure.

Erase Sperm Isolation Kit

A serial dilution was prepared using semen and sterile water. The ratios included 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024. Each liquid dilution (100 µL) was added

to a sterile swab along with 25 µL of liquid female blood and allowed to dry. The samples (~1/4 of swab) were extracted using both the Erase Kit protocol and the current PBSO differential extraction protocol.

Known-Concordance and Accuracy Studies

Lyse&Spin Baskets

Ten previously typed samples (blood stains and buccal swabs) were extracted (~2 mm x 2 mm fabric cuttings or ~1/4 of swab).

Erase Sperm Isolation Kit

Two previously typed blood-semen mixed samples (~2 mm x 2 mm fabric cuttings or ~1/4 of swab) were extracted using both the Erase Kit protocol and the current PBSO differential extraction protocol.

Mixture Studies

Lyse&Spin Baskets

Mixtures of liquid male blood (M) and liquid female blood (F) were prepared as illustrated in Table 1.

Table 1: Preparation of Lyse&Spin Basket mixture study samples

Ratio	Male Blood (µL)	Female Blood (µL)
1:1	200	200
1:2	100	200
1:4	50	200
1:8	25	200
1:16	12.5	200
1:32	6.25	200
2:1	200	100
4:1	200	50
8:1	200	25
16:1	200	12.5
32:1	200	6.25

Each mixture (200 µL) was spotted onto Whatman® filter paper and allowed to dry. Mixture samples (~2 mm x 2 mm cuttings) were extracted in duplicate using the Lyse&Spin Basket protocol.

Erase Sperm Isolation Kit

The samples prepared for the sensitivity study were utilized.

Precision Studies

Lyse&Spin Baskets

Each extraction contained an internal NIST traceable Extraction Positive Control (RCP4).

Erase Sperm Isolation Kit

A serial dilution of liquid semen was prepared to include the following ratios: 1:8, 1:16, 1:32, 1:64, and 1:128. Each liquid dilution (100 µL) was added to a buccal swab from a female donor and allowed to dry. The samples were extracted in duplicate using the Erase Kit protocol. A single replicate was extracted using the current PBSO differential extraction protocol.

DNA Workflow

Extraction: *QIAGEN® Investigator® Lyse&Spin Basket Kit [5]*

Samples were cut (~2 mm x 2 mm filter paper or ~1/4 of swab) and extracted using the QIAGEN® Investigator® Lyse&Spin Basket Kit instructions for use with the QIAGEN® EZ1® DNA Investigator® Kit on the QIAGEN® EZ1® Advanced XL. A master mix was prepared containing 475 µL Buffer G2, 25 µL Proteinase K, and 1 µL carrier RNA (cRNA) per sample. The master mix was added to each sample (500 µL) before incubating for one hour at 900 rpm at 56 °C on a thermomixer. Samples were then centrifuged at 15,000 rpm for one minute, loaded onto the QIAGEN® EZ1® Advanced XL using the QIAGEN® EZ1® DNA Investigator® Kit, purified using the large volume protocol, and eluted in 40 µL TE^{-4}. An Extraction Negative

Control (RCN) and an internal Extraction Positive Control (RCP4) were run with each extraction set.

Extraction: ***PTC Erase Sperm Isolation Kit [8]***

Samples were cut (~2mm x 2 mm fabric or ~1/4 of swab) and differentially extracted using the PTC Erase Sperm Isolation Kit instructions for use with the QIAGEN® EZ1® DNA Investigator® Kit on the QIAGEN® EZ1®Advanced XL. A non-sperm cell master mix was prepared containing 400 µL Extraction Buffer, 7 µL Proteinase K, and 1 µL cRNA per sample. The master mix was added to each sample (400 µL) before incubating for one hour at 850 rpm at 56 °C on a thermomixer. After incubation, sample cuttings were transferred from the Erase dolphin tube into a spin basket using sterile forceps. The spin basket was placed back into the dolphin tube. The tube was centrifuged for five minutes at 15,000 rpm after which the spin basket was removed and discarded. Approximately 340 µL of the non-sperm fraction was removed and set aside for later processing with the QIAGEN® EZ1® DNA Investigator® Kit. Erase solutions #1 and #2 were added to the sperm cell fraction (10 µL each) before incubating for 15 minutes at 850 rpm at 37 °C on a thermomixer. After incubation, 10 µL of Erase solution #3 was added to the sperm cell fraction before incubating for 15 minutes at 850 rpm at 56 °C on a thermomixer. Following the final incubation, 110 µL Buffer G2 was added to the sperm cell fraction and all 200 µL of the sample was used for processing with the QIAGEN® EZ1® DNA Investigator® Kit. Samples were loaded onto the QIAGEN® EZ1® Advanced XL, purified using the trace protocol, and eluted in 40 µL TE^{-4}. An Extraction Negative Control for both the non-sperm cell fraction (RCNS) and the sperm cell fraction (RCSP) were run with each extraction.

Extraction: ***PBSO's Current Differential Extraction Protocol [3]***

Samples were cut (~2 mm x 2 mm fabric or ~1/4 of swab) and differentially extracted using PBSO's current differential extraction protocol. The non-sperm cell master mix contained 240 µL Buffer G2, 10 µL Proteinase K, and 1 µL cRNA and was added to each sample before incubating for 15 minutes at 850 rpm at 56 °C on a thermomixer. After incubation, samples were transferred from the dolphin tube into a spin basket and centrifuged for five minutes at maximum speed. Approximately 200 µL of the liquid from the non-sperm fraction was transferred to a separate tube and set aside. The sperm pellet was washed by adding 500 µL Buffer G2 to the sample and centrifuging for five minutes at maximum speed. Following centrifugation, 500 µL of the liquid from the sperm cell fraction was removed and discarded. This washing process was repeated two more times for a total of three sperm pellet washes. The sperm cell master mix contained 100 µL Buffer G2, 10 µL Proteinase K, 40 µL DTT, and 1 µL cRNA and 150 µL was added to each sperm cell fraction before incubating for ten minutes at 850 rpm at 70 °C on a thermomixer. The total volume of the sperm-cell fraction and the non-sperm cell fraction were purified with the QIAGEN® EZ1® DNA Investigator® Kit on the QIAGEN® EZ1® Advanced XL using the trace protocol and eluted in 40 µL TE^{-4}. An Extraction Negative Control for both the non-sperm cell fraction (RCNS) and the sperm cell fraction (RCSP) were run with each extraction set.

Quantification

Samples were quantified using the Promega® Plexor® HY System (Promega®, Madison, WI). Plates for quantification were prepared as per manufacturer's recommendations [6]. The Plexor® HY Genomic DNA Standard serial dilution was run in duplicate on each quantification plate. Plates were run on the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher

Scientific®, Waltham, MA) and analyzed using the Applied Biosystems® Sequence Detection Software (SDS) v1.2.3 and the Promega® Plexor® Analysis Software v1.5.6.7 using the manufacturer's recommended settings [6].

Amplification

Samples were amplified using the Promega® PowerPlex® 16 System (Promega®, Madison, WI). Plates for amplification were prepared as per manufacturer's recommendations [7]. An Amplification Negative Control (ACN) and Amplification Positive Control (ACP-2800M) were run with each amplification. Between 0.60 and 0.70 ng of DNA was targeted where possible. Up to 19.2 µL of sample was added to each sample well. If less than 19.2 µL of sample was added, enough water was added to bring the final volume up to 19.2 µL. All plates were run on the Applied Biosystems® GeneAmp® PCR System 9700 Thermal Cycler (Thermo Fisher Scientific®, Waltham, MA) using the amplification settings recommended by the manufacturer [7].

Capillary Electrophoresis

A master mix containing 9 µL formamide and 1 µL Internal Lane Standard (ILS) 600 was added to each sample well. Each sample was transferred (1 µL) from the amplification plate to the typing plate using the Eppendorf® epMotion® 5070 automated pipetting system (Eppendorf®, Hamburg, Germany). The PowerPlex® Allelic Ladder Mix (0.5 µL) was manually added to the typing plate. Capillary electrophoresis was performed using the Applied Biosystems® 3130*xl* Genetic Analyzer (Thermo Fisher Scientific®, Waltham, MA) using the manufacturer's recommendations [9] with a three second injection time.

Analysis

Data was analyzed using GeneMapper® ID-*X* v1.3 Software (Thermo Fisher Scientific®, Waltham, MA). An analytical threshold of 50 Relative Fluorescence Units (RFU) and a stochastic threshold of 208 RFU (3130*xl*-A) or 220 RFU (3130*xl*-B) were utilized when analyzing all data.

QIAGEN® Investigator® Lyse&Spin Basket Kit

Results

Sensitivity and Stochastic Study

Results for the blood dilutions show that the Lyse&Spin Basket protocol provided complete profiles for sixteen genetic markers up to a 1:256 dilution. Alleles began dropping below the stochastic threshold (220 RFU) at the 1:128 dilution (one replicate at amelogenin) and alleles began dropping below the analytical threshold (50 RFU) at 1:512. Table 2 summarizes the average quantification results, profiles obtained, and the average RFU height for the sensitivity study data.

Table 2: Summary of sensitivity study results for the QIAGEN® Investigator® Lyse&Spin Basket Kit. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (blood:water)	Average Quant Value (ng/µL)	# Alleles Dropped Out	Average Allele Height (RFU)
1:1	2.40	0	1268 1503 953
1:2	2.07	0	1203 1489 1076
1:4	0.834	0	1554 1685 1185
1:8	0.439	0	1132 1257 868
1:16	0.158	0	1434 1624 1166
1:32	0.170	0	1339 1479 995
1:64	0.084	0	1266 1411 935
1:128	0.054	0	932 1088 756
1:256	0.051	0	503 621 403
1:512	0.011	2	264 297 204
1:1024	0.004	15	81 78 103

These results were compared to the sensitivity data obtained during the original validation of the QIAGEN® EZ1® Advanced XL using the QIAGEN® EZ1® DNA Investigator® Kit. The original validation results showed complete profiles for sixteen genetic markers up to a 1:128 dilution and the average allele heights obtained were similar to those obtained in this study. A summary of the validation results for EZ1® Advanced XL C and D sensitivity study is illustrated in Table 3.

Table 3: Summary of sensitivity results for the performance check of EZ1® Advanced XL C and D. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (blood:water)	# Alleles Dropped Out	Average Allele Height (RFU)
1:1	0	2476
		2917
		2969
1:2	0	2419
		3018
		2693
1:4	0	2604
		2993
		2867
1:8	0	2067
		2614
		2304
1:16	0	1683
		1546
		1887
1:32	0	1378
		1220
		1450
1:64	0	554
		445
		634
1:128	0	836
		821
		1016
1:256	2	561
		540
		457
1:512	2	125
		132
		158
1:1024	9	74
		85
		70

Repeatability, Reproducibility, Known/Concordance, and Accuracy Studies

The same operator and a minimum of two different EZ1® Advanced XL instruments were utilized. The profiles obtained from both extracted sets of sensitivity/stochastic study samples and the known proficiency test samples and staff oral swabs were concordant with the expected profile. All of the DNA profiles provided RFU heights above the stochastic threshold (220 RFU). Quantification values obtained using the Lyse&Spin Basket extraction were comparable to

previously obtained quantification values obtained using the PBSO general extraction protocol (data not shown).

Mixture Study

A discernable major profile was observed in the 1:4 M:F mixture series with minor contributor alleles dropping below analytical threshold (50 RFU) starting at the 1:16 M:F mixture ratio. A discernable major profile was observed at the 4:1 M:F mixture ratio, however it should be noted that at the 4:1 M:F mixture ratio, alleles from the minor contributor dropped below analytical threshold (50 RFU). Alleles began dropping below the stochastic threshold (220 RFU) at the 1:8 and the 8:1 M:F mixture ratios. This is not reflected in Table 4 as the RFU heights listed are averages of all markers in the dye channel. At the 8:1 mixture ratio, the RFU height dropped below the stochastic threshold at various loci in both replicates.

Table 4: Summary of mixture study results for the QIAGEN® Investigator® Lyse&Spin Basket Kit. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Mixture Ratio (male:female)	Average Quant Value (ng/µL)	# Alleles Dropped Out (Minor Contributor)	Average Allele Height Major Contributor (RFU)	Average Allele Height Minor Contributor (RFU)
1:1	8.06	0	1007	880
			1208	996
			986	654
1:2	6.30	0	819	713
			1115	987
			820	824
1:4	4.45	0	1806	647
			1951	709
			1565	593
1:8	6.43	0	992	210
			1338	269
			954	215
1:16	6.02	3	1632	153
			2007	257
			1607	163
1:32	4.96	4	1731	158
			2001	164
			1760	124
2:1	4.54	0	1092	549
			1331	720
			1078	459
4:1	3.12	1	1262	294
			1634	358
			1290	285
8:1	3.32	2	1245	299

			1500	363
			1217	238
16:1	3.33	3	1654	130
			2027	182
			1538	95
32:1	3.35	12	1325	74
			1633	112
			1286	70

These results were compared to the mixture ratio data obtained during the original validation of the QIAGEN® EZ1® Advanced XL using the QIAGEN® EZ1® DNA Investigator® Kit (data not shown). The validation data showed that a discernible major profile was observed at the 1:2 M:F mixture ratio with drop-out of the minor contributor occurring at the 1:8 M:F mixture ratio. Whereas, in the reverse mixture ratios, a discernable major profile was observed at the 4:1 M:F mixture ratio with drop-out of the minor contributor occurring at the 16:1 M:F mixture ratio. The average RFU heights for the major contributor were slightly higher in the EZ1® validation that those observed in this study whereas the average RFU heights for the minor contributor were slightly lower in the EZ1® validation than those observed in this study. This may be a result of the difference in donors used to create the mixture samples as opposed to the extraction method.

Precision Study

Six NIST-traceable RCP4 samples were extracted and typed. All samples gave quantification results averaging 0.98 ng/µL and ranged between 0.61 ng/µL and 1.4 ng/µL with an average coefficient of variation of 47%. All resulting allele calls were concordant with the expected DNA profile for RCP4 (see Table 5).

Table 5: Summary of precision study data results for the QIAGEN® Investigator® Lyse&Spin Basket Kit.

Sample	Quant Value (ng/µL)	# Alleles Below Stochastic Threshold	Complete Expected Profile?
RCP4_4	0.606	0	Yes
LS_sDil_RCP4_12	0.737	0	Yes
LS_sDil_RCP4_25	1.419	0	Yes
LS_RCP4_37	0.997	0	Yes
LS_Mix_RCP4_50	1.395	0	Yes
LS_Mix2_RCP4_63	0.749	0	Yes

Contamination Study

Six extraction negative controls were processed during the validation. DNA was not obtained in any of the Extraction Negative Controls (RCNs).

QIAGEN® Investigator® Lyse&Spin Basket Kit

Discussion and Conclusions

The validation studies that were performed helped to compare the QIAGEN® Investigator® Lyse&Spin Basket Kit to the current general extraction protocol automated on the QIAGEN® EZ1® Advanced XL. The sensitivity data illustrate that the Lyse&Spin Baskets produced a complete profile at sixteen genetic markers up to a 1:256 dilution. It should be noted that the average RFU heights do not decrease sequentially with the quantification values due to normalization performed prior to amplification to target 0.60-0.70 ng of DNA where possible. When the results were compared to the sensitivity data obtained during the original validation of the QIAGEN® EZ1® Advanced XL using the QIAGEN® EZ1® DNA Investigator® Kit, those results showed complete profiles for sixteen genetic markers up to a 1:128 dilution. The RFU heights obtained from the Lyse&Spin Baskets are on average lower than the EZ1® validation for the first half of the sensitivity samples (1:1-1:64) whereas they evened out in the second half (1:128-1:1024) (see Tables 2 and 3). The Lyse&Spin sensitivity samples were typed on 3130*xl*-B (stochastic threshold 220 RFU) and the original EZ1® validation sensitivity samples were run on 3130*xl*-A (stochastic threshold 120 RFU) and on a different thermal cycler (Eppendorf® MasterCycler®). Considering this, although the Lyse&Spin Baskets were able to obtain a full profile at a lower dilution, it cannot be concluded that they are any more sensitive than the existing general extraction method, but are comparable.

For the mixture samples, it was determined that a discernable major and minor profile was observed at a 1:4 M:F mixture ratio and that the minor contributor alleles dropped below analytical threshold (50 RFU) between the 1:4 and a 16:1 M:F mixture ratio. Alleles began dropping below the stochastic threshold (220 RFU) at the 1:8 and the 8:1 M:F mixture ratios. The mixture study results indicate that the Lyse&Spin Baskets are a comparable extraction method for mixture analysis (distinction of a major/minor contributor) with the method that is currently used for general extractions at PBSO. A possibility for the different results displayed by the male:female mixture series could be explained by the fact that the neat liquid male blood quantitated (11.09 ng/µL) almost double the quantification value of the liquid female blood (5.72 ng/µL). It is also possible that the rate of drop-out seen in the minor female contributor was sample dependent (Table 4).

The QIAGEN® Investigator® Lyse&Spin Basket Kit was found to repeatedly produce reliable and reproducible results when used for extraction of DNA from solid substrates. The kit is also able to accurately produce concordant profiles. The validation studies performed also demonstrated that the Lyse&Spin Baskets did not introduce any contamination or foreign alleles into the extraction process.

Advantages of the Lyse&Spin Baskets include: They are ethylene oxide treated, thus eliminating the need for autoclaving and they involve few manual sample manipulation steps. Disadvantages of the Lyse&Spin Baskets include: individual packaging, increased amount of reagents required, and increased incubation time. Individually packaging made it cumbersome and laborious to open each separately packaged tube. The protocol required the use of 2.5 times more Proteinase K and digest buffer than what is currently required and a one hour incubation for sample lysis is required to complete the extraction (current lysis time is 15 minutes), thus the

Lyse&Spin Baskets take at least three times longer to perform than the current extraction method used. In addition, the current method gives analysts the flexibility to use either the EZ1® large volume protocol or the trace protocol whereas, the Lyse&Spin Baskets do not give that option and thus automatically require more reagents to be consumed.

Overall, the results of this validation indicate that the QIAGEN® Investigator® Lyse&Spin Basket Kit produces comparable results to the general extraction method currently used by PBSO. If implemented, the main advantage of the Lyse&Spin Baskets would be the reduction of sample handling steps thus decreasing the chance of contamination from sample handling errors.

PTC Erase Sperm Isolation Kit

Results

Sensitivity, Stochastic, and Mixture Studies

Results for the semen dilutions show that the Erase Sperm Isolation Kit provided complete male DNA profiles for the sperm fractions for sixteen genetic markers up to a 1:256 dilution. Male contributor alleles began dropping below the stochastic threshold (220 RFU) at the 1:256 dilution. This is not reflected in Table 5 as the RFU heights listed are averages of all markers in the dye channel, but the male profile in the 1:256 dilution dropped below stochastic threshold at the genetic marker Penta E. The presence of carry-over from the non-sperm cell fraction was observed at the 1:32 dilution (see Table 5).

Table 5: Summary of sensitivity results for the sperm cell fraction extracting using the PTC Erase Sperm Isolation Kit. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (semen:water)	Quant Value (ng/µL)	Male Quant Value (ng/µL)	# Male Alleles Dropped Out	# Female Carry-Over Alleles	Average Allele Height Male Contributor (RFU)	Average Allele Height Female Contributor (RFU)
1:1	10.44	11.24	0	0	1758	-
					1696	-
					1396	-
1:2	10.47	11.16	0	0	1635	-
					1709	-
					1275	-
1:4	5.83	6.47	0	0	1567	-
					1595	-
					1145	-
1:8	1.15	1.22	0	0	1143	-
					1188	-
					1072	-
1:16	0.587	0.601	0	0	1670	-
					1663	-
					1147	-
1:32	0.545	0.578	0	3	1370	99
					1586	97
					970	-
1:64	0.108	0.106	0	2	1738	61
					1699	140
					1252	-
1:128	0.076	0.076	0	10	1499	110
					1585	101
					1224	56
1:256	0.359	0.041	0	21	264	1252
					303	1501
					255	955
1:512	0.425	0.021	1	21	146	1343
					200	1569
					159	1140
1:1024	0.004	0.004	7	8	78	70
					117	99
					85	82

Results for the non-sperm cell fractions show that the Erase Sperm Isolation Kit provided complete female DNA profiles for the non-sperm fractions for sixteen genetic markers up to the 1:1024 dilution with the presence of male alleles beginning at the 1:1 dilution through the 1:128 dilution. Female contributor alleles dropped below the stochastic threshold (220 RFU) at the 1:32 ratio. This is not reflected in Table 6 as the RFU heights listed are averages of all markers in the dye channel, but the female profile in the 1:32 dilution dropped below stochastic threshold at markers D18S51 and TPOX.

Table 6: Summary of sensitivity results for the non-sperm cell fraction extracting using the PTC Erase Sperm Isolation Kit. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (semen:water)	Quant Value (ng/µL)	Male Quant Value (ng/µL)	# Female Alleles Dropped Out	# Male Carry-Over Alleles	Average Allele Height Female Contributor (RFU)	Average Allele Height Male Contributor (RFU)
1:1	5.57	4.18	0	21	428	1439
					610	1527
					395	1135
1:2	3.05	2.01	0	21	805	1202
					937	1192
					501	912
1:4	4.17	2.08	0	21	924	845
					1260	833
					738	572
1:8	1.24	0.28	0	21	818	651
					1055	599
					550	364
1:16	1.46	0.24	0	21	1126	378
					1333	387
					1119	390
1:32	2.26	0.17	0	20	1414	188
					1743	227
					1158	194
1:64	1.05	0.041	0	20	1567	174
					2015	186
					1428	150
1:128	1.60	0.027	0	15	1926	160
					2410	177
					1614	172
1:256	1.47	0.0062	0	0	2078	-
					2566	-
					1561	-
1:512	2.53	0.002	0	1	1837	56
					2358	-
					1406	-
1:1024	0.97	0.0007	0	0	1762	-
					2200	-
					1318	-

Results for the semen dilutions show that the current PBSO differential extraction method provided complete male DNA profiles for the sperm cell fractions for sixteen genetic markers up to the 1:256 dilution. Male contributor alleles began dropping below the stochastic threshold (220 RFU) at the 1:256 dilution. This is not reflected in Table 7 as the RFU heights listed are averages of all markers in the dye channel, but the male profile in the 1:256 dilution dropped below stochastic threshold at the genetic markers vWA and FGA. The presence of carry-over from the non-sperm fraction was observed beginning at the 1:8 dilution (see Table 7).

Table 7: Summary of sensitivity results for the sperm cell fraction extracting using the current PBSO differential extraction protocol. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (semen:water)	Quant Value (ng/µL)	Male Quant Value (ng/µL)	# Male Alleles Dropped Out	# Female Carry-Over Alleles	Average Allele Height Male Contributor (RFU)	Average Allele Height Female Contributor (RFU)
1:1	13.35	16.25	0	0	1931	-
					1808	-
					1419	-
1:2	6.05	8.10	0	0	2252	-
					2305	-
					1651	-
1:4	2.20	2.90	0	0	2231	-
					2355	-
					1820	-
1:8	1.47	2.01	0	2	1533	60
					1673	-
					1320	56
1:16	0.632	0.934	0	0	2029	-
					2296	-
					1249	-
1:32	0.301	0.460	0	5	2076	94
					2114	105
					1433	140
1:64	0.216	0.312	0	15	1807	100
					2015	148
					1274	100
1:128	0.092	0.111	0	20	1685	138
					1784	224
					1185	119
1:256	0.030	0.039	0	21	501	233
					582	343
					359	213
1:512	0.014	0.006	10	19	85	114
					97	153
					57	101
1:1024	0.019	0.010	2	21	176	159
					170	163
					106	107

Results for the non-sperm cell fractions show that the current PBSO differential extraction method provided complete female DNA profiles for the non-sperm cell fractions for sixteen genetic markers up to the 1:1024 dilution with the presence of male alleles beginning at the 1:1 dilution through the 1:256 dilution. Female contributor alleles dropped below the stochastic threshold (220 RFU) at the 1:512 dilution (see Table 8).

Table 8: Summary of sensitivity results for the non-sperm cell fraction extracting using the current PBSO differential extraction protocol. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (semen:water)	Quant Value (ng/µL)	Male Quant Value (ng/µL)	# Female Alleles Dropped Out	# Male Carry-Over Alleles	Average Allele Height Female Contributor (RFU)	Average Allele Height Male Contributor (RFU)
1:1	7.45	7.99	0	21	359	1466
					409	1542
					257	1267
1:2	3.88	3.45	0	21	739	1745
					882	1752
					529	1325
1:4	2.07	1.47	0	21	746	1144
					931	1127
					480	758
1:8	3.96	2.30	0	21	1298	1087
					1404	974
					869	735
1:16	2.65	1.09	0	21	1243	704
					1407	646
					1076	544
1:32	2.25	0.46	0	21	776	214
					932	234
					572	157
1:64	2.75	0.24	0	20	1478	216
					1724	212
					1139	142
1:128	2.16	0.10	0	10	556	84
					598	61
					371	61
1:256	2.44	0.046	0	8	1704	115
					1929	149
					1054	113
1:512	1.40	0.0054	0	0	322	-
					409	-
					217	-
1:1024	1.66	0.0031	0	0	1921	-
					2186	-
					1214	-

Repeatability, Reproducibility, and Known/Concordance Studies

The same operator and a minimum of two different EZ1® Advanced XL instruments were utilized. The profiles obtained from all samples extracted were concordant with the expected profile. Quantification values obtained using the Erase Sperm Isolation Kit were comparable to or slightly higher than the quantification values obtained using the PBSO differential extraction protocol.

Precision and Accuracy Studies

The Erase Sperm Isolation Kit provided complete male DNA profiles for the sperm cell fractions for sixteen genetic markers up to the 1:128 dilution. Male contributor alleles dropped below the stochastic threshold (208 RFU) at the 1:64 dilution. This is not reflected in Table 9 as the RFU heights listed are averages of all genetic markers in the dye channel, but the male profile in the 1:128 dilution dropped below stochastic threshold at the genetic markers D21S11, Penta E, D13S317, D7S820, amelogenin, D8S1179, and FGA. The appearance of carry-over from the non-sperm cell fraction was observed at the 1:8 dilution (see Table 9). The samples gave average quantification values ranging between 0.07 ng/µL and 0.56 ng/µL with an average coefficient of variation of 62%. The large coefficient of variation may have resulted from the differences in the efficiency of the two quantification runs as opposed to the efficiency of the extractions.

Table 9: Summary of precision and accuracy results for the sperm fraction extracted using the Erase Sperm Isolation Kit. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (semen:water)	Average Quant Value (ng/µL)	# Male Alleles Dropped Out	# Female Carry-Over Alleles	Average Allele Height Male Contributor (RFU)	Average Allele Height Female Contributor (RFU)
1:8	0.56	0	11	820	102
				1454	96
				1005	63
1:16	0.88	0	2	633	-
				1117	54
				738	-
1:32	0.31	0	2	694	58
				1242	58
				894	-
1:64	0.07	0	19	318	148
				670	185
				459	237
1:128	0.14	0	14	576	99
				1060	129
				709	124

The Erase Sperm Isolation Kit provided complete female DNA profiles for the non-sperm cell fractions for sixteen genetic markers up to the 1:128 dilution. Female contributor alleles did

not drop below the stochastic threshold (208 RFU) at any of the dilutions. The appearance of five male alleles appeared in the 1:16 dilution of one of the replicates, but all five alleles were below the stochastic threshold (data not shown).

The current PBSO differential extraction method provided complete male DNA profiles for the sperm cell fractions for sixteen genetic markers up to the 1:128 dilution. Male contributor alleles dropped below the stochastic threshold (208 RFU) at the 1:64 dilution. This is not reflected in Table 10 as the RFU heights listed are averages of all markers in the dye channel, but alleles at various genetic markers in the male profile in the 1:64 dilution dropped below the stochastic threshold (208 RFU). The appearance of carry-over from the non-sperm cell fraction was observed beginning at the 1:8 dilution. These results are summarized in Table 10.

Table 10: Summary of precision and accuracy results for the sperm fraction extracted using the current PBSO differential extraction protocol. Average allele height colors refer to dye channel: blue (FL), green (JOE), yellow (TMR).

Sensitivity Ratio (semen:water)	Quant Value (ng/µL)	Male Quant Value (ng/µL)	# Male Alleles Not Typed	# Female Carry-Over Alleles	Average Allele Height Male Contributor (RFU)	Average Allele Height Female Contributor (RFU)
1:8	1.72	2.04	0	19	1529	109
					2313	152
					1642	176
1:16	0.537	0.49	0	17	1025	145
					1347	162
					758	130
1:32	0.826	0.54	0	19	454	199
					582	296
					355	244
1:64	0.198	0.06	0	19	261	319
					348	468
					206	308
1:128	0.612	0.54	0	19	902	156
					1454	172
					798	171

The current PBSO differential extraction method provided complete female DNA profiles for the non-sperm cell fractions for sixteen genetic markers up to the 1:128 dilution. Female contributor alleles did not drop below the stochastic threshold (208 RFU) at any of the dilutions.

The appearance of one male allele appeared in the 1:16 dilution, but the allele was below the stochastic threshold (data not shown).

Contamination Study

Eighteen extraction negative controls were extracted during the Erase Sperm Isolation Kit validation studies. One RCSP in the sensitivity study contained a partial DNA profile consistent with the Promega® PowerPlex® 16 Allelic Ladder. The RCSP was re-amplified and a negative profile was obtained thus indicating the allelic ladder was introduced as a result of a sample handling error during the 3130*xl* plate set-up. A single allele was called in one RCNS in the precision study. The nine allele at D7S820 was not reproduced after re-amplification. The allele was not consistent with the individual conducting the analysis or any of the samples run with the extraction indicating that the allele at D7S820 resulted from a drop-in event. All other negative extraction controls gave negative results.

PTC Erase Sperm Isolation Kit

Discussion and Conclusions

The validation studies were performed to compare the PTC Erase Sperm Isolation Kit to the current differential extraction method used at PBSO. The results of the sensitivity study demonstrated that the Erase Sperm Isolation Kit produced results that were comparable to the current differential extraction procedure. It was observed that both methods resulted in a clean separation between the sperm and non-sperm fraction for mixed saliva and semen stains down to a lower dilution than the mixed blood and semen samples. The PTC Erase Sperm Isolation Kit was found to repeatedly produce reliable and reproducible results and is also able to accurately produce a profile concordant with the expected profile when used for extraction of DNA from differential samples. The results of the contamination study illustrate the presence of an allele

drop-in event during amplification; however, no contamination was introduced at the extraction step.

Advantages of the Erase Sperm Isolation Kit include: All reagents required for the protocol are contained within the kit, thus eliminating the need for reagent preparation prior to starting the extraction method. Multiple sperm washes are not necessary, requiring fewer sample handling steps thus reducing the chance of sample loss through aspiration of the sperm pellet or contamination. This advantage can be visualized in the male contributor of the 1:512 dilution extracted using a the current PBSO differential extraction protocol with sperm washing steps (Table 7). The RFU heights should be higher than the 1:1024, but they are unexpectedly low. This suggests partial sperm pellet aspiration.

Disadvantages of the Erase Sperm Isolation Kit include: The reagent tubes included in the kit cannot be re-frozen after thawing, thus increasing the amount of wasted reagent especially in small extraction batches. The Erase Sperm Isolation Kit is more expensive than the reagents used in the current extraction method. The approximate cost to extract one sample using the Erase Sperm Isolation Kit is more than three times the cost to extract one sample using the current PBSO differential extraction protocol (see Table 11).

Table 11: Approximate cost of reagents per sample using the Erase Sperm Isolation Kit protocol versus the current PBSO differential extraction protocol.

Reagent	Erase	PBSO Differential*
Erase Kit	$ 9.90	N/A
EZ1® Kit	$ 3.90	$ 3.90
1M DTT (40 µL)	N/A	$ 0.10
Buffer G2 for Lysis and Sperm Washes (1500 µL)	N/A	$ 0.43
Total Cost	$ 13.80	$ 4.43

*Prices of consumables not included

Finally, the Erase Sperm Isolation Kit protocol takes longer to complete than the differential extraction procedure currently used at PBSO. As illustrated in Table 12, the Erase Sperm Isolation Kit protocol takes more than twice as long to complete.

Table 12: Summary of time to complete the Erase Sperm Isolation Kit protocol verses the current PBSO differential extraction protocol.

Process	Erase	PBSO Differential
Non-Sperm Cell Lysis Incubation	60 minutes	15 minutes
Incubation for Nuclease Activity	15 minutes	N/A
Sperm Washes	N/A	~15 minutes*
Sperm Cell Lysis Incubation	15 minutes	10 minutes
Total Time	90 minutes	40 minutes

*Reflects approximate time to complete all three sperm washes (each is approximately five minutes)

Overall, the results of this validation indicate that the Erase Sperm Isolation Kit produces comparable results to the differential extraction method currently used by PBSO. If implemented, the main advantage of the Erase Sperm Isolation Kit protocol would be the reduction in sample handling and processing steps.

Acknowledgements

This project benefitted from the input of Amy McGuckian, Julie Sikorsky, Cathy Cothran, Dr. Cecelia Crouse, Season Seferyn, and Dr. Pamela Staton, all of whom provided assistance and critiques of the presentation and these studies. Thanks are also given to Shanna Saunders who provided support in the production of this paper. The author also thanks the analysts and employees of the Palm Beach County Sheriff's Office Forensic Biology Unit, who have valuable advice, encouragement, and made the experience within their laboratory worthwhile.

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