District of Columbia
Department of Forensic Services
Consolidated Forensic Laboratory

Validation of the QIAGEN EZ1 Advanced XL workstation with the DNA Investigator DNA Extraction Kit

Validation Prepared By:

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Summary:

The EZ1 and EZ1 DNA Investigator kit were validated in accordance with the FBI issued Quality Assurance Standards for Forensic DNA Testing Laboratories, effective September 1, 2011 (1). The internal validation described here includes studies on reproducibility and precision, sensitivity and stochastic studies, a contamination assessment, and use with known and mixed donor samples. Two EZ1 Advanced XL robotic platforms were validated and the results demonstrate that DNA extracted on the EZ1 generates profile results that are accurate, reproducible, precise in sizing and free from contamination.

Background Information:

To achieve the best profile results from biological specimens, it is important to have a DNA extraction method optimized for forensic processing. The extraction method must be able to separate DNA molecules from other cellular material and environmental debris. The extraction method must also be able to purify the DNA from any remaining PCR inhibitors that may be present in the sample, while preserving the condition of the DNA. The EZ1 DNA Investigator Kit is optimized for extraction and purification of DNA from a variety of forensic type samples. The EZ1 Advanced XL workstation (EZ1) was designed to purify nucleic acids from a variety of sample types while minimizing sample handling and contamination risks. The 'Advanced' model of the EZ1 includes additional features to allow barcode reading of sample tubes and reagents, a log file report to be generated, front cover locking during processing and an internal UV light for decontamination. The 'XL' version of the EZ1 can process up to 14 samples simultaneously. Initial cell lysis of the sample is performed off robot on a thermal mixer that simultaneously heats and vortexes the lysate. After lysis, the EZ1 can process between one to fourteen samples in approximately 20 minutes. Nucleic acids in sample lysates are isolated in one step by binding to the silica surface of magnetic particles as other debris is washed away.

The following is a generalized protocol describing the extraction process. Step 1 is performed off-robot, while the remainders of the steps are done by the EZ1 instrument.

1. Cell Lysis – Incubate samples in lysis buffer to lyse cells and release DNA.
2. Bind DNA – Add magnetic particles to lysates and allow DNA to bind. Use magnetic rods to attract magnetic particles to the side of the tubes and while lysate solutions are removed.
3. Wash DNA – Add wash buffer and vortex samples. Use magnetic rods to attract magnetic particles to the side of the tubes while wash buffers are removed.
4. Repeat washes to remove all PCR inhibitors.
5. Elute DNA – Add elution buffer and incubate to release DNA from magnetic particles. Use magnetic rods to attract magnetic particles to the side of the tubes while eluates are transferred to new tubes for downstream processing.

Reagents are supplied in pre-filled EZ1 Reagent Cartridges, which minimizes the analyst's hands on time and reduces risks of contamination. The kit contains lysis buffer for cell lysis, magnetic particles for binding DNA, wash buffers to remove inhibitors and elution buffers for releasing DNA from the magnetic particles.

Three protocols for nucleic acid purification are supplied on pre-programmed EZ1 Cards. The "Trace" protocol extracts samples in a 200 µl lysis volumes. This method is most suitable for liquid samples or substrates with small amounts of DNA that would benefit from using a spin basket to remove all lysate from the sample. The "Tip Dance" protocol uses the "Trace" protocol, except for the initial lysate transfer step can be performed with small substrate still in the lysis tube. The tips move back and forth within the lysis tube to aspirate all lysis volume and not aspirate the substrate. This method is most suitable for small cuttings of swabs, fabrics or FTA paper that contain abundant amounts of DNA. The "Large Volume" protocol extracts samples from a 500 µl lysis volume for substrates requiring a larger lysis volume. These protocols provide both on-screen instructions for the operator and operating commands for the workstation. The resulting extract can be used directly in any downstream application, such as PCR amplification.

Materials and Methods:

A full set of validation samples were run on one of the instruments, indicated in this validation as EZ1-A. An instrument check was performed on the second instrument, indicated in this validation as EZ1-B, which tested sensitivity, accuracy, reproducibility and contamination.

Unless otherwise noted, all experiments in this validation followed protocols described in the EZ1 DNA Investigator Handbook (2).

The following shows the lysis reagent recipes for the protocols used in this validation:

Reagent	Trace and Tip Dance Protocol	Large Volume Protocol	Qiagen Modified Large Volume Protocol
G2	95 µl	245 µl	480 µl
Water	95 µl	245 µl	--
Proteinase K	10 µl	10 µl	20 µl
Total Lysis Volume	*200 µl*	*500 µl*	*500 µl*

All samples, unless otherwise noted, were lysed for 15 minutes at 56°C on a thermomixer shaking at 900 rpm. Samples were immediately transferred to a second incubation at 95°C shaking at 900 rpm for 5 minutes. After incubation, carrier RNA (1 µl) was added to the lysates prior to loading samples onto the EZ1 instrument (3). When the large volume protocol was used, 400 µl of MTL was added to each lysate prior to extraction on the EZ1 instrument.

Sample Preparation and DNA Extraction

The EZ1 Samples were set-up for processing according to the following extraction summaries.

Sensitivity Study: To determine sensitivity, whole blood was diluted in 1X PBS to create the following dilutions: 1:5, 1:10, 1:20, 1:40 and 1:80. The five dilutions, an undiluted blood sample and a reagent blank were extracted in triplicate using 5 µl of each blood dilution. The first two replicates were performed on separate instrument runs using EZ1-A. The third replicate was performed on EZ1-B. The triplicate extractions were performed using both the "Trace" protocol and the "Large Volume" protocol for a total of 42 samples.

Reproducibility and Precision Study: To determine reproducibility. 5 µl of both the neat and the 1:10 dilution of blood were spotted onto 10 cotton swabs each (20 total) and allowed to dry overnight. Samples were extracted in batches of 5 replicates of each dilution of blood with a reagent blank associated with set of 5 samples. Each set of samples was extracted using the "Large Volume" protocol on EZ1-A and EZ1-B for a total of 24 samples. In addition, 5 µl of both the 1:5 and the 1:10 dilutions of blood were spotted into 10 lysis tubes (20 tubes). Samples were extracted in batches of 5 replicates of each dilution of blood with a reagent blank associated with each set of 5 samples. Each set of samples was extracted using the "Trace" protocol on EZ1-A and EZ1-B for a total of 24 samples.

Accuracy and Concordance Study: To determine accuracy, reference type samples with known profiles and samples from the National Institute of Standards and Technology (NIST) with certified profiles were extracted on the EZ1s. Four donors each of buccal swabs and blood on FTA paper were extracted on each EZ1 for a total of 16 reference type samples. Approximately one quarter of a buccal swab was used per extraction. One hole punch from blood on FTA was taken per extraction. In addition to the known reference samples, one 6mm punch from both component E (cells spotted on 903 paper) and component F (cells spotted on FTA) of the NIST Standard Reference Material (SRM) 2391c were extracted on EZ1-A. All samples were extracted using the "Tip Dance" Protocol.

Contamination Assessment: In addition to reagent blanks run throughout the validation in each extraction set, the reference type samples in the accuracy study previously described was run with alternating reagent blanks between every sample. The buccal swabs were extracted first in lanes 1, 3, 5, and 7 with reagent blanks in lanes 2, 4, 6 and 8. The second run extracted reagent blanks in lanes 1, 3, 5, 7 and 9 with blood on FTA samples in lanes, 2, 4, 6 and 8. This study demonstrates samples processed both side by side and one run after the other were free from contamination.

Mixture Study: To demonstrate the EZ1's ability to extract multiple donors from a mixture at the expected ratio, mixtures of male and female whole blood were created using the following ratios: 1:0, 19:1, 9:1, 4:1, 1:1, 1:4, 1:9, 1:19, 0:1. The mixture samples were extracted using both the "Trace" protocol and the "Large Volume" protocol. One reagent blank was extracted with each set of mixture samples for a total of 20 samples.

Mock Evidence Samples: The following table describes the samples for testing casework type samples.

SAMPLE NAME	SAMPLE TYPE	CUTTING SIZE	DIFFERENTIAL
SWA.1	SWAB OF STEERING WHEEL - DONOR A	1/2 SWAB	NO
SWB.1	SWAB OF STEERING WHEEL - DONOR B	1/2 SWAB	NO
IPHONE.1	SWAB OF iPHONE	1/2 SWAB	NO
MOUSEA.1	SWAB OF COMPUTER MOUSE - DONOR A	1/2 SWAB	NO
MOUSEB.1	SWAB OF COMPUTER MOUSE - DONOR B	1/2 SWAB	NO
MOUSEC.1	SWAB OF COMPUTER MOUSE - DONOR C	1/2 SWAB	NO
MOUSED.1	SWAB OF COMPUTER MOUSE - DONOR D	1/2 SWAB	NO
FGAR.1	SWAB OF FINGERNAILS – DONOR A – RIGHT HAND	1/2 SWAB	NO
FGAL.1	SWAB OF FINGERNAILS – DONOR A – LEFT HAND	1/2 SWAB	NO
FGBR.1	SWAB OF FINGERNAILS – DONOR B – RIGHT HAND	1/2 SWAB	NO
FGBL.1	SWAB OF FINGERNAILS – DONOR B – LEFT HAND	1/2 SWAB	NO
BUCCALA.1	BUCCAL SWAB - DONOR A	1/2 SWAB	NO
BUCCALB.1	BUCCAL SWAB - DONOR B	1/2 SWAB	NO
BUCCALC.1	BUCCAL SWAB - DONOR C	1/2 SWAB	NO
BUCCALD.1	BUCCAL SWAB - DONOR D	1/2 SWAB	NO
CIG.1	CIGARETTE BUTT	5X5mm	NO
M11-0017P-3-1.1	BLOODSTAIN	1X1cm	NO
M11-0017P-4-1.1	BLOODSTAIN	5X5mm	NO
M11-0013P-4-1.1	BLOODSTAIN	5X5mm	NO
M11-0006P-3-1.1	BLOODSTAIN	1X1cm	NO
M11-0003P-3-1.1	BLOODSTAIN	5X5mm	NO

All samples from the above sample set were processed using the “Large Volume” protocol with a 15 minute 56°C lysis time.

Additional samples types of the touch and saliva samples from the above set were also processed using the “Large Volume” protocol with a 1 hour 56°C lysis time. The following table describes the samples for testing casework type samples lysed for 1 hour.

SAMPLE NAME	SAMPLE TYPE	CUTTING SIZE	DIFFERENTIAL
MOUSEE.1	SWAB OF COMPUTER MOUSE - DONOR E	1/2 SWAB	NO
MOUSEF.1	SWAB OF COMPUTER MOUSE - DONOR F	1/2 SWAB	NO
MOUSEG.1	SWAB OF COMPUTER MOUSE - DONOR G	1/2 SWAB	NO
MOUSEH.1	SWAB OF COMPUTER MOUSE - DONOR H	1/2 SWAB	NO
FGCR.1	SWAB OF FINGERNAILS – DONOR C – RIGHT HAND	1/2 SWAB	NO
FGCL.1	SWAB OF FINGERNAILS – DONOR C – LEFT HAND	1/2 SWAB	NO
FGDR.1	SWAB OF FINGERNAILS – DONOR D – RIGHT HAND	1/2 SWAB	NO
FGDL.1	SWAB OF FINGERNAILS – DONOR D – LEFT HAND	1/2 SWAB	NO
SWC.1	SWAB OF STEERING WHEEL - DONOR C	1/2 SWAB	NO
SWD.1	SWAB OF STEERING WHEEL - DONOR D	1/2 SWAB	NO
IPHONEB.1	SWAB OF iPHONE – DONOR B	1/2 SWAB	NO
BUCCALA.3	BUCCAL SWAB - DONOR A	1/2 SWAB	NO
BUCCALB.3	BUCCAL SWAB - DONOR B	1/2 SWAB	NO
BUCCALC.3	BUCCAL SWAB - DONOR C	1/2 SWAB	NO
BUCCALD.3	BUCCAL SWAB - DONOR D	1/2 SWAB	NO
CIG.3	CIGARETTE BUTT	5X5	NO

The following table describes the samples for testing casework type samples containing semen.

Sample Name	Sample Type	CUTTING SIZE	DIFFERENTIAL
BS.1	Blood stain with Semen	5X5	YES
M11-0013P-3-1.1	Blood stain with Semen	1X1	YES
M11-0006P-4-1.1	Blood stain with Semen	1X1	YES
M11-0003P-4-1.1	Blood stain with Semen	5X5	YES

The differential samples were separated and extracted following the exact protocol in the EZ1 DNA Investigator Handbook. Epithelial fractions (EF) were processed using the “Large Volume”

protocol and sperm fractions (SF) were processed using the "Trace" protocol. In addition, a modified differential extraction procedure was performed. The modified procedure followed protocol FBS11-*Differential Organic DNA Extraction* steps one through six to separate the epithelial and sperm fractions, as well as wash the sperm pellet. The sperm pellet was then re-suspended in G2 buffer according to the following table and incubated at 56°C overnight. The epithelial fraction was stored at 4°C overnight.

Reagent	**Large Volume Protocol**
G2	240 µl
Water	240 µl
1 M DTT	10 µl
Proteinase K	10 µl
Total Lysis Volume	*500 µl*

Both the epithelial and sperm fractions were processed using the large volume protocol by adding 400 µl of MTL buffer and 1 µl of carrier RNA to each tube. The epithelial fractions were equilibrated to room temperature prior to extraction on the EZ1.

Troubleshooting: A series of troubleshooting steps were performed to determine optimal lysis conditions for a more balanced profile. Qiagen recommended several testing parameters, including different reagent lots, different water and switching lysis buffers. Qiagen optimized a new protocol for MPD that used undiluted G2 (480 µl), increased Proteinase K (20 µl) and no 95°C incubation step after lysis. A set of mock samples was run on both EZ1 instruments to demonstrate that this modified approach improved the overall profile balance of samples.

Additional sample types of the touch and saliva samples from the above set were also processed using the "Large Volume" protocol with a 2 hours 56°C lysis time.

The following table describes the samples for testing casework type samples lysed for 2 hours.

SAMPLE NAME	SAMPLE TYPE	CUTTING SIZE	EZ1
SWC.2	SWAB OF STEERING WHEEL - DONOR C	1/2 SWAB	EZ1-A
IPHONEB.2	SWAB OF iPHONE	1/2 SWAB	EZ1-A
MOUSEE.2	SWAB OF COMPUTER MOUSE - DONOR E	1/2 SWAB	EZ1-A
MOUSEF.2	SWAB OF COMPUTER MOUSE - DONOR F	1/2 SWAB	EZ1-A
MOUSEG.2	SWAB OF COMPUTER MOUSE - DONOR G	1/2 SWAB	EZ1-A
MOUSEH.2	SWAB OF COMPUTER MOUSE - DONOR H	1/2 SWAB	EZ1-A
FGCR.2	SWAB OF FINGERNAILS – DONOR C – RIGHT HAND	1/2 SWAB	EZ1-A
FGCL.2	SWAB OF FINGERNAILS – DONOR C – LEFT HAND	1/2 SWAB	EZ1-A
BUCCALE.1	BUCCAL SWAB - DONOR E	1/2 SWAB	EZ1-A
BUCCALF.1	BUCCAL SWAB - DONOR F	1/2 SWAB	EZ1-B
BUCCALH.1	BUCCAL SWAB - DONOR H	1/2 SWAB	EZ1-B
CIG.4	CIGARETTE BUTT	5X5mm	EZ1-B
M11-0017P-3-1.4	BLOODSTAIN	1X1cm	EZ1-B
M11-0017P-4-1.4	BLOODSTAIN	5X5mm	EZ1-B
M11-0013P-4-1.4	BLOODSTAIN	5X5mm	EZ1-B
M11-0006P-3-1.4	BLOODSTAIN	1X1cm	EZ1-B
M11-0003P-3-1.4	BLOODSTAIN	5X5mm	EZ1-B

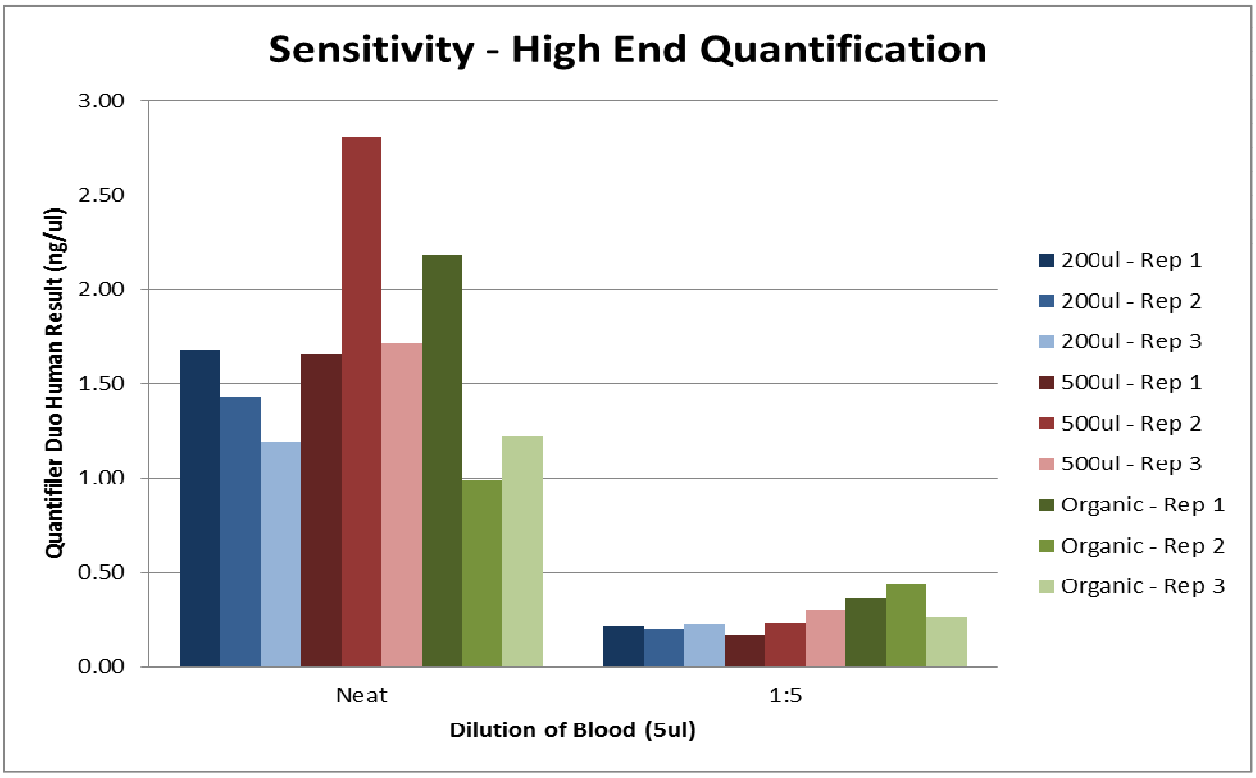
Post Extraction Processing: All extracted DNA were quantitated using Quantifiler Duo Quantification of Human DNA following protocol FBS18 – *Quantitation by Real-Time PCR Using Quantifiler Duo*. The DNA extracts were then amplified with the AmpFℓSTR® Identifiler™ amplification kit in a 25 µl reaction volume following protocol FBS13 – *PCR Amplification Using AmpFlSTR Identifiler Kit*. All samples were setup for fragment separation following protocol FBS14 – *Capillary Electrophoresis Using the AB 3130xl Genetic Analyzer*. The data was analyzed using GMID v 3.2, analysis method "Identifiler_Analysis_Method" and "Identifiler_V2" panels and bins. Samples were analyzed using an analytical threshold of 70 RFU and stochastic threshold of 215 RFU, with an allelic balance requirement of 55%. All data tables can be found following the summary write up.

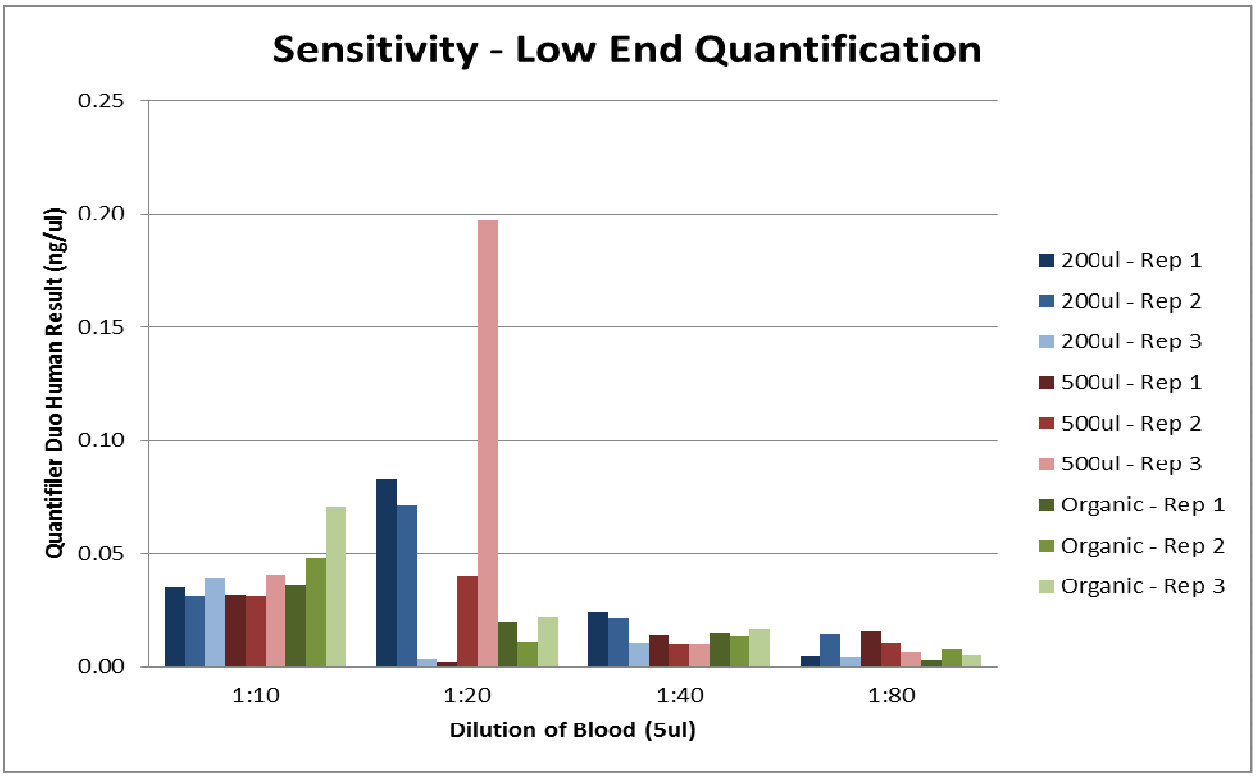
Instrument Error: After starting the first run of the validation on EZ1-A, an instrument error almost immediately occurred. It was observed that the EZ1 had picked up a tip with the tip holder. The instrument crashed and the run was aborted when the instrument tried to go into the cartridge for reagent. It was determined that since no sample had moved yet, the cartridges, lysates and sample tubes were able to be used after restarting the instrument. Sample lysate tubes and elution tubes were removed from the instrument to avoid accidental

contamination when trying to remove the tip holder. The tip and tip holder for the crashed position were removed and the other tips were returned to their original tip holders. A new tip and tip holder for the crashed position was used and the run was started over again and successfully ran to completion. No other instrument errors were observed throughout the validation.

Results

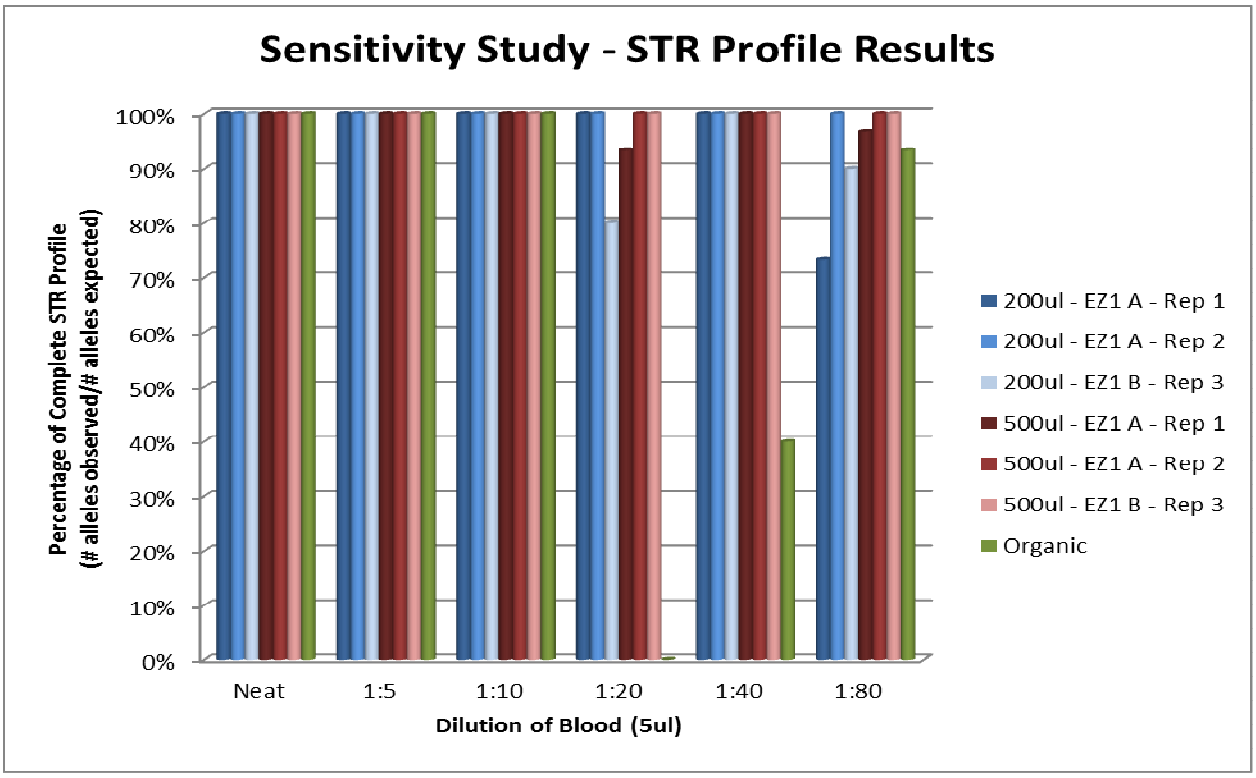
Sensitivity: Quantification and STR profile results were used to determine sensitivity of the EZ1 instrument with the DNA Investigator extraction kit. Based on the results, both EZ1 instruments perform similarly and are comparable to the same sensitivity level obtained using the currently used organic extraction method. In addition, both the Trace protocol and Large Volume protocol on the EZ1 instruments generated similar quantification and profile results. The quantification results are shown in the graphs below and demonstrate that a reduction in yield was observed as the dilution of blood increased.





The blood samples diluted 1:20 demonstrated more scattered results using both EZ1 instruments. Blood that was diluted more than 1:20 were below the sensitivity of the quantification assay and plateaued out in signal. Other than the 1:20 dilution, all replicates of each dilution extracted on the EZ1 instruments were similar in yield to each other as well as to the organic extraction replicates. This indicates that the sensitivity level is reproducible across multiple EZ1 runs, as well as on both EZ1 instruments.

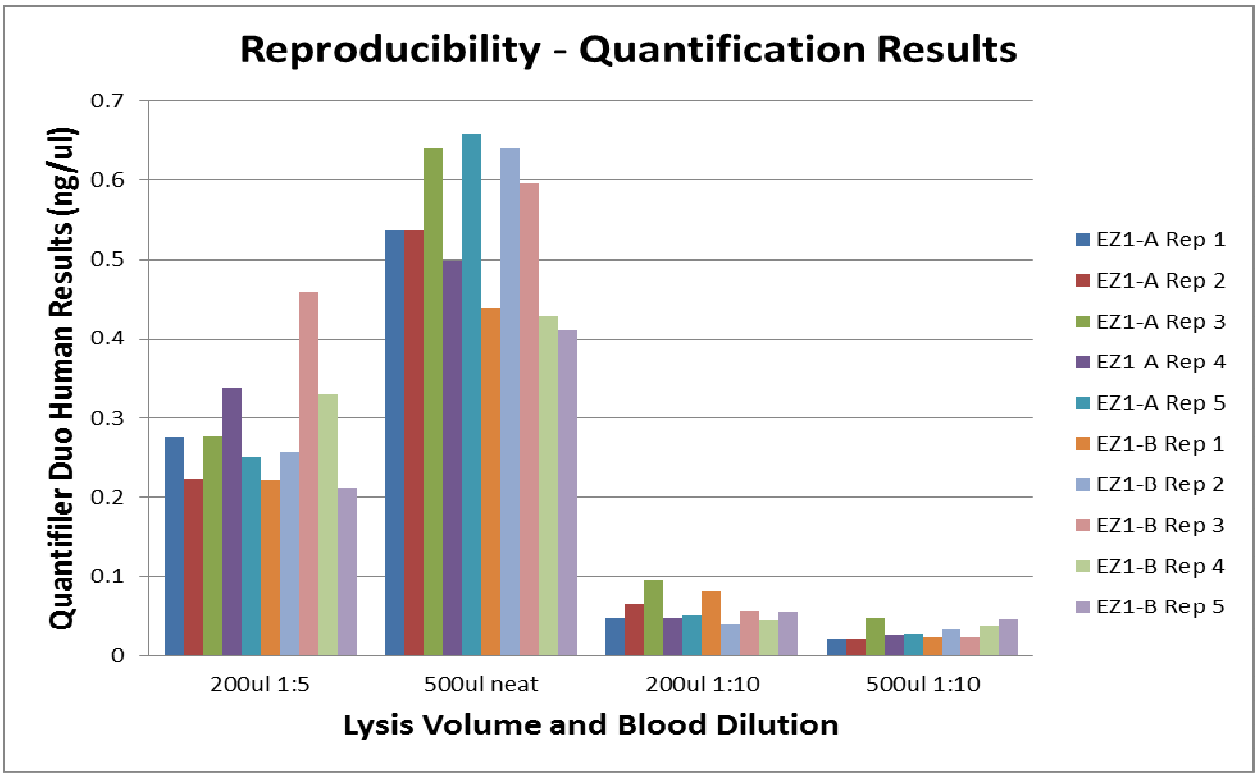
The graph below shows the percentage of complete profiles obtained from each sample. Only one replicate of the sensitivity samples extracted with the organic method was provided for comparison.



Full profiles were obtained from all replicates of both EZ1 and organic extracted samples for the neat, 1:5 and 1:10 samples. The 1:20 samples continue to give the most variable results, with no profile results generated in the organic extraction. The 1:40 dilution generated full STR profiles with all replicates from the EZ1 extraction and only a 40% complete STR profile from the replicate tested from the organic extraction. The 1:80 dilution generated partial profiles from half of the EZ1 extracted replicates and the organic extracted replicate.

Allele and peak height tables for all data generated for this study can be found in Table 1A and Table 1B, respectively.

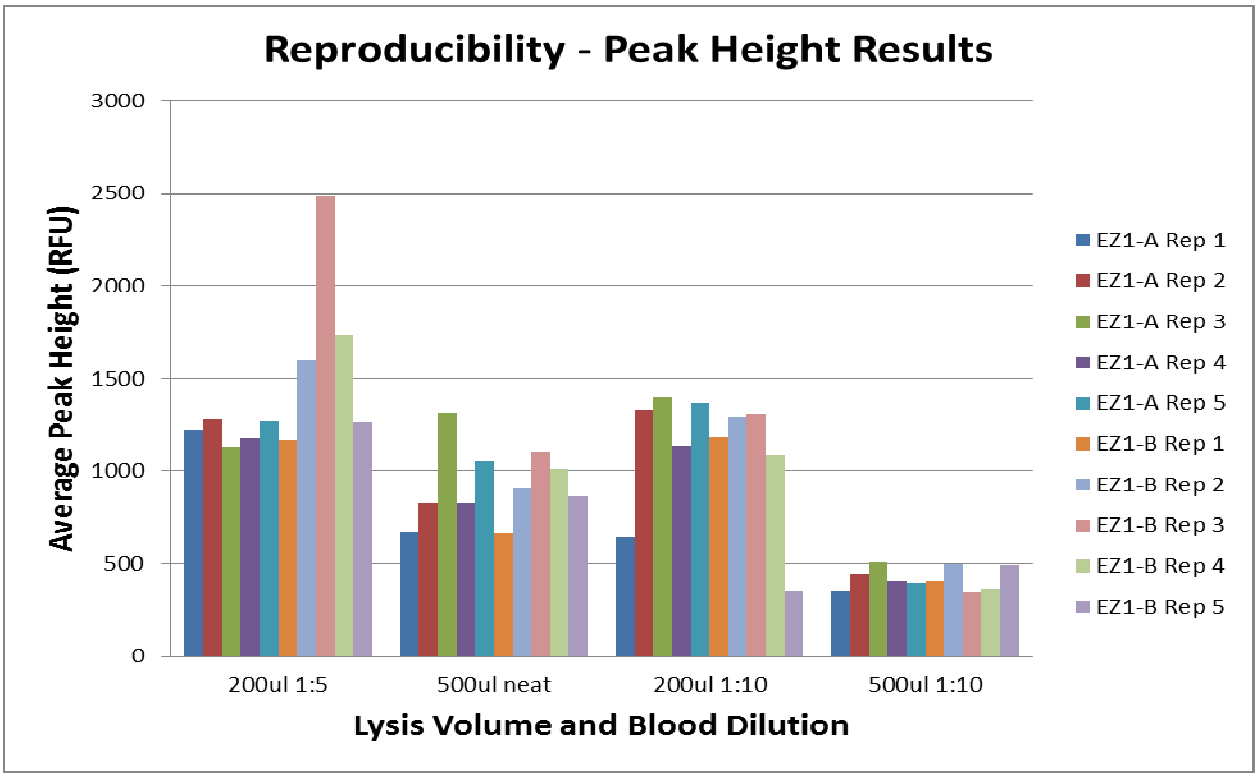
Reproducibility: Quantification and STR profile results were used to determine the reproducibility of the EZ1 instrument with the DNA Investigator extraction kit. Based on the results, the reproducibility both within a single instrument run and across both EZ1 instruments are similar. The 500 µl lysis volume was used to pull blood stains off of cotton swabs and generated the same variability as using the 200 µl lysis volume with liquid blood. The results also indicate that when using the same dilution of blood, the liquid blood generated slightly higher yields over blood dried on cotton swabs. This is an expected result based on the inherent issue of completely removing all cells from a dried stain on a cotton swab. The quantification results are shown in the graph below.



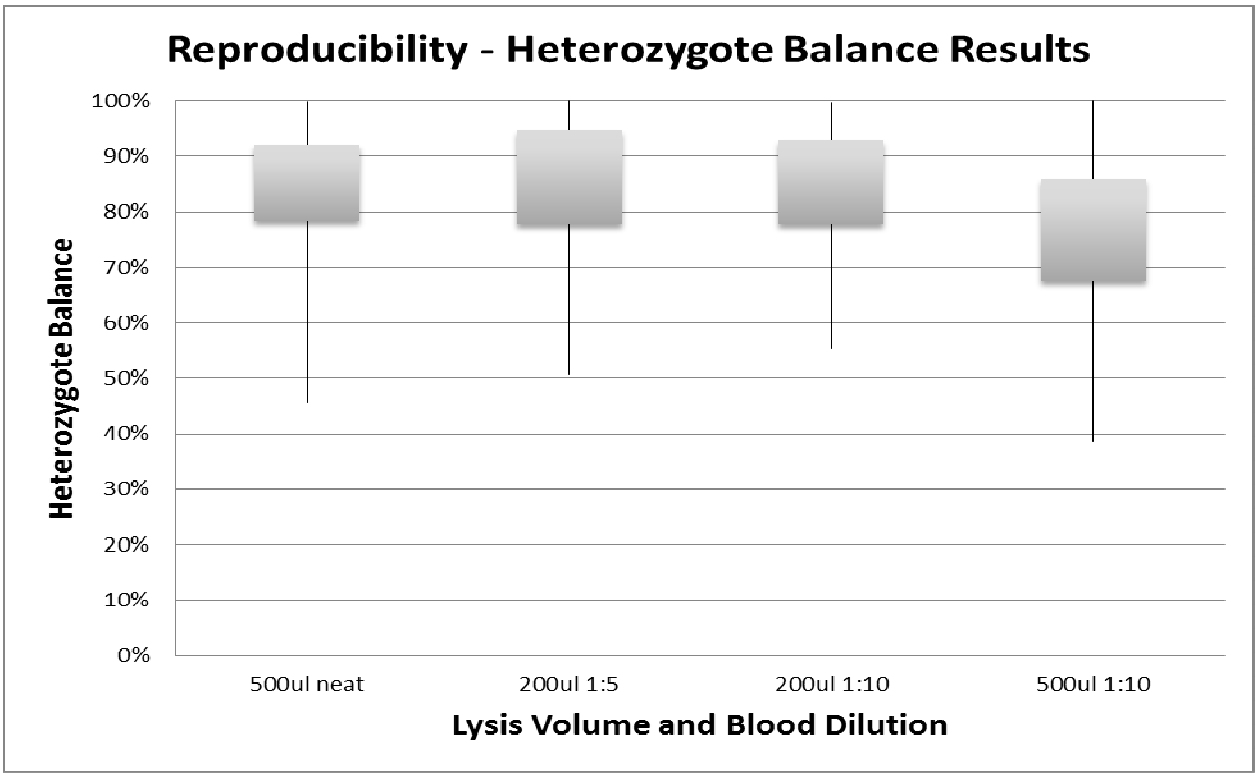
To demonstrate amplification reproducibility independent of quantification results, samples were neither normalized nor concentrated. Instead, all samples of the same dilution were amplified using the same amount of DNA extract listed in the table below.

Dilution of Blood	Volume of Extract in Amplification Reaction
Neat	1 µl
1:5	3 µl
1:10	10 µl

All profiles generated full profiles with all alleles greater than 70 RFU. One sample, replicate 3 of the 500ul lysis of a 1:10 diluted blood stain extracted on EZ1-B, was re-amplified because of allele dropout less than 70 RFU. The second amplification of the extract generated a full profile greater than 70 RFU. The graph below shows that the average profile peak height for each set of samples generates similar peak height results.



The heterozygote balance at each locus was also examined to demonstrate reproducibility. The graph below shows the interquartile range (IQR) as a box with the maximum and minimum observed heterozygote ratio represented by the lines. The 200 µl 1:5 dilution, 200 µl 1:10 dilution and 500 µl neat samples generated tight IQR boxes roughly between 80% and 90%. Three profiles out of the 30 profiles in these sets contained one heterozygote locus balance less than 55%, represented in the lower graph lines for minimum heterozygote ratio. The 500 µl 1:10 dilution samples generated a lower IQR box roughly between 70% and 80%. Eight out of ten samples from the 500 µl 1:10 samples generated profiles with one or more heterozygote locus balance less than 55%. This is explainable due to the average input template amount based on quantification values was 0.25 ng, whereas the other sets averaged 0.5 ng to 0.75 ng.

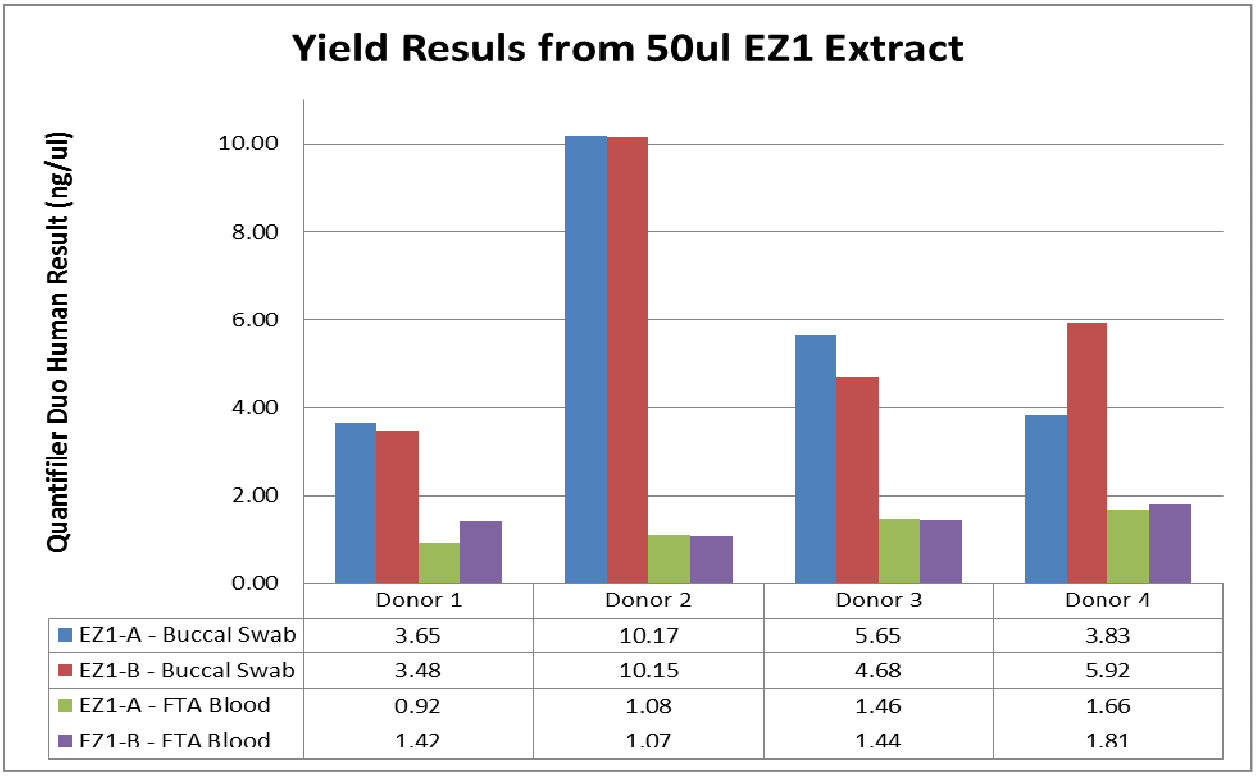


Allele, peak height and heterozygote balance tables for all data generated for this study can be found in Table 2A, Table 2B and Table 2C, respectively.

Precision: The neat, 1:5 and 1:10 samples from the sensitivity study were used to determine if the new extraction method negatively affected allele sizing precision on the 3130. Standard deviations of less than 0.15 base pair are desired for precision, so that three times the standard deviation is less than the required 0.5 base pair size guideline. The maximum observed standard deviation using this set of data was 0.12 and therefore meets this requirement.

Base pair size tables for all data generated in this study can be found in Table 3.

Accuracy and Concordance: Four donors of both blood on FTA and buccal swabs were processed using the "Tip Dance" protocol on both EZ1-A and EZ1-B. In addition, two samples from the NIST SRM 2391c kit, components E and F, were extracted on EZ1-A. All samples generated full STR profiles that accurately matched known profiles. The following graph summarizes the quantification results from the buccal swabs and FTA samples.



	Donor 1	Donor 2	Donor 3	Donor 4
EZ1-A - Buccal Swab	3.65	10.17	5.65	3.83
EZ1-B - Buccal Swab	3.48	10.15	4.68	5.92
EZ1-A - FTA Blood	0.92	1.08	1.46	1.66
EZ1-B - FTA Blood	1.42	1.07	1.44	1.81

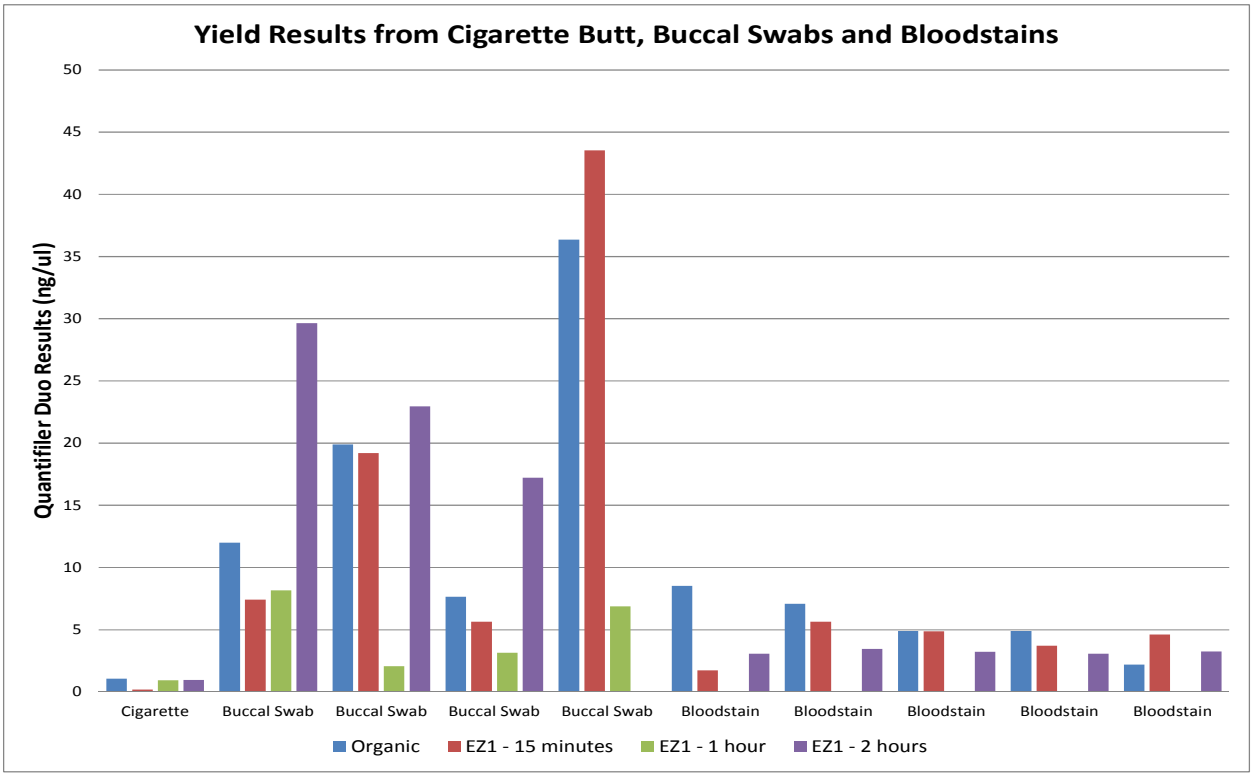
The buccal swabs generated more varied yield results between donors, but consistent yields between the two EZ1 instruments being validated using the same donor. The blood on FTA samples were consistent both between donors, as well as the same donor extracted on both instruments. This is an expected result based on previous knowledge of cell types and substrates. The yields show that a consistent amount of DNA can be extracted from small cuttings of substrates without having to remove the substrate prior to extracting on the EZ1 instrument.

The two samples from the NIST SRM 2391c kit, components E and F, generated quantification results of 3.25 ng/µl and 3.81 ng/µl, respectively. Both samples generated full STR profiles concordant with results published in the SRM Certificate of Analysis.

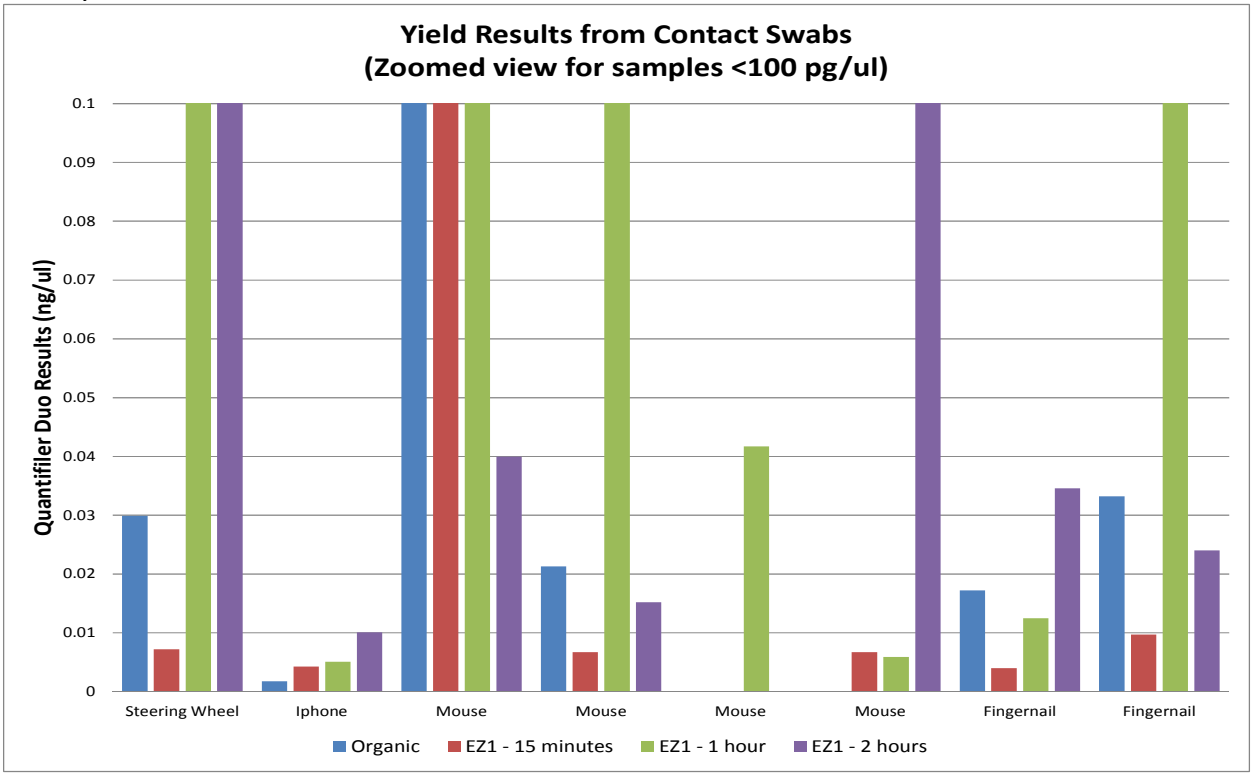
The allele table for all data generated for this study can be found in Table 4.

Mock Evidence-Non-Differential Samples: The first round of processing non-differential mock samples followed the exact protocol in the EZ1 DNA Investigator Handbook using a 15 minute lysis at 56°C. Yields were as expected, but profiles from both buccal swabs and touch samples exhibited extreme locus to locus imbalance, with dropout at the D13S317 locus. Qiagen was contacted for technical support and determined the issue to be due to incomplete lysis and recommended that lysis be increased to one hour (5). After a one hour incubation showed the same imbalanced loci, it was recommended to remove the 95°C step after cell lysis. To do this, samples were lysed for 2 hours to ensure that the proteinase K was no longer active. The following graphs shows the yield results from samples that were extracted on the EZ1 using 15 minute, 1 hour and 2 hour lysis times compared to the same sample types processed using the

organic extraction method. The first graph shows the higher yield samples (cigarette butts, buccal swabs and bloodstains) and the second graph shows the contact swab samples.

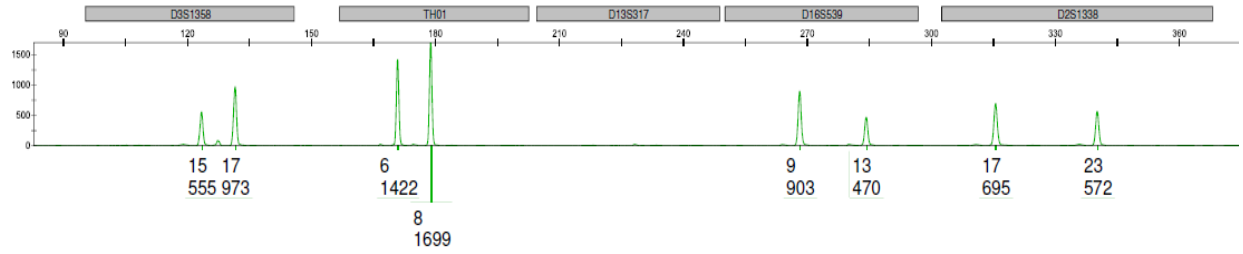


*Only three buccal swabs were tested in the 2 hour lysis. No bloodstains were tested using one hour lysis.

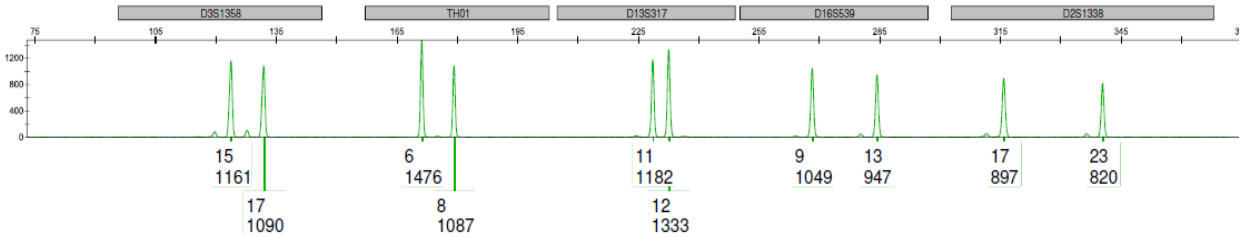


DNA yield from the contact swabs varied greatly and is expected due to the nature of touched items. The bloodstains and cigarette butts exhibited more consistent yields. The following figures show examples of the green dye loci and imbalance initially observed and the improved profile balance with no 95°C step compared to organic extraction.

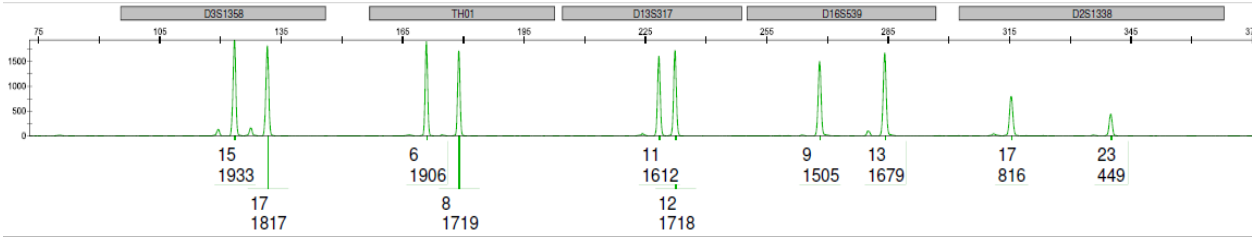
15 minute lysis – 5 minute 95°C step



2 hour lysis – no 95°C step



Organic Extraction



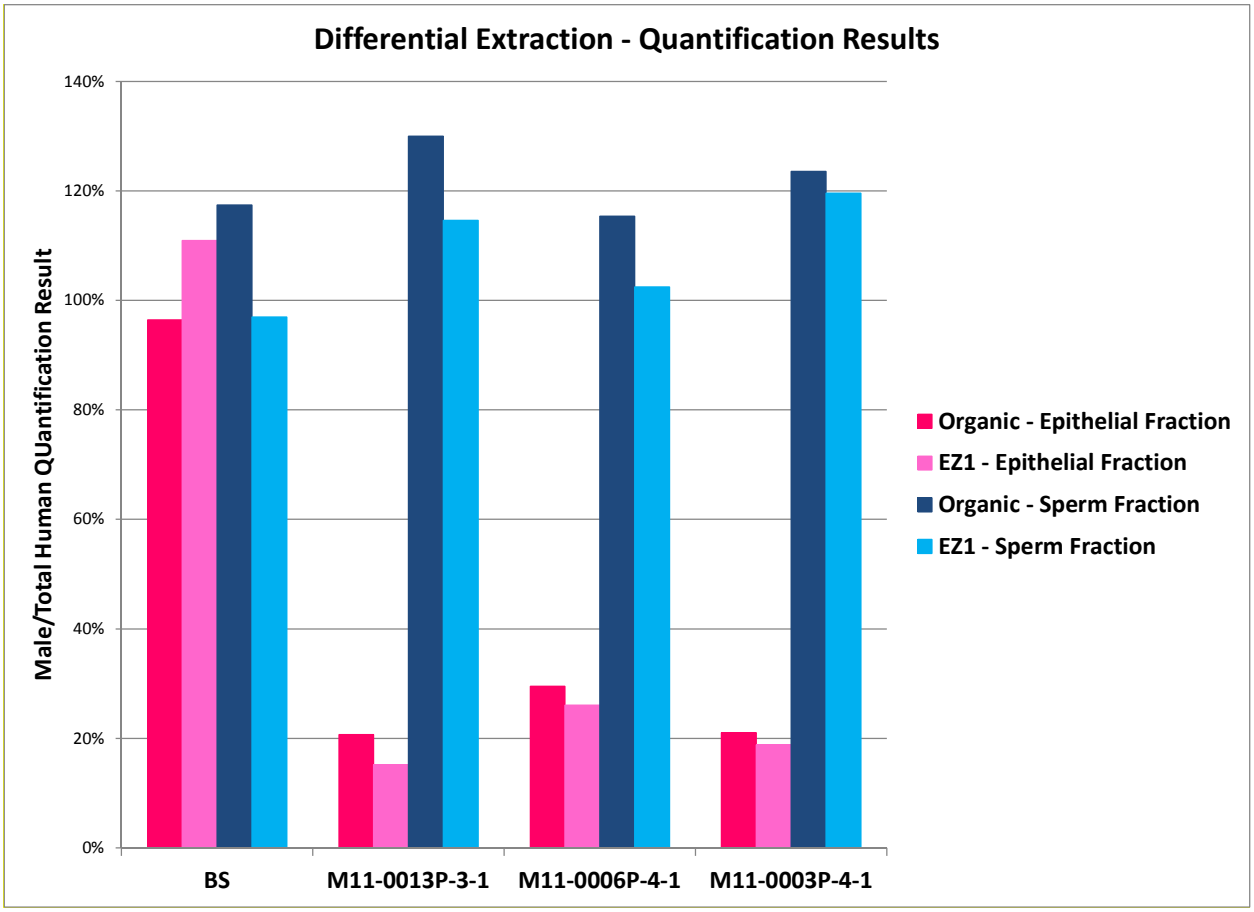
The following table summarizes the profile results from the organic and EZ1 two hour lysis samples. The results show that the EZ1 extraction is equal to or better than the current organic method for the sample types tested.

Sample Type	Organic Results	EZ1 Results
Steering Wheel	1 no profile 1 full profile with heterozygote balance <55%	Full mixture profile – Major donor not expected donor
iphone	No profile	Few alleles
Mouse	1 full profile, 3 no profiles	2 no profiles 2 full profiles
Fingernail swabs	2 no profiles 2 full profiles with heterozygote balance <55%	1 full profile with heterozygote balance <55% 1 High profile – 1 allele dropout and stochastic thresholds not met
Cigarette Butt	1 High profile – 1 allele dropout, heterozygote balance and stochastic thresholds not met	1 Full profile, all thresholds met
Buccal Swabs	4 full profiles	4 full profiles
Bloodstains	5 full profiles	5 full profiles

The allele table for all data generated for this study can be found in Table 5.

Mock Evidence- Differential Samples:
Yields from the sperm fraction of the differential samples processed following the Qiagen manual were much lower than expected. The manufacturer protocol lysed samples with G2 buffer and then the sperm pellet was washed three times with G2 buffer. The modified protocol lysed samples with digestion buffer and the sperm pellet was washed three times with TE^{-4} buffer. The second method is presumably less harsh on the sperm cells and allowed a significantly higher DNA recovery from the sperm fraction.

The profile results obtained using the modified extraction procedure on the EZ1 were equivalent to the organic extraction method for both the epithelial fractions (EF) and sperm fractions (SF). The profile results correlated well with the quantification results with regards to percentage of male within the sample. All non-sperm fractions were mixtures containing minor alleles correlating to the sperm donor profile. The graph below shows that the BS sample was 100% male in the EF. This correlates with the profile results for the EF indicating a mixture of two male donors. The remainder of the samples indicates that the EF fraction contained approximately 20% male and corresponds to the profile results showing minor male donor alleles in the EF. The graph below compares the quantification results for both the EZ1 and organic extraction method.

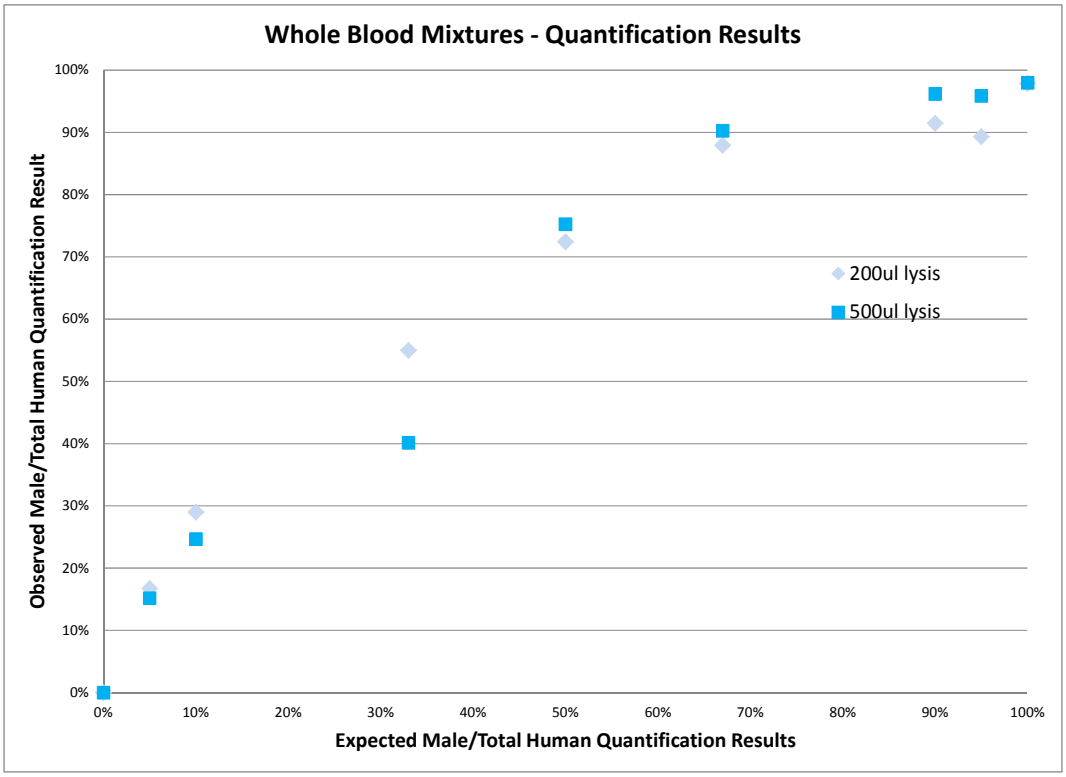


Quantifiler Duo amplifies two separate targets to simultaneously estimate both the human and male DNA quantity. When calculating the percent male in the DNA samples above, many calculations came out to be greater than 100%, most likely due to different estimates for both targets. For the purpose of this study, it is assumed that anything greater than 100% male is actually just 100% male. The SF quantification results indicate clean separation between the sperm and non-sperm donors. The following table shows the number of minor alleles observed from the non-sperm donor for both extraction methods.

Sample Name	# of non-sperm donor alleles in SF		Male Quantification Value (ng/µl)	
	Organic	EZ1	Organic	EZ1
BS	1	0	1.48	0.74
M11-0013P-3-1	1	0	1.01	1.57
M11-0006P-4-1	0	0	1.50	6.16
M11-0003P-4-1	0	0	0.47	0.79

The data demonstrates that the male DNA yield obtained using both extraction methods were similar; especially given different cuttings from samples could contain different amounts of donor cells. The allele table for all data generated for this study can be found in Table 6.

Mixtures: To evaluate mixtures, both quantification data and STR profile results were used. The following graph shows the percentage of male DNA quantified in the Quantifiler Duo assay compared to the total Human DNA quantification value. The ratios listed in the samples were calculated into percentages (i.e. 1:19 is 1/20 or 5%) to determine the expected percentage of donor.



The graph shows that both the 200 µl lysis and the 500 µl lysis generated comparable results with the expected increasing trend in observed male DNA up until the male was at least 90% of the mixture. Based on quantification results, the expected 90% and 95% points were difficult to distinguish from the 100% male samples, most likely due to the estimation of the different targets previously discussed in the mixture results section. The data also indicates that the observed male percentage of the mixture is greater than the expected male percentage of the mixture by at least 33%. This is presumably due to the different blood donors having significantly different cell counts. While the volume of blood mixed can be controlled, the amount of cells in each volume cannot.

The graph below shows the percentage of each donor observed per mixture profile. The percent contribution to the profile was calculated by using the average peak height of the male or female specific donor alleles divided by the total average peak height of unshared alleles. The profile results correlate with observed quantification results in that a higher than expected percentage of male was observed in each mixture level. The 90% and the 95% expected male mixture were most accurate according to percentage of each donor observed in the STR profiles.

The allele table for all data generated for this study can be found in Table 7.

Contamination: All reagent blanks used in this validation were free from contamination. In addition, all negative amplification controls were also free from contamination.

Conclusions:

The EZ1 robotic platform is validated for processing samples using the EZ1 DNA Investigator Kit. The data obtained in the validation demonstrate the EZ1's ability to extract DNA from a variety of forensic sample types. The data show a sensitivity level equal or greater than to the currently used organic extraction method. The results also demonstrate that DNA extracted on the EZ1 generates profile results that are accurate, reproducible, precise sizing and free from contamination.

References

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