July 2026

Validation Report

Developmental validation of the EZ2® DNA Investigator® Sep&Prep Kit on the EZ2 Connect Fx instrument

The EZ2 DNA Investigator Sep&Prep Kit (cat. no. 952134) is designed for automated sample preparation of DNA from sexual assault samples encountered in forensic, human identity and paternity testing applications.

The EZ2 DNA Investigator Sep&Prep Kit is based on the principle of differential extraction (1), which uses the unique properties of sperm cells to separate them from other cells that may be present in sexual assault samples. This enables the generation of a profile specific to the perpetrator(s) from whom the sperm cells originated. The EZ2 DNA Investigator Sep&Prep Kit contains reagents and labware to separate the non-sperm fraction from the sperm fraction and automate the isolation of sperm-derived genomic DNA on the EZ2 Connect Fx instrument.

The validation study was based on the recommendations for total DNA extraction of the European Network of Forensic Science Institutes (ENFSI) (2), and on the validation guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM) (3).

The performance of the EZ2 DNA Investigator Sep&Prep Kit was evaluated with regard to various sample types and conditions commonly encountered in forensic and parentage laboratories. Sensitivity (page 7), linearity (pages 4–5), reproducibility (pages 4–6), accuracy (page 9), repeatability (page pages 10–11), and the absence of cross-contamination (page 13) were tested. Typical sexual assault sample types were extracted and analyzed by qPCR and STR (page 12).

Optimal protocol conditions were established, and the effects of variations in those conditions were assessed. The effect of variations in sample lysis duration (page 22) and the impact of elution volumes on the total yield and the concentration of samples was assessed (pages 23–24). For the Elution Mix, the stability of Reagent UI dilution was explored (page 27) as well as the effect of variations in the Proteinase K concentrations is described (page 29).

Finally, the compatibility of the sperm-derived DNA eluate was tested with various assays from different suppliers (page 30), and the performance of the EZ2 DNA Investigator Sep&Prep Kit was benchmarked against a method in current use, the QIAcube® Connect differential wash procedure (page 32).

Results of developmental validation

The validation study was performed by the QIAGEN® R&D department. For sample processing, protocols were followed as described in the EZ2 DNA Investigator Sep&Prep Kit Handbook, unless stated otherwise. Semen samples were always diluted in water and 10 µL of each dilution was spiked on rayon swabs (Sarstedt) which were subsequently dried overnight. Automated separation of non-sperm DNA from sperm cells and purification of sperm-derived DNA was done on the EZ2 Connect Fx using the EZ2 DNA Investigator Sep&Prep Kit protocol. Quantification of human and male genomic DNA was performed using the Investigator Quantiplex® Pro Kit. STR analysis was performed with the Investigator 24plex QS Kit; capillary electrophoresis was run on the Applied Biosystems® 3500 or 3500XL Genetic Analyzers.

Linearity, sensitivity and reproducibility

The EZ2 DNA Investigator Sep&Prep Kit was evaluated to determine its efficiency in extracting sperm DNA across varying target concentrations while minimizing cross-fraction carryover. Linearity and sensitivity were evaluated by preparing serial dilutions of neat semen (corresponding to dilutions of 1:10, 1:100, 1:1000, 1:10,000) from two different donors, processed with a 50 µL elution volume. For one donor, the analysis was additionally performed using a 100 µL elution volume. Semen dilutions were spiked on swabs, dried at ambient temperature and extracted in 6 replicates for each semen dilution using the EZ2 DNA Investigator Sep&Prep protocol. Each biological replicate was quantified in duplicate. Reproducibility was evaluated by performing these tests across three EZ2 Connect Fx instruments for both donors.

Quantification of the sperm fraction showed a linear correlation between input sample dilution and DNA yield from both high (donor 1) and low (donor 2) DNA semen samples (Figure 1 and Figure 2). No evidence of PCR inhibition was observed in any of the quantification results across all samples and dilutions. Reproducibility tests across different instruments with the EZ2 DNA Investigator Sep&Prep Kit show high agreement and low variability across technical and biological replicates (Figure 1, Figure 2, Figure 3). Sensitivity was evaluated by yield and allele recovery of the dilution series (Figure 4). The EZ2 DNA Investigator Sep&Prep sperm fraction eluates, used for STR amplification with the Investigator 24plex QS Kit, showed recovery of full profiles (100% allele recovery) with input amounts as low as 20 pg. Stochastic effects (allelic dropout) were observed exclusively in the highest dilution category (1:10,000), most likely due to the extremely low amount of input DNA. Such low-template conditions are known to increase stochastic effects during STR amplification.

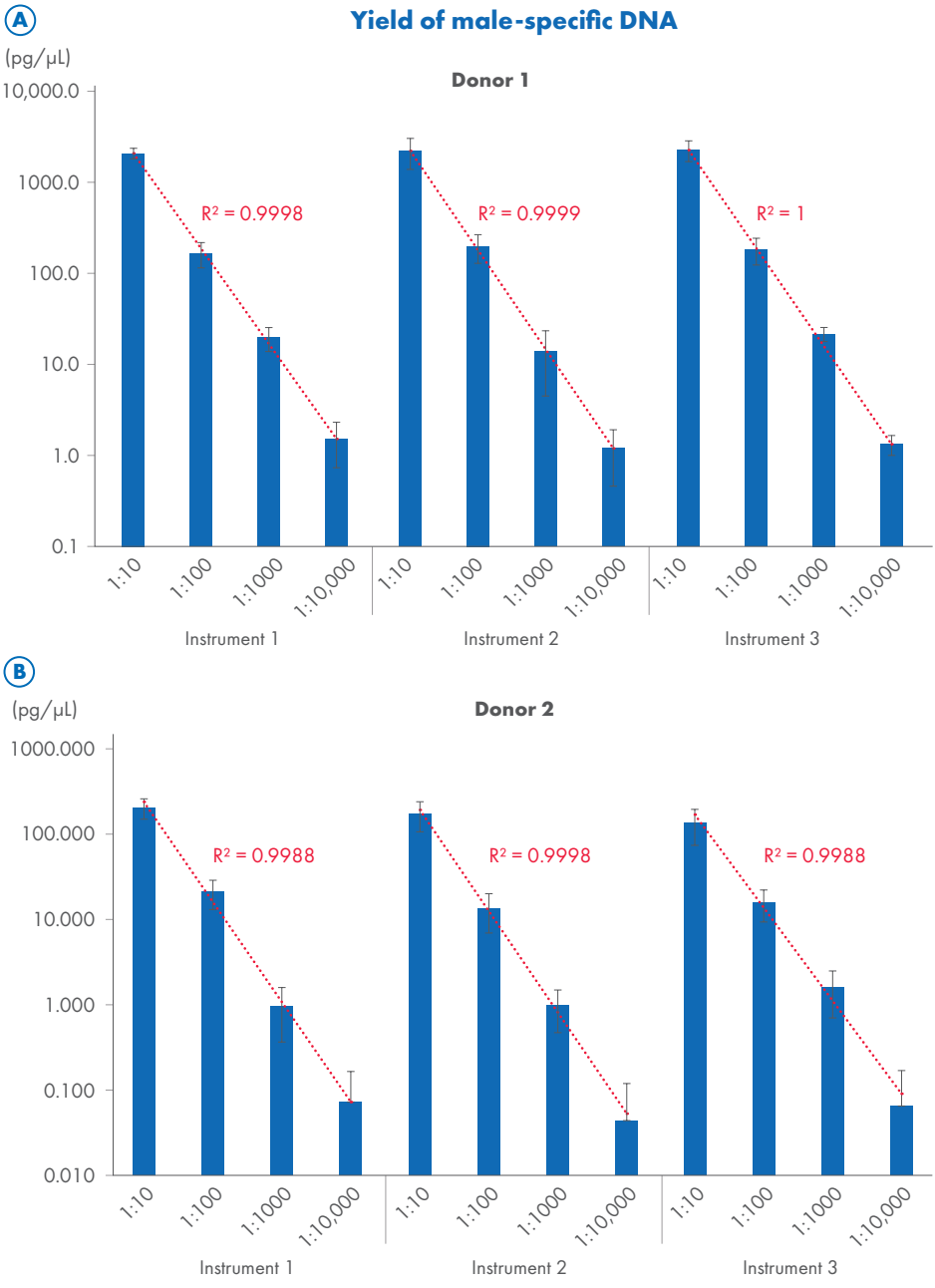

Figure 1. Linearity and reproducibility. Neat semen from two different donors was diluted (1:10, 1:100, 1:1000, 1:10,000), spiked on swabs and extracted in six replicates, each quantified in duplicate. The elution volume was 50 µL. Quantification based on the male target for Y-specific DNA. Donor 1 (**A**), Donor 2 (**B**).

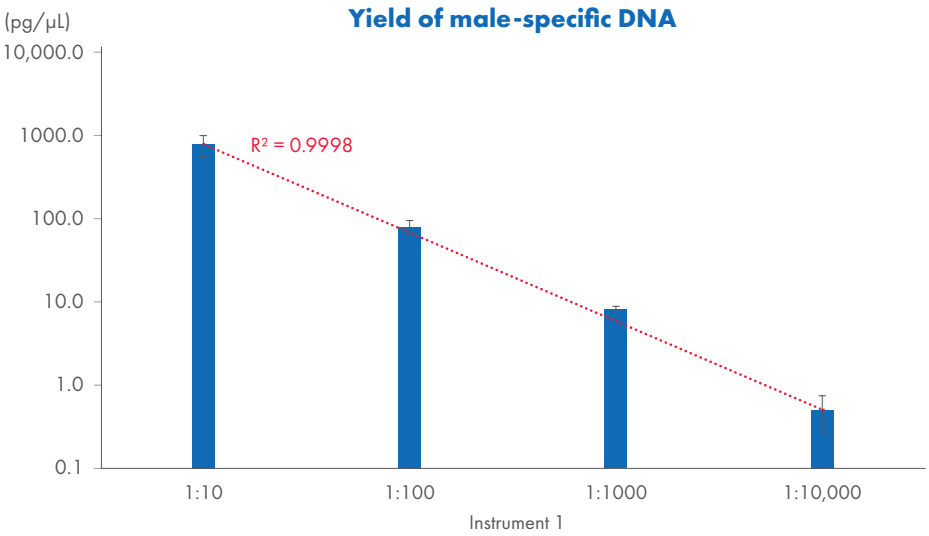

Figure 2. Linearity. Neat semen from one donor was diluted (1:10, 1:100, 1:1000, 1:10,000), spiked on swabs and extracted in 6 replicates each that were quantified in duplicates. The elution volume was 100 µL. Quantification based on the male target for Y-specific DNA.

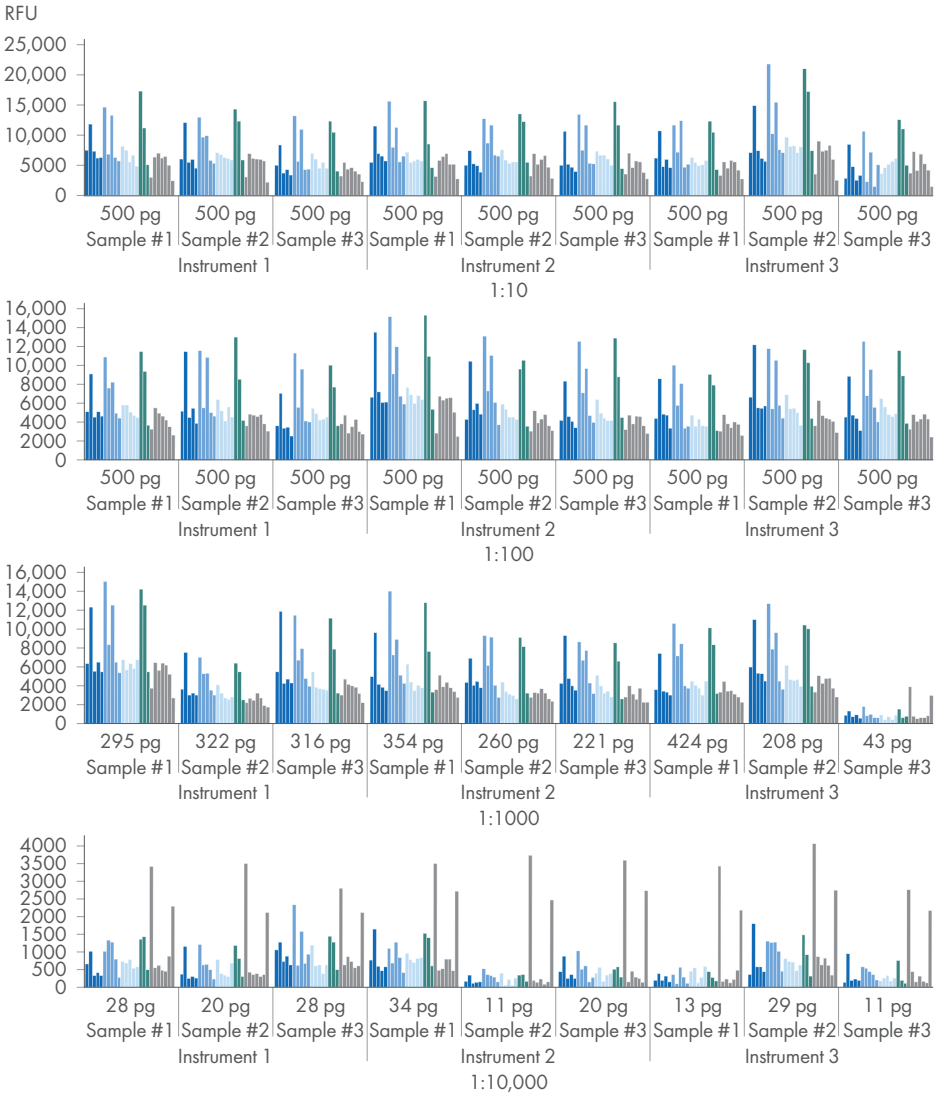

Figure 3. Average STR peak heights (RFU) for sperm-derived DNA from semen dilutions processed on three different instruments. The figure shows the average STR peak heights (RFU) for samples run in biological triplicates and amplified with the Investigator 24plex QS Kit. Samples of semen dilutions 1:10, 1:100 and 1:1000 were analyzed using a threshold of 200 RFU while samples of semen dilutions 1:10,000 were analyzed using a threshold of 50 RFU. The total DNA amount [pg] used per single amplification for each replicate is indicated along the X-axis.

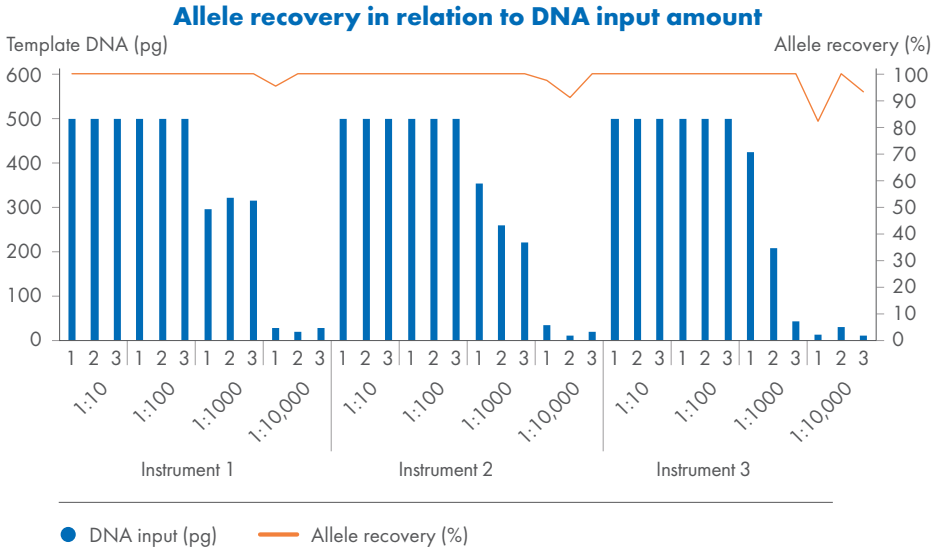

Figure 4. Correlation between template DNA for STR profiling and allele recovery. Allele recovery [%] for three biological replicates is shown in relation to the input DNA amount [pg]. Samples were amplified with the Investigator 24plex QS Kit. Samples of semen dilutions 1:10, 1:100 and 1:1000 were analyzed using a threshold of 200 RFU while samples of semen dilutions 1:10,000 were analyzed using a threshold of 50 RFU.

Accuracy

The efficiency of separating the male component from mixed biological samples determines whether the male contributor(s) can be reliably identified.

A key indicator of successful separation of the sperm fraction from the mixed sample is the human-to-male DNA ratio. For optimal STR profile generation, a human:male DNA ratio of 1:1 would be ideal to obtain a single-source DNA profile of the male contributor. Ratios exceeding this threshold indicate a carry-over of female-originated DNA and may result in mixed autosomal STR profiles that are more difficult to interpret. Ratios falling below this threshold indicates variation between the quantification targets for the 'human' value and the 'male' value as different regions are targeted in the genome. Minor deviations from the ideal ratio of 1 are also partly attributable to inherent variability of the human-to-male ratio, even in single-source male profiles as previous validation of the Investigator Quantiplex Pro Kit reported a mean human-to-male target ratio of 1.04 ± 0.16 across populations, consistent with the theoretical value of 1 (see HB-2496 Developmental validation of the Investigator Quantiplex Pro Kit).

To investigate separation effectiveness, 23 biological replicates, consisting of vaginal swabs spiked with 1:200 semen dilution were prepared. DNA was eluted in 50 µL and quantified using the Investigator Quantiplex Pro Kit. The human:male DNA ratio was determined for each biological replicate.

The results demonstrate a consistent human:male ratio close to the target value of 1 across all replicates, with a mean ratio of 0.94 (Figure 5), demonstrating that all data points fall within the expected range in which exclusively male profiles are anticipated. This observation was further confirmed by STR analysis, which revealed clearly distinct male major components in 19 of 23 profiles. For 4 of 23 samples, a slightly elevated level of female carryover was shown; however, the male DNA remained the major component (data not shown).

The vaginal swab samples contained a substantial excess of female-specific DNA, demonstrated by the autosomal DNA yield of about 10 µg DNA in the non-sperm fraction (Figure 26). This excess of female carryover DNA is effectively removed by the EZ2 DNA Investigator Sep&Prep Kit (Figure 23).

Overall, these results indicate a high accuracy of differential separation and effective enrichment of the male DNA fraction while minimizing the carryover of non-sperm DNA.

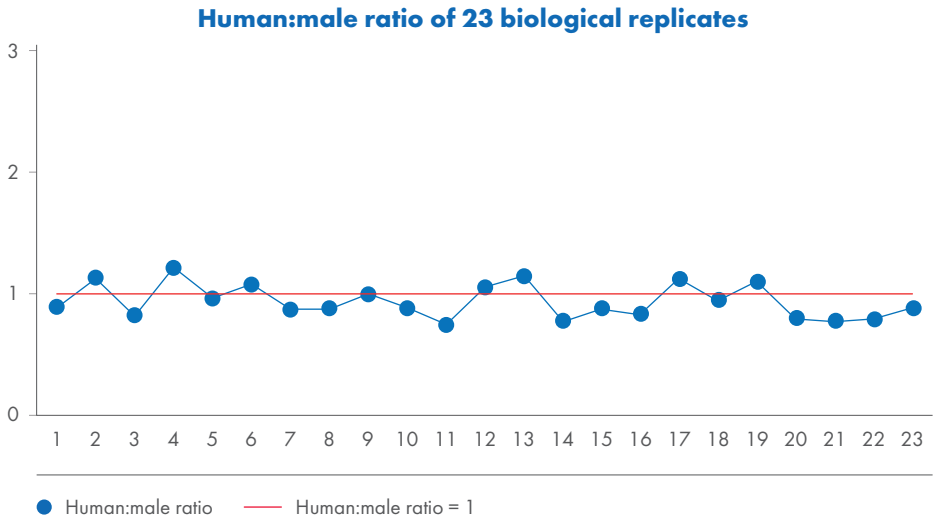

Figure 5. Separation accuracy of differential DNA extraction. Extraction was performed using the EZ2 DNA Investigator Sep&Prep protocol with 50 µL elution. The human:male DNA ratio was determined for 23 biological replicates of vaginal swabs spiked with semen (1:200).

Repeatability

A repeatability study was performed to assess intra-run variation among biological replicates within an EZ2 DNA Investigator Sep&Prep run on the EZ2 Connect Fx. A total of 24 vaginal swabs from a single female donor were prepared. For each of four male donors, six swabs were spiked with 1:200 diluted semen, resulting in six biological replicates per male donor. All samples were processed in a single EZ2 DNA Investigator Sep&Prep run, eluted in 100 µL and quantified in duplicate.

The quantification results show consistent male DNA yield within the biological replicates of each male donor, with only minor variation between biological replicates and small error ranges of technical replicates. Across three of the four male donors, the coefficient of variation (CV) values for male DNA remain consistently below 20%, indicating good repeatability of the workflow. This low level of variability across replicates demonstrates that the method provides stable and reproducible male DNA recovery under the tested conditions. For male donor 4, increased variability is observed (CV of approximately 34%). This is driven by a single lower outlying replicate combined with overall lower male DNA recovery, which amplifies relative variation at low concentration levels. As expected, differences in absolute DNA yield were observed between different male donors (Figure 6).

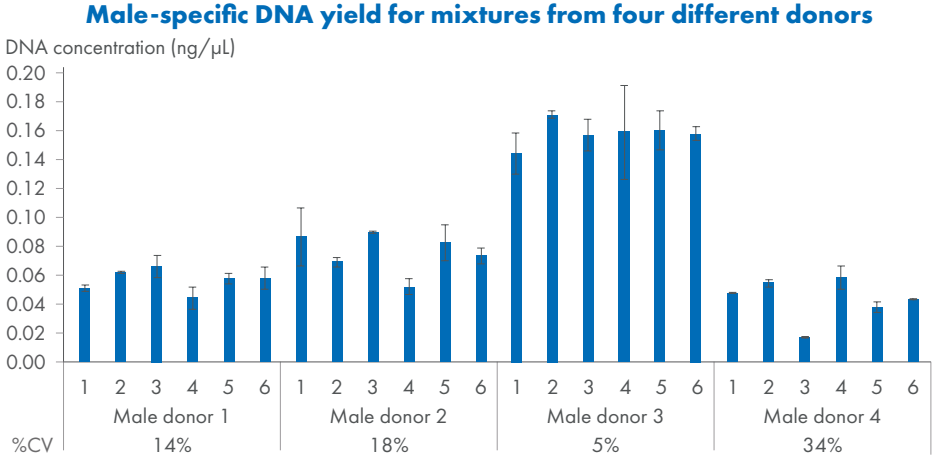

Figure 6. Repeatability of six biological replicates. Extraction of 24 vaginal swabs of one single female donor spiked with 1:200 diluted semen from four male donors was performed using the EZ2 DNA Investigator Sep&Prep protocol with 100 µL elution in one run. Quantification based on the male target for Y-specific DNA (n=2). The coefficient of variation (%CV) describes the variability among the six biological replicates, quantified in duplicates.

The autosomal target ("human") reflects the sum of both male and female DNA present in the sample. This value varies with the amount of sperm DNA present, as well as the efficiency of the non-sperm DNA removal by differential extraction. Quantification of vaginal swabs spiked with semen dilution, but without differential separation, would be expected to show human DNA amounts several orders of magnitude higher than the male fraction. While there is some variation in the amount of total human DNA seen from sperm fractions obtained from all 1:200 sample mixtures, the efficiency of the non-sperm DNA removal can be seen in that the human DNA values are close to the male DNA values for all four samples (Figure 7). Overall, the data demonstrate low intra-run variation and high repeatability.

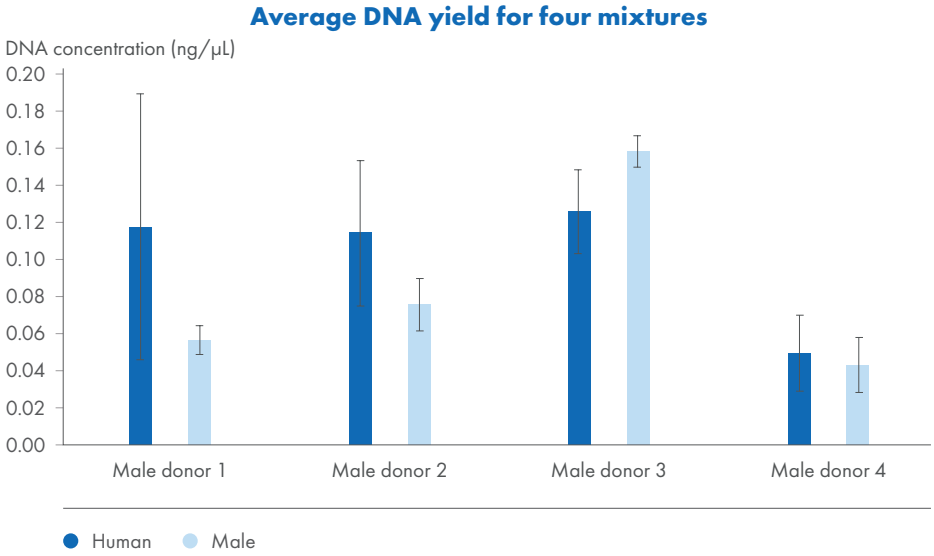

Figure 7. Average DNA yield from four mixtures. Vaginal swabs of one single female donor were spiked with 1:200 diluted semen from four male donors in 6 biological replicates and processed with the EZ2 DNA Investigator Sep&Prep Kit. The sperm fraction was quantified in duplicates. Mean DNA concentrations (ng/µL) of autosomal ("human") and Y-specific ("male") targets are shown per donor.

The STR analysis confirms the quantification results (Figure 8). The STR profiles of the male donors 3 and 4 show mixed profiles with distinct major profiles of the male donor, with minute proportions of the female minor component present in the background. Regarding the male donors 1 and 2, mixed DNA profiles with varying contributor proportions were observed. Profiles ranged from balanced mixtures with comparable peak heights of both contributors to profiles with a dominant male contributor. Overall, the results demonstrate that the EZ2 DNA Investigator Sep&Prep Kit consistently enables recovery of male contributor information.

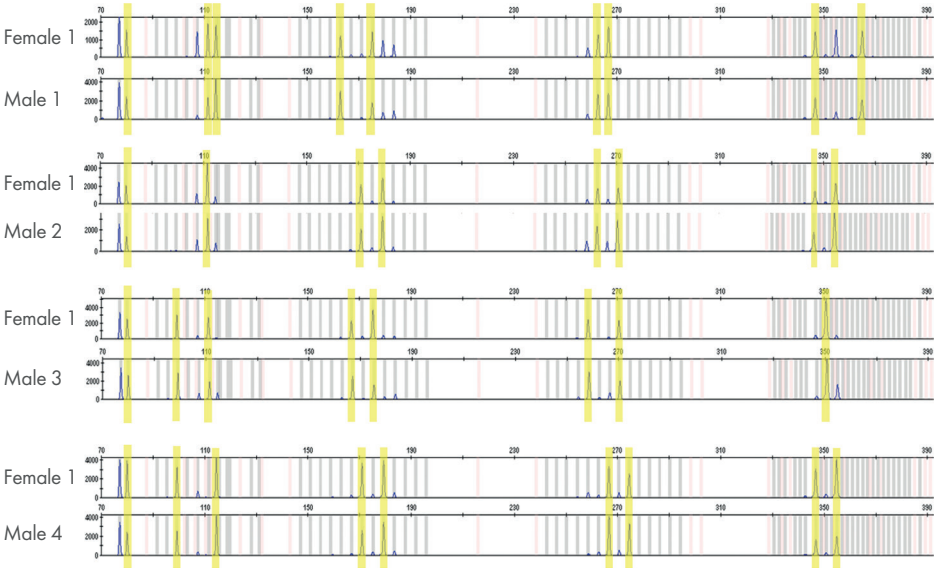

Figure 8. Representative electropherogram excerpts from sperm fractions. Vaginal swabs of one single female donor were spiked with 1:200 diluted semen from four male donors in 6 biological replicates and processed with the EZ2 DNA Investigator Sep&Prep Kit. 6-FAM dye channels of the STR electropherograms from Investigator 24plex QS Kit are displayed. For visualization purposes, male-specific and shared alleles are highlighted within yellow bars.

Freedom from cross-contamination

A cross-contamination study was performed to ensure no contamination between samples was observed. 12 vaginal swabs of one female donor, spiked with a 1:200 semen dilution of one male donor were extracted, alternating with a total of 12 blank positions within the EZ2 Connect Fx. The method was repeated for both the 50 µL elution volume and 100 µL elution volume. Amplification of the 24 negative control sperm fraction eluates was performed using the maximum input volume for amplification (15 µL) with the Investigator 24plex QS Kit. No alleles were confirmed in the electropherograms for any of the negative control sperm fraction eluates, using an analytical threshold of 50 RFU. The presence of Quality Sensor peaks confirmed successful amplification in the negative control samples.

Casework sample types

The performance of the EZ2 DNA Investigator Sep&Prep workflow was evaluated using a selection of typical case-type samples occurring in sexual assault investigations; postcoital samples, semen spiked on buccal and vaginal swabs, semen spiked on vaginal swabs containing menstrual blood, and semen samples (either neat or mixed with saliva) spiked on different fabrics. Samples were extracted using the EZ2 DNA Investigator Sep&Prep protocol with elution in 50 µL, and DNA quantification was performed using the Investigator Quantiplex Pro Kit.

Postcoital samples

Post-coital vaginal swabs were collected at defined time points (24 h, 48 h, 72 h, 96 h and 120 h) after sexual intercourse from a voluntary donor couple with known reference profiles. After four years of storage at ambient temperature, samples were processed in triplicate, except at the 48 h time point, for which only two swabs were

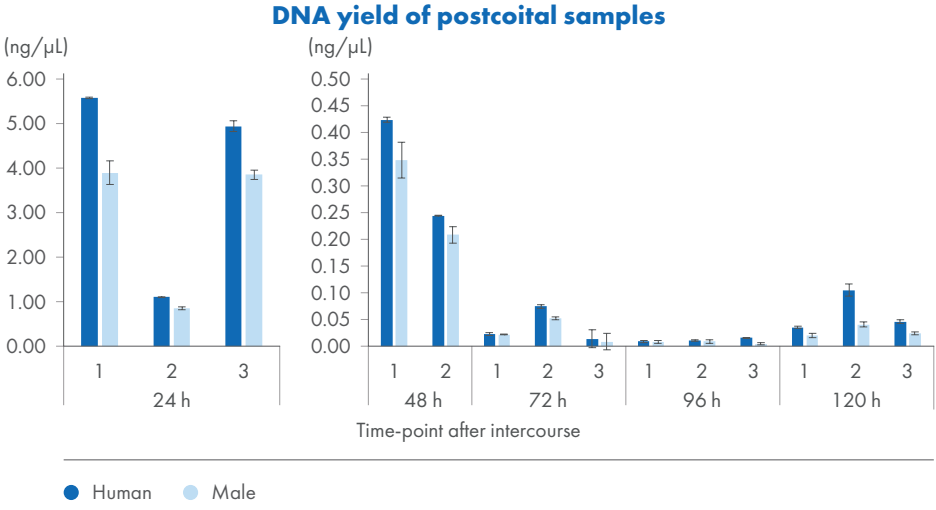

Figure 9. Time-dependent DNA recovery from postcoital vaginal swabs. Postcoital vaginal swabs were collected at 24, 48, 72, 96, and 120 hours after intercourse and stored for four years. Extraction was performed using the EZ2 DNA Investigator Sep&Prep protocol with elution in 50 µL. Each time point includes biological triplicates, except for 48 hours, which has duplicates. Quantification was done in duplicates. A broken y-axis is used to allow simultaneous visualization of high DNA concentrations at early time points and low DNA concentrations at later time points.

available. Eluates were quantified with Investigator Quantiplex Pro and STR profiles were generated with Investigator 24plex QS. A threshold of 50 RFU was used for STR interpretation.

Time-dependent recovery of total human DNA and male-specific DNA is shown in Figure 9. At early post coital time points (24 h and 48 h), high concentrations of both human and male DNA were recovered. With increasing time after intercourse, DNA concentrations decreased markedly, reaching very low template levels at 96 h and 120 h. Importantly, despite this pronounced decrease in DNA quantity, male-specific DNA remained detectable at all investigated time points.

The efficiency of differential separation over time was assessed using the human:male DNA ratio. At early time points (24–72 h), mean ratios across all samples remained close to the target value of 1, indicating effective isolation of DNA from the male contributor with limited female carryover (Figure 10). At later time points (96 h and 120 h), both the mean ratios (Figure 10 A) and the dispersion of individual values increased while both median and mean ratios shift to higher values (Figure 10 B), reflecting a relative increase in DNA originating from the female contributor under low male template conditions.

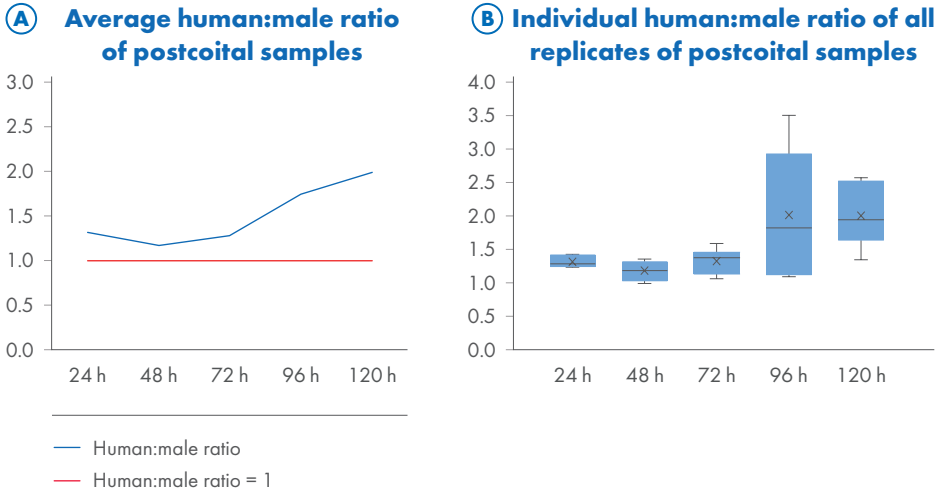

Figure 10. Human-to-male DNA ratios from postcoital vaginal swabs. (**A**) Mean human:male DNA ratios across samples collected 24–120 h after intercourse. (**B**) Box plot of human:male DNA ratios for all biological replicates at each time point. The mean values are indicated by crosses. The central line within each box represents the median, dividing the dataset into two equal halves. The box corresponds to the interquartile range (IQR; 25th–75th percentile), encompassing 50% of the data. Whiskers extend to the minimum and maximum values. Each time point includes biological triplicates, except for 48 hours, which has duplicates.

This shift in human:male ratios can be attributed to a biological reduction in recoverable spermatozoa with increasing time since intercourse. This effect has been described in early persistence studies and leads to a decreasing proportion of male genetic material in postcoital samples (Sharpe, 1963; Davies & Wilson, 1974). This significantly reduces the amount of sperm DNA in sexual assault samples collected a long time after intercourse, while the efficiency of depletion of female carryover DNA by the EZ2 DNA Investigator Sep&Prep Kit remains stable. As a result, the human:male ratio shifts to values greater than 1, as the ratio at later time points is more heavily

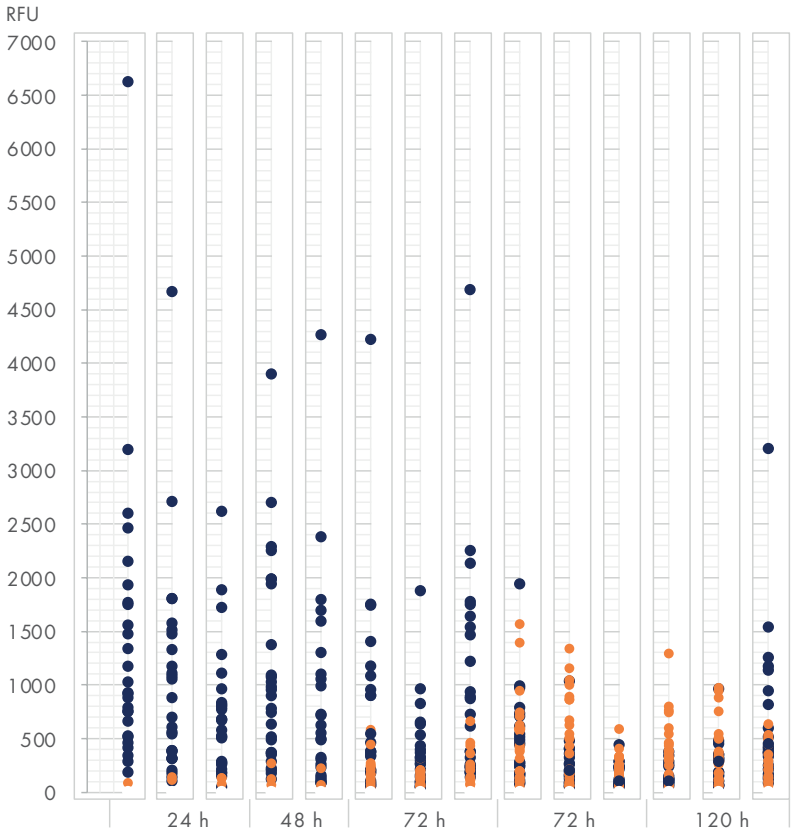

Figure 11. Individual RFU for each male- and female-specific allele of postcoital samples. Postcoital samples collected at defined time intervals (24 h, 48 h, 72 h, 96 h and 120 h) were processed using the EZ2 DNA Investigator Sep&Prep workflow. Using the respective reference profiles of the DNA donors, each allele of the STR profiles was assigned to the correct donor and highlighted according to origin: Female donor (orange dot) or male donor alleles (blue dot). Shared alleles present in both individuals were not included in the analysis. One vertical strip represents one sample, i.e., one specific sperm fraction STR profile. For STR profile analysis, the analytical threshold was set to 50 RFU and the locus-specific minimum peak height threshold to RFU ≥9% of the highest allele within the STR locus.

influenced by residual female DNA than at earlier time points. This is reflected consistently across readouts, including higher total human DNA quantities relative to male-specific DNA during quantification (Figure 9), elevated human:male DNA ratios at later time points (Figure 10), and an increased contribution of female alleles in the corresponding STR profiles (Figure 11 and Figure 12). This is illustrated by the following example: A female-specific allele in the green dye channel (highlighted in yellow, Figure 12) is absent at 24 h and 48 h, detectable at 72 h at low RFU levels, with increasing prominence at 96 h and 120 h. Despite the combined impact of these effects, the observed ratios remained within a range compatible with successful autosomal STR analysis.

Table 1. Number of male-specific alleles vs. female-specific alleles of postcoital samples.

Postcoital interval	24 h			48 h		72 h			96 h			120 h		
Biological replicate	**1**	**2**	**3**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Male-specific alleles	27	27	27	27	26	25	24	27	26	21	20	15	21	23
Female-specific alleles	1	2	3	3	2	13	20	14	26	27	24	23	23	22

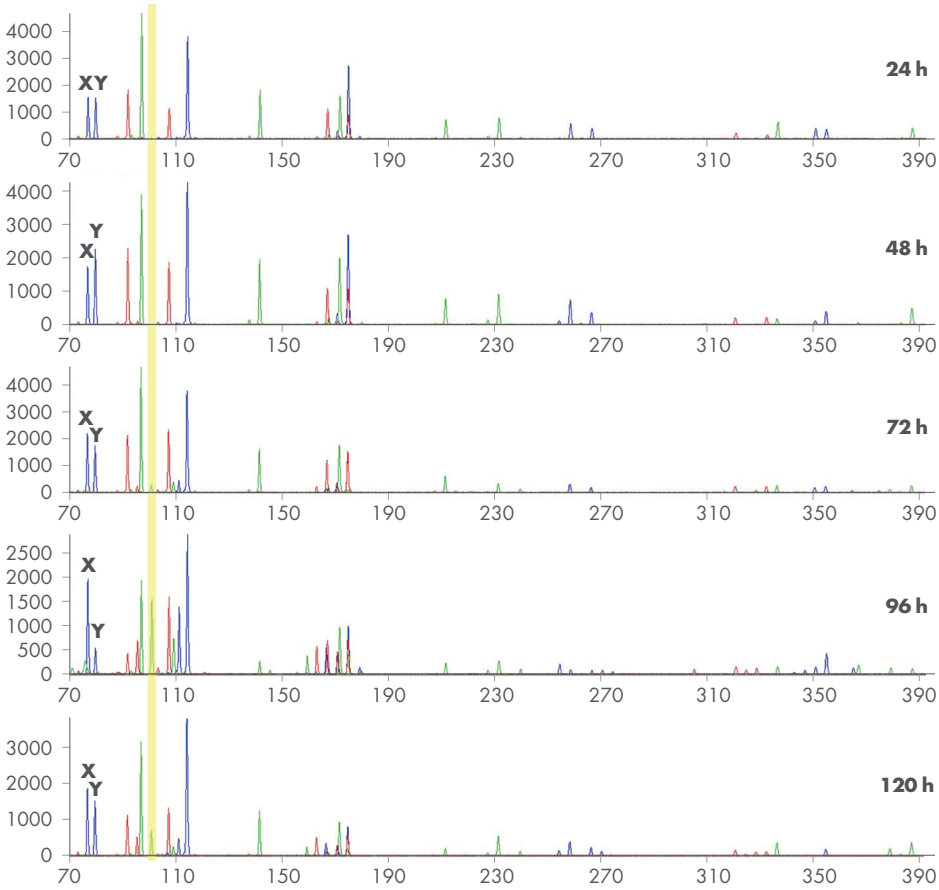

Figure 12. STR profiles from postcoital vaginal swabs. Representative electropherograms generated using the DNA Investigator 24plex QS Kit from samples collected 24–120 h post-intercourse and processed with the EZ2 DNA Investigator Sep&Prep Kit. Blue, red and green dye channels are shown as overlay. For improved visualization, Amelogenin alleles X and Y are annotated. Exemplarily, one female-specific allele in the green channel is highlighted in yellow.

Across postcoital samples collected between 24 h and 120 h, the EZ2 DNA Investigator Sep&Prep workflow enabled the generation of interpretable sperm-fraction STR profiles throughout the analyzed time course (Figure 11 and Figure 12, Table 1). At early time points, full STR profiles of the male donor were obtained using an analytical threshold of 50 RFU (Figure 11 and Table 1), with 100% recovery of male-specific alleles (27 alleles per replicate) and minimal female-specific allele detection (1–3 allele out of 29 female-specific alleles).

With increasing post-coital interval, a progressive reduction in peak heights of male-derived alleles was observed in the electropherograms (Figure 11). This was accompanied by a decrease in the number of detected male-specific alleles (from 27 at 24 h to 15–23 alleles at 120 h) and an increasing relative contribution of alleles from the female contributor at later time points, particularly at 72 h to 120 h (up to 27 alleles at 96 h; Table 1). Despite this shift in relative contributions, male-derived alleles consistently showed higher RFU values compared with female-derived alleles up to 72 h (Figure 11) and male-specific alleles remained detectable up to 120 h post intercourse (Figure 11 and Figure 12). The slightly higher DNA yield (Figure 9) and lower number of female-specific alleles (Table 1) observed at 120 h compared to 96 h represents a stochastic sampling effect at very low template levels rather than a systematic increase in DNA recovery.

The STR electropherograms show a pronounced ski-slope effect for alleles derived from the male contributor (Figure 12). Importantly, this effect is already evident at early post-coital intervals (24 h and 48 h) and is therefore not correlated with the post-coital interval. Rather, the degradation of larger amplicons is attributed to DNA fragmentation resulting from the four-year ambient storage period of the sample matrix prior to extraction.

These results demonstrate that the EZ2 DNA Investigator Sep&Prep workflow enables effective differential separation and generation of interpretable STR results even for aged, low-template sexual assault samples, highlighting its suitability for the analysis of challenging casework samples.

Mock casework samples

A selection of other typical casework sample types was prepared and processed as described before. 1:100 and 1:600 diluted semen were spiked on buccal and vaginal swabs and processed in triplicate (Figure 13 A). 1:10 and 1:100 diluted semen samples were added to empty swabs and processed as triplicates within the same test (Figure 13 B).

Vaginal swabs containing menstrual blood from a single female donor were used as high-background samples and spiked with a semen mixture from three male donors, each contributing approximately equal DNA input amounts. The mixture was diluted 1:2 in water for application to vaginal/menstrual blood swabs (sample 'Semen mix 1:2 on vaginal/blood swab') and 1:10 dilution to buccal swabs collected from a second donor ('Semen mix 1:10 on buccal swab'), see Figure 13 C.

To further evaluate the performance of the EZ2 DNA Investigator Sep&Prep workflow on alternative casework-relevant substrates, approximately 1 cm^2 sections of different fabrics were tested, including denim (jeans), polyester, cotton (towel, underwear, and bedsheet), and nylon. Each substrate was spiked with either 1:200 diluted semen (Figure 13 D) or a 1:1 mixture of semen diluted 1:200 with undiluted saliva from a female donor (Figure 13 E).

Quantification revealed an effective isolation of male-specific DNA across all substrate types (Figure 13). No inhibition was observed in any of the samples, as indicated by the internal quantification control.

STR profiling confirmed that genetic contributions of all three male donors were retained in approximately equal proportions, consistent with the initial mixture composition. Female-specific alleles were only sporadically detected and limited to low-intensity background peaks (data not shown), further supporting the high specificity of male DNA isolation observed.

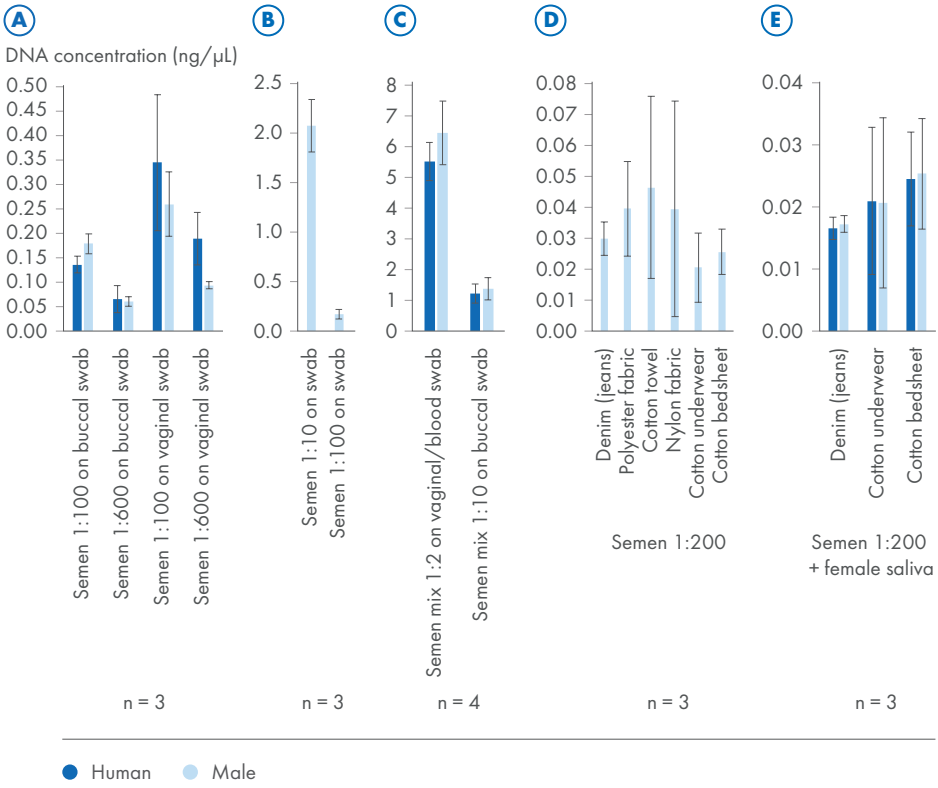

Figure 13. DNA concentration from typical case-type samples. 10 µL of various semen dilutions was spiked on different types of swab or fabrics. (**A**) 1:100 and 1:600 diluted semen on buccal and vaginal swabs. (**B**) 1:10 and 1:100 diluted semen on empty swabs. (**C**) 1:2 and 1:10 diluted semen mix on vaginal/blood swabs and buccal swabs. (**D**) Semen diluted 1:200 that was applied to approximately 1 cm² pieces of denim (jeans), polyester, cotton towel, nylon, cotton underwear, and cotton bedsheet. (**E**) Semen diluted 1:200 that was applied as a 1:1 mixture with undiluted female saliva to approximately 1 cm² pieces of denim (jeans), cotton underwear, and cotton bedsheet. For samples composed solely of semen (i.e., without female donor addition), only the male DNA target is displayed.

Duration of sample lysis

In order to define the optimal lysis duration for differential DNA extraction, the impact of lysis time on DNA yield was systematically evaluated. Differential lysis was performed using varying incubation times (20 min, 1 h, 2 h, overnight) and tested on two sample matrices: semen on vaginal swabs and semen on buccal swabs.

Therefore, a 1:100 and 1:600 semen dilution was spiked on 24 vaginal and 24 buccal swabs from two different female donors per swab type. Differential lysis was done in triplicates per sample type and lysis duration. Samples were processed on the EZ2 Connect Fx and eluted in 50 µL, and quantified with the Investigator Quantiplex Pro Kit.

The results demonstrate that lysis duration has a measurable effect on male and human DNA yield (Figure 14). Short lysis (20 minutes) resulted in reduced DNA yield for sperm on vaginal swabs, while extended lysis (2 hours and overnight incubation) did not lead to a substantial improvement. For sperm on buccal swabs, no substantial differences were noted across any of the lysis times.

Overall, a lysis duration of 1 hour was identified as the optimal condition, providing the most favorable balance between efficient DNA recovery for both sample types and overall processing time.

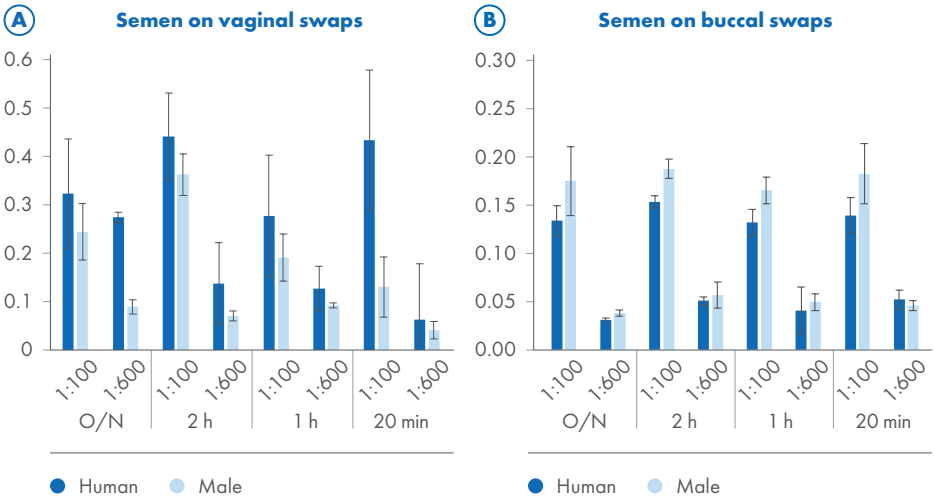

Figure 14. Differential lysis time evaluation. DNA yield was quantified after differential lysis for 20 min, 1 h and 2 h using vaginal (A) and buccal (B) swabs spiked with semen (1:100 and 1:600) from three biological replicates each. Male and human DNA from the sperm-fraction was quantified using the Investigator Quantiplex Pro Kit. A lysis duration of 1 h showed the best balance between DNA yield and processing time.

Impact of elution volume on DNA concentration and yield

The EZ2 DNA Investigator Sep&Prep Kit enables elution of the sperm fraction in both 50 µL and 100 µL. Lower elution volumes increase the sensitivity by providing higher concentrations of extracted DNA, while a higher elution volume can improve the workflow for high template samples and allow multiple downstream applications. To evaluate the impact of the elution volume on total sperm-derived DNA yield, results obtained using the different elution volume protocols were systematically compared.

Dilutions of 1:10, 1:100, 1:1000 and 1:10,000 of neat semen spiked on swabs were extracted in 6 replicates per elution volume (50 µL and 100 µL) on the same EZ2 Connect Fx instrument (Figure 15). Recovered eluate volumes were measured using a pipette. Both the 50 µL and 100 µL elution volume protocols show consistent elution volumes with low variation.

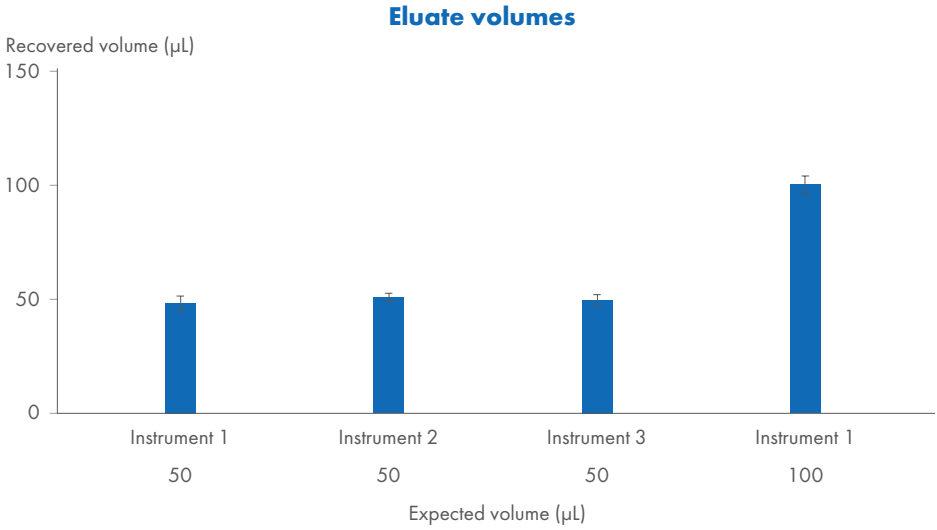

Figure 15. Eluate volumes. Extraction was performed using the EZ2 DNA Investigator Sep&Prep protocol with elution in 50 µL and 100 µL. 24 replicates were run for each elution volume and EZ2 Connect Fx instrument. Elution in 50 µL has been tested on three instruments, while elution in 100 µL has been tested on one instrument.

Across all dilutions, elution in 50 µL increased the sensitivity by providing higher concentrations of extracted autosomal DNA compared to elution in 100 µL (Figure 16). Autosomal DNA concentrations decreased proportionally with increasing dilution for both elution volumes, demonstrating linear behavior across the tested input range.

Total autosomal DNA yields were comparable between elution volumes and decreased proportionally with increasing dilution (Figure 17).

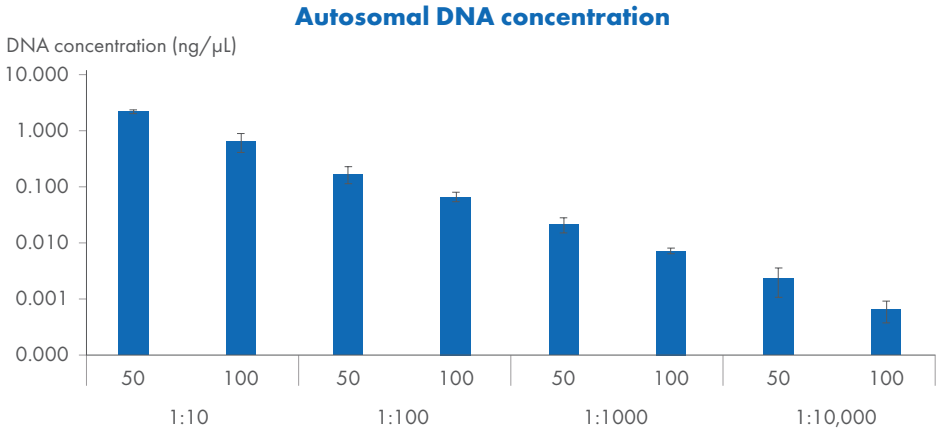

Figure 16. Correlation between DNA concentration and elution volumes. Extraction was performed using the EZ2 DNA Investigator Sep&Prep protocol with elution in 50 µL and 100 µL. 24 replicates were run for each elution volume. Quantification shows the autosomal DNA concentration (ng/µL, log scale) obtained from semen dilutions of 1:10 to 1:10,000.

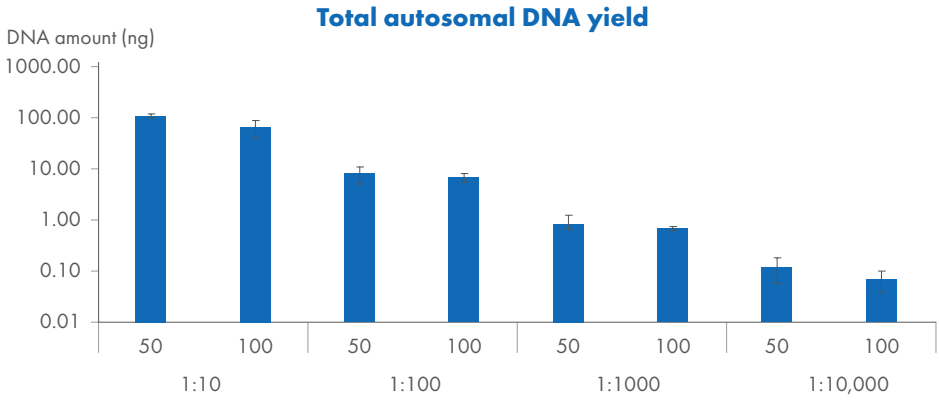

Figure 17. Correlation between total DNA yield and elution volumes. Extraction was performed using the EZ2 DNA Investigator Sep&Prep protocol with elution in 50 µL and 100 µL. 24 replicates were run for each elution volume. Quantification shows the total autosomal DNA amount (ng, log scale) recovered from semen dilutions of 1:10 to 1:10,000.

Stability of Proteinase K and Reagent UI Dilution

Proteinase K

To assess the stability of diluted Proteinase K, it was diluted 1:10 with water as specified in the handbook and stored at room temperature for 14 days. The diluted Proteinase K was used for sample processing at four time points: immediately after preparation (T0), after 3 (T1), 7 (T2), 14 (T3) and 28 days (T4).

At the beginning of the study, vaginal swabs from a single female donor were spiked with a 1:50 semen dilution, followed by overnight drying. At each test time point (T0–T4), six swabs were processed using the EZ2 DNA Investigator Sep&Prep Kit, applying the respective stored diluted Proteinase K solution to the Elution Mix.

The results show no time-dependent reduction in DNA yield for either the autosomal or Y-specific target over 14 days of storage of the 1:10 diluted Proteinase K at room temperature (Figure 18). These results indicate that sperm cell lysis remains efficient when using stored diluted Proteinase K. In addition, the removal of female-specific DNA remains effective when applying stored diluted Proteinase K within the EZ2 DNA Investigator Sep&Prep workflow. However, when adhering to good lab practices making reagent dilutions should be done as close to the time of need for best performance.

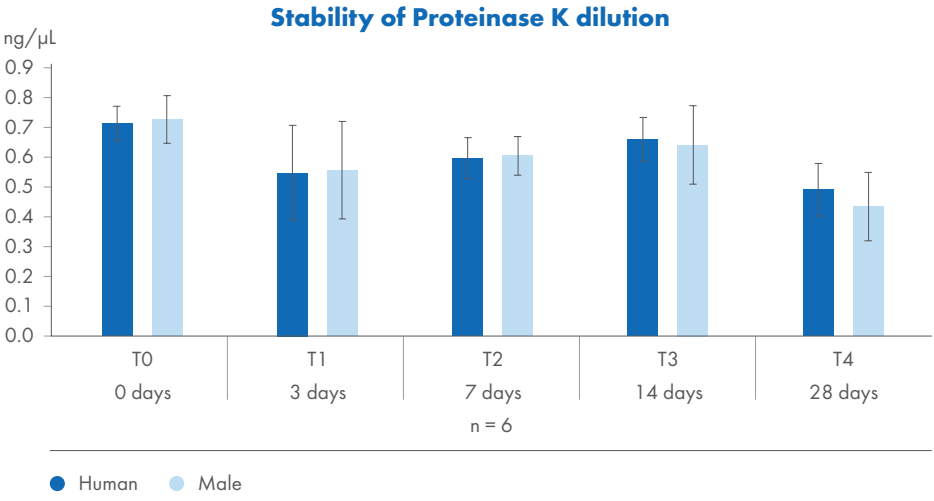

Figure 18. Stability of 1:10 diluted Proteinase K stored at room temperature for 28 days. Mean DNA concentrations (ng/µL) of autosomal ("human") and Y-specific ("male") targets obtained from six spiked vaginal swabs per time point using diluted Proteinase K for the Elution Mix at T0 (0 days), T1 (3 days), T2 (7 days), T3 (14 days) and T4 (28 days).

Reagent UI

To assess the stability of diluted Reagent UI with respect to its ability to efficiently lyse sperm cells over time, the reagent was diluted 1:100 as specified in the kit handbook and used for sample processing at three time points: immediately after preparation (T0), after 3 days (T1) and 5 days (T2) stored at room temperature protected from light.

Vaginal swabs from one female donor were spiked with semen from one male donor diluted 1:50 in water and dried overnight. At each test time point (T0–T2), six swabs were processed using the EZ2 DNA Investigator Sep&Prep Kit, applying the respective stored diluted Reagent UI to the Elution Mix.

As shown in Figure 19, male DNA yields across all time points (T0–T2) are overall comparable. No consistent decrease in male DNA recovery is observed with increasing storage time of the diluted Reagent UI. Inter-replicate variability is present at all time points. At T1, one markedly elevated value is observed (sample 5), representing an outlier that is not consistent with the general distribution and is therefore not indicative of a systematic effect.

Overall, the results indicate that storage of Reagent UI diluted 1:100 at room temperature protected from light over the tested period does not impair sperm lysis efficiency, as reflected by stable male DNA recovery across time points. These findings demonstrate that diluted Reagent UI remains sufficiently active to support reliable sperm cell lysis within the tested timeframe. As previously mentioned, when adhering to good lab practices making reagent dilutions should be done as close to the time of need for best performance.

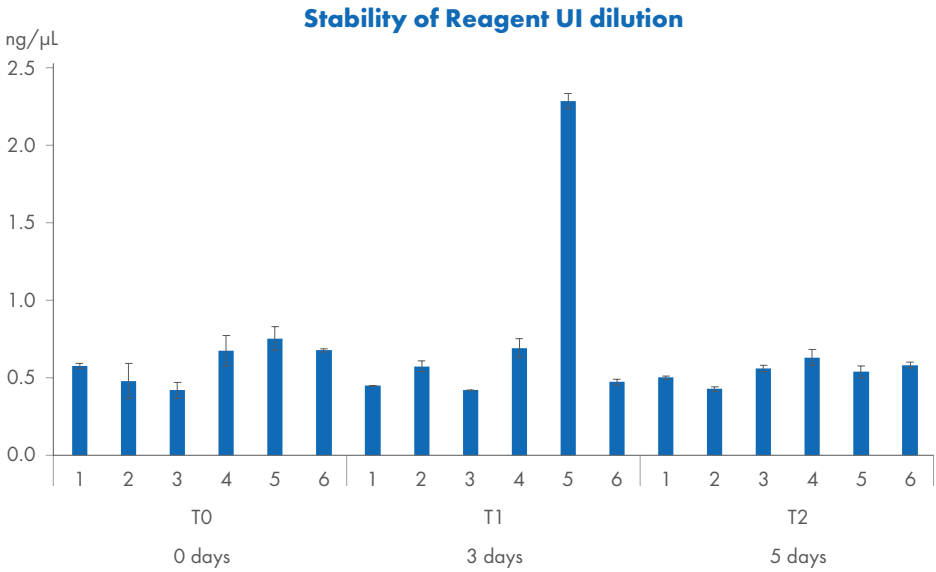

Figure 19. Stability of 1:100 diluted Reagent UI stored at room temperature protected from light for 5 days. Mean DNA concentrations (ng/µL) of Y-specific ("male") target obtained from six spiked vaginal swabs per time point after the EZ2 DNA Investigator Sep&Prep processing using diluted Reagent UI at T0 (after preparation of UI dilution), T1 (3 days) and T2 (5 days).

Equivalence study of Proteinase K dilution

The Investigator EZ2 DNA Investigator Sep&Prep Kit handbook permits the use of Proteinase K (PK) in both diluted and undiluted form, with the condition that DNA extracts generated using undiluted PK may require dilution prior to STR amplification. However, the standard protocol recommends the use of PK diluted 1:10.

To demonstrate that comparable STR results are obtained regardless of PK dilution, semen samples from three donors were processed at two semen dilutions (1:10 and 1:200). For each dilution, the EZ2 DNA Investigator Sep&Prep Elution Mix was set up with undiluted PK and 1:10 diluted PK.

The dilution of Proteinase K had no measurable impact on DNA yield (Figure 20). Comparable recovery was observed across all conditions, indicating that efficient sperm cell lysis is maintained even at reduced enzyme concentrations. As expected, the 1:200 diluted semen samples yielded substantially lower DNA quantities compared to the 1:10 dilutions. Consequently, DNA extracts from the 1:10 semen samples required additional dilution prior to STR-PCR setup, whereas extracts from the 1:200 samples required minimal or no further dilution.

The resulting STR profiles obtained using undiluted and diluted PK showed no significant differences (Figure 21).

Comparison of DNA yield using undiluted and diluted Proteinase K

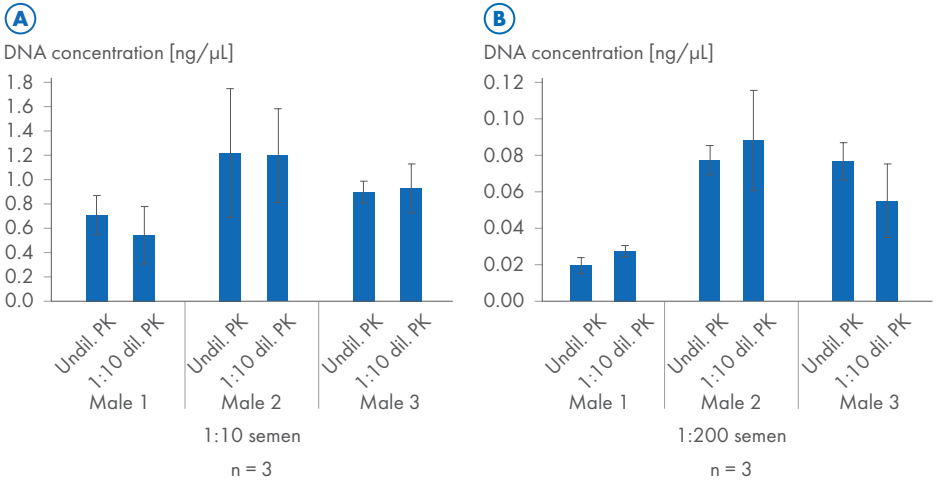

Figure 20. Quantification of sperm fractions processed with undiluted and diluted Proteinase K in the Elution Mix. Mean DNA concentrations (ng/µL) of Y-specific ("male") target. Semen samples of male donors 1, 2 and 3 were diluted 1:10 (**A**) and 1:200 (**B**) respectively. Samples were run in biological triplicates using either undiluted Proteinase K (PK) or 1:10 diluted PK.

Influence of Proteinase K dilution on STR profiling

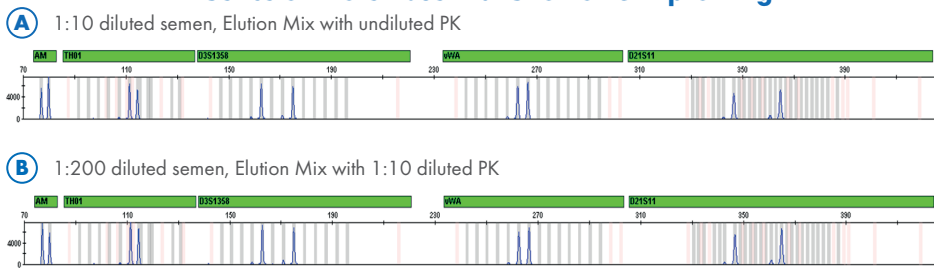

Figure 21. STR profiles of sperm fractions processed with undiluted and diluted Proteinase K in the Elution Mix. Representative electropherogram excerpts of the blue dye channel generated with Investigator 24plex QS Kit are shown for donor 1 from semen samples diluted 1:10 (**A**) and 1:200 (**B**) . Following quantification, the 1:10 semen sample was further diluted 1:10 in nuclease-free prior to STR-PCR reaction. Total DNA input for STR-PCR were, 503 pg (sample A) and 426 pg (sample B).

Compatibility studies

Compatibility studies are essential to demonstrate that DNA from the sperm fraction generated with the EZ2 DNA Investigator Sep&Prep Kit is fully compatible with established downstream forensic DNA analysis systems used in routine workflows.

Therefore, identical samples—including both non-template controls (NTCs) and DNA-containing EZ2 DNA Investigator Sep&Prep eluates (1:10 diluted semen of one male donor spiked on vaginal swabs of one female donor, processed via EZ2 DNA Investigator Sep&Prep Kit and eluted in 50 µL)—were pooled and analyzed using multiple qPCR and STR kits from different suppliers. Compatibility was assessed by analyzing quantification and STR results of the following kits using identical pooled eluates of the EZ2 DNA Investigator Sep&Prep:

- QIAGEN Investigator Quantiplex Pro Kit, Investigator Quantiplex Pro RGQ Kit, Investigator 24plex QS and Investigator Argus Y-28 QS
- Thermo Fisher Scientific Quantifiler™ Trio DNA Quantification Kit, GlobalFiler™ PCR Amplification Kit, AmpFLSTR™ NGM SElect™ PCR Amplification Kit and Yfiler™ Plus PCR Amplification Kit
- Promega PowerQuant® System, PowerPlex® Fusion 6C System and PowerPlex® Y23 System

Autosomal STR results (Figure 22), gonosomal STR and quantification results (data not shown) confirm the compatibility of the EZ2 DNA Investigator Sep&Prep eluates with all qPCR and STR kits listed above when processed according to their respective handbooks.

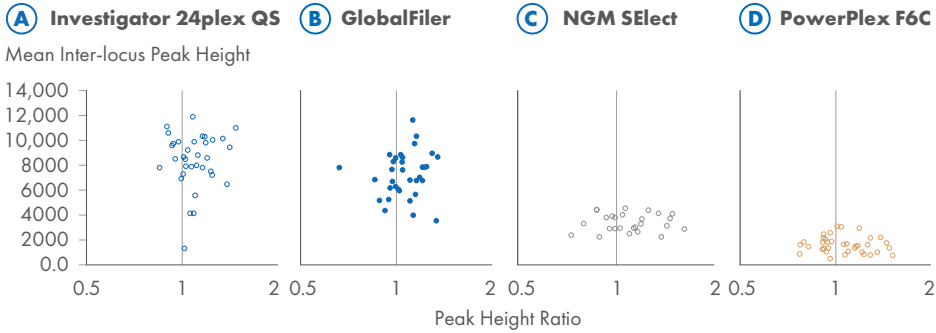

Figure 22. STR peak heights of four autosomal STR kits. Graphs show the mean inter-locus peak height and the peak height ratio calculated for all alleles per STR marker. (**A**) Investigator 24plex QS Kit, (**B**) GlobalFiler PCR Amplification Kit, (**C**) AmpFLSTR NGM SElect PCR Amplification Kit, (**D**) PowerPlex Fusion 6C System.

Benchmarking against QIAcube Connect differential wash protocol

To benchmark the performance of the EZ2 DNA Investigator Sep&Prep Kit, the workflow was compared with the commonly used QIAcube Connect Differential Wash protocol (QIAcube). Vaginal swabs from a single female donor, spiked with a 1:200 semen dilution were used. For the differential wash procedure on the QIAcube, swab samples were lysed in 480 µL Buffer ATL with 20 µL Proteinase K and incubated for 2 h at 56 °C at 900 rpm. Following lysis, automated separation of sperm and non-sperm fractions was performed on the QIAcube using protocols 5. Separation Lysis 12 A and 12 B, which were run sequentially, each processing up to 12 samples. Washing steps were carried out using Buffer G2, applying 4 consecutive wash cycles to the sperm pellet. The sperm fraction was lysed with the lysis mix of 130 µL G2 buffer, 8.7 µL Proteinase K and 34.6 µL DTT for 1 h at 56 °C and 900 rpm and purified on the EZ2 Connect Fx with the EZ1&2 DNA Investigator Kit using the Trace protocol (1.5 mL sample tubes) with elution in 50 µL TE buffer. The non-sperm fractions from both workflows were purified with the EZ1&2 DNA Investigator Kit using the Large Volume protocol and elution in 50 µL TE buffer. DNA concentration and degradation were assessed using Investigator Quantiplex Pro RGQ and evaluated separately for the sperm and non-sperm fractions. STR raw data were evaluated using a threshold of 200 RFU. For calculation of allele recovery and allele distribution (Figure 28 and Table 2), a locus-specific minimum peak height threshold of 9% relative to the highest RFU allele at each locus was used. For profile deconvolution, the software STRmix was used in cooperation with Sam Houston State University (Figure 30). STRmix analysis was performed with the number of contributors set to two, samples conditioned on the female contributor and evaluated under the propositions ‘female and male’ versus ‘female and unknown unrelated person’. For STRmix analysis, the marker SE33 was ignored as excessive shoulder peaks and noise at this locus made it difficult to process with STRmix.

In the sperm fraction, the EZ2 DNA Investigator Sep&Prep Kit produced higher male DNA concentrations than the QIAcube workflow (Figure 23), indicating better isolation of DNA originating from sperm cells. Furthermore, the EZ2 DNA Investigator Sep&Prep Kit displayed consistently lower human DNA concentrations than the QIAcube workflow, indicating more effective depletion of non-male DNA from the

sperm fraction. Degradation indices (DI) in the sperm fraction were predominantly at or below the reference value of 1 for the EZ2 DNA Investigator Sep&Prep Kit (mean male DI of 0.9 and mean human DI of 0.8), consistent with largely non-degraded DNA. While the QIAcube-processed samples more frequently exhibited ratios above 1 for both autosomal (mean DI of 1.2) and Y-specific DNA (mean DI of 1.1, Figure 24), these values are still consistent with largely non-degraded DNA.

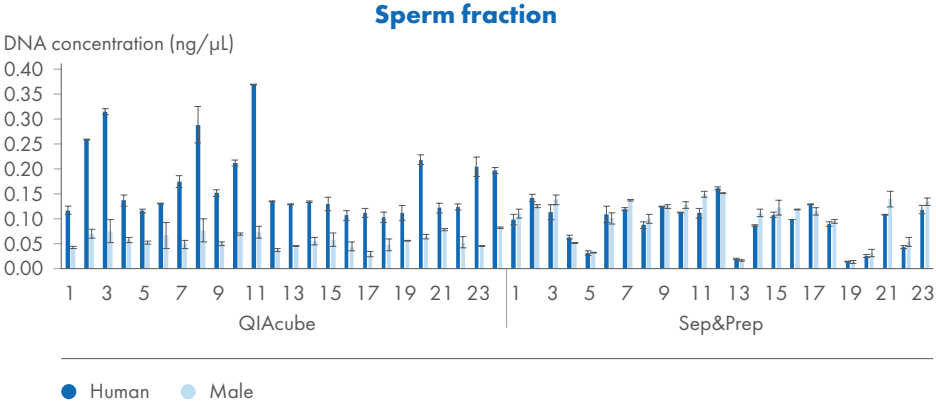

Figure 23. Comparison of DNA concentrations from sperm fractions. Biological replicates of 1:200 semen dilutions of vaginal swabs were processed with the QIAcube Connect Differential Wash protocol and purified with Investigator Trace protocol on the EZ2 Connect Fx (n=24, left) or the EZ2 DNA Investigator Sep&Prep Kit (n=23, right). Male and human DNA concentrations (ng/µL) are shown (n=2 technical replicates).

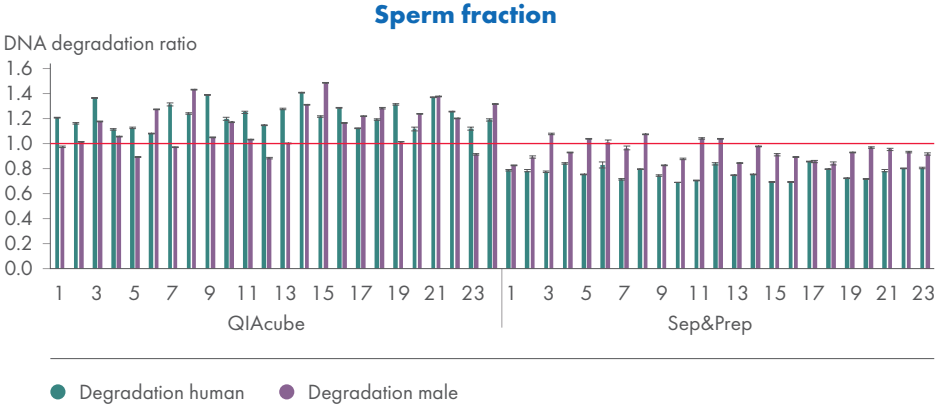

Figure 24. Comparison of DNA degradation ratios from sperm fractions. Biological replicates of 1:200 semen dilutions of vaginal swabs have been processed with QIAcube Connect Differential Wash protocol and purified with Investigator Trace protocol on the EZ2 Connect Fx (n=24, left) or the EZ2 DNA Investigator Sep&Prep Kit (n=23, right). Autosomal and male DNA degradation ratios are shown; the red horizontal line indicates the degradation ratio reference threshold of 1 where DNA is intact (n=2 technical replicates).concentrations (ng/µL) are shown (n=2 technical replicates).

The human-to-male DNA ratio further emphasized the superior separation of non-sperm DNA from sperm derived DNA when using the EZ2 DNA Investigator Sep&Prep Kit (Figure 25). A human:male DNA ratio close to 1 indicates that the DNA in the sperm fraction originates almost entirely from the male contributor and is therefore expected to yield predominantly male STR profiles or mixed profiles with the male contributor as the major component. The EZ2 DNA Investigator Sep&Prep Kit produced human:male ratios consistently close to 1, reflecting efficient removal of female contributor DNA from the male contributor in the sperm fraction. In contrast, QIAcube-processed samples yielded higher and more variable human:male ratios, including multiple samples with ratios between 2 and 5, indicating increased carryover of human DNA from the female contributor into the sperm fraction.

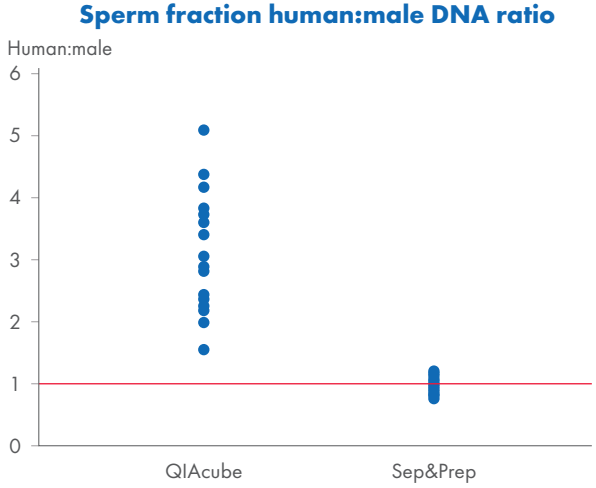

Figure 25. Human to male DNA ratios in the sperm fraction after differential extraction with the QIAcube workflow and the EZ2 DNA Investigator Sep&Prep Kit. Human:male DNA ratios are shown for individual biological replicates (n=24 for QIAcube sample, n=23 for EZ2 DNA Investigator Sep&Prep samples).

In the non-sperm fraction, both methods yielded similarly high human DNA concentrations (Figure 26). Male DNA concentrations remained low across both workflows, with the EZ2 DNA Investigator Sep&Prep Kit showing lower concentrations (1.6 pg/µL) than QIAcube-processed samples (3 pg/µL). Human DNA degradation ratios in the non-sperm fraction were close to the reference value of 1 for both methods (mean human DI of 1.4 for both methods), indicating mainly intact DNA. In contrast, male-DNA degradation ratios were more variable and the EZ2 DNA Investigator Sep&Prep-treated samples show more samples of increased degradation index

(mean male DI of 2.8) compared to QIAcube-treated samples (mean male DI of 2.1, Figure 27). This can be explained by the fact that DNA from male epithelial or damaged sperm is more likely to be degraded and DNA of these sources will be separated into the non-sperm fraction by the EZ2 DNA Investigator Sep&Prep Kit. Male DNA of the QIAcube method might be less degraded because it has probably lost intact sperm cells to the non-sperm fraction.

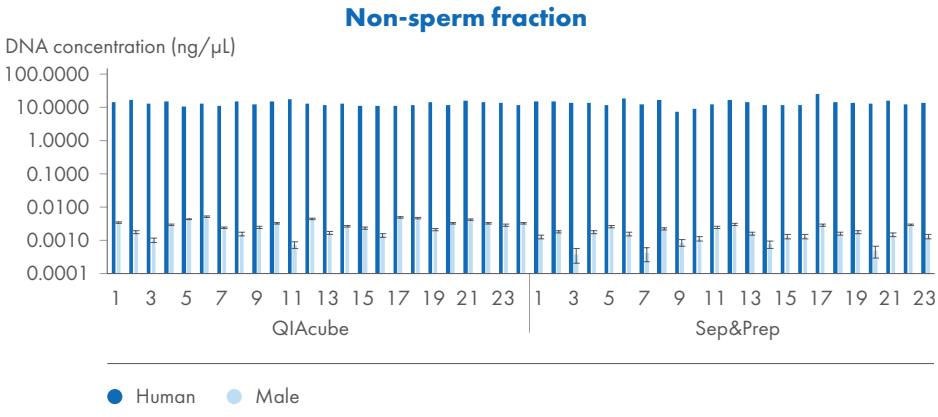

Figure 26. Comparison of DNA concentrations from non sperm fractions. Biological replicates of 1:200 semen dilutions of vaginal swabs have been processed with the QIAcube Connect Differential Wash protocol (n=24, left) or the EZ2 DNA Investigator Sep&Prep Kit (n=23, right). Log scaled human and male DNA concentrations (ng/µL) are shown (n=1).

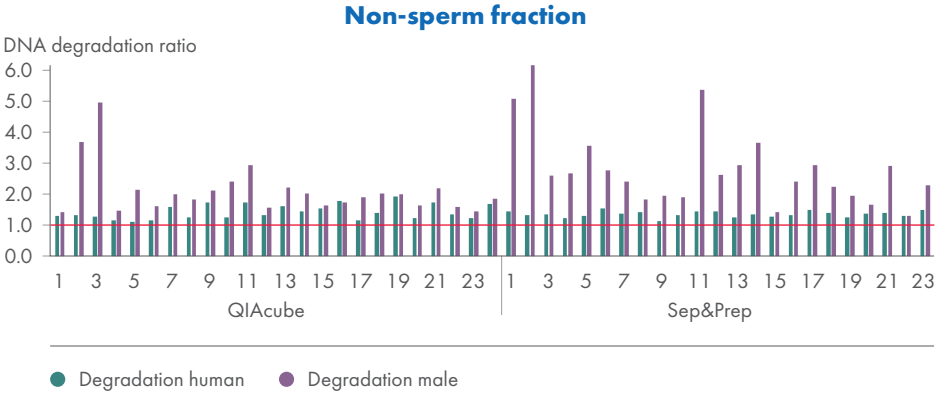

Figure 27. Comparison of DNA degradation ratios from non sperm fractions. Biological replicates of 1:200 semen dilutions of vaginal swabs have been processed with the QIAcube Connect Differential Wash protocol (n=24, left) or the EZ2 DNA Investigator Sep&Prep Kit (n=23, right). Autosomal and male DNA degradation ratios are shown; the red horizontal line indicates the degradation ratio reference threshold of 1 where DNA is intact (n=1).

Overall, the most relevant method-dependent differences from the quantification results were observed in the sperm fraction. The EZ2 DNA Investigator Sep&Prep Kit more effectively separated female-derived human DNA from the sperm fraction, consistently yielded near-ideal human:male ratios in the sperm fraction, and maintained DNA integrity. These differences between samples processed with the QIAcube workflow and the EZ2 DNA Investigator Sep&Prep Kit were also observed by STR typing (Figure 28, Figure 29 and Figure 30). The EZ2 DNA Investigator Sep&Prep Kit enabled a more distinct separation of sperm-derived DNA from mixed samples, as reflected by profiles containing fewer alleles, markedly decreased female-derived RFU signals, and higher RFU from alleles originating from the male donor. This results in a distinct separation between the female-specific and male-specific genetic information (Figure 28). STRmix calculations demonstrated that the proportion of male-specific DNA in the mixed profiles was significantly higher in Sep & Prep–processed samples compared to QIAcube-processed samples (Figure 30). All biological replicates processed using the EZ2 DNA Investigator Sep&Prep Kit and the QIAcube workflow yielded complete male donor STR profiles, corresponding to the recovery of 19 male-specific alleles, with the exception of a single QIAcube-processed replicate in which one male-specific allele was missing. All QIAcube-processed replicates showed full recovery of all 22 female-specific alleles. In contrast, only four replicates that were processed with the EZ2 DNA Investigator Sep&Prep Kit exhibited complete recovery of all female-specific alleles (Table 2).

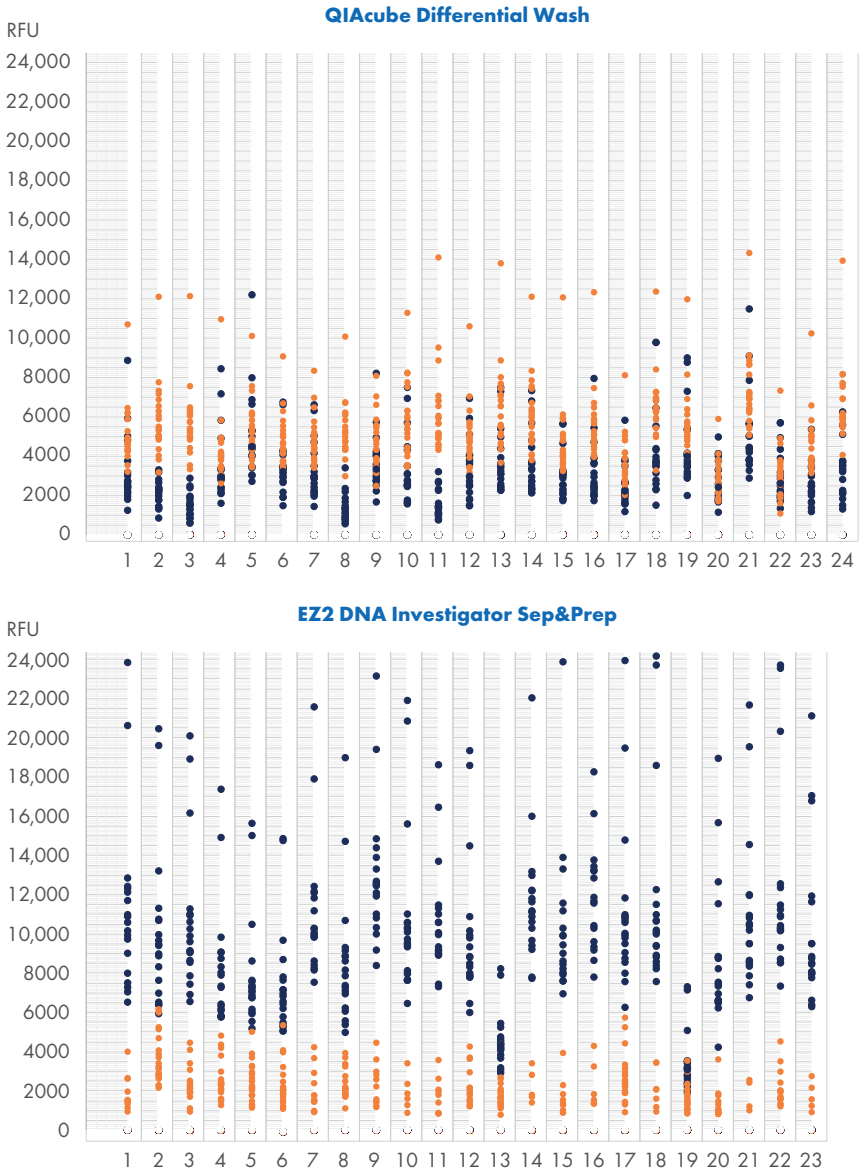

Figure 28. Allele comparison of sperm fractions. Biological replicates of 1:200 semen dilutions of vaginal swabs were processed with the QIAcube Connect Differential Wash protocol (upper panel) or the EZ2 DNA Investigator Sep&Prep Kit (lower panel). Using the respective reference profiles of the DNA donors, each allele of the STR profiles was assigned to the correct donor and highlighted according to origin: Female donor (orange dot), or male donor (blue dot). Shared alleles present in both individuals were not included in the analysis. One strip = one sample, i.e., one specific sperm fraction STR profile (coming from one biological replicate). For STR profile analysis, the analytical threshold was set to 200 RFU and the locus-specific minimum peak height threshold to RFU ≥9% of the highest allele within the STR locus.

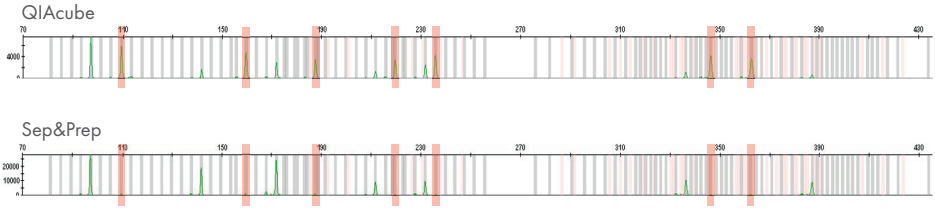

Figure 29. Representative STR profiles of sperm fractions processed with the QIAcube workflow and the EZ2 DNA Investigator Sep&Prep Kit. Only the green fluorescence channel of the Investigator 24plex QS Kit is displayed. For visualization purposes, female-specific alleles were highlighted with red bars.

Table 2. Number of male-specific alleles vs. female-specific alleles found in the sperm fraction from samples processed with the EZ2 DNA Investigator Sep&Prep Kit or the QIAcube Connect Differential Wash protocol.

	QIAcube Differential Wash		EZ2 DNA Investigator Sep&Prep Kit	
Biological replicate	Male-specific alleles	Female-specific alleles	Male-specific alleles	Female-specific alleles
1	19	22	19	11
2	19	22	19	22
3	19	22	19	18
4	19	22	19	21
5	19	22	19	21
6	19	22	19	20
7	19	22	19	13
8	19	22	19	21
9	19	22	19	11
10	19	22	19	6
11	19	22	19	9
12	19	22	19	16
13	19	22	19	22
14	19	22	19	5
15	19	22	19	8
16	19	22	19	6
17	19	22	19	22
18	19	22	19	7
19	19	22	19	22
20	18	22	19	13
21	19	22	19	4
22	19	22	19	14
23	19	22	19	5
24	19	22		

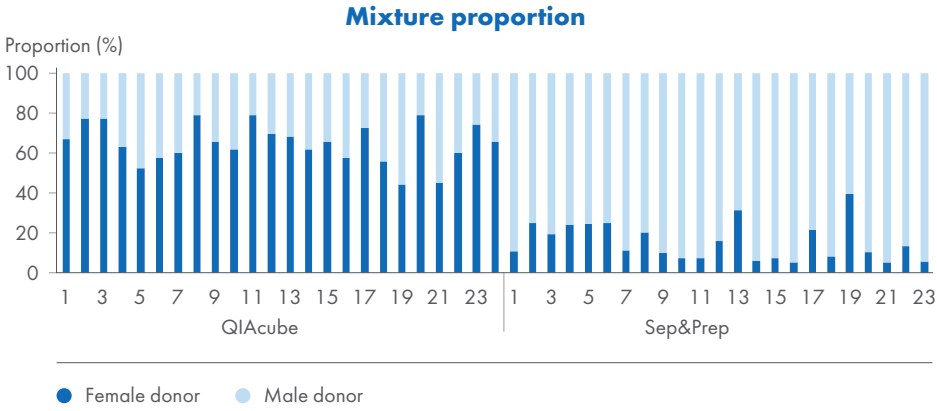

Figure 30. Mixture proportions of the female and male donors, calculated by the STRmix software. Biological replicates of 1:200 semen dilutions of vaginal swabs were processed with the EZ2 DNA Investigator Sep&Prep Kit (right) or the QIAcube Connect Differential Wash protocol (left). For STR profile analysis, the analytical threshold was set to 200 RFU. Using the respective reference profiles of the DNA donors, the proportions of male- and female-specific alleles on each mixed profile were calculated by the STRmix software.

In conclusion, the EZ2 DNA Investigator Sep&Prep workflow is highly effective and demonstrated robust performance for challenging samples characterized by high levels of female DNA and low male DNA input, whereas the QIAcube Connect differential wash method is already reaching its limits under these conditions.

Conclusion

The validation studies confirmed the following:

- The EZ2 DNA Investigator Sep&Prep Kit is capable of robust and reliable separation of sperm-derived DNA from female-derived DNA in sexual assault samples
- The kit provides reliable results from a wide range of sample materials and input amounts that simulate what might be found in sexual assault samples.
- The kit exhibits no cross-contamination when processed on the EZ2 Connect Fx instrument.
- The kit shows superior performance to current differential wash procedures.

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Ordering Information

Product	Contents	Cat. no.
EZ2 DNA Investigator Sep&Prep Kit (48)	For 48 preps: Reagent Cartridges (EZ2 DNA Investigator Sep&Prep Kit), Disposable Filter-Tips and Tip-Holders, Tubes, QIAshredder, Reagent UI, Lysis Buffer, Elution Buffer, Proteinase K, and Nuclease-free Water	952134
EZ2 Connect Fx	Robotic instrument for automated purification of nucleic acids from up to 24 samples, 1 year warranty on parts and labor	9003220

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Document Revision History

Date	Changes
07/2026	Initial release

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