Emphasizing accuracy and precision of pipette systems for human identification applications

Goal

The goal of this study was to establish the accuracy and precision of the **Thermo Scientific™ Finnpipette™ F1 multichannel pipettes** with **Thermo Scientific™ ART™ Barrier pipette tips** and the **Thermo Scientific™ F1-ClipTip™ system** for use in Human Identification (HID) and to further provide data to support in-house comparison studies as warranted by the FBI (Federal Bureau of Investigation) Director's Quality Assurance Standards (QAS), Scientific Working Group on DNA Analysis Methods (SWGDAM) and European Network of Forensic Science Institutes (ENFSI) guidelines.

Introduction

Forensic labs use protocols for human identification that play a critical role in the criminal justice system by aiding law enforcement agencies in the identification of human samples collected from crime scenes. Due to increasing demands on time and resources, forensic labs are under constant pressure to increase their efficiency. The accuracy, precision and reproducibility of the data generated in forensic labs are of utmost importance given the scope of the results generated and how they are used in the legal system.

The protocols, materials, and instruments used in forensic labs are held to a strict standard and require validation and review. Forensic labs in North America performing DNA testing or utilizing the Combined DNA Index System (CODIS) are required to follow the Quality Assurance Standards (QAS), which places specific requirements on the laboratory to obtain and maintain accreditation[1]. The labs also routinely follow validation guidelines for DNA analysis methods outlined by the Scientific Working Group on DNA Analysis Methods (SWGDAM)[2]. Forensic laboratories in the European Union are required to adhere to the guidelines provided by the European Network of Forensic Science Institutes (ENFSI)[3]. All these guidelines are derived from ISO17025. Any change in materials (new chemistry or instrumentation: sources or brands) used in the lab undergo validation before implementation into the lab's HID workflow. Accuracy, precision, and sensitivity determinations are standard components of method validations that help describe the performance of the method or product.

Pipette systems are a critical tool used in forensic laboratories for DNA testing. The results of the tests often are the key to solving a case or investigation. The aspiration and dispensing of samples and related reagents are critical to generating accurate and precise data for irreplaceable samples. Therefore, pipettes and tips need to deliver exact volumes and must have low retention properties. Error in pipetting volumes can greatly affect the results.

The present study is a comparison between four different pipette/tip combinations, which include the F1-ClipTip/ClipTip pipette tips, F1-Finnpipette/ART Barrier pipette tips, Rainin™ LTS/**SoftFit-L™** tips and Rainin LTS /Rainin LTS tips. Results demonstrate that all four pipetting systems exhibit a high degree of accuracy, precision and reproducibility for forensic applications such as DNA quantification, short tandem repeats (STR) amplification, and genotyping assays.

Generally, forensic laboratories will use the recommended 0.5 ng to 1 ng DNA input per sample for HID STR amplifications. In cases where a sample has limited DNA, laboratories may use inputs less than 100 pg of DNA. However, inputs of 100 pg or less can result in stochastic effects observed in the sample profile. This study aimed to examine the accuracy and precision of the pipette and tip systems, using DNA concentrations both above and below the recommended input for downstream STR amplification.

Materials and methods

Table 1. List of pipettes, associated pipette tips, and tip volumes assessed in testing.

Pipettes	Tips	Tip Models
Rainin LTS	Rainin LTS pipette tips, filtered, low-retention, sterile	LTS10, LTS20, LTS200, and LTS1000
	Thermo Scientific SoftFit-L pipette tips, filtered, low-retention, sterile	20L, 200L, 1000L
F1-Finnpipette	Thermo Scientific ART Barrier pipette tips, low-retention, sterile	ART 10 REACH, ART 20P, ART 200, ART 1000
F1-ClipTip	Thermo Scientific ClipTip pipette tips, filtered, sterile	ClipTip 12.5 EXT, ClipTip 20, ClipTip 200, ClipTip 1000

The below-described methods were executed by Thermo Fisher Scientific HID Professional Services (HPS), who are experts that offer services designed to help forensic DNA labs integrate, validate, and implement new methodologies and technologies.

Determination of accuracy by standard curve

Standard curve dilutions series were created according to the Applied Biosystems™ Quantifiler™ HP and Trio DNA Quantification Kits User Guide. Using the DNA standard, included in the **Quantifler Trio DNA Quantification Kit** (Applied Biosystems™, 4476135), five 10-fold dilutions were made, from 50 ng/µL to 0.005 ng/µL. Before running the quantification plate, two standard curve dilution series were prepared separately, using each pipette/tip combination. For quantification plate setup, 18 µL of the prepared master mix (PCR Reaction Mix + Primer Mix) from the DNA quantification kit was added into the appropriate wells of a barcoded 96-well plate (MicroAmp Optical 96-well plate), followed by dispensing 2 µL of the diluted DNA standard. The quantification plate was run on a **Applied Biosystems™ QuantStudio™ 5 using the HID Real-Time PCR** analysis software (version 1.3.1).

Standard curve metrics were recorded for each of the two curves on each plate and analyzed individually. Standards were independently created using the Rainin LTS/LTS tips, Rainin pipettes/SoftFit-L™ tips, F1-Finnpipette/ART Barrier tips, and the F1-ClipTip/ClipTip tips. Master Mix was also created using the respective pipette and tip combination for each set of standards. For this study, a total of 16 standard curves across the 4 combinations tested were generated and data were used for analyses. All TE buffer (Invitrogen, 12090-015) used for controls, sample dilutions, and amplification controls were from a single aliquot when possible. All aliquots used were tracked and tested for any possible contamination by quantification and/or amplification.

Determination of precision by serial dilution

The Quantifiler Trio DNA Quantification Kit was designed to reliably quantify unknown samples within the range of the standard dilution series. This study assessed the variation of samples within and outside this range by diluting a known amount of human genomic DNA. It is essential to understand the implications of these measurements and how they can affect downstream STR processing. The protocol was run according to the kit manufacturer.

A two-fold serial dilution series was prepared using the Rainin LTS/LTS tips, ranging from 64 ng/µL to 0.008 ng/µL of human genomic DNA (InnoGenomics), and used as the test samples. Using each of the pipette tip combinations, the following were performed. Two independent standard curves were prepared, consisting of five 10 -fold dilutions ranging from 50 ng/µL to 0.005 ng/µL, and added (2 µL) to the plate in duplicates. Test samples were also added (2 µL) to the plate in triplicates. Separate master mixes were prepared and 18 µL added to each well. This method was used to show that any variation observed can be attributed to pipetting variability.

Measuring sensitivity by STR amplification and fragment analysis

Short Tandem Repeat (STR) analysis compares allele repeats at specific loci in DNA between two or more samples making them effective for human identification purposes. The variable (polymorphic) nature of the STR regions analyzed for forensic testing can discriminate between one DNA profile and another.

Small autosomal target quantification results for the samples of the serial dilution series detailed in the methods above were used for the targeted amplification of these samples using the **Applied Biosystems™ GlobalFiler PCR Amplification Kit** (Thermo Scientific, 4476135). The samples were normalized so that amplification input ranged from 4 ng to 0.016 ng. For each sample, a master mix containing PCR Master Mix, DNA, and TE buffer was prepared for four reactions, then 25 µL was aliquoted into each of the three-replicate amplification tubes. Amplification was done on the **Applied Biosystems™ Proflex PCR System** using the thermal cycling parameters listed in Figure 1. Each sample from the dilution series was typed using the **Applied Biosystems™ 3500xL capillary electrophoresis (CE) instrument**. The CE master mix was prepared using 9.6 µL of Applied Biosystems™ Hi-Di formamide as an injection solvent along with 0.4 µL of Gene Scan 600 LIZ Size Standard v2.0. One microliter (1 µL) of PCR amplified product or allelic ladder was added to the appropriate wells. Default injection conditions (1.2kV for 24 seconds) were used to perform fragment analysis of the sensitivity series samples. The resulting sample electropherograms were used to determine the heterozygous peak heights for each sample replicate for the pipette/tip combinations. All data analysis was performed using the Local Southern sizing method with analytical thresholds of 25 RFU for all color channels.

Initial incubation step	Cycle (29 Cycles)		Final extension	Final hold
	Denature	Anneal/Extend		
95°C, 1 minute	94°C, 10 seconds	59°C, 90 seconds	60°C, 10 minutes	4°C, Up to 24 hrs

Figure 1. Thermal Cycling Parameters for GlobalFiler Amplifications.

Results and discussion

Comparing accuracy by standard curve

As defined in the SWGDAM Validation Guidelines for DNA Analysis Methods (2016): accuracy is the degree of conformity to a measured quantity to its actual (true) value. Precision characterizes the degree of mutual agreement among a series of individual measurements, values, and/or results. When pipettes are accurate and precise, the mean volume is the set volume, and there is little to no variation between different pipetting events, providing reliable results. The standard curves prepared with each pipette/tip combination were analyzed for quality metrics to define the reliability of the different pipette and tip combinations.

The slope, y-intercept, and R^2 values for each target and each pipette/tip combination are listed in Table 2. The results for the standard curve metrics encompass four separate standard curves prepared by each pipette/tip combination, two curves from the quantification study and two from the sensitivity dilution study, with each pair prepared at different instances. The relative standard deviations for all the metrics were minimal (ranging from 0.10% to 1.37%) as noted in Table 2, demonstrating repeatability and reproducibility across all pipette/tip combinations.

R-squared (R^2) values are the measure of fit that indicates the amount of variance for a dependent variable. An R^2 value greater than or equal to 0.99 indicates a close fit between the standard curve and the individual data points, indicating less variance and high accuracy. An R^2 value below 0.98 indicates errors were likely introduced resulting in less accuracy, since pipetting errors in the dilution series are likely to result in a non-linear response. The average R^2 values for all the standard curves generated for this comparison were greater than 0.99 indicating that the dispensing of the pipettes was accurate.

The slope of a standard curve is a measure of the efficiency of the PCR reaction, with a slope of -3.3 indicating 100% efficiency. Slopes between -3.0 and -3.6 for the small autosomal target and Y target and between -3.1 and -3.7 for the large autosomal target are within the typical range defined in the Quantifiler Trio DNA Quantification Kit User Guide. Quality metrics for the standard curves created using each pipette/tip combination were averaged and compared. The average slope of standard curves created by each pipette/tip combination ranged from -3.207 (F1-ClipTip/ClipTip tips -small autosomal) to -3.444 (F1-Finnpipette/ART Barrier tips- large autosomal). The overall relative standard deviations for the slopes of the standard curves across all pipette/tip combinations ranged from 1.39% to 2.29%, indicating minimal variation between pipetting systems.

The Y- Intercept indicates the expected CT (Cycle Threshold) value or the sensitivity of the PCR reaction for a given sample with quantity = 1 (for example, 1 ng/µL). In this study, the Y-intercept for all standard curves prepared using each pipette/tip combination ranged between 26.97 – 27.399 for the small autosomal, 25.038 – 25.43 for the large autosomal and 25.969 – 26.34 for the Y loci, with an overall relative standard deviation of 0.30%, 0.47% and 0.23% respectively. The Y-Intercept values were shown to be highly comparable for all the pipette/tip combinations. Since CT values are directly proportional to the amount of input DNA in the sample, this demonstrates that there were equal amounts of DNA detected in all the samples from each pipette/tip combination.

Table 2: Comparison of pipettes and tips with average slope, Y-intercept, and R^2 values of the three Quantifiler Trio targets. Blue indicates the lowest variation across pipette / tip combinations. Red indicates the highest variation across pipette and tip combinations.

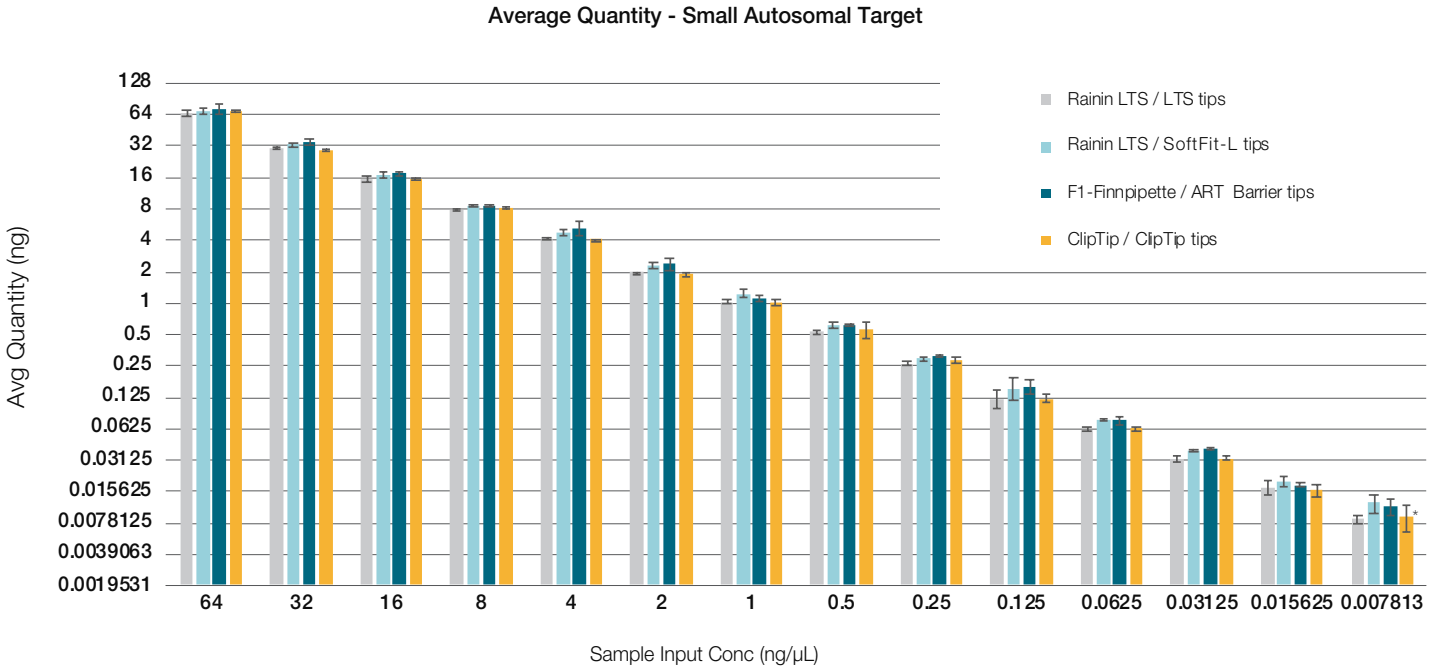
		Small Autosomal Slope			Large Autosomal			Y		
		Slope	Y-intercept	R^2	Slope	Y-intercept	R^2	Slope	Y-intercept	R^2
Rainin LTS / LTS tips	Average	-3.238	26.974	0.999	-3.345	25.054	0.999	-3.287	25.969	0.999
	Standard Deviation	0.019	0.138	0.001	0.039	0.159	0.001	0.037	0.094	0.001
F1-Finnpipette / ART Barrier tips	Average	-3.329	27.399	0.999	-3.444	25.43	0.999	-3.344	26.34	0.998
	Standard Deviation	0.041	0.052	0.001	0.051	0.091	0.001	0.046	0.037	0.001
F1-Cliptip / ClipTip tips	Average	-3.207	26.971	0.999	-3.273	25.038	1	-3.305	26.002	1
	Standard Deviation	0.05	0.066	0.001	0.012	0.114	0.001	0.048	0.033	0.001
Rainin LTS / SoftFit-L tips	Average	-3.331	27.159	0.999	-3.429	25.224	0.999	-3.345	26.153	0.999
	Standard Deviation	0.026	0.066	0.001	0.022	0.111	0.001	0.039	0.078	0.001
Overall Average		-3.276	27.125	0.999	-3.372	25.186	0.999	-3.32	26.116	0.999
Overall Standard Deviation		0.034	0.08	0.0007	0.031	0.118	0.0007	0.042	0.06	0.001
Overall Relative Standard Deviation		1.10%	0.30%	0.10%	0.92%	0.47%	0.08%	1.28%	0.23%	0.10%

Comparing precision by serial dilution series

The Quantifiler Trio assay is designed to detect a short PCR amplicon (small autosomal), a long PCR amplicon (large autosomal) and a target-specific human male (Y) DNA. Small autosomal data provides a more accurate DNA estimation compared to large autosomal data and was used for STR amplification (Figure 2). The Y target quantification results are typically used for Y-STR amplification.

Average DNA quantities were assessed for the small autosomal target by analyzing samples of a serial dilution series in triplicate. Variation of replicate concentrations for each pipette/tip combination were shown to be similar across the dilution series, with relative standard deviations below 20% for concentrations of 20 pg/µL or greater. The highest variation was observed at the lowest DNA concentration of 7.8 pg/µL. It should be noted that this is 10-fold lower than the 1 ng DNA input threshold used for routine forensic STR amplifications. Furthermore, DNA input amounts less than 100 pg for STR amplification in forensic applications are likely to produce results below the stochastic threshold for standard interpretation[4].

The Quantifiler Trio kit can detect concentrations < 5 pg/µL; however, it is routinely accepted that samples at this range can contribute to more significant variability in results and any variations noted at concentrations below 10 pg/µL are anticipated and acceptable. Samples with DNA concentrations greater than 4 ng/µL are subject to competition during quantification from the IPC (Internal Positive Control), small autosomal, large autosomal, and Y target amplification reactions. Samples with high concentration input (> 4 ng/µl) are also subject to critical reagent consumption. These factors can inhibit all assays.



* indicates the sample, concentration, and pipette/tip combination at which the highest variation in relative standard deviation across the three replicates for each input was observed (37%)

Figure 2: Average quantity results for the small autosomal target across the three sensitivity replicates for each input. Error bars represent +/- one standard deviation.

Comparing the sensitivity by STR amplification and fragment analysis

The small autosomal quantification results from the samples of the serial dilution series were used for amplification with the GlobalFiler PCR Amplification Kit, with DNA input targets between 4 ng and 16 pg. The electropherograms were examined for each replicate and the number of alleles detected in each sample was recorded by pipette/tip combination and listed in Table 3. This study can be used to determine the acceptable limit of DNA input and when it is reasonable to expect a complete profile to be generated from a sample. Complete profiles were observed for the human DNA sample when at least 0.063 ng of DNA was amplified using the Rainin/Rainin, Rainin/SoftFit-L, and F1-Finn/ART Barrier pipettes and tips. The F1-ClipTip/ ClipTip tip combination was shown to detect all 42 alleles for each replicate at 0.031 ng DNA input but had 1 allele below the analysis threshold for two of the replicates at 0.063 ng DNA input. This was attributed to stochastic effects, such as sample input variability during amplification.

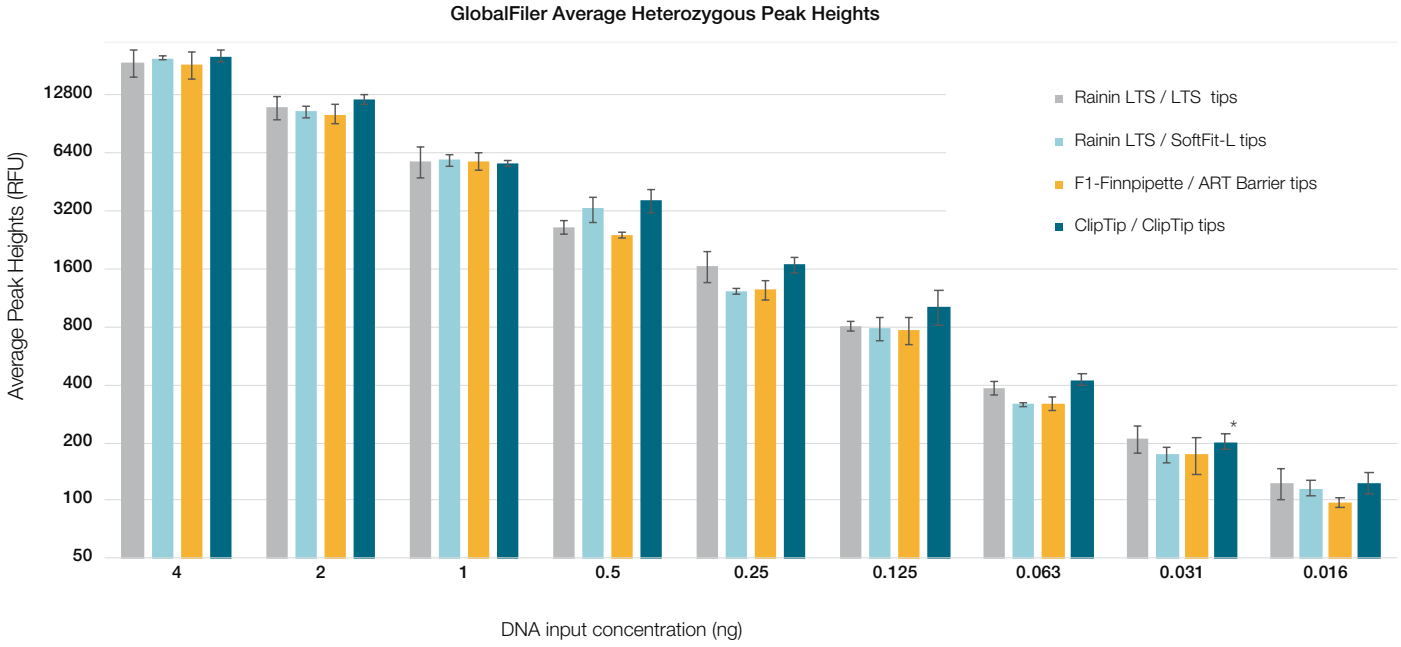
Table 3: Comparison of total allele counts for the Dilution Series using GlobalFiler PCR Amplification Kit (42 alleles represent a complete profile for the male DNA stock sample used for the Sensitivity Series).

Sample input (ng)	Rainin LTS/ LTS tips			Rainin LTS/SoftFit-L Tips			F1-Finnipettes/ART Barrier Tips			F1-ClipTip/ClipTip tips		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
4	42	42	42	42	42	42	42	42	42	42	42	42
2	42	42	42	42	42	42	42	42	42	42	42	42
1	42	42	42	42	42	42	42	42	42	42	42	42
0.5	42	42	42	42	42	42	42	42	42	42	42	42
0.25	42	42	42	42	42	42	42	42	42	42	42	42
0.125	42	42	42	42	42	42	42	42	42	42	42	42
0.063	42	42	42	42	42	42	42	42	42	41*	42	41*
0.031	39	42	39	40	39	38	38	42	39	42	42	42
0.016	39	38	37	32	28	32	31	30	30	33	34	37

*The one missing allele in these replicates can be attributed to stochastic effects, such as sample input variability during amplification.

Average heterozygous peak heights were calculated for each DNA input and pipette/tip combination obtained from the sample electropherograms and typing results. The average RFU values for the 1 ng target inputs ranged from 5674 RFU for the ClipTip data to 5902 RFU for the Rainin/SoftFit-L data, which are close in range and within the expected range for the specific instrument used in this study. The average peak heights were similar across all pipette/tip combinations when compared for each DNA amplification input, as illustrated in Figure 3. The graph also illustrated an approximate 2-fold stepdown for the average peak height RFU values between input DNA concentrations in the dilution series, trending as expected.

The variation of replicate average peak height calculations for each pipette and tip combination was similar across the dilution series, with relative standard deviations close to or below 20%. The highest variation was observed at 0.031 ng. The differences observed in this data set are within the expected variation associated with pipetting technique and electrokinetic injection of the detection systems.



* Indicates the sample, concentration, and pipette/tip combination at which the highest variation in relative standard deviation across the three replicates was observed (>20%). The 4ng DNA input does not show a 2-fold increase unlike the other concentrations as that amount of input tends to saturate the instrument.

Figure 3: Comparison of average heterozygous peak heights of Dilution Series using GlobalFiler PCR Amplification Kit with y-axis presented with a logarithmic scale at base 2. Error bars represent +/- one standard deviation.

Conclusions

The preparation and processing of standard curves using the various pipette/tip combinations and analysis of their quality metrics presented a minimal variation in overall standard deviation and % CV (between 0.1 and 1.2%). These results indicated comparable performances across all the pipette/tip combinations accessed in this study, and any minimal variation observed was well within the ranges expected from user operation.

The serial dilution series prepared and processed using the four pipette/tip combinations yielded consistent results for the average small autosomal quantity and relative standard deviations of replicate samples. The average concentrations were also consistent between the pipette/tip combinations. The similarity in total allele counts and average heterozygous peak heights across all pipette/tip combinations for each DNA sample indicated consistency in terms of precision and accuracy.

The study shows no discernable trends for higher accuracy or precision for any one pipette/tip combination. The Rainin LTS pipettes with Rainin LTS tips, Rainin LTS pipette with Thermo Scientific SoftFit-L tips, Thermo Scientific F1-Finnpipette with Thermo Scientific ART Barrier tips, Thermo Scientific F1-ClipTip pipette with Thermo Scientific ClipTips produced comparable data in terms of accuracy, precision, and sensitivity.

In conclusion,

- The F1-Finnpipette with ART Barrier Tips and F1 ClipTip pipette with Cliptip filtered tips perform equivalent to Rainin LTS in terms of accuracy and precision with no discernable variation.
- The data from this study shows that these products would pass the in-house comparison studies that forensic labs would need to perform as warranted by the QAS, SWGDAM and ENFSI if a switch in pipetting systems is necessary.

References

1. Quality Assurance Standards for Forensic DNA Testing Laboratories: 2020 July Quality Assurance Standards for Forensic DNA Testing Laboratories — FBI
2. Scientific Working Group on DNA Analysis Methods:2016 Validation Guidelines. SWGDAM
3. Guideline for the single laboratory Validation of instrumental and Human Based Methods in Forensic Science approved by European Network of Forensic Science Institutes (ENFSI): 10/11/2014 Guidance-QCC-VAL-002.pdf (enfsi.eu)
4. Validity of Low Copy Number Typing and Applications to Forensic Science. DOI: 10.3325/cmj.2009.50.207. Bruce Budowle1,2, Arthur J. Eisenberg1,2, Angela van Daal3

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