July 2024

Validation Report

Developmental validation of the Investigator® Quantiplex® Pro FLX

The Investigator Quantiplex Pro FLX Kit is intended for molecular biology applications in forensic, human identity, and paternity testing. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Human identification is commonly based on the analysis of short tandem repeats (STRs), or single nucleotide polymorphisms (SNPs). The choice of assay depends on the demands of the examination and on the sample quality. These types of multiplex assays used for human identification are complex systems that require a defined range of template input.

The Investigator Quantiplex Pro FLX Kit was developed for the quantification of total human genomic and human male DNA in a sample, using quantitative real-time PCR. The kit is designed to confirm whether a sample contains sufficient DNA to enable DNA fingerprinting analysis (e.g., STR or SNP analysis) and to enable the adjustment of the level of input DNA to the STR PCR for optimal performance. It also establishes whether a sample contains inhibitors that may interfere with downstream applications, thus necessitating further sample purification. Furthermore, the integrity of the DNA is assessed, such as whether the DNA has become degraded or fragmented due to temperature and humidity, among other factors.

The validation study was based on the recommendations of the European Network of Forensic Science Institutes (ENFSI) (1) and, where applicable, on the Revised Validation Guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM) (2).

The optimum amplification conditions for the Investigator Quantiplex Pro FLX Kit are given on page 4. A target validation was performed in an internal and external study (page 6). The kit was validated for reproducibility, repeatability (page 6), and sensitivity (page 11). It was tested for cross-reactivity with other species (page 16), and its performance with inhibitors (page 19) and contamination (page 28) was assessed. The quantification of male:female mixtures was also tested (page 33).

Validation of the Investigator Quantiplex Pro FLX Kit showed that it yielded robust and reproducible results within the normal range of conditions expected in forensic casework. The results of this study show that the kit is suitable for forensic casework, paternity testing, and other human identity testing applications.

Principle and procedure

The Investigator Quantiplex Pro FLX Kit is a lyophilized ready-to-use system for the detection of human and male DNA and parallel assessment of DNA degradation using quantitative real-time PCR. The kit provides fast and accurate quantification of human DNA in forensic database and casework samples.

The kit contains reagents and a DNA polymerase lyophilized in the wells of a 96-well optical PCR plate for specific amplification of 4NS1C®, which is a 91 bp proprietary multicopy region present on several autosomes of the human genome. It was selected to give high sensitivity with high reliability within different individuals and populations. The target region was validated in an internal and external study.

Furthermore, the kit detects a longer autosomal amplification product (353 bp) targeting the same locus (4NS1C) as the 91 bp autosomal target. Due to the differently sized autosomal targets, the longer autosomal target is more susceptible to DNA degradation, allowing for a precise assessment of the degradation status of the DNA.

The target region for male DNA quantification was selected in order to reliably give the same high sensitivity within different individuals and populations and in the presence of mixed DNA samples.

In addition, the Investigator Quantiplex Pro FLX Kit contains a balanced internal amplification control that is used to test successful amplification and identify the presence of PCR inhibitors.

The validation of the human targets used is described in the validation report of the Investigator Quantiplex Pro Kit (**qiagen.com/HB-2496**).

Detection of amplification is performed using TaqMan® probes and a novel, fast PCR chemistry. Dual-labeled probes, such as TaqMan probes, contain a fluorescent reporter and a quencher at their 5' and 3' ends, respectively. During the extension phase of PCR, the 5' and 3' exonuclease activity of the DNA Polymerase cleaves the fluorophore from the quencher. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

Instrumentation for validation

All of the validation experiments in this Validation Report were performed on the following instruments:

- Applied Biosystems 7500 Real-Time PCR System for Human Identification
- QuantStudio 5 Real-Time PCR System

Amplification conditions

The amplification conditions developed during validation are shown in Tables 1 and 2 (pages 4 and 5). The assay allows for flexible input volumes of 1–18 µL. Reaction conditions were established for optimal performance in terms of sensitivity, specificity, and reproducibility.

For the Applied Biosystems 7500 Real-Time PCR System for Human Identification, HID Real-Time PCR Analysis software version 1.2 was used in Custom Assays mode. For the Applied Biosystems 7500 Real-Time PCR System, SDS Software version 1.4.0.25 was used, and for QuantStudio 5 Real-Time PCR System, the QuantStudio Design and Analysis Software version 1.5.2 was used.

Table 1. Reaction setup

Component	1 reaction
Quantiplex Pro FLX Reaction Mix and Primer Mix	Lyophilized cake
Sample	Variable (1–18 µL)
Water	Fill up to 18 µL
Total reaction volume	**20 µL***

* Lyophilized cake + 18 µL input volume will yield a final PCR reaction volume of 20 µL.

Table 2. Cycling protocol

Step	Temperature (°C)	Time	No. of cycles	Remarks
Initial PCR activation step	98	3 min	–	PCR requires an initial incubation at 98°C to activate the DNA polymerase
Denaturation	98	5 s	40	–
Combined annealing/extension	65	35 s		Perform fluorescence data collection

Note: Always use a compression pad if using the entire plate. If using 8-tube strips, please follow the PCR cycler manufacturer's recommendation for the use of strips.

Results of developmental validation

Reproducibility and repeatability

Reproducibility and repeatability (or intra-run precision) are critical in forensic analysis to ensure consistency of results. These were tested to ensure sample-to-sample reproducibility.

Following the ENFSI guidelines, we tested reproducibility (the variation in average measurements obtained when 2 or more people measure the same parts or items, using the same measuring technique) and repeatability (the variation in measurements obtained when one person measures the same unit, with the same measuring equipment).

Reproducibility and repeatability were tested on the Applied Biosystems 7500 Real-Time PCR System for Human Identification and on the QuantStudio 5 Real-Time PCR System. This was done by taking 5 replicates of the 4 standard dilutions and the no-template control (NTC), and 5 replicates of 3 male and 3 female DNAs.

Dilutions were made using the QuantiTect® Nucleic Acid Dilution buffer. Each sample was quantified twice using the same instrumentation by the same operator (repeatability) and by a second operator (reproducibility).

The runs were set up independently. Tables 3–6 (starting on the next page) show the data from the study. The mean quantity and standard deviation (σ) were calculated for each sample dilution.

The reproducibility and repeatability of the DNA quantification using the Investigator Quantiplex Pro FLX Kit was demonstrated for both instruments.

Table 3. Results comparing 2 different runs performed by 2 different operators on the same Applied Biosystems 7500 Real-Time PCR System for Human Identification

		Operator 1		Operator 2	
Target	**DNA sample**	**Conc. (ng/µL) ± σ**	**CV**	**Conc. (ng/µL) ± σ**	**CV**
Degradation	Male 1	4.47 ± 0.56	12.5%	4.2 ± 0.52	12.4%
	Male 2	17.5 ± 1.01	5.8%	15.54 ± 1.13	7.3%
	Male 3 (NIST 2372a)	0.78 ± 0.08	10.3%	0.83 ± 0.09	10.8%
	Female 1	3.67 ± 0.21	5.7%	3.3 ± 0.3	9.1%
	Female 2	10.13 ± 0.75	7.4%	8.75 ± 0.86	9.8%
	Female 3 (NIST 2372a)	0.89 ± 0.02	2.2%	0.87 ± 0.05	5.7%
Human	Male 1	4.58 ± 0.76	16.6%	4.29 ± 0.69	16.1%
	Male 2	18.15 ± 1.85	10.2%	15.72 ± 1.69	10.8%
	Male 3 (NIST 2372a)	0.8 ± 0.11	13.8%	0.88 ± 0.1	11.4%
	Female 1	3.98 ± 0.62	15.6%	3.53 ± 0.48	13.6%
	Female 2	11.53 ± 0.97	8.4%	10.28 ± 1.07	10.4%
	Female 3 (NIST 2372a)	0.92 ± 0.07	7.6%	0.91 ± 0.06	6.6%
Male	Male 1	3.85 ± 0.43	11.2%	3.76 ± 0.39	10.4%
	Male 2	13.72 ± 0.69	5.0%	12.91 ± 1.02	7.9%
	Male 3 (NIST 2372a)	0.64 ± 0.06	9.4%	0.65 ± 0.08	12.3%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372a)	NA	NA	NA	NA

Table 4. Results comparing 2 different runs performed by 2 different operators on the same QuantStudio 5 Real-Time PCR System

		Operator 1		Operator 2	
Target	**DNA sample**	**Conc. (ng/µL) ± σ**	**CV**	**Conc. (ng/µL) ± σ**	**CV**
Degradation	Male 1	4.33 ± 0.17	3.9%	4.34 ± 0.24	5.5%
	Male 2	16.62 ± 1.5	9.0%	16.77 ± 1.31	7.8%
	Male 3 (NIST 2372a)	0.89 ± 0.04	4.5%	0.87 ± 0.03	3.4%
	Female 1	3.58 ± 0.34	9.5%	3.52 ± 0.3	8.5%
	Female 2	10.65 ± 0.43	4.0%	10.48 ± 0.43	4.1%
	Female 3 (NIST 2372a)	0.83 ± 0.08	9.6%	0.78 ± 0.07	9.0%
Human	Male 1	4.62 ± 0.17	3.7%	4.85 ± 0.27	5.6%
	Male 2	16.08 ± 1.6	10.0%	17.02 ± 1.8	10.6%
	Male 3 (NIST 2372a)	0.95 ± 0.04	4.2%	0.96 ± 0.05	5.2%
	Female 1	3.84 ± 0.32	8.3%	4.05 ± 0.16	4.0%
	Female 2	11.49 ± 0.45	3.9%	11.43 ± 1	8.7%
	Female 3 (NIST 2372a)	0.87 ± 0.07	8.0%	0.9 ± 0.07	7.8%
Male	Male 1	3.72 ± 0.32	8.6%	3.7 ± 0.33	8.9%
	Male 2	14.08 ± 1.24	8.8%	13.67 ± 0.79	5.8%
	Male 3 (NIST 2372a)	0.71 ± 0.07	9.9%	0.7 ± 0.04	5.7%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372a)	NA	NA	NA	NA

Table 5. Results comparing 2 different runs performed by the same operator using the same Applied Biosystems 7500 Real-Time PCR System for Human Identification

		Operator 1/Run 1		Operator 1/Run 2	
Target	**DNA sample**	**Conc. (ng/µL) ± σ**	**CV**	**Conc. (ng/µL) ± σ**	**CV**
Degradation	Male 1	4.47 ± 0.56	12.5%	4.25 ± 0.56	13.2%
	Male 2	17.5 ± 1.01	5.8%	16 ± 1.25	7.8%
	Male 3 (NIST 2372a)	0.78 ± 0.08	10.3%	0.84 ± 0.11	13.1%
	Female 1	3.67 ± 0.21	5.7%	3.29 ± 0.28	8.5%
	Female 2	10.13 ± 0.75	7.4%	9.37 ± 1.2	12.8%
	Female 3 (NIST 2372a)	0.89 ± 0.02	2.2%	0.85 ± 0.04	4.7%
Human	Male 1	4.58 ± 0.76	16.6%	4.31 ± 0.66	15.3%
	Male 2	18.15 ± 1.85	10.2%	15.6 ± 2.07	13.3%
	Male 3 (NIST 2372a)	0.8 ± 0.11	13.8%	0.85 ± 0.1	11.8%
	Female 1	3.98 ± 0.62	15.6%	3.4 ± 0.43	12.6%
	Female 2	11.53 ± 0.97	8.4%	10.55 ± 1.47	13.9%
	Female 3 (NIST 2372a)	0.92 ± 0.07	7.6%	0.89 ± 0.06	6.7%
Male	Male 1	3.85 ± 0.43	11.2%	3.61 ± 0.43	11.9%
	Male 2	13.72 ± 0.69	5.0%	13.37 ± 0.78	5.8%
	Male 3 (NIST 2372a)	0.64 ± 0.06	9.4%	0.66 ± 0.11	16.7%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372a)	NA	NA	NA	NA

Table 6. Results comparing 2 different runs performed by the same operator using the same QuantStudio 5 Real-Time PCR System

		Operator 1/Run 1		Operator 1/Run 2	
Target	**DNA sample**	**Conc. (ng/µL) ± σ**	**CV**	**Conc. (ng/µL) ± σ**	**CV**
Degradation	Male 1	4.33 ± 0.17	3.9%	4.43 ± 0.23	5.2%
	Male 2	16.62 ± 1.5	9.0%	16.77 ± 1.56	9.3%
	Male 3 (NIST 2372a)	0.89 ± 0.04	4.5%	0.83 ± 0.03	3.6%
	Female 1	3.58 ± 0.34	9.5%	3.54 ± 0.36	10.2%
	Female 2	10.65 ± 0.43	4.0%	10.57 ± 0.45	4.3%
	Female 3 (NIST 2372a)	0.83 ± 0.08	9.6%	0.78 ± 0.05	6.4%
Human	Male 1	4.62 ± 0.17	3.7%	4.73 ± 0.19	4.0%
	Male 2	16.08 ± 1.6	10.0%	16.68 ± 1.76	10.6%
	Male 3 (NIST 2372a)	0.95 ± 0.04	4.2%	0.9 ± 0.02	2.2%
	Female 1	3.84 ± 0.32	8.3%	3.96 ± 0.21	5.3%
	Female 2	11.49 ± 0.45	3.9%	11.93 ± 0.55	4.6%
	Female 3 (NIST 2372a)	0.87 ± 0.07	8.0%	0.84 ± 0.03	3.6%
Male	Male 1	3.72 ± 0.32	8.6%	3.74 ± 0.26	7.0%
	Male 2	14.08 ± 1.24	8.8%	14.66 ± 1.09	7.4%
	Male 3 (NIST 2372a)	0.71 ± 0.07	9.9%	0.65 ± 0.03	4.6%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372a)	NA	NA	NA	NA

Sensitivity and linearity

The Investigator Quantiplex Pro FLX Kit is designed to detect a broad range of DNA quantities. Figures 1–6 show a serial dilution of Male Control DNA M1 from 50 ng/µL to 0.015625 pg/µL. The sample input volume was 2 µL and the standard conditions specified in the Investigator Quantiplex Pro FLX Kit Handbook have been used for both instruments (Applied Biosystems 7500 Real-Time PCR Systems for Human Identity and QuantStudio 5 Real-Time PCR System). Triplicates were done for the range 50 ng/µL to 0.005 ng/µL and 6 replicates for DNA concentration below 0.005 ng/µL. The optimal linear dynamic range of the assay is in the range of 50 ng/µL to 0.5 pg/µL total DNA. On the Applied Biosystems 7500 Real-Time PCR Systems for Human Identity, detection limits have been found to be 0.25 pg/µL for human and degradation, and 0.5 pg/µL for male. On the QuantStudio 5 Real-Time PCR System, DNA could be detected for the degradation and human target down to 0.125 pg/µL, and for the male target, down to 0.25 pg/µL.

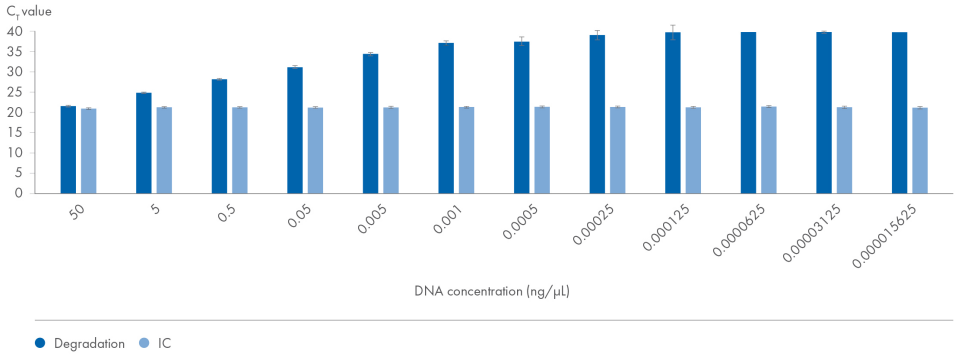

Figure 1. Detection of the degradation target in male DNA down to 0.015625 pg/µL using the Investigator Quantiplex Pro FLX Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation.

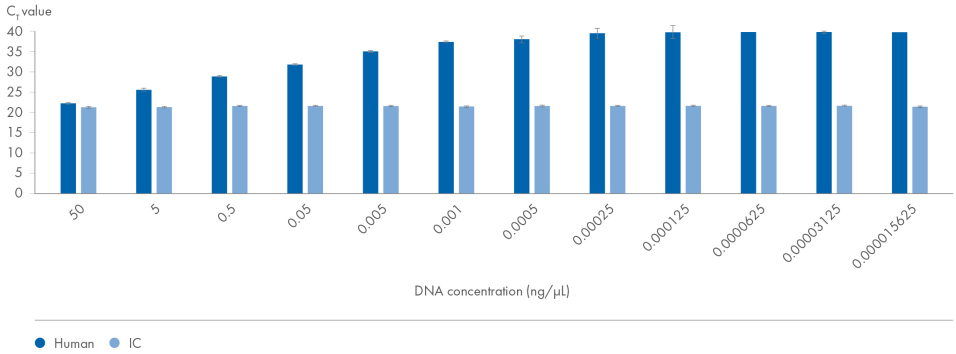

Figure 2. Detection of human target in male DNA down to 0.015625 pg/µL using the Investigator Quantiplex Pro FLX Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation.

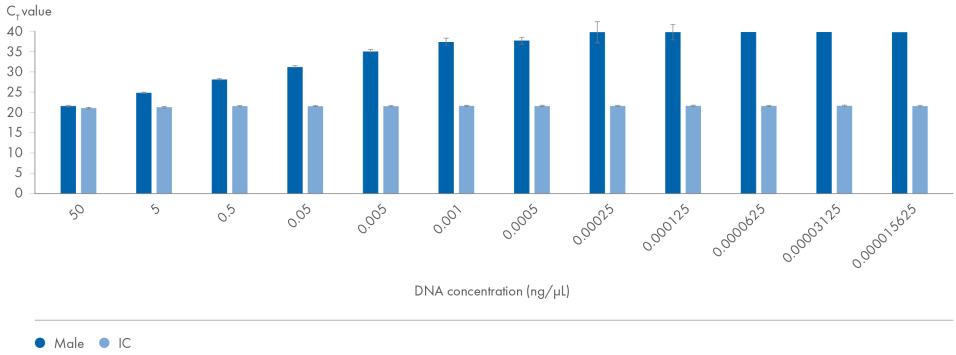

Figure 3. Detection of the male target in male DNA down to 0.015625 pg/µL using the Investigator Quantiplex Pro FLX Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation.

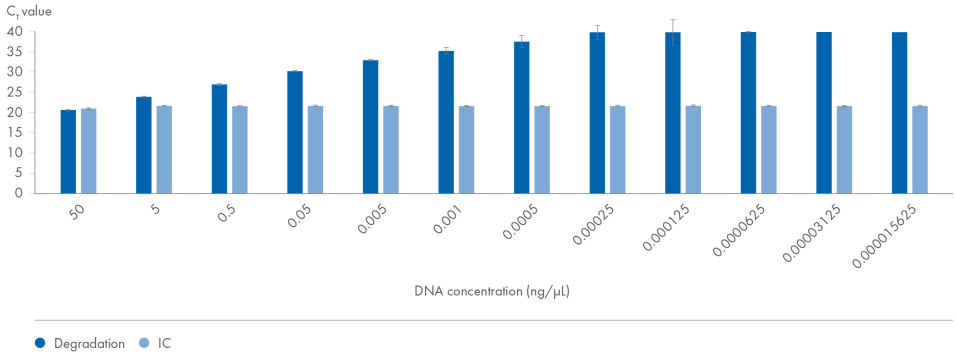

Figure 4. Detection of the degradation target in male DNA down to 0.015625 pg/µL using the Investigator Quantiplex Pro FLX Kit on QuantStudio 5 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

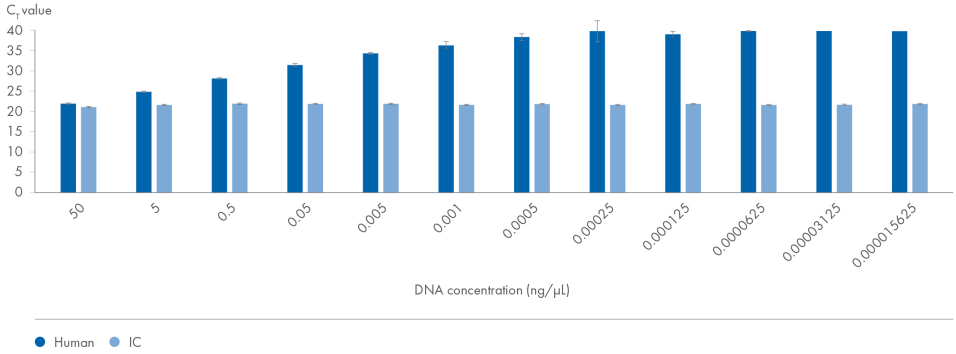

Figure 5. Detection of human target in male DNA down to 0.015625 pg/µL using the Investigator Quantiplex Pro FLX Kit on QuantStudio 5 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

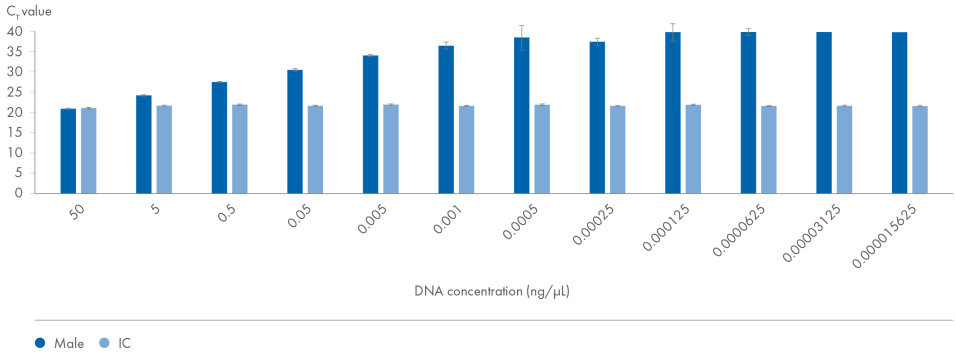

Figure 6. Detection of the male target in male DNA down to 0.015625 pg/µL using the Investigator Quantiplex Pro FLX Kit on QuantStudio 5 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

The Quantiplex Pro FLX Kit can be used with flexible sample input volumes, which allows to adjust sensitivity to the needs of a laboratory. In order to investigate the correlation of sample input and quantified amount of DNA, increasing volumes from 2–16 µL of a purified DNA were amplified. Linear correlation was observed across all 3 quantification targets. The concentration determined for the sample is unaffected (Figure 7, next page). Note that since the quantification standard series concentration values refer to 2 µL input volume, correction factor needs to be applied for samples for any input volume other than 2 µL. A table with correction factors is provided in the *Investigator Quantiplex Pro FLX Kit Handbook* (**qiagen.com/HB-3538**).

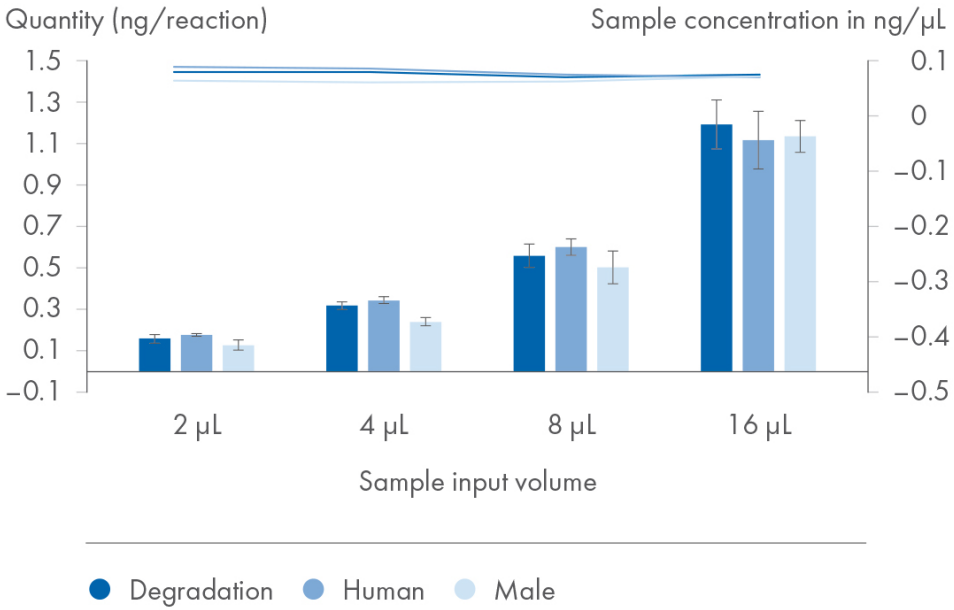

Figure 7. Correlation of sample input volume and quantification result. Extracted male DNA was quantified with input volumes of 2–16 µL on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. Bars show the total amount of DNA quantified per reaction, lines show the DNA concentration of the sample itself, calculated using the corresponding correction factors.

Species specificity

Non-human DNA is commonly present in forensic casework samples. It is critical that quantification assays show no cross-reactivity between species to provide an accurate determination of total human DNA within a sample.

To verify Investigator Quantiplex Pro FLX Kit's species specificity, 2.5 ng of DNA from vertebrate species, commonly found at crime scenes, was examined. As a positive control, 2.5 ng of Male Control DNA M1 was used.

No cross-reactivity was shown for DNA from the tested common vertebrates, as shown in Figure 8.

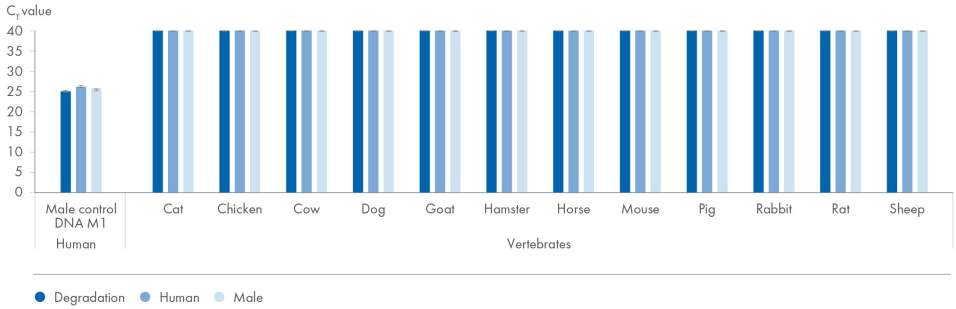

Figure 8. Results of a cross-reactivity study on common vertebrate species. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

Some primates, including gorillas, chimpanzees, bonobo, orangutans, and macaques were also examined, as described above. Due to the evolutionary proximity of these primates to humans, positive results were observed for these species' DNA (Figure 9, next page).

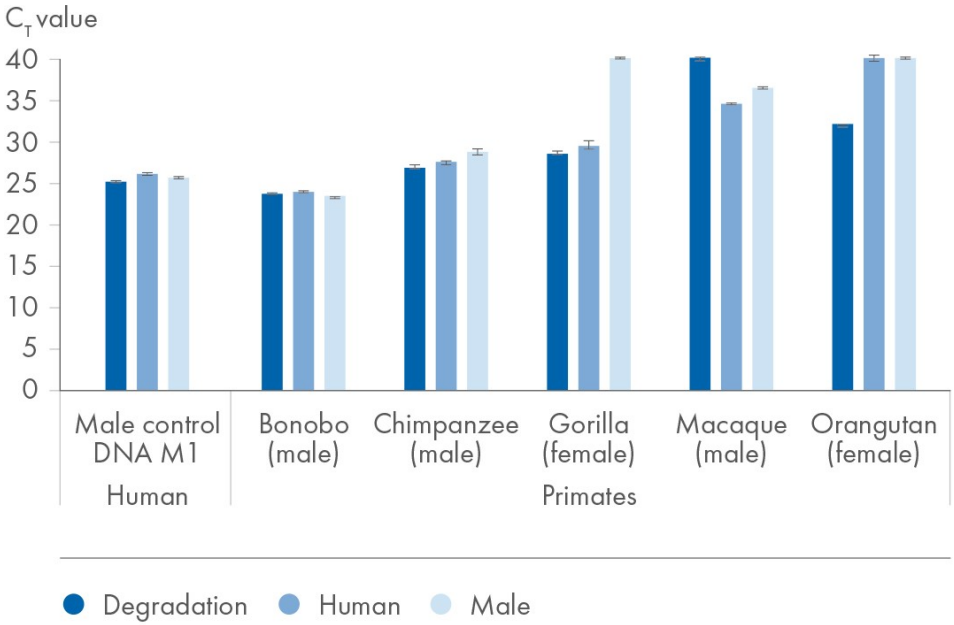

Figure 9. Results of a cross-reactivity study on primates. The figure shows the average C_T ± standard deviation.
NTC: No-template control.

Crime scene stains are frequently contaminated with bacteria and fungi. Therefore, it is critical that these species do not interfere with the accurate determination of total human DNA. DNA samples from *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Staphylococcus aureus* (2.5 ng of each) were tested, with 2.5 ng Male Control DNA M1 as a positive control. None of the tested microbial species yielded detectable DNA, as shown in Figure 10 (next page).

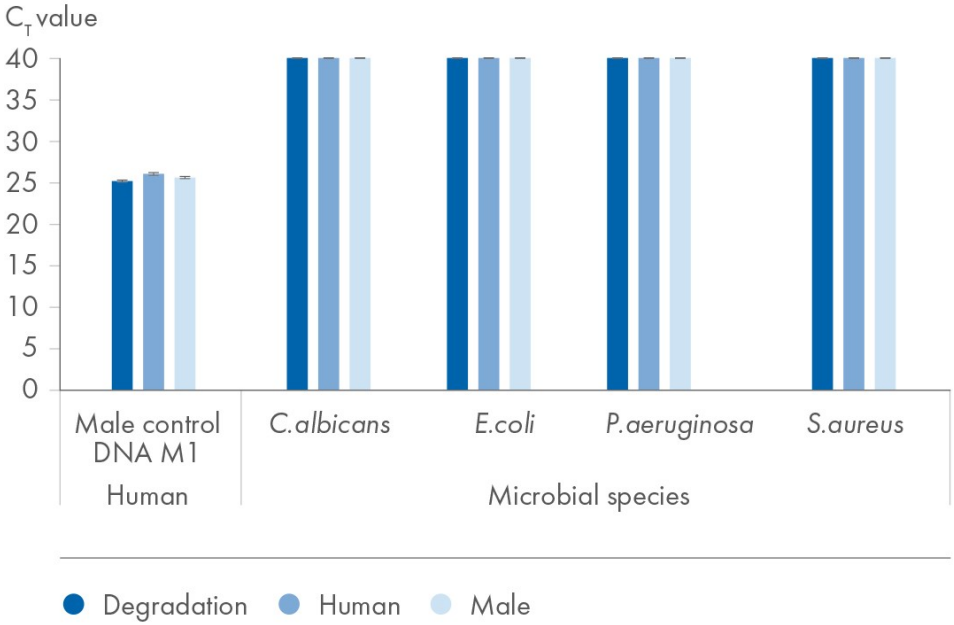

Figure 10. Results of a cross-reactivity study on microbial species. No cross-reactivity could be shown for the tested microbes. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

The results show that the Investigator Quantiplex Pro FLX Kit assay provides a determination of total DNA specific to humans and some primates.

In conclusion, these experiments show that the Investigator Quantiplex Pro FLX Kit assay offers a robust quantification solution for DNA with high specificity for humans.

Performance with simulated inhibition

QIAGEN's sample preparation technology is recommended for extraction because it yields pure DNA free of inhibitors. If DNA is extracted from forensic casework samples using inappropriate methods, STR assay performance may be compromised.

The Investigator Quantiplex Pro FLX Kit contains a 434 bp internal control that was developed to provide information about the presence of inhibitors within a sample. The change in C_T value of the internal control in comparison to non-inhibited samples, such as standard curve samples, provides the user with information regarding the likelihood of successful STR amplification. Note that the correlation of quantification internal control response and STR assay impact depends on the input volume used in both assays. We recommend to determine this correlation within the internal validation study laboratories conduct. Input volume of 2 µL has been used in this study, with 66 pg Male Control DNA M1 per reaction. Inhibitor concentrations stated are final concentrations in 20 µL PCR reaction volume. Note that using higher sample input volumes in general increases the risk of observing inhibition during quantification.

Humic acid

Humic acid, a principal component of humic substances, has an inhibitory effect on PCR. It is often co-purified and co-extracted from forensic samples collected from soil.

To test the robustness of the kit, the assay was run in the presence of 0, 25, 33.33, 41.67, 50, 58.33, and 66.67 ng/µL humic acid (Acros®; cat. no. 120860050). The results are shown in Figures 11 and 12.

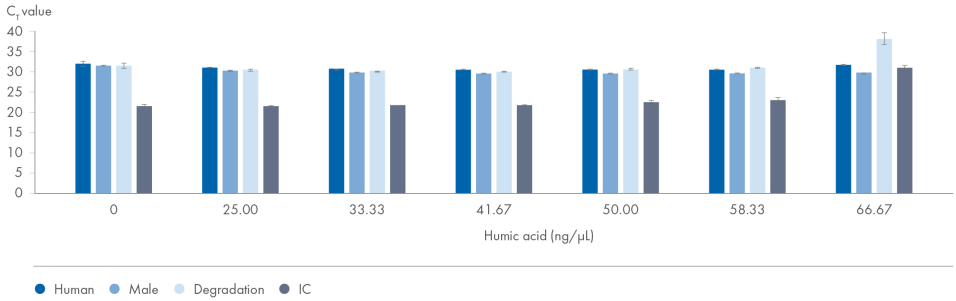

Figure 11. Performance with simulated humic acid inhibition on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control (IC) reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 66.67 ng/µL. The degradation target is susceptible to humic acid. The figure shows the average C_T ± standard deviation.

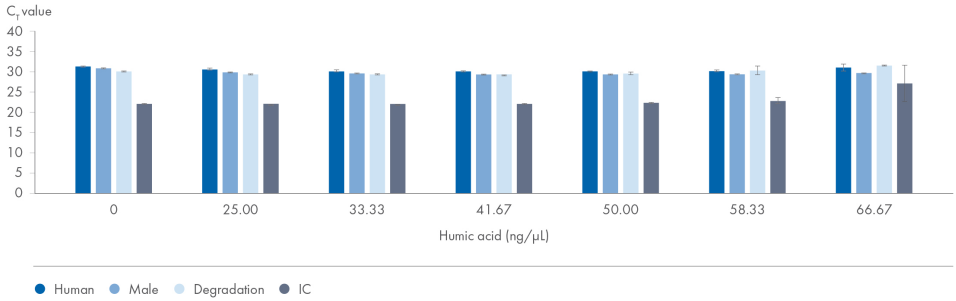

Figure 12. Performance with simulated humic acid inhibition on the QuantStudio 5 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 66.67 ng/µL. The degradation target is susceptible to humic acid. The figure shows the average C_T ± standard deviation.

It was shown that the internal control acts as a quality sensor and reports the presence of the inhibitor with a C_T shift while quantification for the human and male targets remains reliable up to a final humic acid concentration of 66.67 ng/µL in the PCR. This corresponds to a concentration in the DNA sample of 1334 ng/µL when using 2 µL DNA sample in the assay. Similar inhibitor resistance was confirmed for both validated instruments. Humic acid can have an impact on the large human autosomal target due to the large amplicon size, but the presence of humic acid was reliably reported by the IC.

Hematin

Hematin is formed by the oxidation of heme, the main component of blood. It has been identified as a PCR inhibitor in DNA samples extracted from bloodstains. Its interfering effect is related to the inhibition of polymerase activity.

To test the robustness of the kit, the assay was run in the presence of 0, 125, 166.67, 208.33, 250, 291.67, and 333.33 µM hematin (ICN Biomedicals Inc.; cat. no. 198969). The results are shown in Figures 13 and 14.

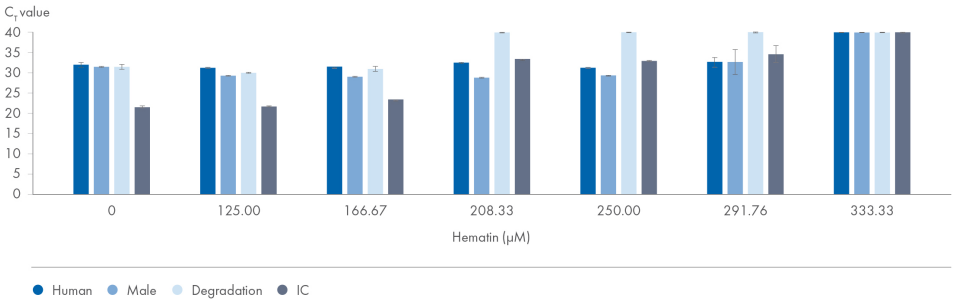

Figure 13. Performance with simulated hematin inhibition on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 250 µM. The degradation target is susceptible to hematin at higher concentrations. The figure shows the average C_T ± standard deviation.

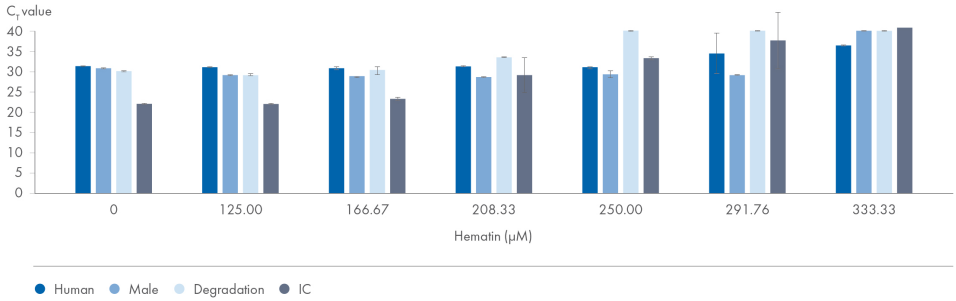

Figure 14. Performance with simulated hematin inhibition on the QuantStudio 5 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 250 µM. The degradation target is susceptible to hematin at higher concentrations. The figure shows the average C_T ± standard deviation.

It was shown that the internal control acts as quality sensor and reports the presence of the inhibitor with a C_T shift while quantification for the human and male targets remains reliable up to a hematin concentration of 250 µM (final concentration in the reaction). This corresponds to a concentration in the DNA sample of 6666 µM using 2 µL DNA sample in the assay. Hematin can have an impact on the large human autosomal target due to the large amplicon size, but the presence of hematin was reliably reported by the IC. Similar inhibitor resistance was confirmed for both validated instruments.

Calcium

Calcium is a major inorganic component of bones and teeth. Inhibition by calcium reduces the efficiency of the amplification and shows evidence of limiting reagents (3).

To test the robustness of the kit, the assay was run in the presence of 0, 0.5, 0.67, 0.83, 1, 1.17, and 1.33 mM calcium hydrogen phosphate (VWR®; cat. no. 83524.290). The results are shown in Figures 15 and 16. No inhibitory effect could be observed for the tested concentrations.

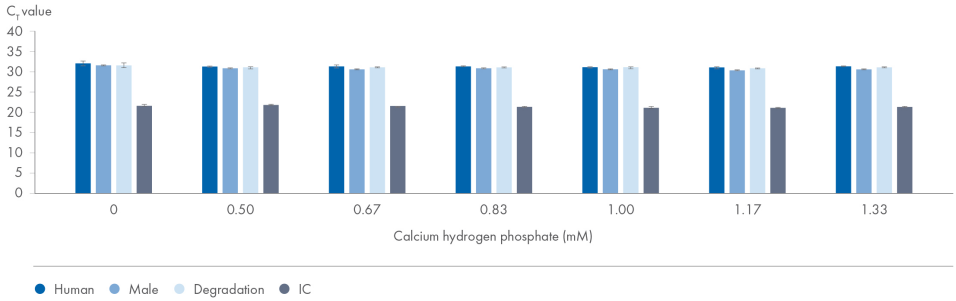

Figure 15. Performance with simulated inhibition effect of calcium hydrogen phosphate on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The quantification is reliable up to a concentration of 1.33 mM. The figure shows average C_T ± standard deviation.

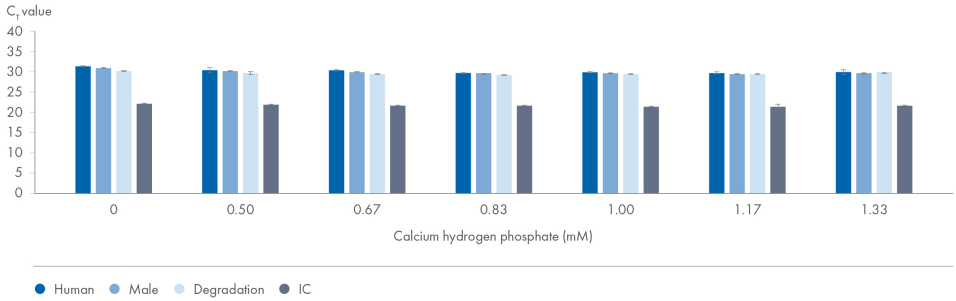

Figure 16. Performance with simulated inhibition effect of calcium hydrogen phosphate on the QuantStudio 5 Real-Time PCR System. The quantification is reliable up to a concentration of 1.33 mM. The figure shows average C_T ± standard deviation.

Tannic acid

Tannic acid is an agent found in leather, as well as in certain types of plant material. It may also be encountered in samples that have been exposed to leaf litter. Tannic acid is supposed to be a DNA polymerase inhibitor that also affects availability of the DNA template (3).

To test the robustness of the kit, the assay was run in the presence of 0, 333.33, 500, 666.67, 833.33, 1000, and 1166.67 ng/µL tannic acid (Sigma-Aldrich®; cat. no. 403040). The results are shown in Figures 17 and 18.

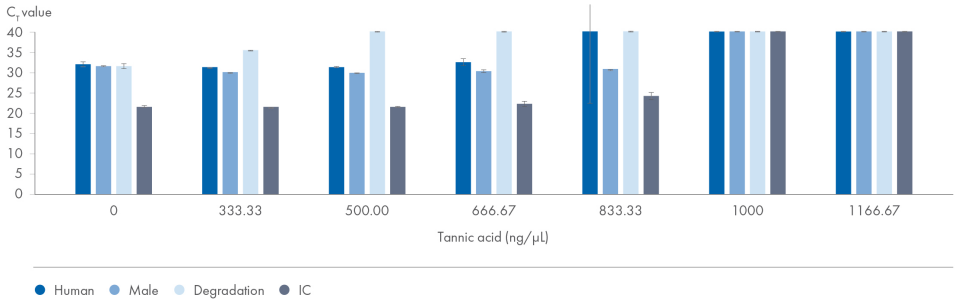

Figure 17. Performance with simulated inhibition effect of tannic acid on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 666.67 ng/µL. The degradation target is susceptible to tannic acid. The figure shows average C_T ± standard deviation.

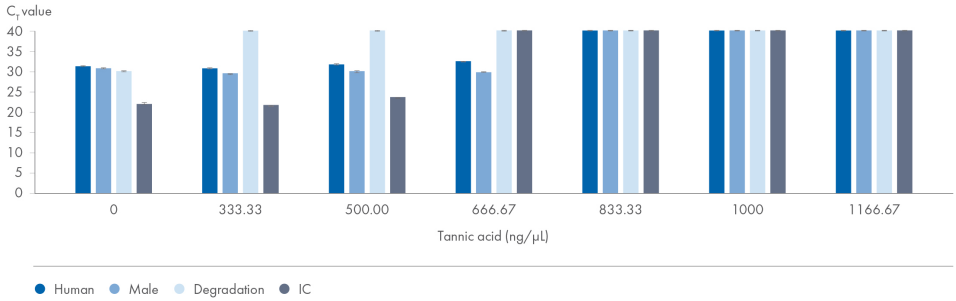

Figure 18. Performance with simulated inhibition effect of tannic acid on QuantStudio 5 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 666.67 ng/µL. The degradation target is susceptible to tannic acid at concentrations higher than 333.33 ng/µL. The figure shows average C_T ± standard deviation.

It was shown that the internal control acts as a quality sensor and reports the presence of the inhibitor with a C_T shift while quantification for the human and male targets remains reliable up to a final tannic acid concentration of 666 µM (final concentration in the reaction). Tannic

acid can have an impact on the large human autosomal target due to the large amplicon size, but the presence of tannic acid was reliably reported by the IC.

Collagen

Collagen is the main protein compound of many tissues. Collagen is supposed to inhibit DNA polymerase activity.

To test the robustness of the kit, the assay was run in the presence of 0, 33.33, 41.67, 50, 58.33, 66.67, and 75 ng/µL collagen (Sigma-Aldrich; cat. no. 403040).

The effect of collagen is shown in Figures 19 and 20. No inhibitory effect could be observed for the concentrations tested.

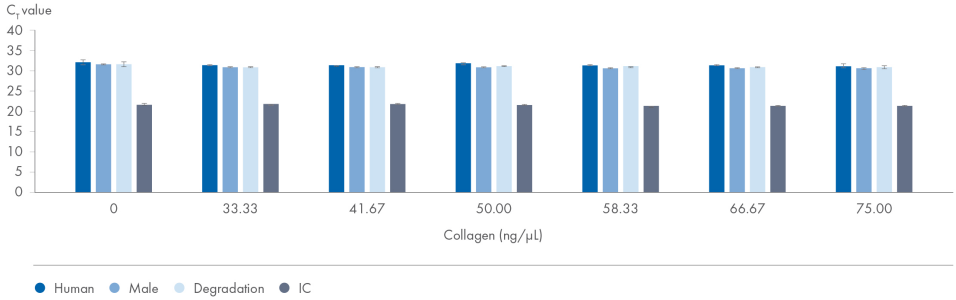

Figure 19. Performance with simulated inhibition effect of collagen on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The quantification is reliable up to a concentration of 75 ng/µL. The figure shows average C_T ± standard deviation.

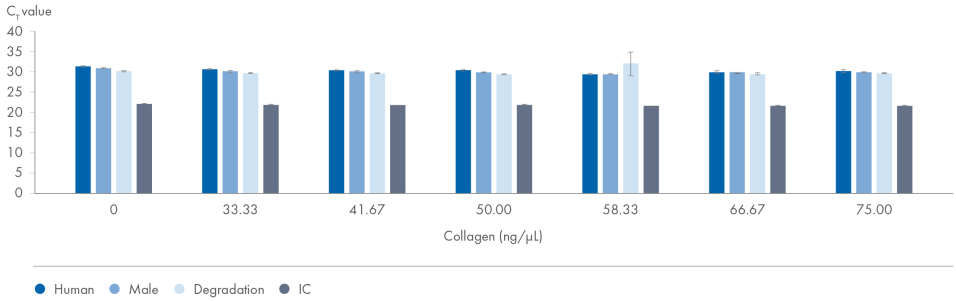

Figure 20. Performance with simulated inhibition effect of collagen on the QuantStudio 5 Real-Time PCR System. The quantification is reliable up to a concentration of 75 ng/µL. The figure shows average CT ± standard deviation.

Ethanol

Ethanol is a potential carryover of the DNA extraction methods. To test the robustness of the kit, the assay was run in the presence of 0, 0.17, 0.33, 0.67, 0.89, 1.14, and 1.39% ethanol. The results are shown in Figure 21 and 22. No inhibitory effect could be observed for the concentrations tested.

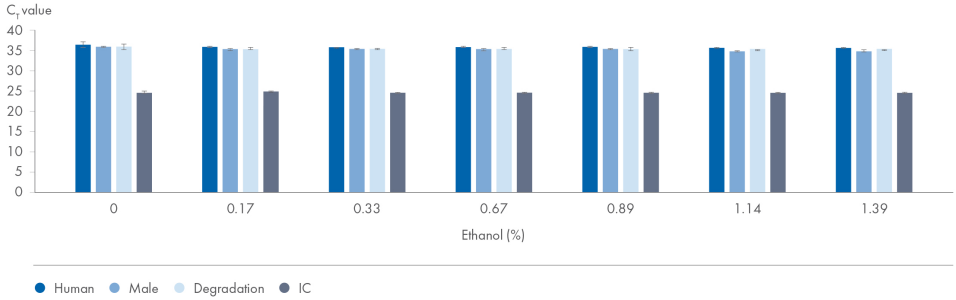

Figure 21. Performance with simulated inhibition effect of ethanol on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The quantification is reliable up to a concentration of 1.39% ethanol. The figure shows average C_T ± standard deviation.

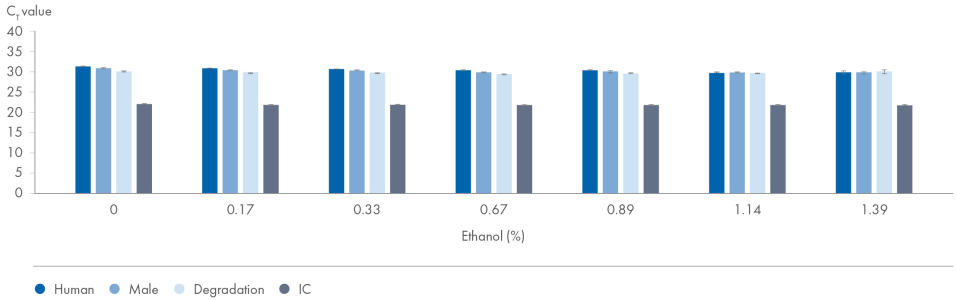

Figure 22. Performance with simulated inhibition effect of ethanol on the QuantStudio 5 Real-Time PCR System. The quantification is reliable up to a concentration of 1.39% ethanol. The figure shows average C_T ± standard deviation.

Contamination of reagents

Laboratory contamination of one of the reagents contained in the Investigator Quantiplex Pro FLX Kit may result in a false positive in the quantification reaction. Contamination studies were performed to exclude reagent contamination. One run is shown as an example (Figure 23). In total, 94 no-template controls and 2 positive controls (Male Control DNA M1; 50 ng/µL) were analyzed.

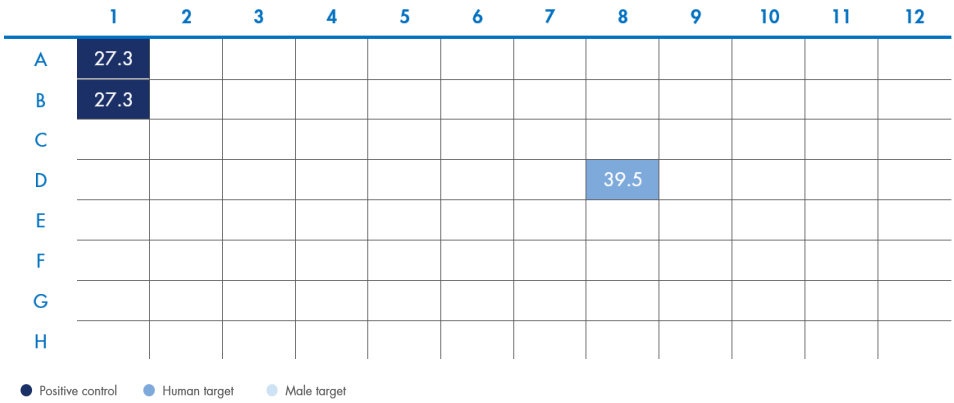

Figure 23. Results of the NTC run. Depicted are the C_T values for each detected target and their position on the PCR plate. Positive controls are in A1 and B1.

One sample did produce a detectable C_T value for the human target close to the detection limit. Presence of detectable human DNA was not confirmed with all 3 targets for human, degradation, and male in any of the samples, except for the positive controls.

Stability

Stability of the lyophilized chemistry

The Quantiplex Pro FLX Kit provides the reaction chemistry lyophilized in the wells of a PCR plate. When exposed to air, the lyophilized chemistry (called "cake") can start to rehydrate, a process that strongly depends on the level of humidity. We do not recommend to use the assay at a humidity of more than 60%. Plates should always be stored in the aluminum bag with desiccant. In order to test the impact of collapsed cakes on assay performance, an open plate was incubated in a climate chamber at a humidity of 90% for 90 min. Alternatively, closed plates in the aluminum bag with desiccant were stored over a period of 4 weeks at a humidity of 90%. Only open plates were visibly affected by the humidity. Collapsed cakes were redissolved by prolonged incubation with added sample until no visible residues remained (Figure 24 on the next page). PCR results did not reveal any difference to reactions that had not been exposed to high humidity (Figure 25 on page 31).

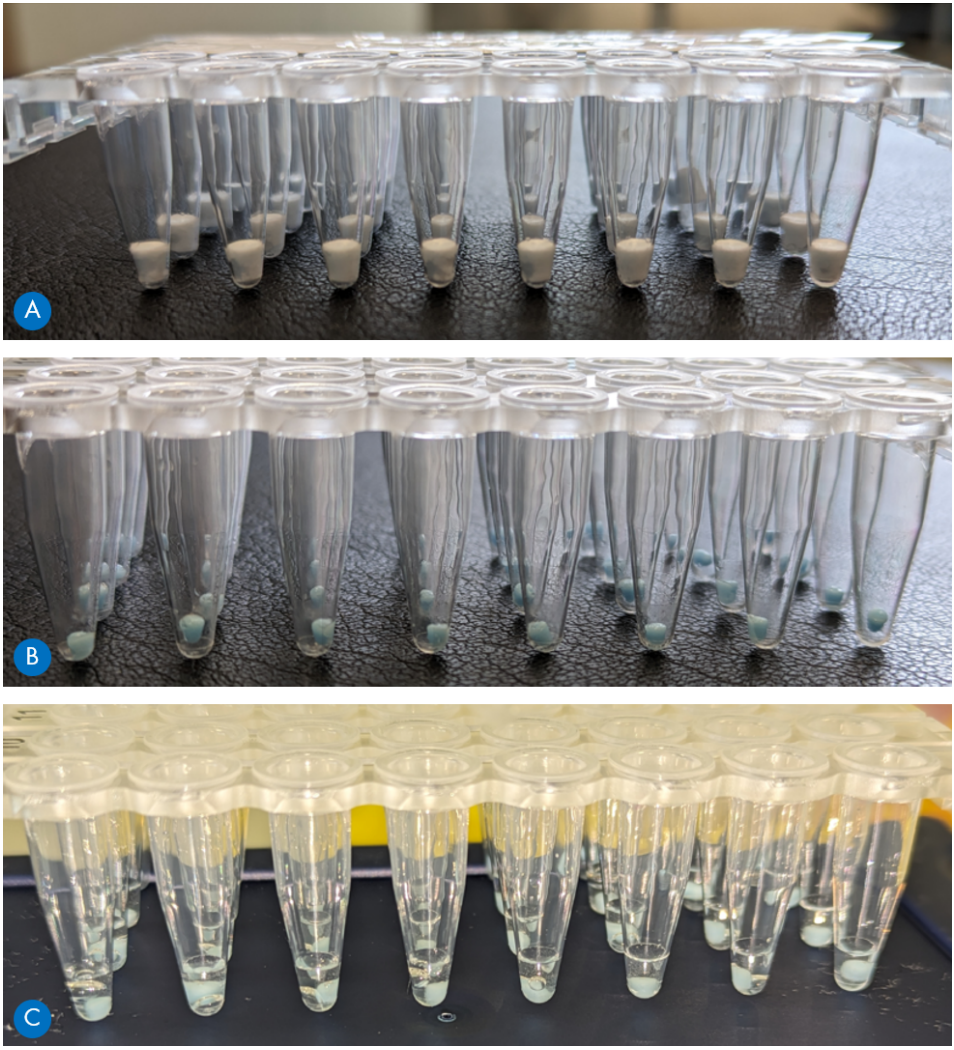

Figure 24. Shape of lyophilized chemistry (cakes) when exposed to humidity. The pictures show representative examples of cakes. **A)** No exposure. **B)** Open plate exposed to 90% humidity for 90 min. **C)** Directly after addition of sample to collapsed cakes. Note that the cake is still partly unsolved.

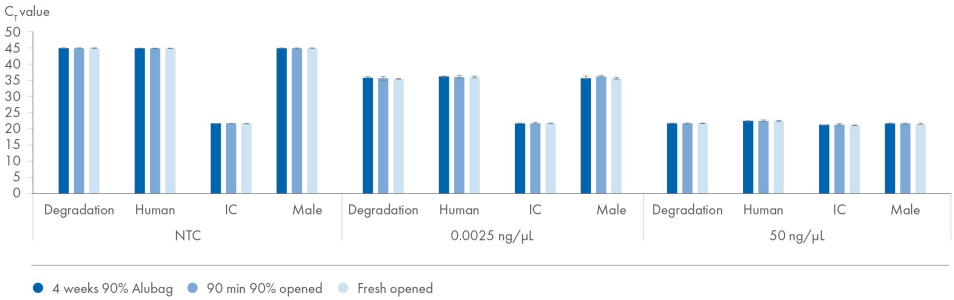

Figure 25. PCR results of reactions exposed to humidity. The results show no relevant differences whether cakes have collapsed due to humidity, or not exposed. The figure shows average C_T ± standard deviation.

Stability of reactions

In particular when PCR setup is done on automated systems, time can pass between setup and start of the cycling protocol. Here we tested the potential impact of incubations of final reactions on performance. Fully set-up plates were left for up to 6 hours at room temperature, or 24 hours at 2–8°C. No significant changes of C_T values for any of the targets were observed, and no artificial amplification happened in negative controls (Figure 26 below).

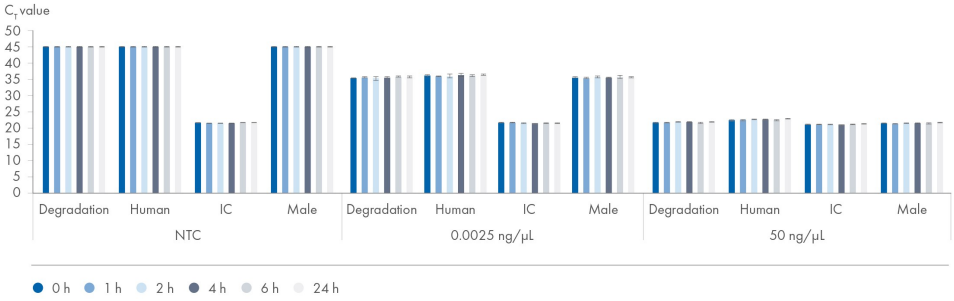

Figure 26. PCR results of reconstituted reactions with incubations before PCR cycling was started. The results show no relevant differences up to 6 hours room temperature, or 24 hours at 2–8°C. The figure shows average C_T ± standard deviation.

Mixture studies

Evidence samples are frequently composed of more than one individual's DNA. For the correct setup of the downstream STR analysis, it is important to detect even low amounts of male DNA in the presence of high amounts of female background. Mixture samples were created by mixing male and female DNA in ratios of 1:0, 1:10, 1:1000, 1:20,000, 1:200,000, 1:400,000, 1:1,000,000, and 1:2,000,000 (Table 7). Sample input was 2 µL. Highly accurate quantification results were obtained in all cases for the human and male targets (Figures 27 and 28, page 34).

Table 7. Amounts of DNA template in the mixtures

Male:female ratio	Male component (pg)	Female component (ng)
1:0	1	–
1:10	1	0.01
1:1000	1	1
1:20,000	1	20
1:200,000	1	100
1:400,000	1	200
1:1,000,000	1	1000
1:2,000,000	1	2000

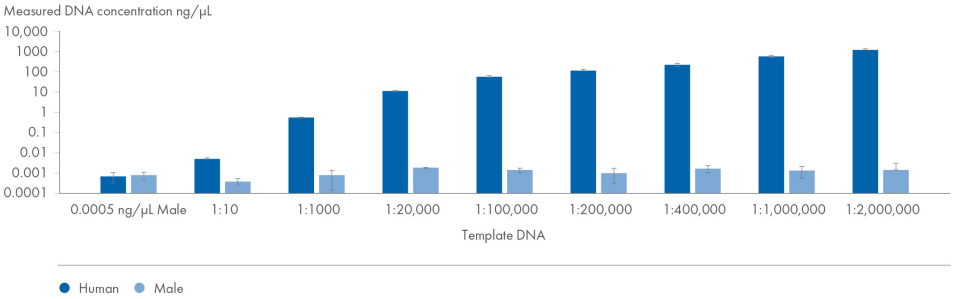

Figure 27. Detection of mixtures using the Investigator Quantiplex Pro FLX Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

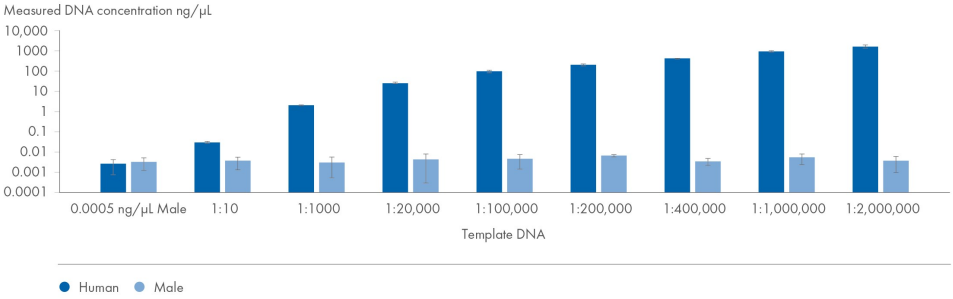

Figure 28. Detection of mixtures using the Investigator Quantiplex Pro FLX Kit on the Quantstudio 5. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

Degraded DNA Samples

Environmental degradation may occur with forensic casework samples and is a challenge in routine genetic fingerprinting. The kit detects a longer autosomal amplification product (353 bp) targeting the same locus (4NS1C) as the 91 bp autosomal target. Due to the differently sized autosomal targets, the longer autosomal target is more susceptible to DNA degradation, allowing for a precise assessment of the degradation status of the DNA. The Investigator Quantiplex Pro FLX Kit was tested for performance on degraded DNA samples on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. Male genomic DNA was sheared with a Covaris® S220 Focused-ultrasonicator to average fragment sizes of 500, 300, and 150 bp. Each fragmented DNA (0.46 ng) was tested according to the kit handbook instructions. The degradation index (DI) was calculated using the QIAGEN Quantification Assay Data Handling and STR Setup Tool v2.01. Reliable detection of the degradation status of the DNA was obtained (Figure 29, next page). The calculated degradation index (DI = quantification value for small human autosomal or for large human autosomal) is depicted in Table 8 (next page).

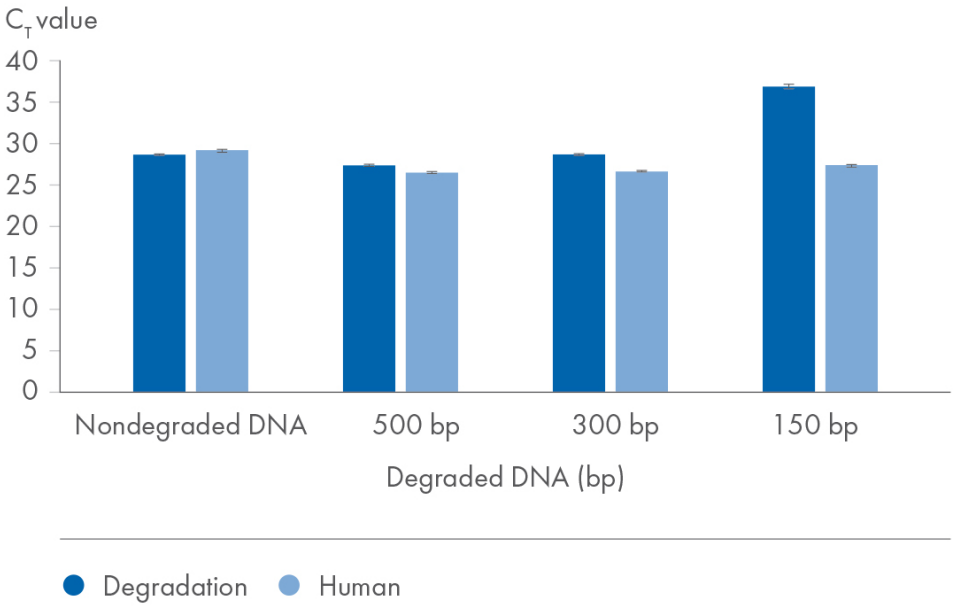

Figure 29. Detection of the degraded DNA using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The results show the detection of DNA degradation indicated by the increase in C_T values for the degradation target. The figure shows average C_T ± standard deviation.

Table 8. Calculated degradation index (DI)

	Degradation index
Nondegraded DNA	1.06
500 bp	2.93
300 bp	6.01
150 bp	1428.91

Link between quantification results and genetic profile

The quantification reaction is performed in order to enhance the rate of first-time success in the STR reaction. Therefore, it is imperative that the quantification result correlates with the downstream application. For screening applications, such as to identify sexual assault samples suitable for differential wash, it is important to not miss samples due to insufficient sensitivity. The Quantiplex Pro FLX Kit allows for flexible sample input volumes of up to 18 µL, which can be used to increase sensitivity.

Dilutions of semen were applied to swabs and dried. Swabs were lysed in 300 µL Investigator Casework GO! Buffer according to the kit handbook. Volumes of 2 µL and 18 µL were used for quantification on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. Autosomal and Y-STRs were setup based on the quantification result using the Investigator 24plex QS Kit and the Investigator Argus Y-28 QS Kit.

Table 9. Quantification results using 2 µL or 18 µL sample

	2 µL sample input volume		18 µL sample input volume	
Semen concentration	**Measured human DNA (pg/µL)**	**Measured male DNA (pg/µL)**	**Measured human DNA (pg/µL)**	**Measured male DNA (pg/µL)**
1:10	1286.979	1362.873	1206.716	1856.981
1:100	83.802	70.576	62.493	90.669
1:1000	3.696	4.190	3.397	5.874
1:5000	0.332	0.378	0.510	0.874
1:10,000	0.168	0.075	0.135	0.287
1:20,000	0.126	0.076	0.047	0.065
1:40,000	0.073	0.077	0.017	0.082

Table 9. Quantification results using 2 µL or 18 µL sample (continued)

	2 µL sample input volume		18 µL sample input volume	
Semen concentration	**Measured human DNA (pg/µL)**	**Measured male DNA (pg/µL)**	**Measured human DNA (pg/µL)**	**Measured male DNA (pg/µL)**
1:80,000	0.000	0.000	0.016	0.016
1:160,000	0.000	0.000	0.009	0.000

Using 2 µL as sample input, the 1:80,000 and 1:160,000 dilutions could not be detected anymore, whereas detection of the human target was possible down to the highest dilution when 18 µL sample was quantified. The male target dropped out at the highest dilution.

Based on the quantification results obtained, samples were used for STR analysis. The dilutions 1:10, 1:100, and 1:1000 provided full profiles in both assays. Using the Investigator 24plex QS Kit, partial profiles were obtained for all remaining dilutions, with low percentages of detected alleles for the 3 highest dilutions. With the Investigator Argus Y-28 QS Kit, partial profiles were obtained down to the 1:80,000 dilution, with low percentages of detected alleles for the 1:20,000 and 1:40,000 dilutions. These results demonstrate the correlation between DNA quantification and STR profile quality.

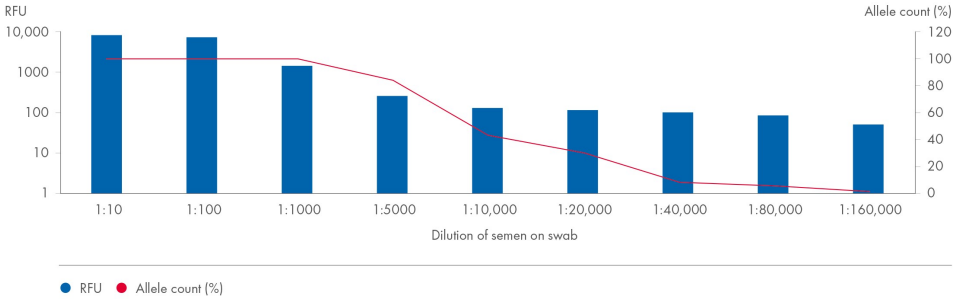

Figure 30. STR results showing the mean profile peak height in RFU and the percentage of detected alleles. If available, 500 pg DNA was used; otherwise, the maximum input of 15 µL sample was applied to Investigator 24plex QS Kit. Data were analyzed with an analytical threshold of 50 RFU.

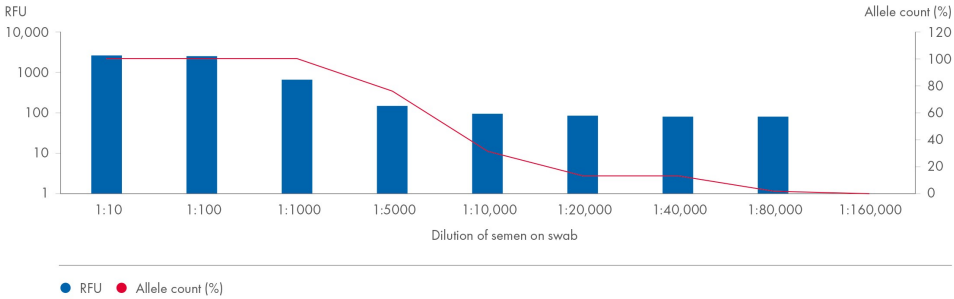

Figure 31. Y-STR results showing the mean profile peak height in RFU and the percentage of detected alleles. If available, 500 pg DNA was used; otherwise, the maximum input of 15 µL sample was applied to Investigator Argus Y-28 QS Kit. Data were analyzed with an analytical threshold of 50 RFU.

References

1. ENFSI Standing Committee for Quality and Competence (QCC). Validation and Implementation of (New) Methods. Ref. Code: QCC-VAL-002, Issue No. 001, 10 November 2014. **https://enfsi.eu/wp-content/uploads/2017/06/Guidance-QCC-VAL-002.pdf**.
2. Scientific Working Group on DNA Analysis Methods (SWGDAM). Validation Guidelines for DNA Analysis Methods, 12 May 2016. **https://www.swgdam.org/_files/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf**.
3. Opel, K.L., Chung, D., and McCord, BR. (2010) A study of PCR inhibition mechanisms using real time PCR. J. Forensic Sci. *55*, 25.

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Pro FLX Kit (576)	Quantiplex Pro FLX Plates, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387516
Related product		
Investigator Quantiplex Pro Calibration Kit	For use on Applied Biosystems Real-Time Systems: Calibration Standard FAM (60 µL), Calibration Standard JOE (60 µL), Calibration Standard ATTO 550 (60 µL), Calibration Standard ROX (60 µL), Calibration Standard ATTO 647N (60 µL), Quanti-plex Pro Calibration Buffer (30 mL)	387416

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Service or your local distributor.

Document Revision History

Date	Description
07/2024	Initial release

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