Validation Report November 2018

Developmental validation of the Investigator® Quantiplex® Pro Kit

The Investigator Quantiplex Pro 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), single nucleotide polymorphisms (SNPs) or deletion/insertion polymorphisms (DIPs). The choice of assay depends on the demands of the examination and on the sample quality. These 3 types of multiplex assays used for human identification are complex systems that require a defined range of template input.

The Investigator Quantiplex Pro 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 (i.e., STR, DIP 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 status of the DNA (whether the DNA has become degraded/fragmented due to temperature/humidity/other factors) is assessed.

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 Kit are given on page 4. A target validation was performed in an internal and external study (page 5). The kit was validated for reproducibility, repeatability (page 9) and sensitivity (page 15). It was tested for cross-reactivity with other species (page 19), and its performance with inhibitors (page 21) and contamination (page 35) was assessed. The quantification of male:female mixtures was also tested (page 38).

Validation of the Investigator Quantiplex Pro 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 Kit is a 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 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. The human target is detected using the FAM™ dye channel on Applied Biosystems® 7500 Real-Time PCR Systems or the yellow channel on the Rotor-Gene® Q.

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. The male quantification target region is detected as an 81 bp fragment using the Cy5® dye channel on Applied Biosystems instruments or the green channel on the Rotor-Gene Q.

In addition, the Investigator Quantiplex Pro Kit contains a balanced internal amplification control that is used to test successful amplification and identify the presence of PCR inhibitors. This heterologous amplification system is detected as a 434 bp internal control (IC) in the JOE™ dye channel on Applied Biosystems instruments.

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 larger autosomal quantification target region is detected as a 353 bp fragment using the TAMRA dye channel on Applied Biosystems instruments or the red channel on the Rotor-Gene Q.

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 QuantiNova® 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:

- Rotor-Gene Q (only used for target validation study)
- Applied Biosystems 7500 Real-Time PCR System for Human Identification
- Applied Biosystems 7500 Real-Time PCR System

Amplification conditions

The amplification conditions developed during validation are shown in Tables 1–3 (pages 4 and 5). An input volume of 2 µl sample, control DNA or standard is used per reaction. Reaction conditions were established for optimal performance in terms of sensitivity, specificity and reproducibility.

For the Rotor-Gene Q, all the data for the target validation presented in this validation report were obtained using Rotor Gene Q Software version 2.2.3 or higher.

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, and for the Applied Biosystems 7500 Real-Time PCR System, SDS Software version 1.4.0.25 was used.

Table 1. Master mix for DNA quantification

Component	Volume per 20 µl reaction	Final concentration
Reaction Mix	9 µl	1x
Primer Mix	9 µl	1x
Total volume of master mix	**18 µl**	

Table 2. Cycling conditions for the Rotor-Gene Q (only for target validation)

Temperature	Temp	Time	Number of cycles	Additional comments
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C
Two-step cycling:				
Denaturation	95°C	5 s	40	Perform fluorescence data collection using the green, yellow and red channels with auto-gain optimization
Combined annealing / extension	60°C	20 s		

Table 3. Cycling conditions for the Applied Biosystems 7500 Real-Time PCR Systems

Temperature	Temp	Time	Number of cycles	Additional comments
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C for 3 min
Two-step cycling:				
Denaturation	95°C	5 s	40	Perform fluorescence data collection
Combined annealing / extension	60°C	35 s		

Results of developmental validation

Human target validation study

For a precise quantification of total human and human male DNA, it is crucial to have a target sequence that is equal in all individuals.

In an internal and external study, the copy number of the human target sequence was analyzed. The first target was the small human autosomal target in the Investigator Quantiplex Pro Kit. The second target was the large human autosomal target in the Investigator Quantiplex Pro Kit. The sequence detected by the primers used in this study for both targets refer to multicopy targets in the human genome.

Reaction efficiency and linearity using different template amounts are important parameters to be able to compare the simultaneous amplification of all targets. The amplification of all systems was confirmed to be in the linear range with efficiency >90% and an R^2 value >0.99 using DNA concentrations between 50 and 0.08 ng/µl. An example obtained on the Rotor-Gene Q is shown in Figure 1 (page 6).

Participants were asked to use between 1 and 10 ng human DNA per 20 µl reaction, which lies within the linear reaction range. The reaction was performed using the QIAGEN® Quantiplex Pro Reaction Mix.

The ready-to-use Primer Oligo Mix containing primers and TaqMan probes was provided by QIAGEN. The reactions were performed using the Rotor-Gene Q.

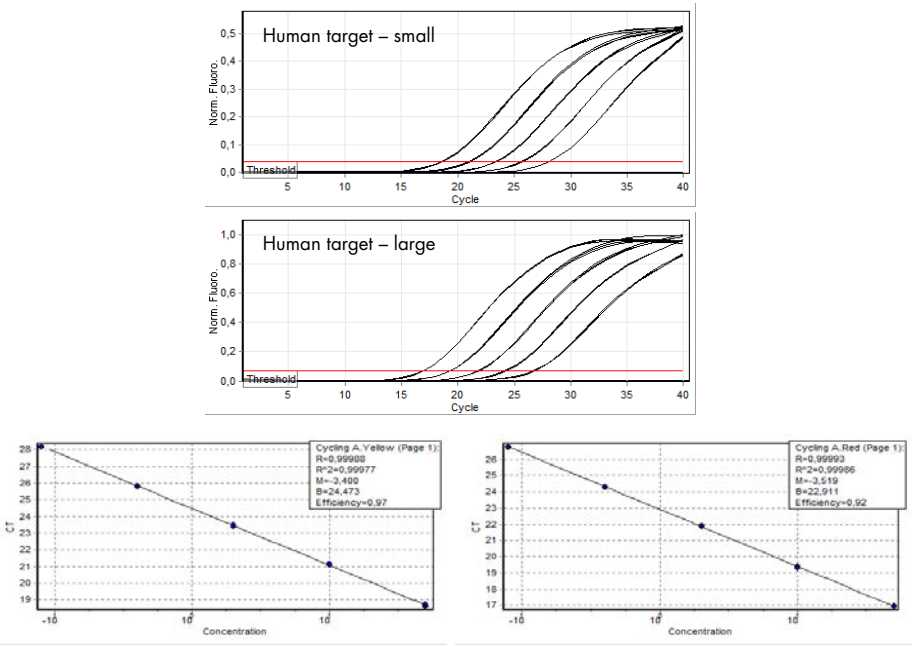

Figure 1. PCR efficiency and linearity of the target validation qPCR for the small and large human autosomal targets. These parameters are comparable for both yellow (human small) and red (human large) channels using DNA concentrations between 50 and 0.08 ng/µl.

In the study, DNA from 317 individuals was examined to ensure reproducibility across the four main human population groups: African-American, Asian, Caucasian and Hispanic. Figure 2 shows the ratio of the DNA quantification values for the small human autosomal target/large human autosomal target and represents the degradation index (DI). The

average value for the DI for all populations in this study is 1.10±0.14. The theoretical ideal ratio for the DI is 1. These results demonstrate that there is a consistent copy number across the four population groups studied.

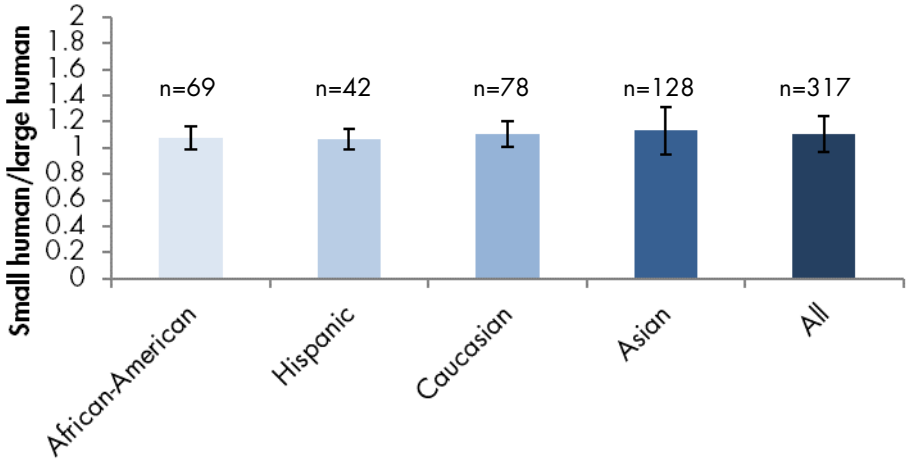

Figure 2. Comparable DI values for the human targets were detected for the 4 main human population groups. The figure shows the average DI ± standard deviation.

Male target validation study

In an internal and external study, the copy number of the male target sequence was analyzed. The first target analyzed was the small human autosomal target in the Investigator Quantiplex Pro Kit, and the second target analyzed was the male gonosomal target in the Investigator Quantiplex Pro Kit. The sequence detected by the primers used in this study for both targets refer to multicopy targets in the human genome. Reaction efficiency and linearity using different template amounts are important parameters to be able to compare the simultaneous amplification of all targets. The amplification of all systems was confirmed to be in the linear range with efficiency >90% and an R^2 value >0.99 using DNA concentrations between 50 and 0.08 ng/µl. An example obtained on the Rotor-Gene Q is shown in Figure 3 (page 8). Participants were asked to use between 1 and 10 ng human DNA per 20 µl

reaction, which lies within the linear reaction range. The reaction was performed using the QIAGEN Quantiplex Pro Reaction Mix. The ready-to-use Primer Oligo Mix containing primers and TaqMan probes was provided by QIAGEN. The reactions were performed using the Rotor-Gene Q.

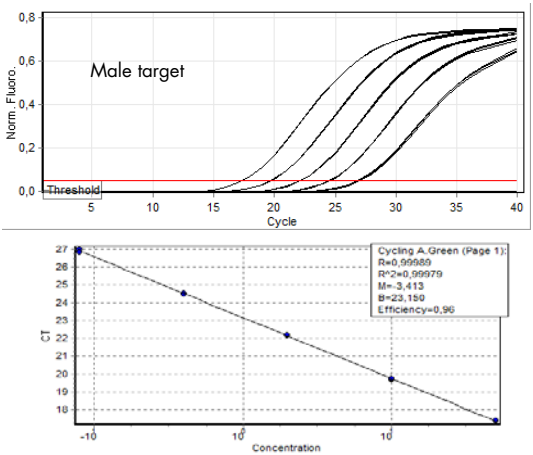

Figure 3. PCR efficiency and linearity of the male target validation qPCR for the male target. These parameters are comparable to the parameters obtained for the small human autosomal and large human autosomal targets in the red and yellow channel between 50 and 0.08 ng/µl.

In the study, DNA from 317 individuals was examined to ensure reproducibility across the four main human population groups: African-American, Asian, Caucasian and Hispanic. Figure 4 shows the human/male ratio of the DNA quantification values for the small human autosomal target/male gonosomal target. The average value for the ratio across all populations in this study is 1.04±0.16. The theoretical ideal ratio is 1. These results demonstrate that there is a consistent copy number across the four population groups studied.

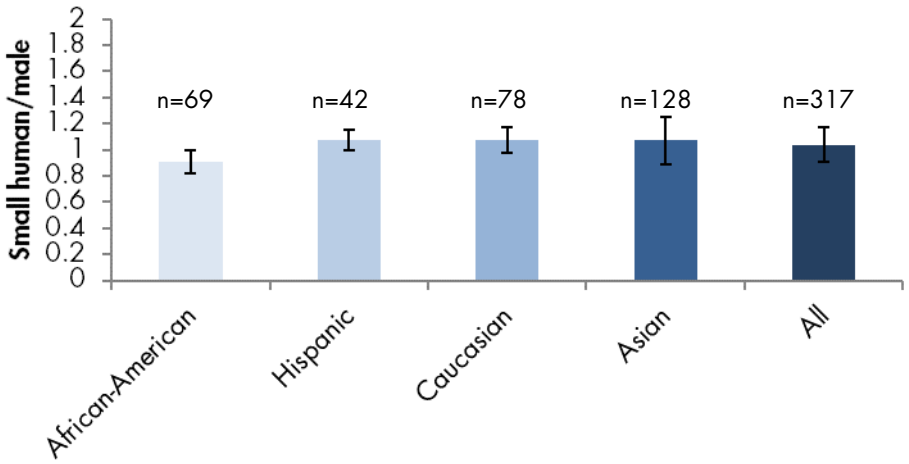

Figure 4. Comparable human/male ratios were detected for the four main human population groups. The figure shows the average ratios ± standard deviation.

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 two 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). All analysis was set up using the QIAgility® system for automated liquid handling.

Reproducibility and repeatability were tested on the Applied Biosystems 7500 Real-Time PCR System for Human Identification and 7500 Real-Time PCR Systems, 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 4–7 (pages 11–14) show the data from the study. The mean quantity and standard variation were calculated for each sample dilution.

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

Table 4. Highly reproducible 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**	**Concentration (ng/µl) ± standard deviation**	**CV**	**Concentration (ng/µl) ± standard deviation**	**CV**
Degradation	Male 1	2.47 ± 0.05	2.0%	3.06 ± 0.2	7.0%
	Male 2	1.76 ± 0.02	1.0%	2.15 ± 0.04	2.0%
	Male 3 (NIST 2372)	0.6 ± 0.03	4.0%	0.77 ± 0.02	3.0%
	Female 1	2.89 ± 0.12	4.0%	3.59 ± 0.17	5.0%
	Female 2	1.64 ± 0.07	4.0%	2.1 ± 0.18	9.0%
	Female 3 (NIST 2372)	0.53 ± 0.02	4.0%	0.64 ± 0.05	8.0%
Human	Male 1	2.56 ± 0.13	5.0%	3.18 ± 0.13	4.0%
	Male 2	1.79 ± 0.1	5.0%	2.19 ± 0.11	5.0%
	Male 3 (NIST 2372)	0.58 ± 0.03	5.0%	0.76 ± 0.04	5.0%
	Female 1	2.96 ± 0.15	5.0%	3.73 ± 0.15	4.0%
	Female 2	2.02 ± 0.04	2.0%	2.61 ± 0.12	5.0%
	Female 3 (NIST 2372)	0.52 ± 0.02	4.0%	0.65 ± 0.06	9.0%
Male	Male 1	2.31 ± 0.13	6.0%	2.85 ± 0.24	8.0%
	Male 2	1.68 ± 0.05	3.0%	2.11 ± 0.07	3.0%
	Male 3 (NIST 2372)	0.53 ± 0.05	9.0%	0.68 ± 0.03	4.0%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372)	NA	NA	NA	NA

Table 5. Highly reproducible results comparing 2 different runs performed by 2 different operators on the same Applied Biosystems 7500 Real-Time PCR System

		Operator 1		Operator 2	
Target	**DNA sample**	**Concentration (ng/µl) ± standard deviation**	**CV**	**Concentration (ng/µl) ± standard deviation**	**CV**
Degradation	Male 1	2.56 ± 0.29	11.0%	2.53 ± 0.19	7.0%
	Male 2	1.72 ± 0.07	4.0%	1.67 ± 0.03	2.0%
	Male 3 (NIST 2372)	0.59 ± 0.07	12.0%	0.55 ± 0.05	9.0%
	Female 1	3.21 ± 0.08	2.0%	3.18 ± 0.19	6.0%
	Female 2	1.63 ± 0.13	8.0%	1.66 ± 0.09	5.0%
	Female 3 (NIST 2372)	0.55 ± 0.03	5.0%	0.53 ± 0.04	8.0%
Human	Male 1	2.63 ± 0.12	4.0%	2.56 ± 0.06	2.0%
	Male 2	1.82 ± 0.14	8.0%	1.74 ± 0.15	8.0%
	Male 3 (NIST 2372)	0.6 ± 0.02	3.0%	0.55 ± 0.02	4.0%
	Female 1	3.24 ± 0.13	4.0%	3.31 ± 0.3	9.0%
	Female 2	2.07 ± 0.11	6.0%	2.05 ± 0.11	5.0%
	Female 3 (NIST 2372)	0.58 ± 0.04	7.0%	0.54 ± 0.03	5.0%
Male	Male 1	2.63 ± 0.26	10.0%	2.54 ± 0.15	6.0%
	Male 2	1.82 ± 0.09	5.0%	1.79 ± 0.12	7.0%
	Male 3 (NIST 2372)	0.63 ± 0.02	3.0%	0.59 ± 0.05	8.0%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372)	NA	NA	NA	NA

Table 6. Highly repeatable 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		Operator 2	
Target	**DNA sample**	**Concentration (ng/µl) ± standard deviation**	**CV**	**Concentration (ng/µl) ± standard deviation**	**CV**
Degradation	Male 1	2.47 ± 0.05	2.0%	2.24 ± 0.14	6.0%
	Male 2	1.76 ± 0.02	1.0%	1.58 ± 0.1	6.0%
	Male 3 (NIST 2372)	0.6 ± 0.03	4.0%	0.55 ± 0.03	5.0%
	Female 1	2.89 ± 0.12	4.0%	2.82 ± 0.08	3.0%
	Female 2	1.64 ± 0.07	4.0%	1.55 ± 0.06	4.0%
	Female 3 (NIST 2372)	0.53 ± 0.02	4.0%	0.47 ± 0.04	8.0%
Human	Male 1	2.56 ± 0.13	5.0%	2.41 ± 0.15	6.0%
	Male 2	1.79 ± 0.1	5.0%	1.66 ± 0.14	9.0%
	Male 3 (NIST 2372)	0.58 ± 0.03	5.0%	0.58 ± 0.02	3.0%
	Female 1	2.96 ± 0.15	5.0%	2.99 ± 0.08	3.0%
	Female 2	2.02 ± 0.04	2.0%	1.94 ± 0.07	4.0%
	Female 3 (NIST 2372)	0.52 ± 0.02	4.0%	0.49 ± 0.03	6.0%
Male	Male 1	2.31 ± 0.13	6.0%	2.21 ± 0.23	10.0%
	Male 2	1.68 ± 0.05	3.0%	1.55 ± 0.07	5.0%
	Male 3 (NIST 2372)	0.53 ± 0.05	9.0%	0.52 ± 0.03	7.0%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372)	NA	NA	NA	NA

Table 7. Highly repeatable results comparing 2 different runs performed by the same operator using the same Applied Biosystems 7500 Real-Time PCR System

		Operator 1		Operator 2	
Target	**DNA sample**	**Concentration (ng/µl) ± standard deviation**	**CV**	**Concentration (ng/µl) ± standard deviation**	**CV**
Degradation	Male 1	2.56 ± 0.29	11.0%	2.69 ± 0.26	10.0%
	Male 2	1.72 ± 0.07	4.0%	1.91 ± 0.1	5.0%
	Male 3 (NIST 2372)	0.59 ± 0.07	12.0%	0.62 ± 0.06	10.0%
	Female 1	3.21 ± 0.08	2.0%	3.53 ± 0.18	5.0%
	Female 2	1.63 ± 0.13	8.0%	1.76 ± 0.18	10.0%
	Female 3 (NIST 2372)	0.55 ± 0.03	5.0%	0.59 ± 0.03	5.0%
Human	Male 1	2.63 ± 0.12	4.0%	2.72 ± 0.17	6.0%
	Male 2	1.82 ± 0.14	8.0%	1.98 ± 0.11	5.0%
	Male 3 (NIST 2372)	0.6 ± 0.02	3.0%	0.61 ± 0.05	7.0%
	Female 1	3.24 ± 0.13	4.0%	3.63 ± 0.2	5.0%
	Female 2	2.07 ± 0.11	6.0%	2.21 ± 0.13	6.0%
	Female 3 (NIST 2372)	0.58 ± 0.04	7.0%	0.59 ± 0.04	8.0%
Male	Male 1	2.63 ± 0.26	10.0%	2.66 ± 0.35	13.0%
	Male 2	1.82 ± 0.09	5.0%	2.09 ± 0.12	6.0%
	Male 3 (NIST 2372)	0.63 ± 0.02	3.0%	0.62 ± 0.06	9.0%
	Female 1	NA	NA	NA	NA
	Female 2	NA	NA	NA	NA
	Female 3 (NIST 2372)	NA	NA	NA	NA

Sensitivity

The Investigator Quantiplex Pro Kit is designed to detect a broad range of DNA quantities. Figures 5, 6 and 7 show a serial dilution of Male Control DNA M1 from 50 ng/µl to 0.015625 pg/µl. The optimal linear dynamic range of the assay is in the range of 50 ng/µl to 0.5 pg/µl total DNA. DNA could be detected with the degradation and human target down to 0.015625 pg/µl, using triplicates for the range 50 ng/µl to 0.005 ng/µl and 6 replicates for DNA concentration below 0.005 ng/µl. Furthermore, the standard conditions specified in the *Investigator Quantiplex Pro Kit Handbook* have been used for both validated instruments (Applied Biosystems 7500 Real-Time PCR Systems for Human Identity and Applied Biosystems 7500 Real-Time PCR Systems).

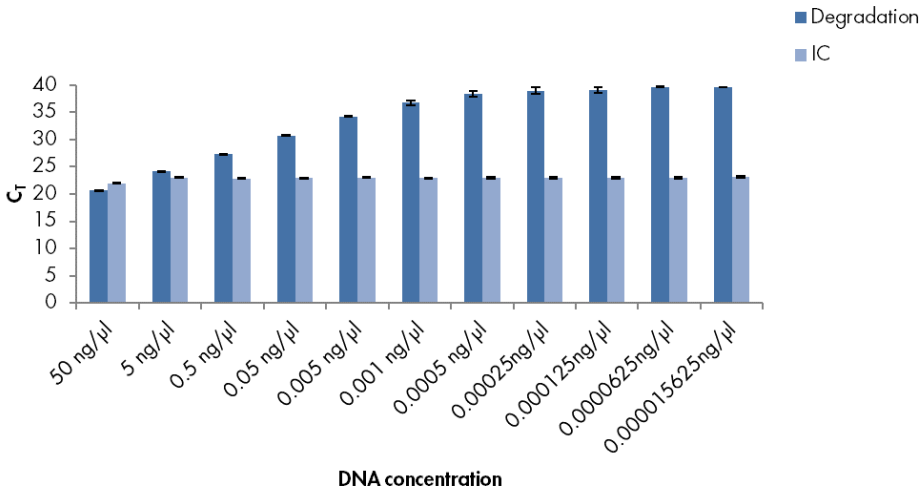

Figure 5. Detection of the degradation target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation.

The sensitivity of the Investigator Quantiplex Pro Kit has been tested for the male DNA component. Figures 7 and 10 show the quantification of the male component of a serial

dilution of Male Control DNA M1 from 50 ng/µl to 0.015625 pg/µl male DNA. The optimal linear dynamic range of the assay is in the range of 50 ng/µl to 0.5 pg/µl of male DNA. DNA could be detected down to 0.015625 pg/µl, using triplicates for DNA concentration above 0.005 ng/µl and six replicates for DNA concentrations below 0.005 ng/µl. Furthermore, standard conditions specified in the *Investigator Quantiplex Pro Kit Handbook* have been used for both validated instruments (Applied Biosystems 7500 Real-Time PCR Systems for Human Identity and Applied Biosystems 7500 Real-Time PCR Systems).

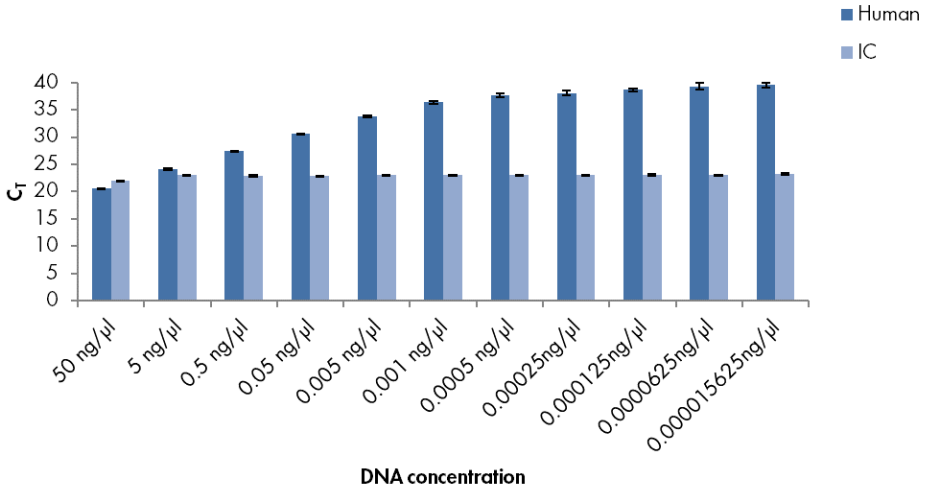

Figure 6. Detection of human target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation.

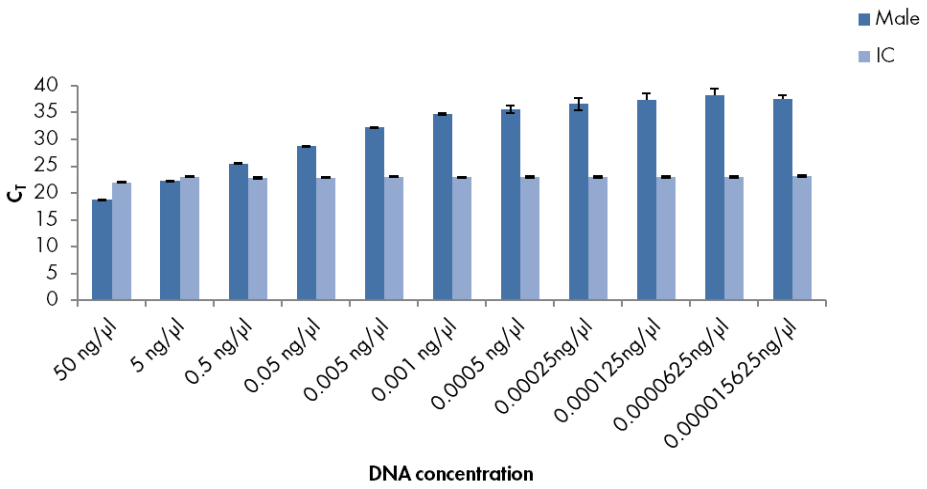

Figure 7. Detection of the male target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation.

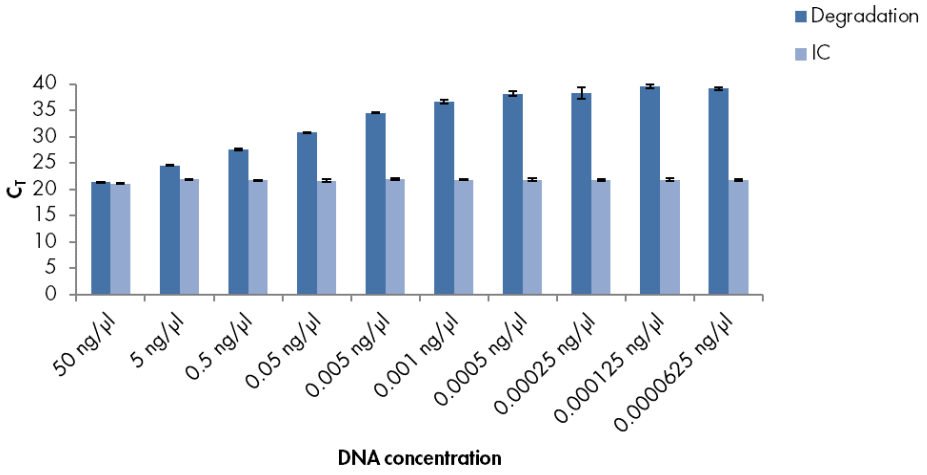

Figure 8. Detection of the degradation target in male DNA down to 0.0625 pg/µl using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

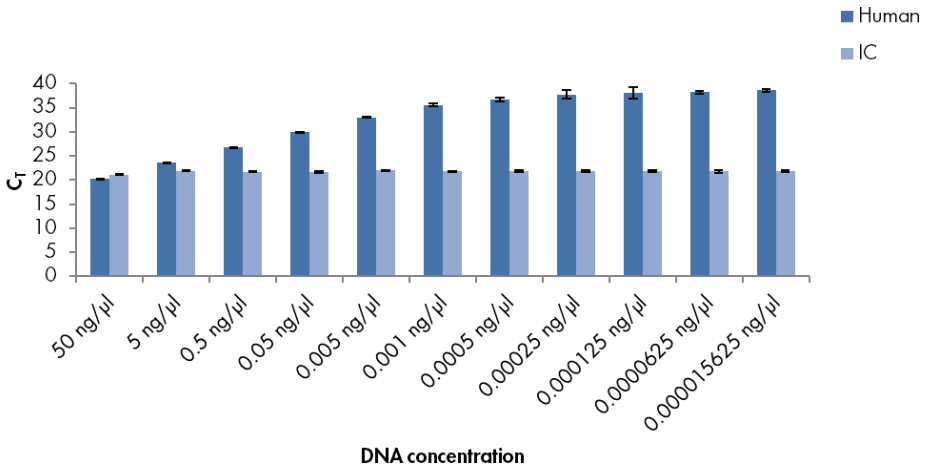

Figure 9. Detection of human DNA down to 0.015625 pg /µl using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

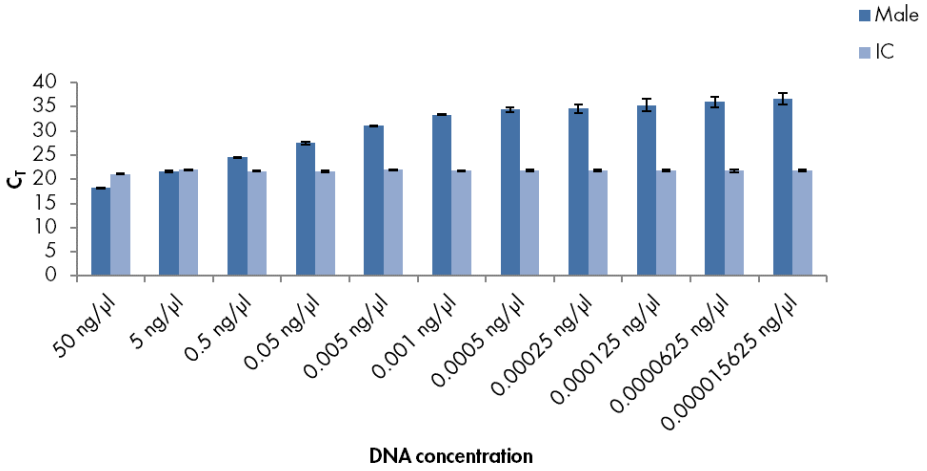

Figure 10. Detection of male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

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 Kit species specificity, 2.5 ng of DNA from vertebrate species, commonly found at crime scenes, was examined. Each was tested following the standard assay protocol with 2.5 ng of Male Control DNA M1 as a positive control.

No cross-reactivity was shown for DNA, from the tested common vertebrates, under standard conditions, as shown in Figure 11.

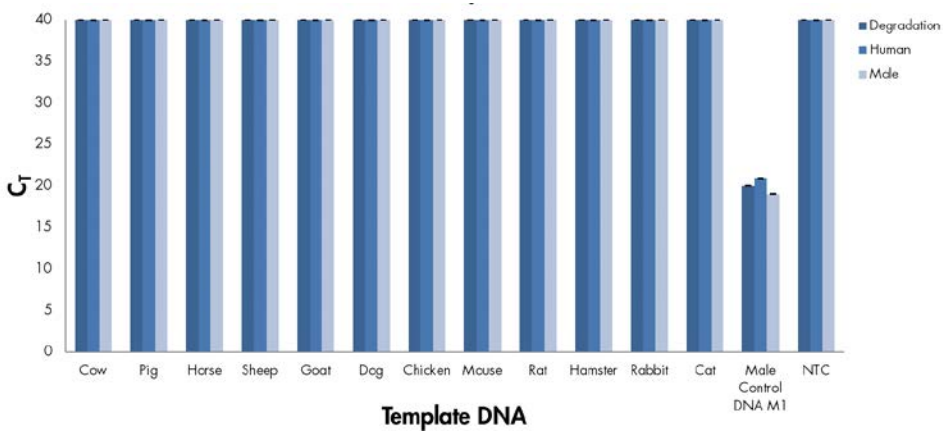

Figure 11. Results of a cross-reactivity study on common vertebrate species. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

Some primates, including gorillas, chimpanzees, bonobo, orangutans and macaque were also examined, as described above. Due to the evolutionary proximity of the chimpanzee, bonobo, orangutan, macaque and gorilla to humans, positive results were observed for these species DNA (Figure 12).

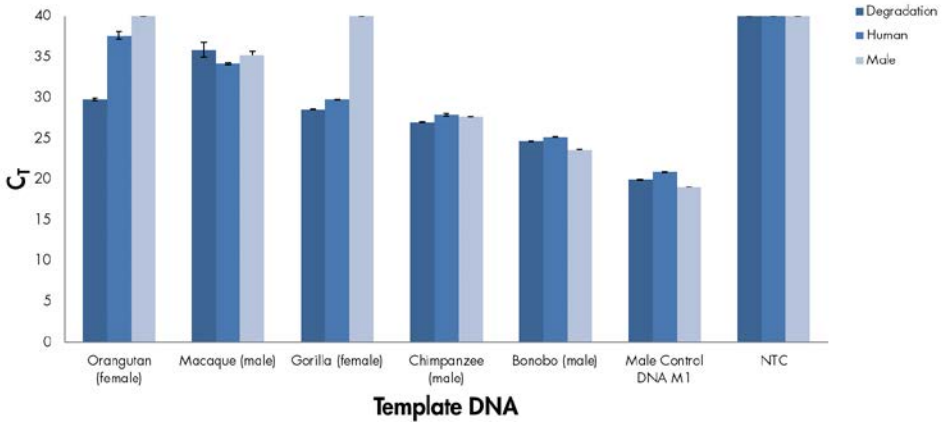

Figure 12. Results of a cross-reactivity study on primates. The figure shows the average C_T ± standard deviation. **NTC:** No-template control.

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 from *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Staphylococcus aureus* (2.5 ng of each) was tested following the standard assay protocol, with 2.5 ng Male Control DNA M1 as a positive control. None of the tested microbial species yielded detectable DNA under standard conditions, as shown in Figure 13.

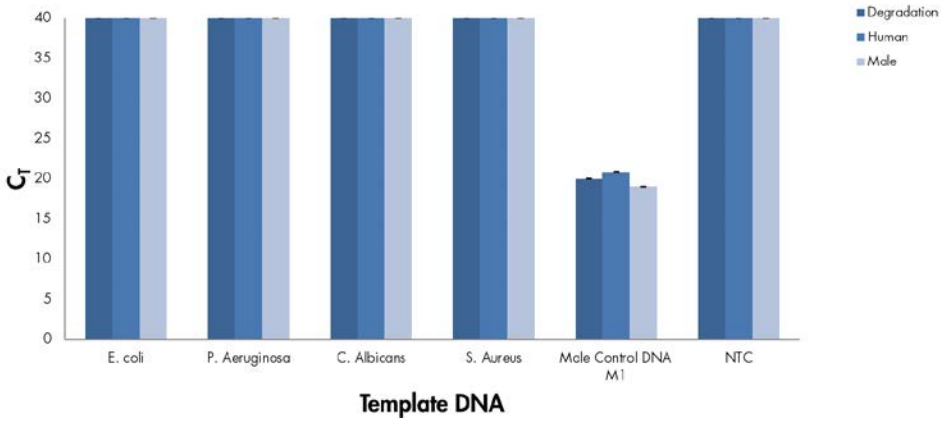

Figure 13. Results of a cross-reactivity study on microbial species. No cross-reactivity could be shown for the tested microbes. The figure shows the average C_T ± standard deviation. **NTC:** No-template control.

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

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

Performance with simulated inhibition

QIAGEN 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 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.

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) under standard conditions as described in the *Investigator Quantiplex Pro Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figures 14 and 15.

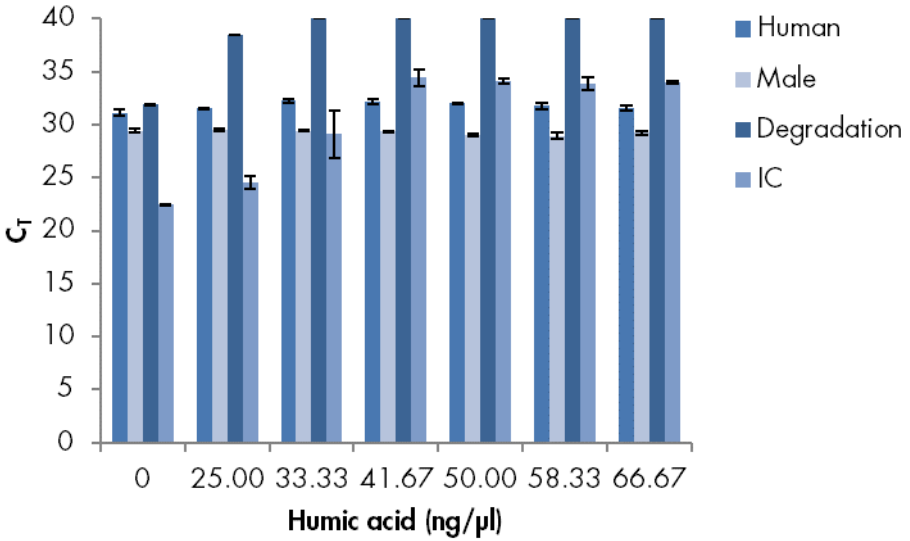

Figure 14. Performance of the Investigator Quantiplex Pro Kit with simulated humic acid inhibition on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control (IC) reports the presence of the inhibitor (C_T shift) while the quantification for the human and male 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.

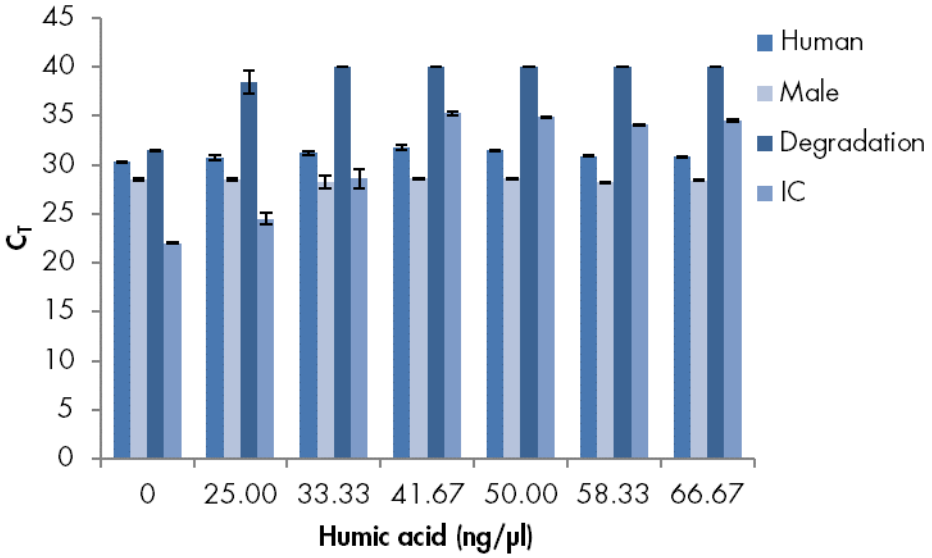

Figure 15. Performance of the Investigator Quantiplex Pro Kit with simulated humic acid inhibition on the Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration 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 (using 2 µl DNA sample in the assay, as recommended). The same 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.

When using STR kits, two different parameters must be considered when analyzing inhibited samples: the DNA sample volume to be added to the reaction and the inhibitor resistance of the STR kit. STR kits, such as the Investigator ESSplex SE QS Kit and the Investigator 24plex QS Kit, are very flexible with regard to reaction setup as a broad range of DNA sample

volumes (up to 15 µl) may be added to the reaction. The Investigator ESSplex SE QS Kit and the Investigator 24plex QS Kit show resistance to humic acid from 500 pg DNA up to 200 ng/µl (final concentration in the reaction). The internal control of the Investigator Quantiplex Pro Kit acts as a quality sensor and reports the presence of a broad range of humic acid with a broad C_T shift. Therefore, further internal laboratory validation should be performed to determine criteria for obtaining a full DNA profile without inhibition using the Investigator ESSplex SE QS and the Investigator 24plex QS kits.

See the Developmental Validation Reports for the Investigator ESSplex SE QS and the 24plex QS kits for more information.

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) under the standard conditions described in the *Investigator Quantiplex Pro Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figures 16 and 17.

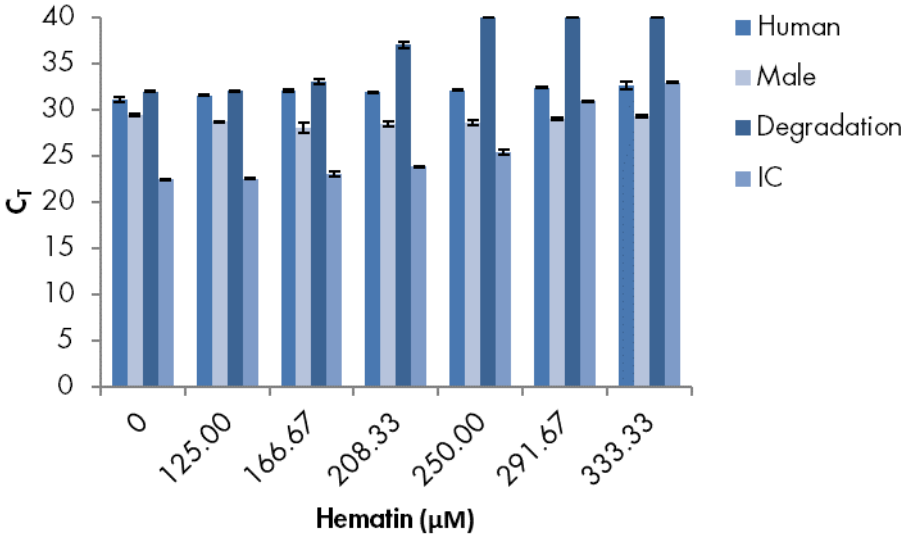

Figure 16. Performance of the Investigator Quantiplex Pro Kit with simulated hematin inhibition on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 333.33 µM. The degradation target is susceptible to hematin at concentrations higher than 125 µM. The figure shows the average C_T ± standard deviation.

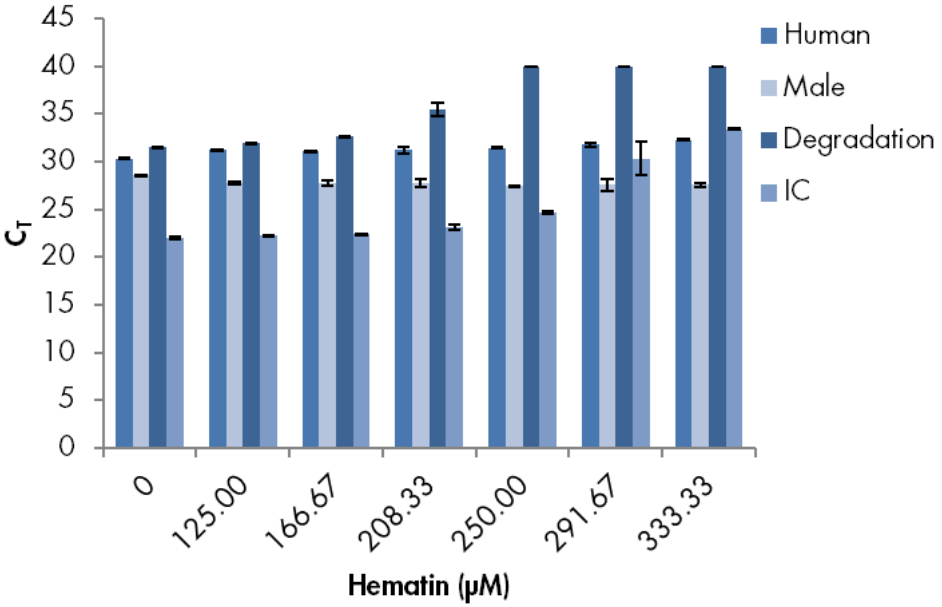

Figure 17. Performance of the Investigator Quantiplex Pro Kit with simulated hematin inhibition on the Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 333.33 µM. The degradation target is susceptible to hematin at concentrations higher than 125 µM. 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 final hematin concentration of 333.33 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, as recommended). 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.

The same inhibitor resistance was confirmed for both validated instruments.

The internal control of the Investigator Quantiplex Pro Kit acts as a quality sensor and reports the presence of a broad range of hematin with a broad C_T shift. Therefore, further internal laboratory validation should be performed to determine criteria for obtaining a full DNA profile without inhibition, using the Investigator ESSplex SE QS and the Investigator 24plex QS kits.

See the Developmental Validation Reports for the Investigator ESSplex SE QS and the Investigator 24plex QS kits for more information.

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) under standard conditions, as described in the *Investigator Quantiplex Pro Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figures 18 and 19.

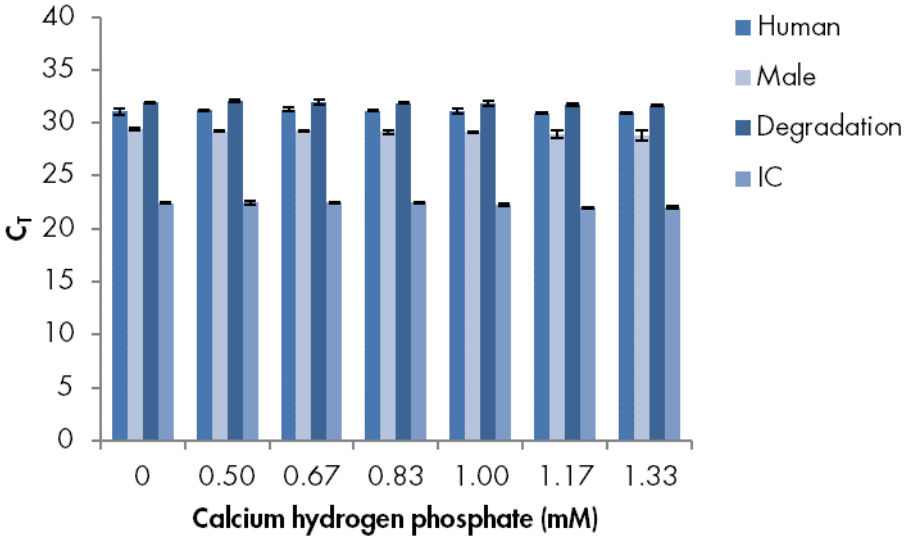

Figure 18. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of calcium hydrogen phosphate on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The quantification is reliable up to a concentration of 1.33 mM. The figure shows average C_T ± standard deviation.

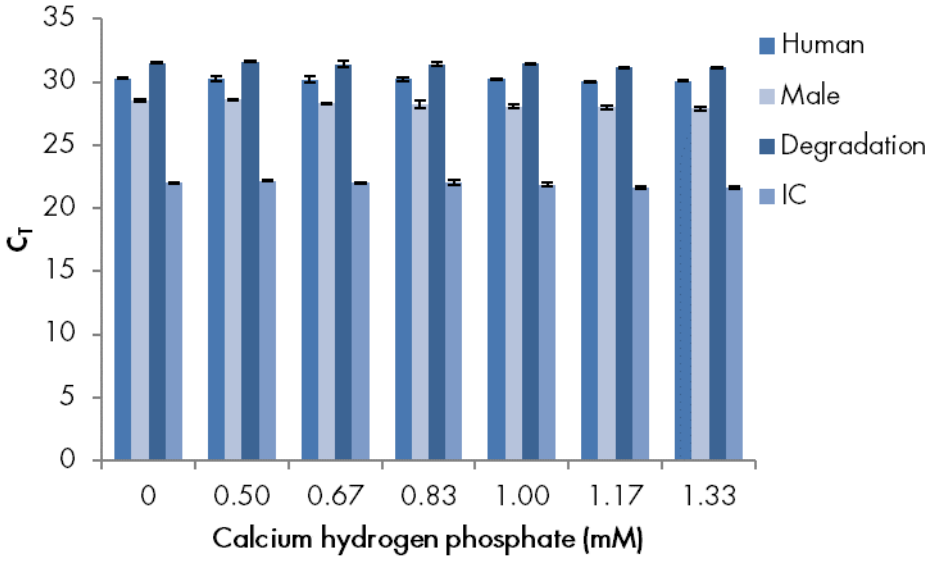

Figure 19. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of calcium hydrogen phosphate on the Applied Biosystems 7500 Real-Time PCR System. The quantification is reliable up to a concentration of 1.33 mM. The figure shows average C_T ± standard deviation.

Tannic acid

Tannic acid is an agent found in leather, as well as in some types of plant material. It may also be encountered in samples that have been exposed to leaf litter. Tannic acid is proposed 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), under standard conditions, as described in the *Investigator Quantiplex Pro Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figures 20 and 21.

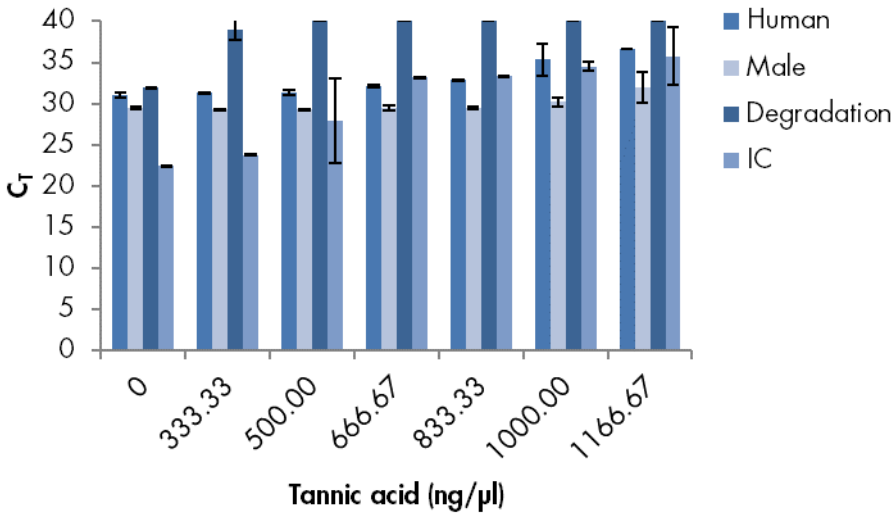

Figure 20. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of tannic acid on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 500 ng/µl. The degradation target is susceptible to tannic acid. The figure shows average C_T ± standard deviation.

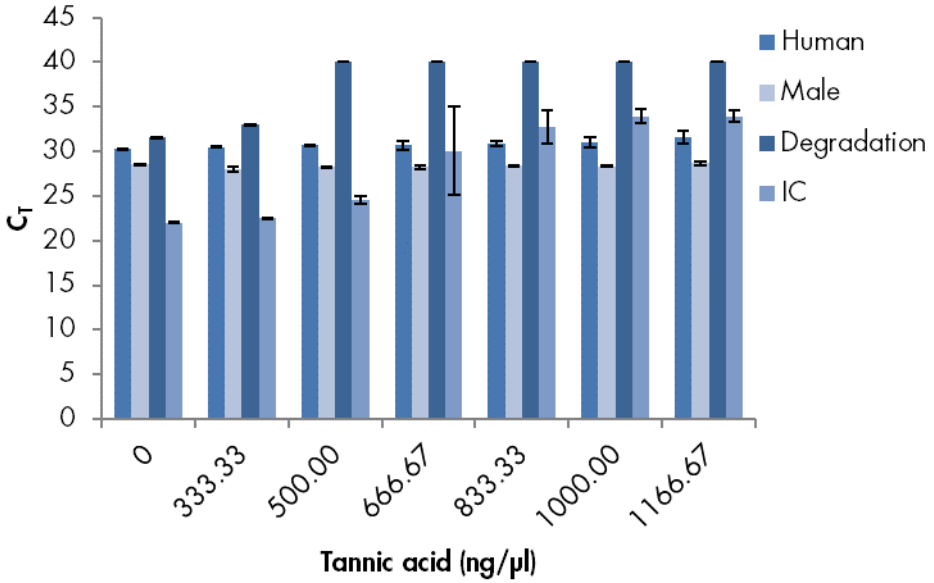

Figure 21. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of tannic acid on the Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human and male targets is reliable up to a concentration of 833.33. 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 500–833.33 µM (final concentration in the reaction; this value is instrument-dependent and must be validated). 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.

Further internal laboratory validation should be performed to determine criteria for obtaining a full DNA profile without inhibition using the Investigator ESSplex SE QS and the Investigator 24plex QS kits.

See the Developmental Validation Reports for the Investigator ESSplex SE QS and the Investigator 24plex QS kits for more information.

Collagen

Collagen is the main protein compound of many tissues. Collagen is proposed 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) under standard conditions, as described in the *Investigator Quantiplex Pro Kit Handbook* (66 pg Male Control DNA M1).

The effect of collagen is shown in Figures 22 and 23.

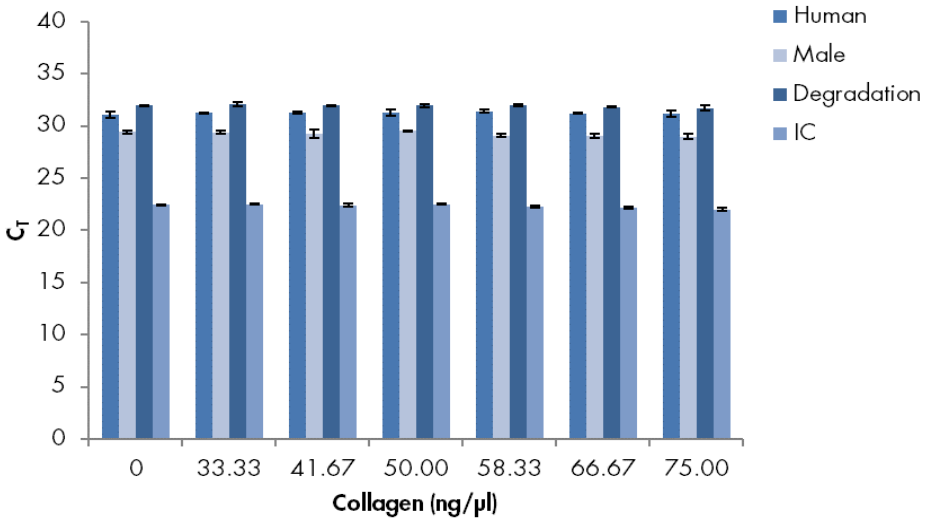

Figure 22. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of collagen on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The quantification is reliable up to a concentration of 75 ng/µl. The figure shows average C_T ± standard deviation.

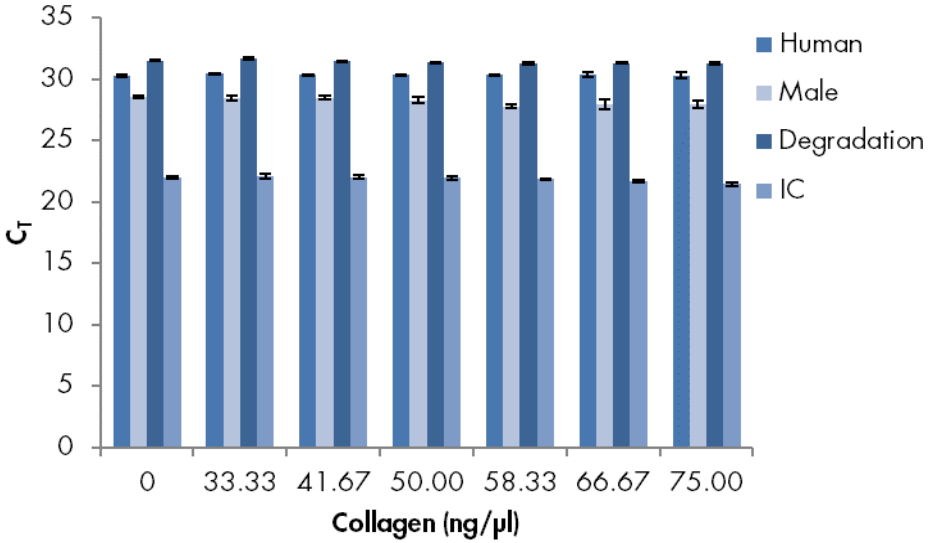

Figure 23. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of collagen on the Applied Biosystems 7500 Real-Time PCR System. The quantification is reliable up to a concentration of 75 ng/µl. The figure shows average C_T ± standard deviation.

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, under standard conditions, as described in the *Investigator Quantiplex Pro Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figures 24 and 25.

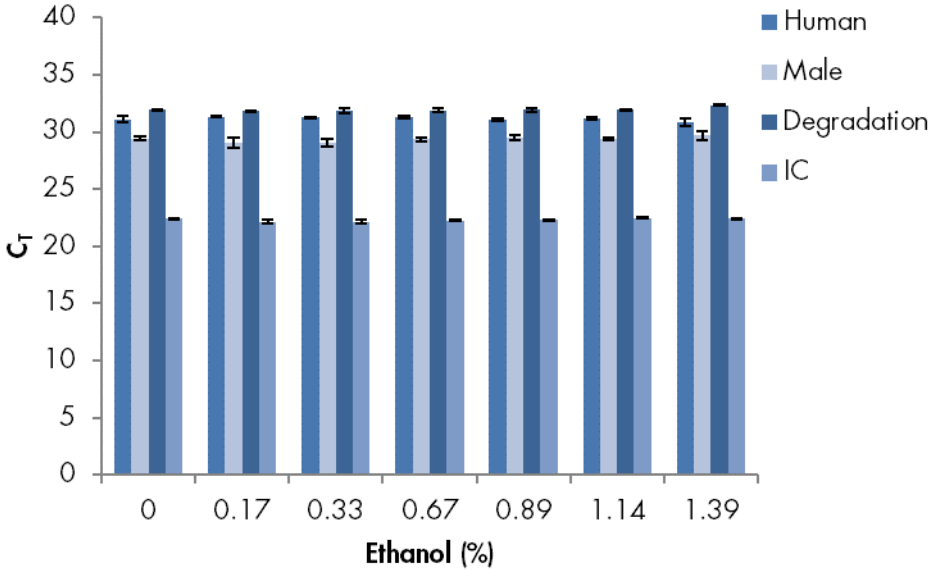

Figure 24. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of ethanol on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The quantification is reliable up to a concentration of 1.39% ethanol. The figure shows average C_T ± standard deviation.

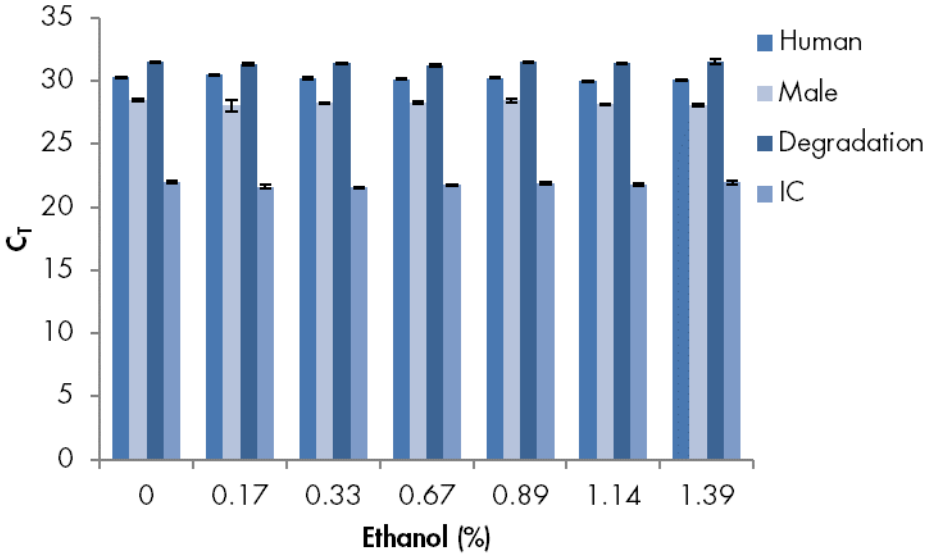

Figure 25. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of ethanol on the Applied Biosystems 7500 Real-Time PCR System. The quantification is reliable up to a concentration of 1.39% ethanol. The figure shows average C_T ± standard deviation.

Contamination of reagents

Laboratory contamination of one of the reagents contained in the Investigator Quantiplex Pro 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 26). In total, 94 no-template controls and 2 positive controls (Male Control DNA M1; 50 ng/µl) were analyzed.

	1	2	3	4	5	6	7	8	9	10	11	12
A								39.4		39.1		
B												
C												
D												
E									40.0	38.3		
F	38.4					38.2						
G							38.2				39.9	
H												

Positive Control
Human Target
Male Target

Figure 26. 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 A2. C_T values of 20.3 and 20.0 (A1and A2) were detected for the degradation target, 19.9 and 19.6 (A1 and A2) for the human target and 18.0 and 17.7 for the male target.

Most of the samples did not produce any detectable C_T values for any of the three targets (degradation/human/male), and in the rare case where a detectable C_T value was generated, it was either for the human target or for the male target, only. Presence of detectable human DNA was not confirmed with all three targets for human, degradation and male in any of the samples, excepted for the positive controls. The outlier signals detected with either the human target or the male target are possibly due to ambient DNA specific to these PCR wells. Further laboratory validation studies should be performed to determine the C_T threshold that will produce an interpretable STR profile.

Stability

Stability of the Male Control DNA M1 dilution series

In a forensic laboratory, the maximum number of reactions of a kit may not be performed in a single day. The possibility to set up the dilution series for the Male Control DNA M1 for a whole week is a real advantage. Therefore, the stability of the serial dilutions of the Male Control DNA M1 at 4°C was tested.

The dilutions were performed using QuantiTect Nucleic Acid Dilution Buffer in untreated plastic 1.5 ml tubes. The dilutions were tested directly after dilution (Day 0) and after 1, 3, 6 and 7 days storage at 4°C. The tests were run on an Applied Biosystems 7500 Real-Time PCR System for Human Identification following the standard reaction protocol. For each dilution point, 3 replicates were tested (Figure 27).

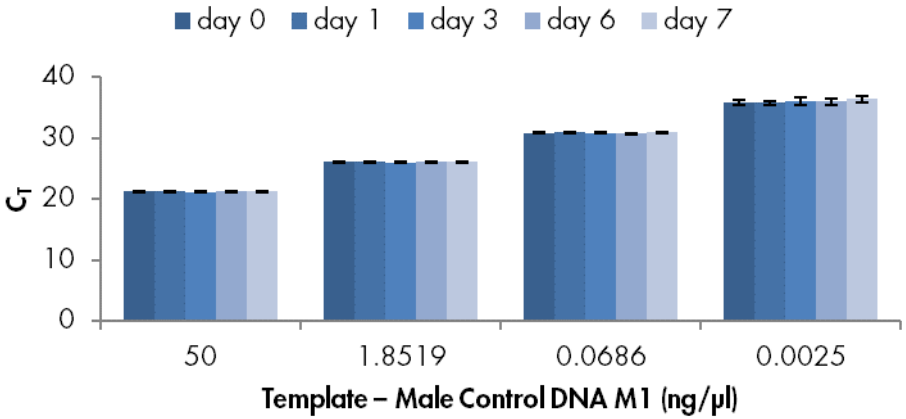

Figure 27. Detection of the degradation target on Male Control DNA M1 dilution series, using the QuantiTect Nucleic Acid Dilution Buffer in untreated tubes, before storage and after storage for 1, 3, 6 and 7 days at 4°C. The results show no relevant differences, even in the low DNA range. The figure shows average C_T ± standard deviation.

The results demonstrate that the dilution series using the QuantiTect Nucleic Acid Dilution Buffer is stable at 4°C for at least 7 days, without any effect on performance. This buffer was developed to provide optimal storage conditions for nucleic acids, even at very low concentrations.

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. To test for cross-reactivity of the male Y-chromosomal target in samples with high concentrations of female DNA, different female single-source DNA samples were tested in four replicates each. No cross-reactivity of the male Y-chromosomal target with female DNA was observed in any of the samples tested (Table 8).

Table 8. Testing for cross-reactivity of the male Y-chromosomal target in single-source DNA samples with high concentrations of female DNA

	Human (ng/µl)	SD	Degradation (ng/µl)	SD	Male (ng/µl)
Female 1 ~250 ng/µl	229.2	9.9	220.2	12.0	NA
Female 2 ~250 ng/µl	221.3	13.4	208.6	14.1	NA
Female 3 ~250 ng/µl	220.1	6.1	216.3	18.3	NA

Mixture samples were created by mixing male and female DNA in ratios of 1:0, 1:10, 1:1000, 1:20000, 1:200000 and 1:400000. The amount of male DNA used in this study was either 2.5 pg/µl or 0.5 pg/µl; a 1:400000 mixture contained 0.5 pg/µl of the male component DNA and 200 ng/µl of the female component (Table 9). Highly accurate quantification results were obtained in all cases for the human and male targets on both validated cyclers (Figures 28 and 29).

Table 9. Amounts of DNA template in the mixtures

Male:female ratio	Male component	Female component
1:0	0.5 pg/µl	0
1:0	2.5 pg/µl	0
1:10	2.5 pg/µl	25 pg/µl
1:1000	2.5 pg/µl	2.5 ng/µl
1:20.000	2.5 pg/µl	50 ng/µl
1:200.000	0.5 pg/µl	100 ng/µl
1:400.000	0.5 pg/µl	200 ng/µl

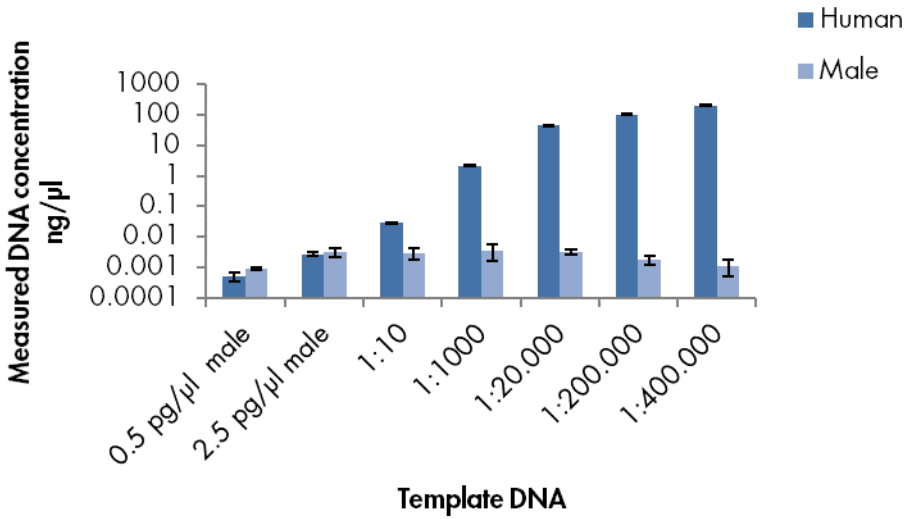

Figure 28. Detection of mixtures using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

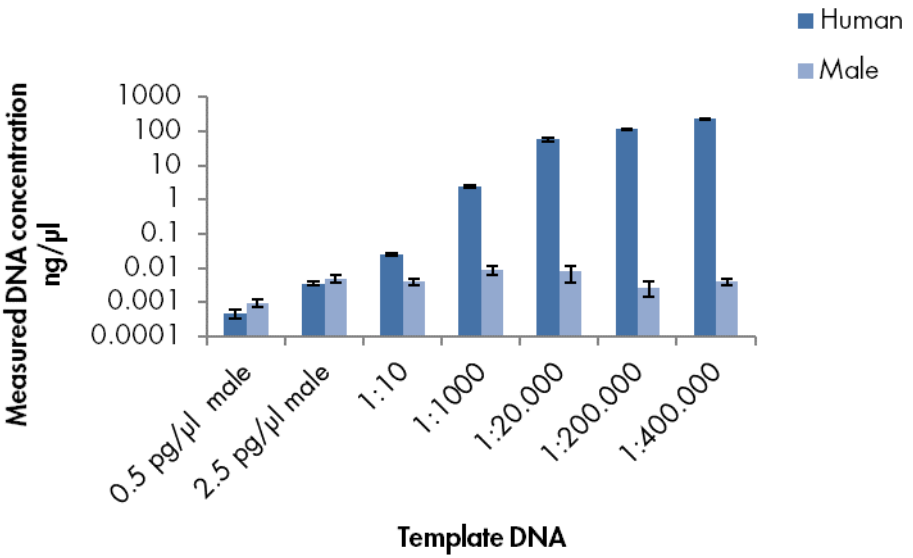

Figure 29. Detection of mixtures using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

Degraded DNA Samples

Environmental degradation may occur with forensic casework samples and is a classic 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 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 fragment size (0.46 ng) was tested with the Investigator Quantiplex Pro Kit, 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 30). The calculated degradation index (DI=quantification value for small human autosomal/quantification value for large human autosomal) is depicted in Table 10.

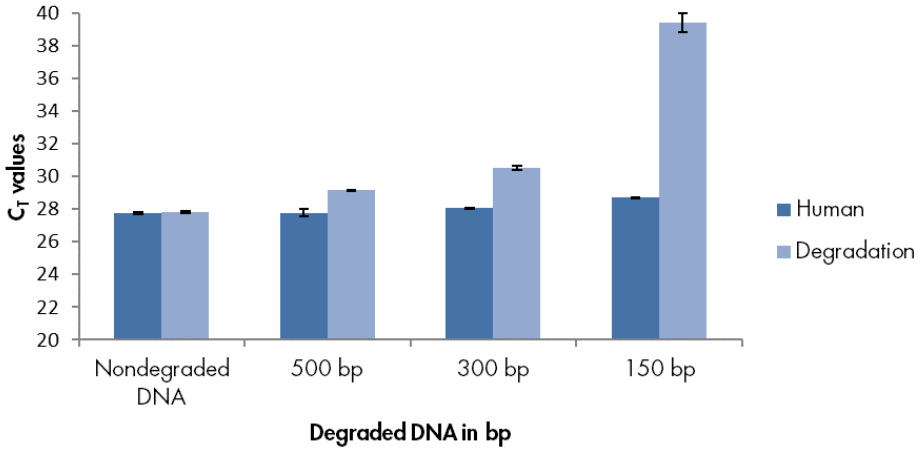

Figure 30. Detection of the degraded DNA using the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The results show the detection of DNA degradation indicated by the increase in C_T values for the degradation target. The figure shows average C_T ± standard deviation.

Table 10. Calculated degradation index (DI)

	Degradation index
Nondegraded DNA	1.08
500 bp	2.77
300 bp	5.95
150 bp	2073.01

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.

One possible application of the Investigator Quantiplex Pro Kit is sexual assault samples. In the case of a DNA mixture, the autosomal STRs may be inconclusive. A possible option is then the use of gonosomal STR markers (such as Y-STRs).

In order to test the link between the male quantification and the results using gonosomal markers, different samples were created by mixing male and female DNA in a ratio of 1:5. This mixed DNA was then diluted into a series of 9 samples. The total amount of DNA contained in sample 1 was 300 pg/µl, and different dilution factors were used for the remaining samples (Table 11).

Highly accurate quantification results were obtained for both the total human and the male component (Table 11). STR reactions were set up using the Investigator Argus Y-12 QS Kit (cat. no. 383615), according to the quantification of the male component.

Table 11. Amounts of DNA template in the mixtures

	Theoretical human DNA (pg/µl)	Measured human DNA (pg/µl)	Theoretical male DNA (pg/µl)	Measured male DNA (pg/µl)	DNA in Y-STR (pg)
Sample 1	300	275.81	50.00	54.23	500.00
Sample 2	18.75	20.90	3.13	3.85	65.10
Sample 3	4.69	5.38	0.78	0.81	13.61
Sample 4	2.34	1.98	0.39	0.35	5.91
Sample 5	1.17	1.11	0.20	0.18	3.03
Sample 6	0.59	1.06	0.10	0.18	3.12
Sample 7	0.29	0.30	0.05	0.07	1.21
Sample 8	0.15	0.11	0.03	0.22	3.79
Sample 9	0.07	0.12	0,01	0.00	0.00

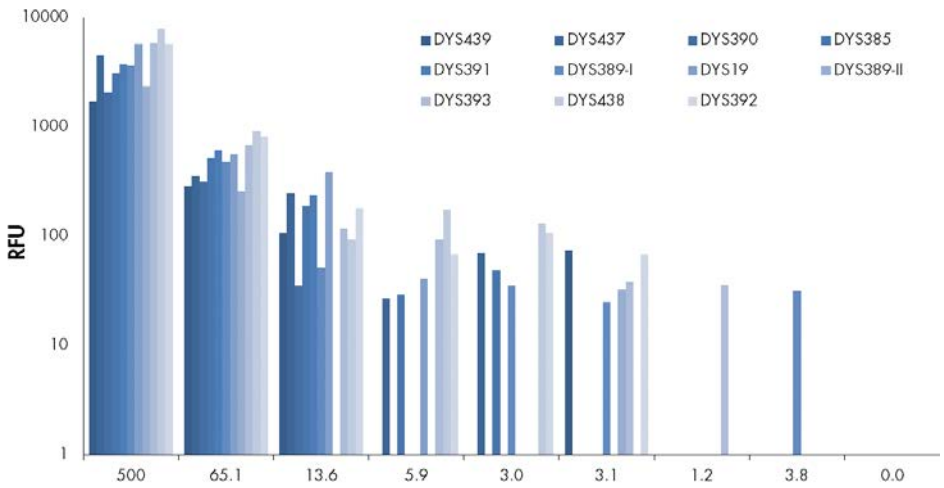

Figure 31. STR results showing the mean peak height in RFU. The results show the correlation of the quantification using the Investigator Quantiplex Pro Kit to the STR results using the Investigator Argus Y-12 QS Kit. The figure shows average RFU ± standard deviation.

With decreasing amounts of DNA, the average peak heights decreased (Figure 31). It was not possible to detect a full profile using DNA amounts lower than approximately 65 pg

(samples 2–9). Sporadic alleles could be detected using an input DNA amount between 13.6 pg and 3.8 pg (samples 4–8) due to stochastic effects. For sample 9, no male DNA was detected during quantification and no alleles were detected in the Y-STR reaction. These results demonstrated the correlation between DNA quantification and STR profile quality.

References

Cited references

1. ENFSI Standing Committee for Quality and Competence (QCC). Validation and Implementation of (New) Methods. Ref. Code: QCC-VAL-001, Issue No. 001, 4 November 2006. **www.enfsi.eu/get_doc.php?uid=144**.
2. Revised Validation Guidelines of Scientific Working Group on DNA Analysis Methods (SWGDAM) Forensic Science Communications, July 2004, Volume 6, Number 3. **www.cstl.nist.gov/strbase/validation/SWGDAM_Validation.doc**.
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 Kit (200)	Quantiplex Pro Reaction Mix, Quantiplex Pro Primer Mix, Quantiplex Pro Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387216

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	
R1 10/2017	First version of document
R2 11/2018	Addition of data regarding cross-reactivity of the male Y-chromosomal target in samples with high concentrations of female DNA (page 38 and Table 8)

1115505 11/2018