QIAGEN Validation Report

Developmental validation of the Investigator® Quantiplex HYres Kit

The Investigator Quantiplex HYres 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 assay used for human identification are complex systems that require a defined range of template input.

The Investigator Quantiplex HYres 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). It also establishes whether a sample contains inhibitors that may interfere with downstream applications, thus necessitating further sample purification.

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 optimal amplification conditions for the Investigator Quantiplex HYres Kit are given on page 4. A target validation was performed in an external study (page 6). The kit was validated for reproducibility, repeatability (page 11), and sensitivity (page 15). It was tested for cross-reactivity with other species (page 20), and its performance with inhibitors (page 23) and contamination (page 36) was assessed. The quantification of male:female mixtures was also tested (page 39).

The validation of Investigator Quantiplex HYres Kit showed that it yielded robust and reproducible results within the normal range of conditions expected in forensic casework.

Principle and procedure

The Investigator Quantiplex HYres Kit is a ready-to-use system for the quantification of human DNA using real-time PCR. It provides fast and accurate quantification of total human and human male DNA in forensic database and casework samples. The assay provides sensitivity down to <1 pg/µl, with accurate quantification below 5 pg/µl, where the standard curve shows linearity.

The kit contains reagents and a DNA polymerase for specific amplification of a 146 bp multicopy region on different autosomal chromosomes of the human genome. The proprietary multicopy region was selected in order to give high sensitivity with high reliability within different individuals and populations. The target region was validated in an external study. The human target is detected using the green channel on the Rotor-Gene® Q or the FAM™ dye channel on Applied Biosystems® 7500 Real-Time PCR Systems.

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. This target region is detected as a 129 bp fragment using the red channel on the Rotor-Gene Q or the Cy™5 dye channel on Applied Biosystems instruments.

In addition, the Investigator Quantiplex HYres Kit contains a balanced internal amplification control that is used to test for successful amplification and detect PCR inhibitors. This heterologous amplification system is detected as a 200 bp internal control (IC) in the yellow channel on the Rotor-Gene Q or in the VIC® dye channel on Applied Biosystems 7500 Real-Time PCR Systems.

Detection of amplification is performed using Scorpions® primers and a novel, fast chemistry. Scorpions primers are bifunctional molecules containing a PCR primer covalently linked to a probe (Figure 1, page 3). The fluorophore in this probe interacts with a quencher, also incorporated into the probe, which reduces fluorescence. During PCR, when the probe binds to the amplicon, the fluorophore and quencher become separated. This leads to an increase in fluorescence in the reaction tube.

Scorpions primers are well known for their rapid hybridization to the target sequence, via the intramolecular reaction (3). The reaction chemistry was carefully optimized to further support the rapid mechanism.

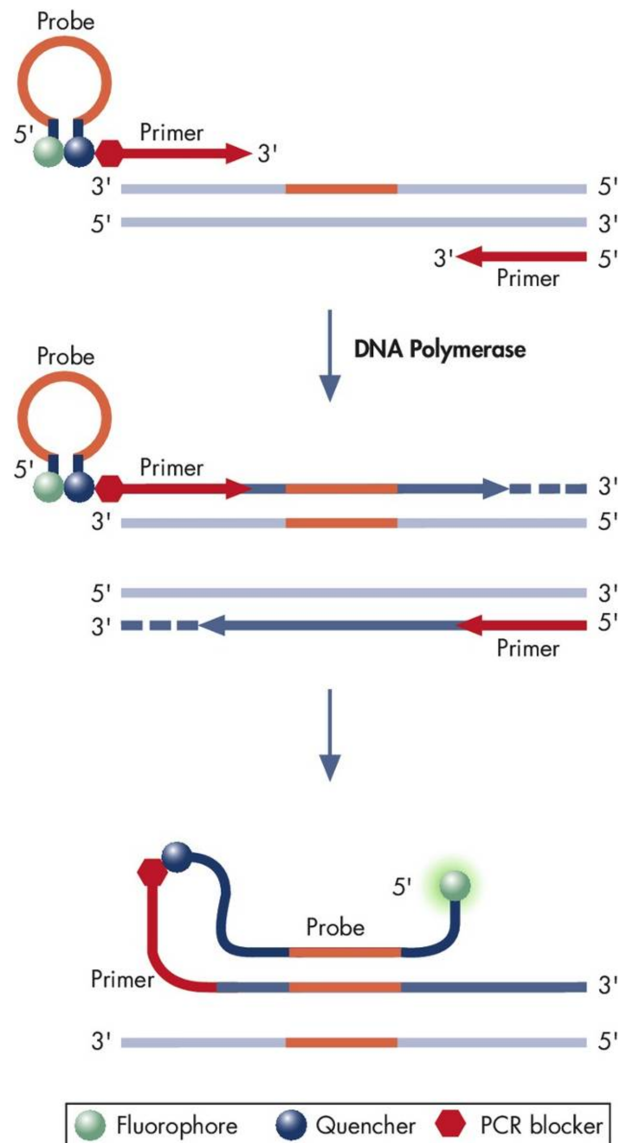

Figure 1. Scorpions primers and their function.

Instrumentation for validation

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

- Rotor-Gene Q
- 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 presented in this Validation Report were obtained using 0.1 ml Strip Tubes on the 72-well rotor (cat. no. 981103 or 981106). Rotor-Gene Q Software version 2.0.2 (Build 4) was used.

For both the Applied Biosystems 7500 Real-Time PCR System for Human Identification and the Applied Biosystems 7500 Real-Time PCR System, all the data presented in this Validation Report were obtained using optical plates (cat. no. N801-0560). For the Applied Biosystems 7500 Real-Time PCR System for Human Identification, HID Real-Time PCR Analysis software version 1.1 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 FQ	9 µl	1x
Primer Mix IC YQ	9 µl	1x
Total volume of master mix	**18 µl**	

Table 2. Cycling conditions for the Rotor-Gene Q

Temperature	Temp	Time	Number of cycles	Additional comments
Initial PCR activation step	95°C	1 min	–	PCR requires an initial incubation at 95°C for 1 min
Two-step cycling:				
Denaturation	95°C	5 s	40	Perform fluorescence data collection using the green, red and yellow channels with auto-gain optimization
Combined annealing/ extension	60°C	10 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 external study, the copy number of the target sequence was analyzed. The participants were asked to perform real-time PCR for 2 different targets in the same reaction vessel. The first target was a nonvariable region of the *KRAS* gene on chromosome 12 (12p12.1). The sequence detected by the primers used in this study refers to a single-copy target in the human genome. The second target was the target used in the Investigator Quantiplex HYres Kit. The ΔC_T values (the difference between the C_T value of the first and second targets) describe the difference in copy number between the 2 targets.

Reaction efficiency and linearity using different template amounts are important parameters in order to be able to compare the simultaneous amplification of both targets. The amplification of both systems was confirmed to be in the linear range with efficiency >90% and a R^2 value >0.99 using DNA concentrations between 50 and 0.05 ng/µl. An example obtained on the Rotor-Gene Q is shown in Figure 2 (page 7). The same values were also confirmed on the Applied Biosystems 7500 Real-Time PCR System (data not shown).

Participants were asked to use between 1 and 10 ng human DNA per 25 µl reaction, which lies within the linear reaction range. The reaction was performed using the QIAGEN® QuantiTect® Multiplex PCR Kit, following the manufacturer's instructions.

The ready-to-use Scorpions Oligo Mix containing primers and Scorpions primers was provided by QIAGEN. The reactions were performed using the Rotor-Gene Q and the Applied Biosystems 7500 Real-Time PCR System.

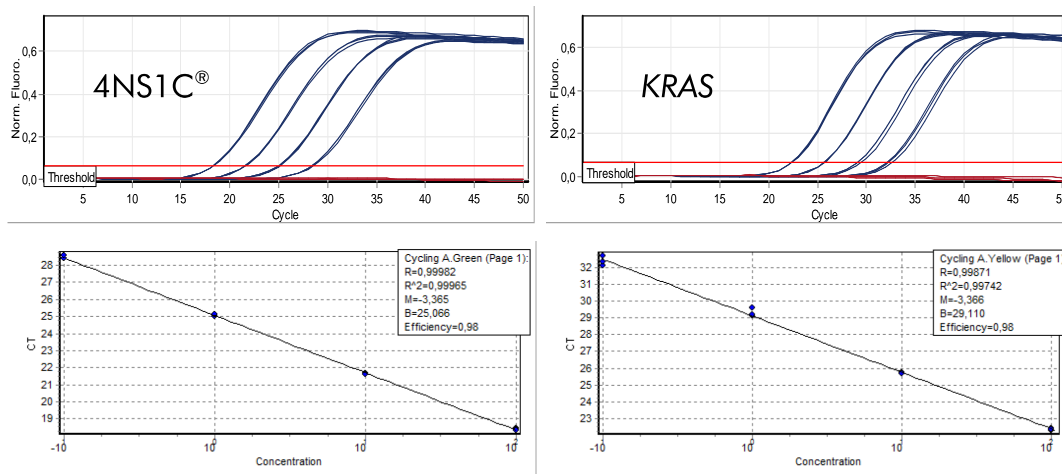

Figure 2. PCR efficiency and linearity of the target validation duplex qPCR for the human target. These parameters are comparable for both green and yellow channels using DNA concentrations between 50 and 0.05 ng/μl.

In the study, DNA from 169 men and 19 women was examined to ensure reproducibility across genders. Figure 3 shows a comparison of ΔC_T values generated in this study, demonstrating that there is a consistent copy number between males and females. The mean ΔC_T value and standard deviation were calculated for each population.

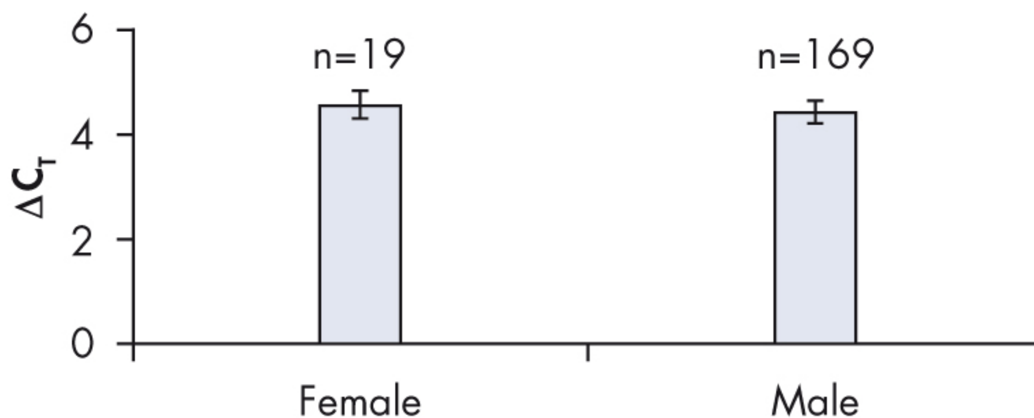

Figure 3. Comparable ΔC_T values (highly stable copy number) were detected for both male and female subjects. The figure shows the average $\Delta C_T \pm$ standard deviation.

Figure 4 shows a comparison of 189 samples representing the 4 main human population groups: African American, Asian, Caucasian, and Hispanic. Comparing the ΔC_T of each population there is a consistent copy number across the 4 population groups studied.

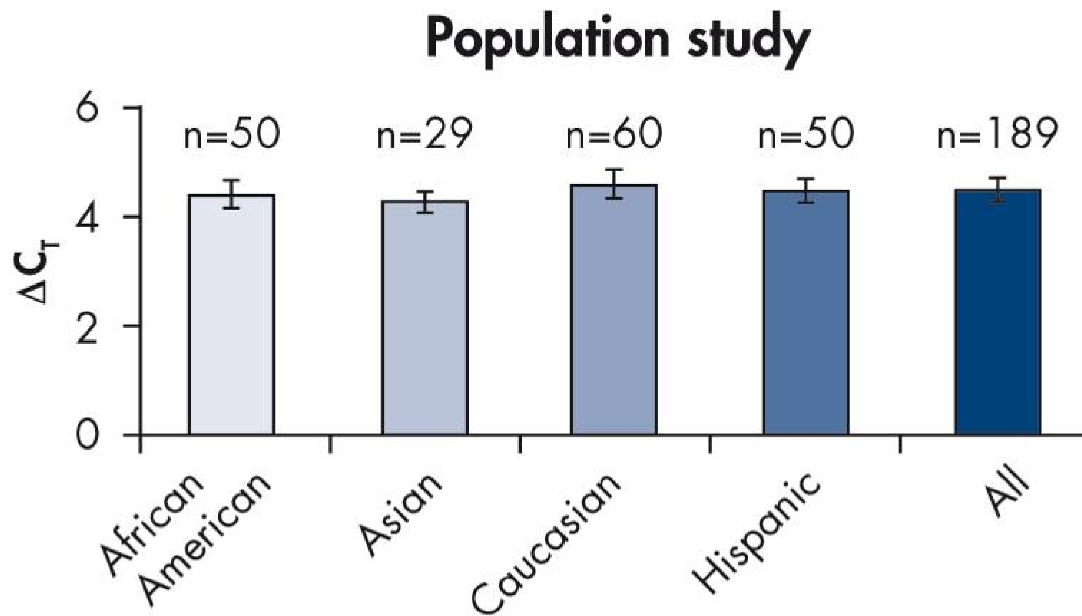

Figure 4. Comparable ΔC_T values (highly stable copy number) for the human target were detected for the 4 main human population groups. The figure shows the average ΔC_T ± standard deviation.

The average ΔC_T for the 189 samples analyzed was 4.45 ± 0.27. This value corresponds to a copy number for the Investigator Quantiplex HYres human target of about 20, considering a haploid genome (40: diploid genome).

Male target validation study

In an external study, the copy number of the male target sequence was analyzed. Again, the participants were asked to perform a real-time PCR for 2 different targets in the same reaction vessel. The first target was the *KRAS* gene, as described above. The sequence detected by the primers used in this study refers to a single-copy target in the human genome. The second target was the male target used in the Investigator Quantiplex HYres Kit. The ΔC_T values (the difference between the C_T value of the first and second targets) describe the difference in copy number between the 2 targets.

Reaction efficiency and linearity using different template amounts are important parameters in order to be able to compare the simultaneous amplification of both targets. The amplification of both systems was confirmed to be in the linear range with efficiency >90% and a R^2 value >0.99 using DNA concentrations between 20 and 0.02 ng/µl. An example obtained on the Rotor-Gene Q is shown in Figure 5. The same values were also confirmed on the Applied Biosystems 7500 Real-Time PCR System (data not shown).

Participants were asked to use between 1 and 10 ng human DNA per 25 µl reaction, which lies within the linear reaction range. The reaction was performed using the QIAGEN QuantiTect Multiplex PCR Kit, following the manufacturer's instructions.

The ready-to-use Scorpions Oligo Mix containing primers and Scorpions primers was provided by QIAGEN. The reactions were performed using the Rotor-Gene Q and the Applied Biosystems 7500 Real-Time PCR System.

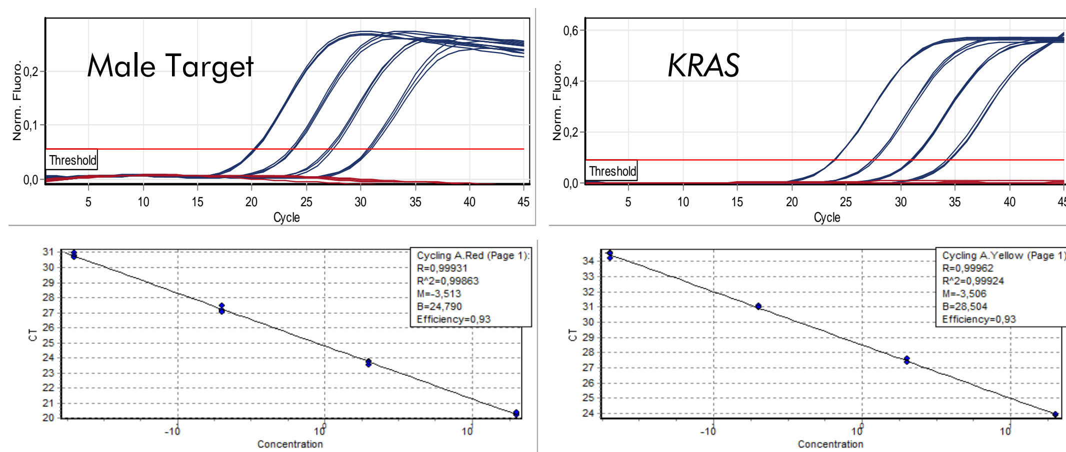

Figure 5. PCR efficiency and linearity of the target validation duplex qPCR for the male target. These parameters are comparable for both green and yellow channels using DNA concentrations between 20 and 0.02 ng/µl.

In the study, DNA from 469 men and 9 women was examined to ensure reproducibility. No female DNA gave a signal for the male target. Figure 6 shows a comparison of 469 samples representing the 4 main human population groups: African American, Asian, Caucasian, and Hispanic. Comparing the ΔC_T of each population there is a consistent copy number across the 4 population groups studied.

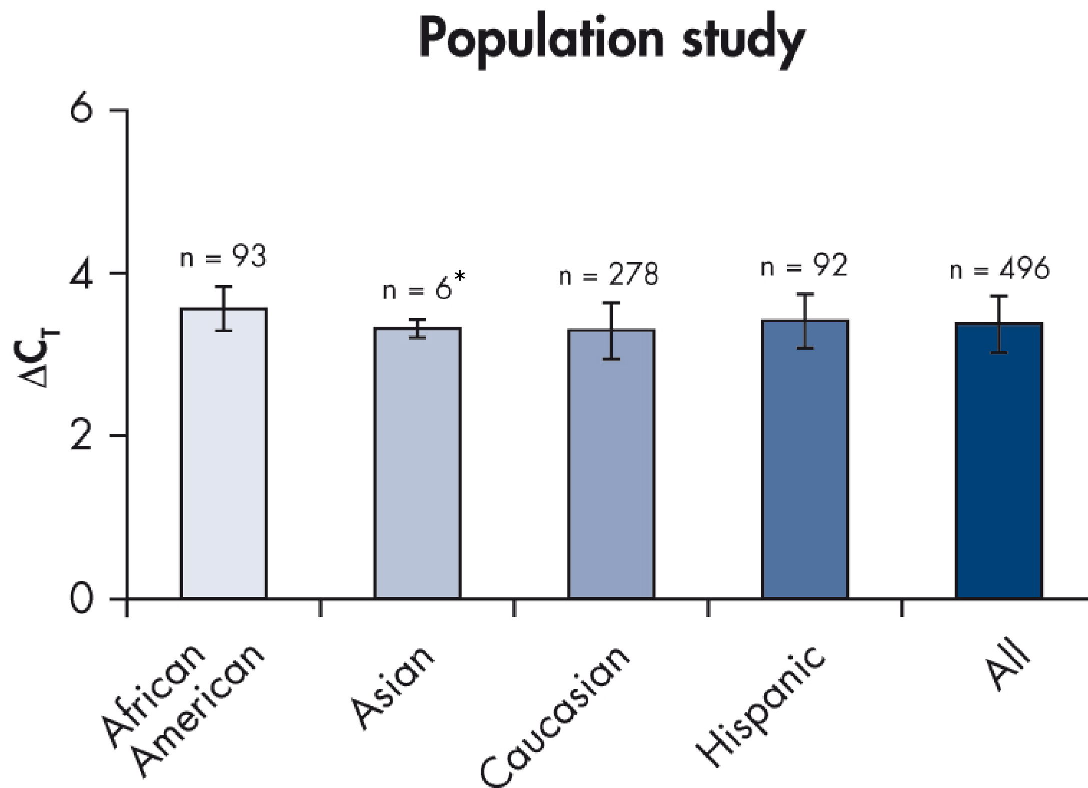

Figure 6. Comparable ΔC_T values (highly stable copy number) for the male target were detected for the 4 main human population groups. The figure shows the average $\Delta C_T \pm$ standard deviation. * More data coming soon.

The average ΔC_T for the 469 samples analyzed was 3.36 ± 0.35. This value corresponds to a copy number for the Investigator Quantiplex HYres male target of about 18 per cell.

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 Rotor-Gene Q, on the Applied Biosystems 7500 Real-Time PCR System for Human Identification and 7500 Real-Time PCR Systems by taking 5 replicates of the 7 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 4–9 (pages 12–14) show the data from the study. The mean quantity and standard variation were calculated for each sample dilution.

The reproducibility of the DNA quantification using the Investigator Quantiplex HYres Kit was demonstrated for all 3 validated instruments.

Table 4. Highly reproducible results comparing 2 different runs performed by 2 different operators using the same Rotor-Gene Q

DNA sample	Operator 1 Concentration (ng/μl) ± standard deviation		Operator 2 Concentration (ng/μl) ± standard deviation	
	Human	Male	Human	Male
Male 1 (~0.5 ng/μl)	0.53±0.09	0.52±0.09	0.60±0.20	0.52±0.08
Male 2 (~0.5 ng/μl)	0.65±0.18	0.59±0.07	0.56±0.22	0.52±0.05
Male 3 (~0.5 ng/μl)	0.62±0.09	0.65±0.03	0.51±0.10	0.56±0.03
Female 1 (~0.5 ng/μl)	0.61±0.13	–	0.55±0.22	–
Female 2 (~0.5 ng/μl)	0.49±0.13	–	0.66±0.17	–
Female 3 (~0.5 ng/μl)	0.63±0.05	–	0.74±0.07	–

Table 5. 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

DNA sample	Operator 1 Concentration (ng/μl) ± standard deviation		Operator 2 Concentration (ng/μl) ± standard deviation	
	Human	Male	Human	Male
Male 1 (~0.5 ng/μl)	0.34±0.09	0.57±0.06	0.52±0.14	0.62±0.08
Male 2 (~0.5 ng/μl)	0.71±0.10	0.79±0.11	0.83±0.05	0.86±0.09
Male 3 (~0.5 ng/μl)	0.49±0.15	0.73±0.10	0.68±0.14	0.82±0.12
Female 1 (~0.5 ng/μl)	0.69±0.17	–	0.90±0.18	–
Female 2 (~0.5 ng/μl)	0.68±0.12	–	0.77±0.06	–
Female 3 (~0.5 ng/μl)	0.64±0.16	–	0.76±0.19	–

Table 6. Highly reproducible results comparing 2 different runs performed by 2 different operators on the same Applied Biosystems 7500 Real-Time PCR System

DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation		Operator 2 Concentration (ng/µl) ± standard deviation	
	Human	Male	Human	Male
Male 1 (~0.5 ng/µl)	0.68±0.06	0.74±0.04	0.53±0.02	0.78±0.04
Male 2 (~0.5 ng/µl)	0.71±0.10	0.72±0.03	0.56±0.04	0.81±0.06
Male 3 (~0.5 ng/µl)	0.79±0.07	0.73±0.02	0.56±0.02	0.80±0.05
Female 1 (~0.5 ng/µl)	0.38±0.06	–	0.24±0.02	–
Female 2 (~0.5 ng/µl)	0.45±0.12	–	0.28±0.08	–
Female 3 (~0.5 ng/µl)	0.57±0.03	–	0.34±0.02	–

The repeatability of the DNA quantification using the Investigator Quantiplex HYres Kit was demonstrated for all 3 validated instruments.

Table 7. Highly repeatable results comparing 2 different runs performed by the same operator using the same Rotor-Gene Q

DNA sample	Run 1 Concentration (ng/µl) ± standard deviation		Run 2 Concentration (ng/µl) ± standard deviation	
	Human	Male	Human	Male
Male 1 (~0.5 ng/µl)	0.53±0.09	0.52±0.09	0.49±0.14	0.53±0.06
Male 2 (~0.5 ng/µl)	0.65±0.18	0.59±0.07	0.50±0.18	0.54±0.08
Male 3 (~0.5 ng/µl)	0.62±0.09	0.65±0.03	0.45±0.03	0.59±0.04
Female 1 (~0.5 ng/µl)	0.61±0.13	–	0.57±0.19	–
Female 2 (~0.5 ng/µl)	0.49±0.13	–	0.56±0.13	–
Female 3 (~0.5 ng/µl)	0.63±0.05	–	0.64±0.07	–

Table 8. 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

DNA sample	Run 1 Concentration (ng/µl) ± standard deviation		Run 2 Concentration (ng/µl) ± standard deviation	
	Human	Male	Human	Male
Male 1 (~0.5 ng/µl)	0.34±0.09	0.57±0.06	0.53±0.13	0.57±0.07
Male 2 (~0.5 ng/µl)	0.71±0.10	0.79±0.11	0.84±0.08	0.82±0.06
Male 3 (~0.5 ng/µl)	0.49±0.15	0.73±0.10	0.68±0.14	0.79±0.11
Female 1 (~0.5 ng/µl)	0.69±0.17	–	0.88±0.19	–
Female 2 (~0.5 ng/µl)	0.68±0.12	–	0.77±0.08	–
Female 3 (~0.5 ng/µl)	0.64±0.16	–	0.79±0.15	–

Table 9. Highly repeatable results comparing 2 different runs performed by the same operator using the same Applied Biosystems 7500 Real-Time PCR System

DNA sample	Run 1 Concentration (ng/µl) ± standard deviation		Run 2 Concentration (ng/µl) ± standard deviation	
	Human	Male	Human	Male
Male 1 (~0.5 ng/µl)	0.68±0.06	0.74±0.04	0.67±0.05	0.87±0.07
Male 2 (~0.5 ng/µl)	0.71±0.10	0.72±0.03	0.68±0.04	0.83±0.04
Male 3 (~0.5 ng/µl)	0.79±0.07	0.73±0.02	0.69±0.05	0.86±0.07
Female 1 (~0.5 ng/µl)	0.38±0.06	–	0.32±0.04	–
Female 2 (~0.5 ng/µl)	0.45±0.12	–	0.36±0.06	–
Female 3 (~0.5 ng/µl)	0.57±0.03	–	0.47±0.05	–

Sensitivity

The Investigator Quantiplex HYres Kit is designed to detect a broad range of DNA quantities. Figures 7, 9, and 11 show a serial dilution of Control DNA Z1 from 20 ng/µl to 0.08 pg/µl. The optimal linear dynamic range of the assay is in the range of 20 ng/µl to 4.88 pg/µl total DNA. DNA could be detected down to 0.08 pg/µl using triplicates and the standard conditions specified in the *Investigator Quantiplex HYres Handbook* using all 3 validated instruments (Rotor-Gene Q and Applied Biosystems 7500 Real-Time PCR Systems for Human Identity and Applied Biosystems 7500 Real-Time PCR Systems).

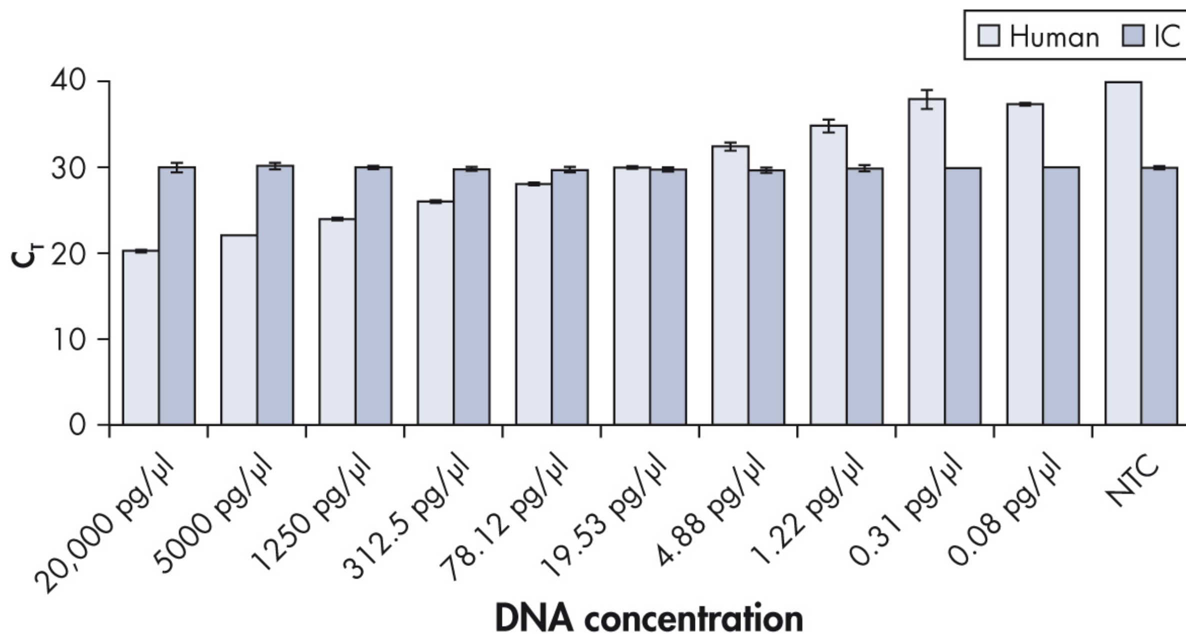

Figure 7. Detection of human DNA in Control DNA Z1 down to 0.08 pg/µl using the Investigator Quantiplex HYres Kit on the Rotor-Gene Q. The figure shows the average CT ± standard deviation. **NTC**: No-template control.

The sensitivity of Investigator Quantiplex HYres Kit has been tested for the male DNA component. The Control DNA Z1 contains a mixture of male and female DNA, which mimics the situation of a mixed sample — 1 µl of Control DNA Z1 contains 20 ng of human DNA, which includes 6.67 ng of male DNA.

Figures 8, 10, and 12 show the quantification of the male component of a serial dilution of Control DNA Z1 from 6.67 ng/µl to 0.03 pg/µl male DNA. The optimal linear dynamic range of the assay is in the range of 6.67 ng/µl to 6.5 pg/µl male DNA. DNA could be detected down to 0.1 pg/µl using triplicates and the standard conditions specified in the *Investigator Quantiplex HYres Handbook* using all 3 validated instruments (Rotor-Gene Q and Applied Biosystems 7500 Real-Time PCR Systems for Human Identity and Applied Biosystems 7500 Real-Time PCR Systems).

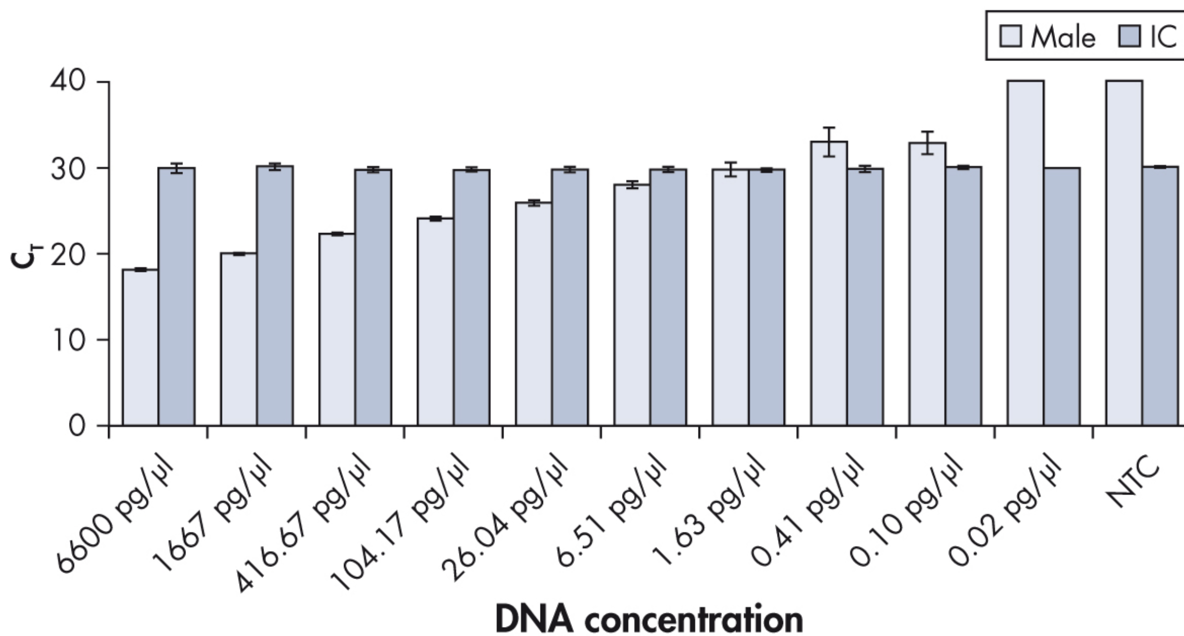

Figure 8. Detection of male DNA down to 0.1 pg/µl using the Investigator Quantiplex HYres Kit on the Rotor-Gene Q. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

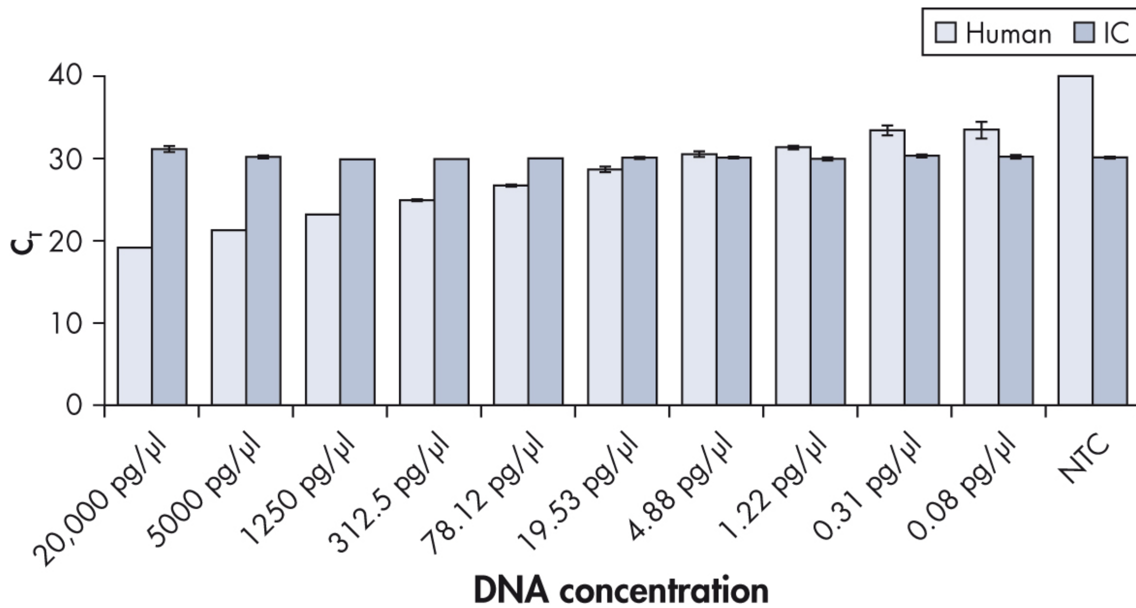

Figure 9. Detection of human DNA down to 0.08 pg/µl using the Investigator Quantiplex HYres Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

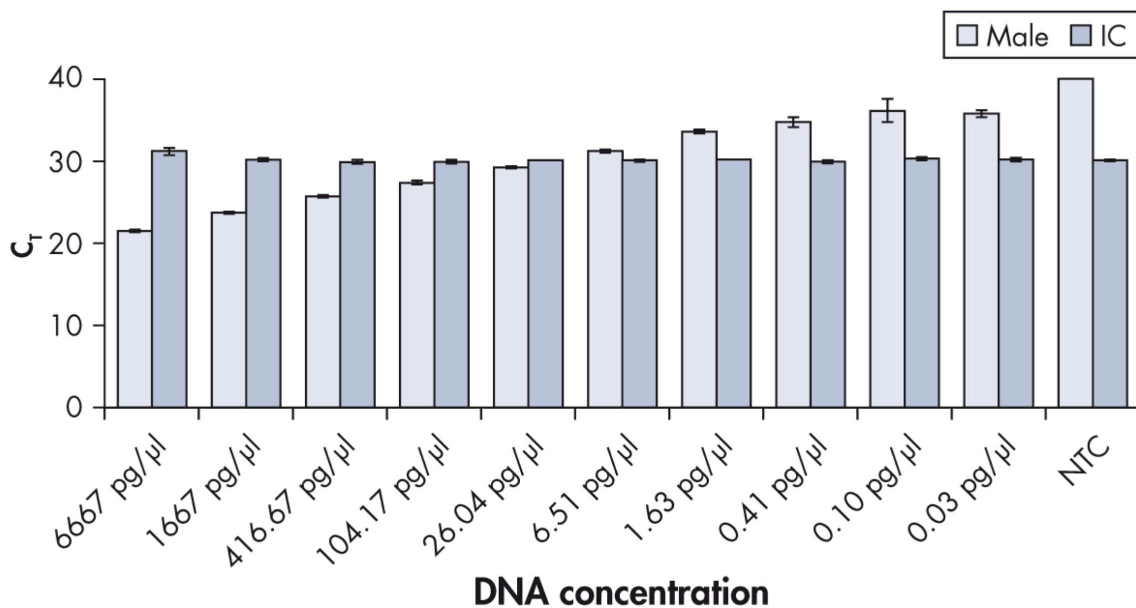

Figure 10. Detection of male DNA down to 0.03 pg/µl using the Investigator Quantiplex HYres Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

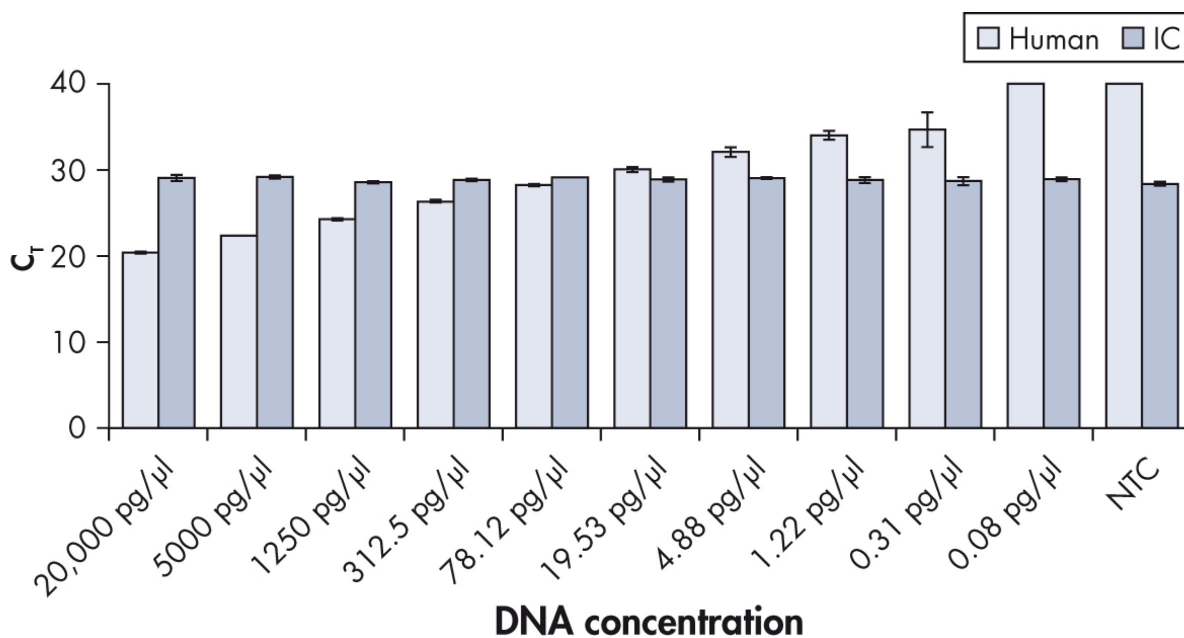

Figure 11. Detection of human DNA down to 0.15 pg/µl (data point not shown) using the Investigator Quantiplex HYres Kit on the Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

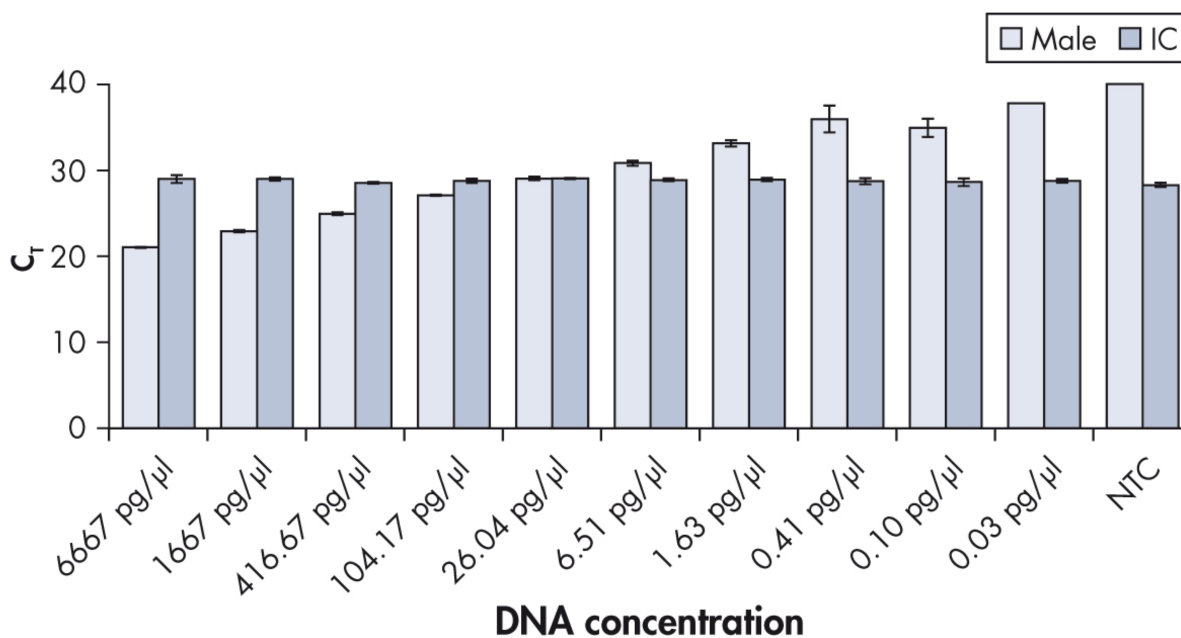

Figure 12. Detection of male DNA down to 0.03 pg/µl using the Investigator Quantiplex HYres Kit on the Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

Compatibility with high amounts of DNA

A very high concentration of DNA above the range of the standard curve (>20 ng/µl) may result from the isolation of DNA from reference samples, such as buccal swabs. This high concentration of DNA may interfere with the amplification of the internal control, due to competition effects. Therefore, the Investigator Quantiplex HYres internal control was tested using high amounts of DNA on the Rotor-Gene Q.

Control DNA Z1 (100 ng, 50 ng, 25 ng, 12.5 ng, and 6.25 ng) was used in the Investigator Quantiplex HYres reaction. Figure 13 shows that the C_T value of the internal control is stable, even where high amounts of human DNA are present in the sample. The quantification of the DNA is reliable over the full range tested, even at concentrations above the standard curve.

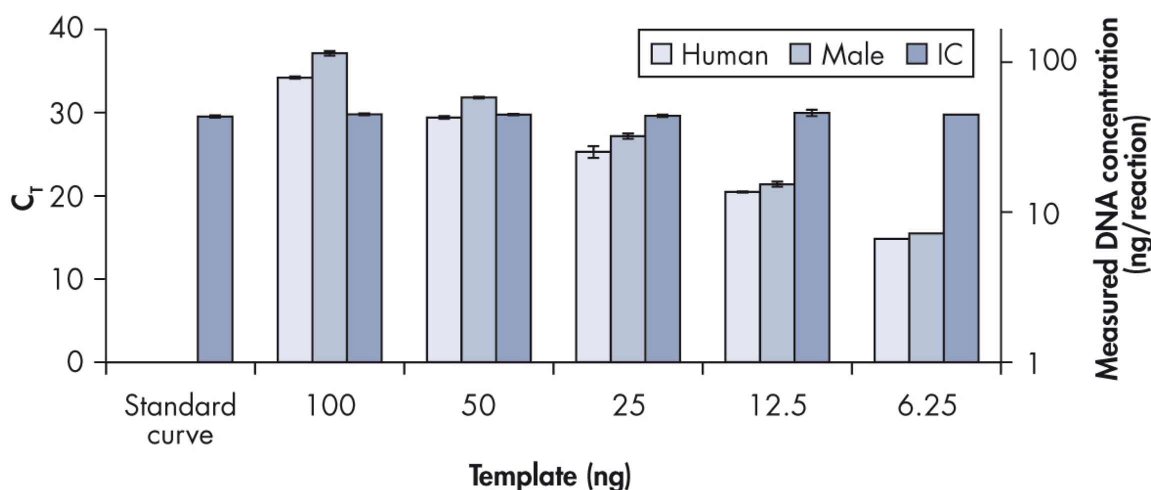

Figure 13. DNA overload test on the Rotor-Gene Q. The figure shows the average C_T ± standard deviation for the internal control and the measured human and male DNA concentration (ng/reaction). The C_T value for the internal control is stable, even at high DNA concentrations.

Species specificity

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

To verify Investigator Quantiplex HYres 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 Control DNA Z1 as a positive control.

No cross-reactivity was shown for DNA from the tested common vertebrates under standard conditions, as shown in Figure 14.

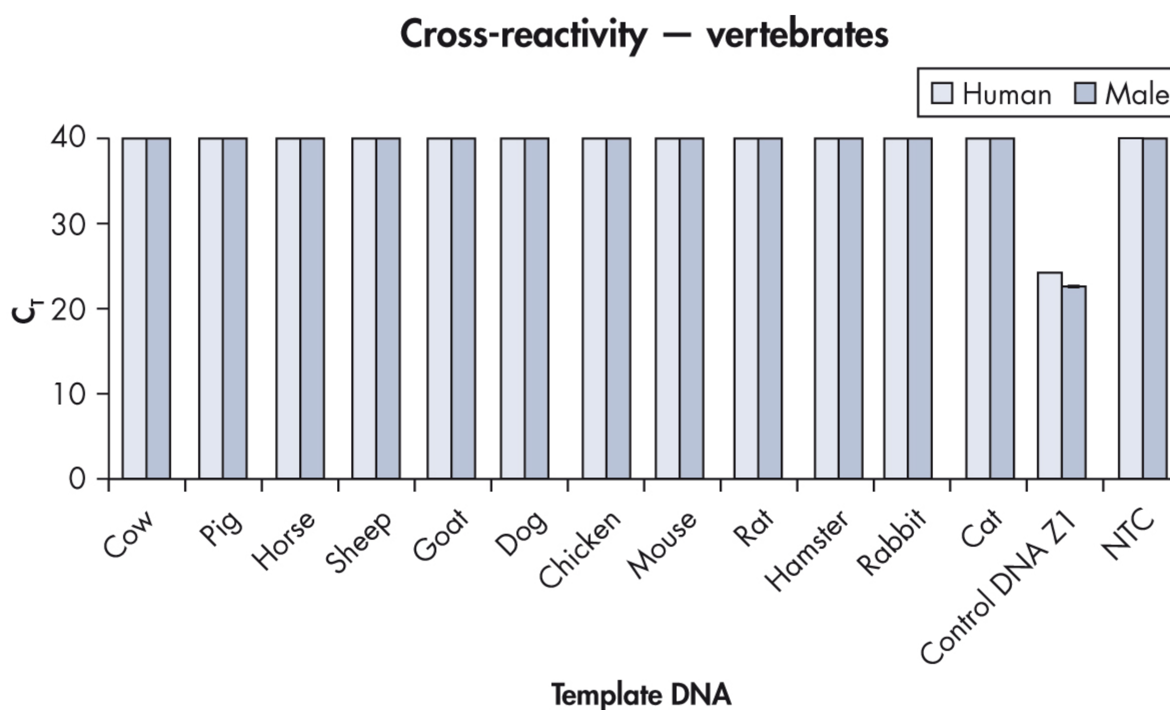

Figure 14. Results of a cross-reactivity study on common vertebrate species. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

Some primates, including gorilla, chimpanzee, bonobo, orangutan, and macaque were also examined, as described above. Due to the evolutionary proximity of chimpanzee, bonobo, and gorilla to humans, positive results were observed for these species. No cross-reactivity was found for orangutan and macaque DNA (Figure 15).

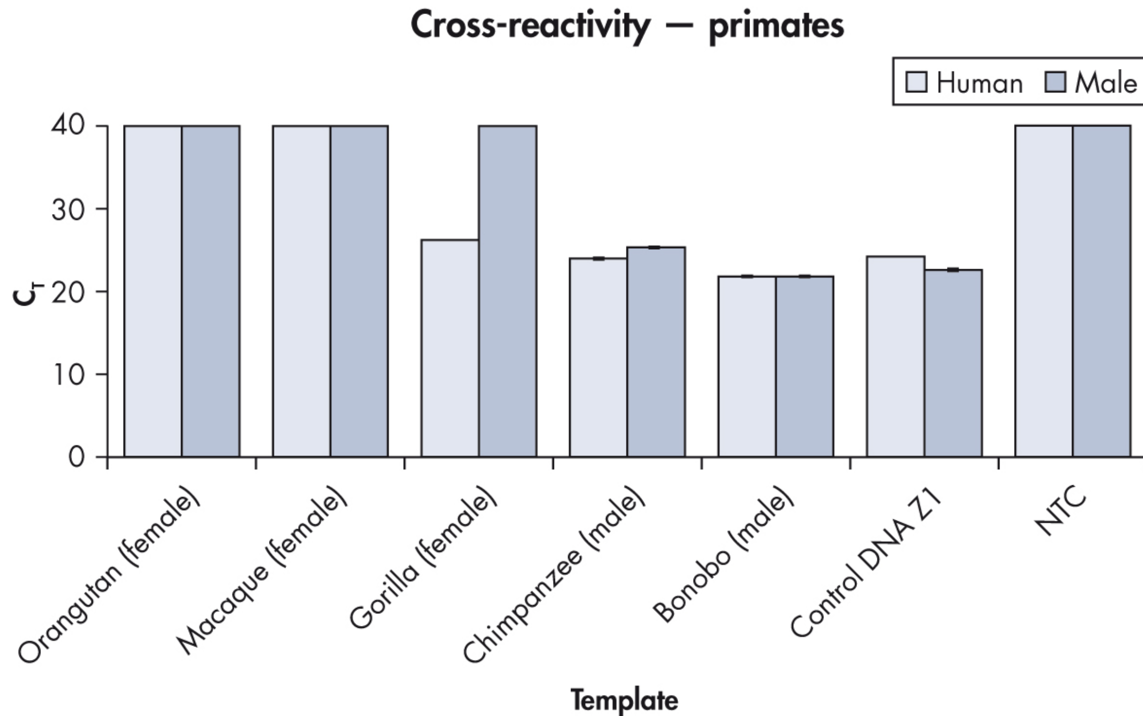

Figure 15. Results of a cross-reactivity study on primates. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

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) were tested following the standard assay protocol, with 2.5 ng Control DNA Z1 as a positive control. None of the tested microbial species yielded detectable DNA under standard conditions, as shown in Figure 16.

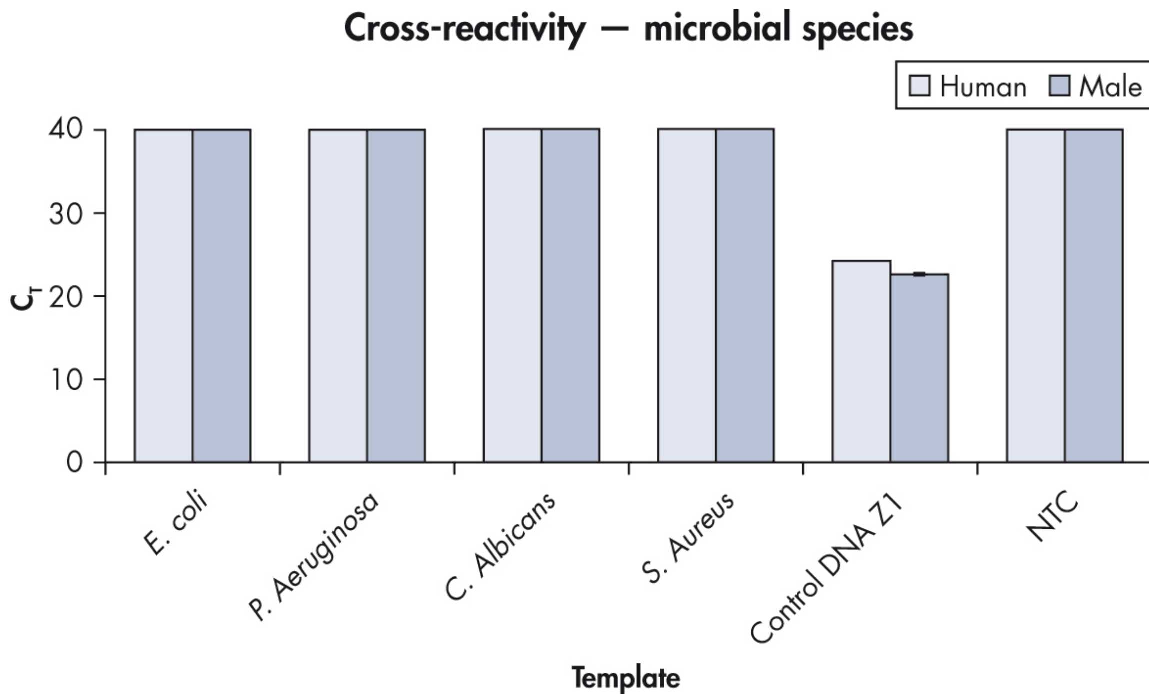

Figure 16. Results of a cross-reactivity study on microbial species. No cross-reactivity could be shown for the tested microbes. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

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

In conclusion, these experiments show that the Investigator Quantiplex HYres 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 HYres assay contains a 200 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, 5, 10, 12.5, 15, 17.5, 20, 25, 30, 40, and 50 ng/µl humic acid (Acros; cat. no. 120860050) under standard conditions as described in the *Investigator Quantiplex HYres Handbook* (1 ng Control DNA Z1). The results are shown in Figures 17–20.

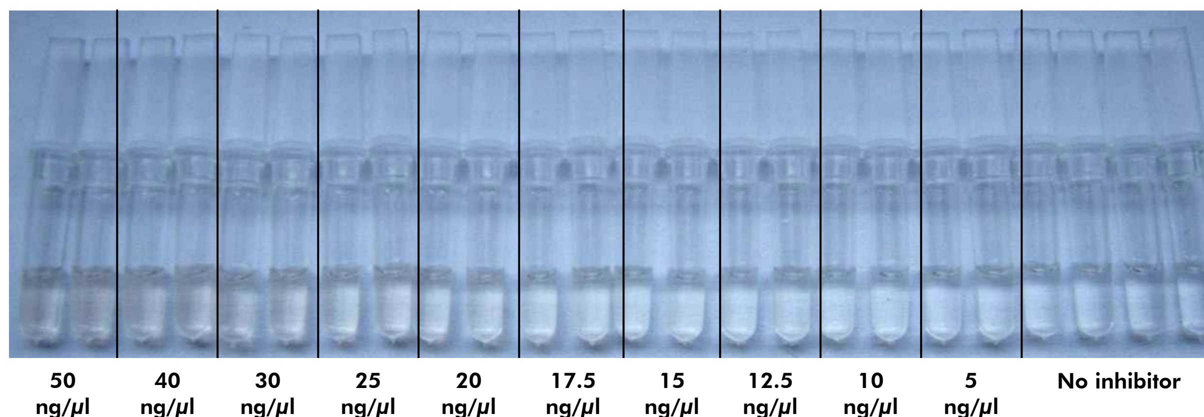

Figure 17. Rotor-Gene Q tubes containing reactions with 0 to 50 ng/µl humic acid.

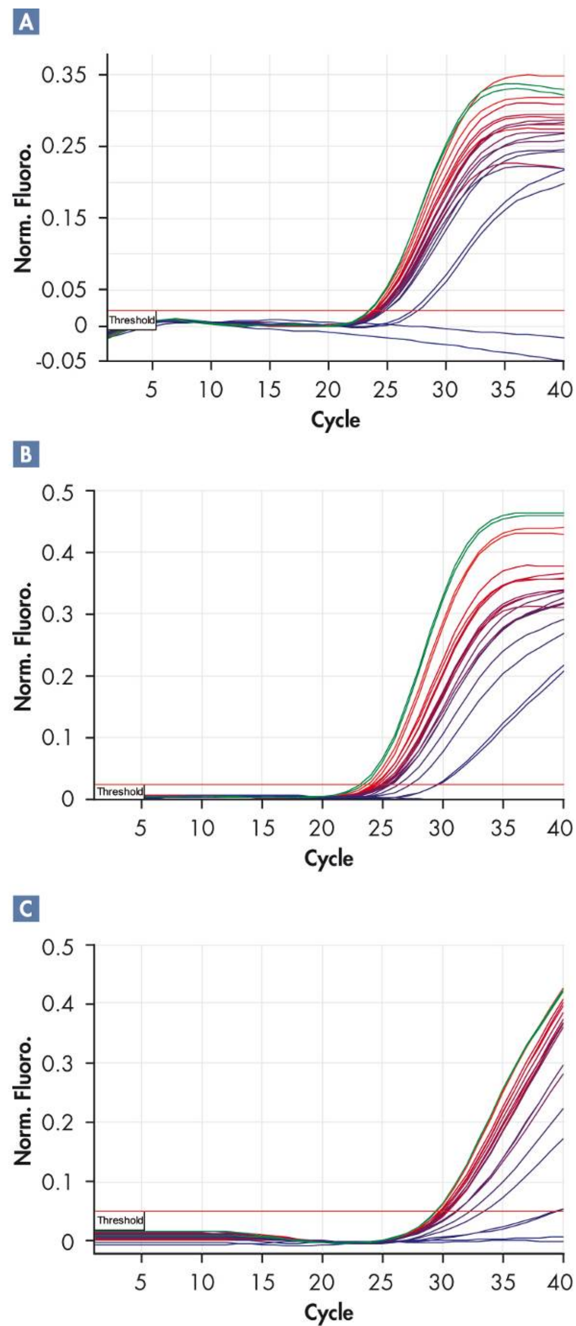

Figure 18. Response to increasing concentrations of humic acid. Amplification plots were produced using the Rotor-Gene Q and the Investigator Quantiplex HYres Kit with increasing concentrations of humic acid from 0 to 50 ng/µl (final concentration in the reaction). **A** Green channel **B** Red channel **C** Yellow channel. Red lines indicate increasing concentrations from 5–15 ng/µl. Purple lines indicate increasing concentrations from 17.5–30 ng/µl. Blue lines indicate increasing concentrations from 40–50 ng/µl. Green lines indicate no inhibitor present.

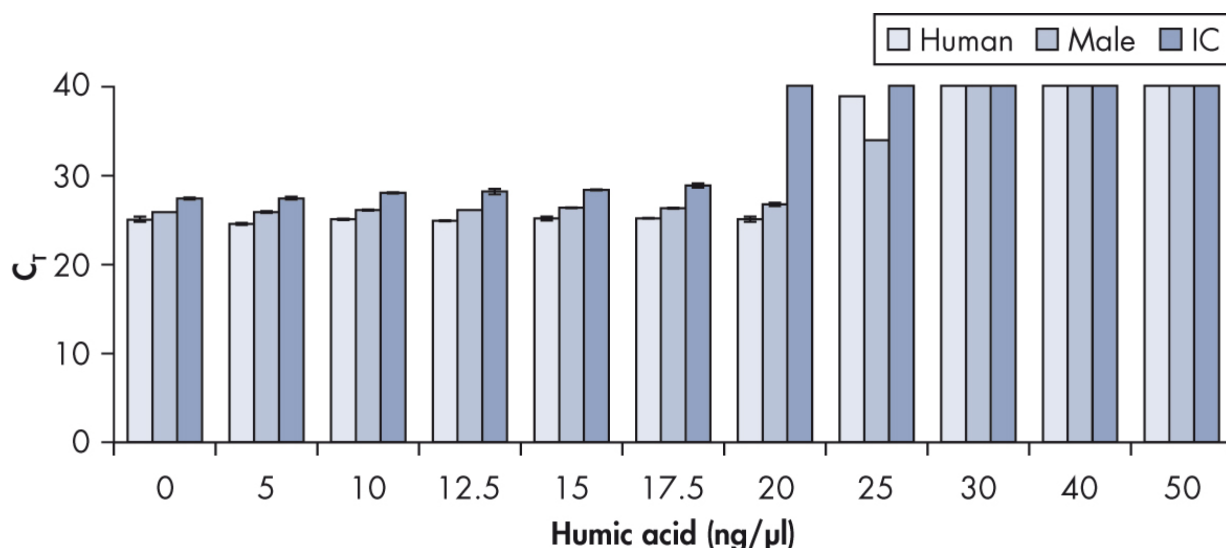

Figure 19. Performance of the Investigator Quantiplex HYres Kit with simulated humic acid inhibition on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 17.5 ng/μl. The figure shows the average C_T ± standard deviation.

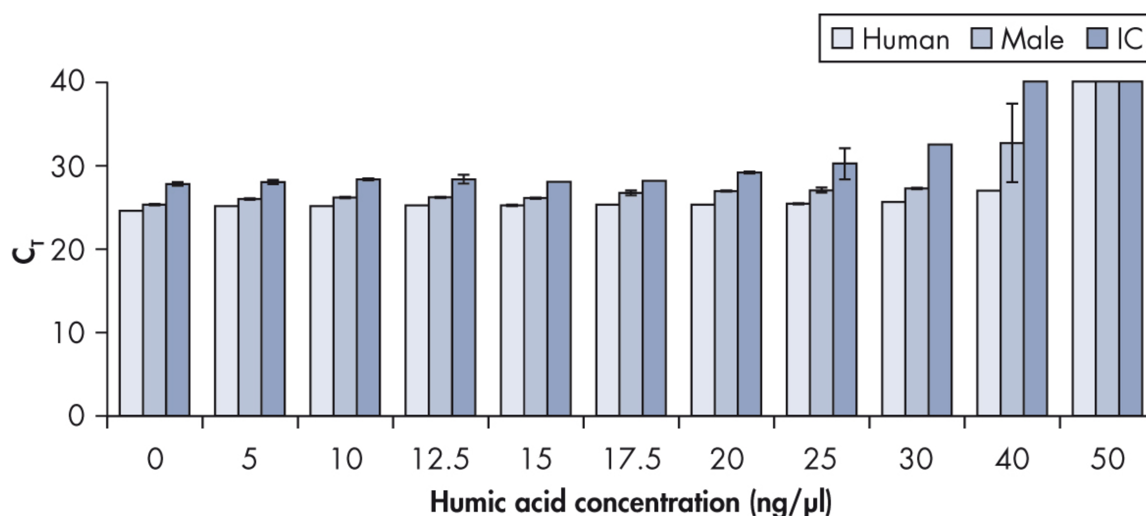

Figure 20. Performance of the Investigator Quantiplex HYres Kit with simulated humic acid inhibition on the Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 20 ng/μl. The 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 remains reliable up to a final humic acid concentration of 17.5 to 20 ng/µl in the PCR (this value is instrument-dependent and must be validated). This corresponds to a concentration in the DNA sample of 175 ng/µl (using 2 µl DNA sample in the assay, as recommended). Approximately the same inhibitor resistance was confirmed for all 3 validated instruments (data not shown).

When using STR kits, 2 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 Plus 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 Plus Kit shows resistance to humic acid of 500 pg DNA up to 200 ng/µl (final concentration in the reaction). Therefore, it is possible to use up to 15 µl of an inhibited DNA sample (containing 175 ng/µl humic acid) and still obtain a full DNA profile without inhibition using the Investigator ESSplex Plus Kit.

See the *Developmental Validation Report for the Investigator ESSplex Plus Kit* 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, 25, 50, 75, 80, 90, 100, 110, 120, 130, 140, and 150 µM hematin (ICN Biomedicals Inc.; cat. no. 198969) under the standard conditions described in the *Investigator Quantiplex HYres Handbook* (1 ng Control DNA Z1). The results are shown in Figures 21–24.

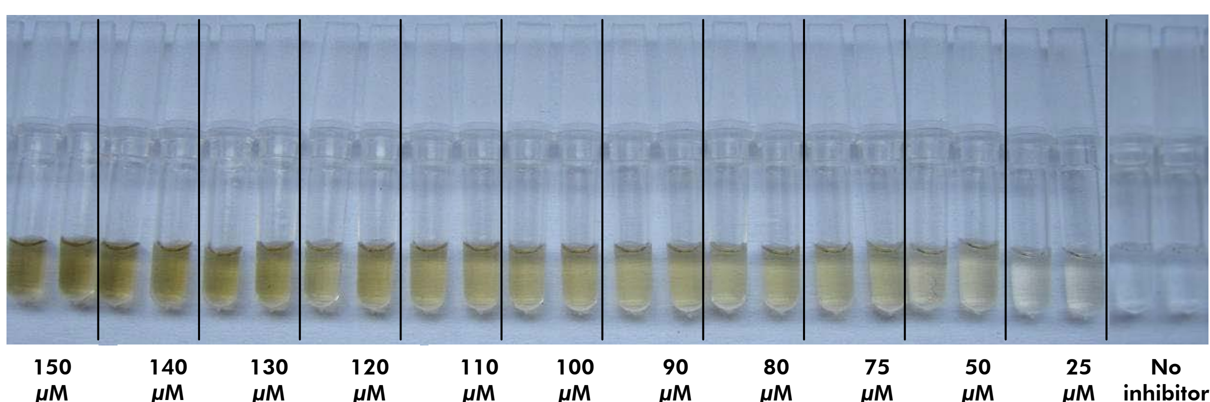

Figure 21. Rotor-Gene Q tubes containing reactions with 0 to 150 µM hematin.

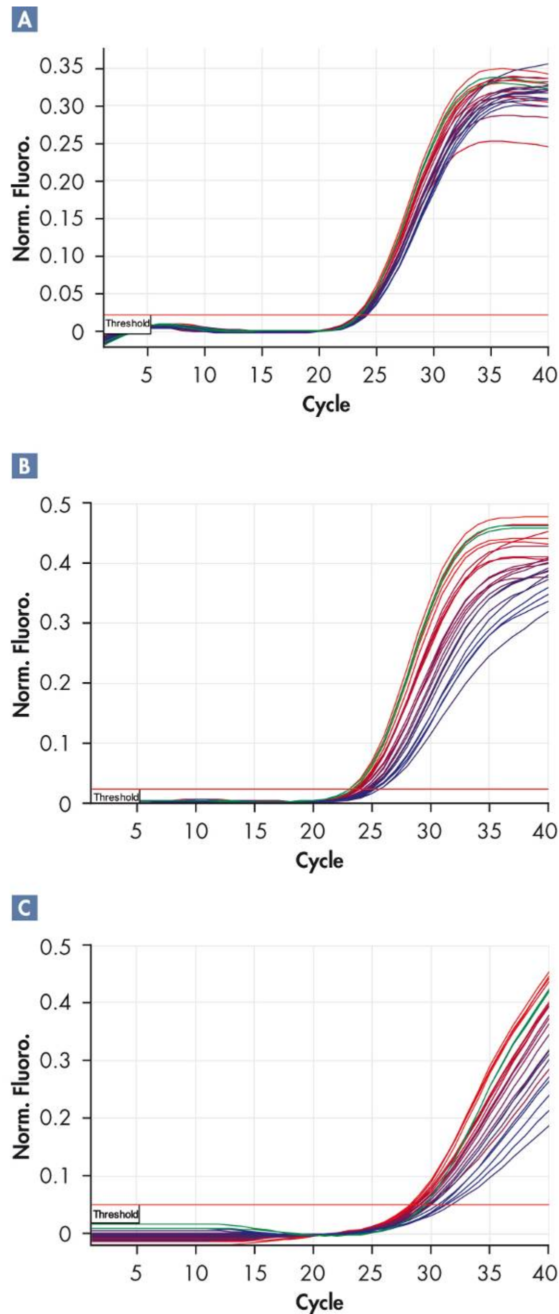

Figure 22. Response to increasing concentrations of hematin. Amplification plots were produced using the Rotor-Gene Q and the Investigator Quantiplex HYres Kit with increasing concentrations of hematin from 0 to 150 µM (final concentration in the reaction). **A** Green channel **B** Red channel **C** Yellow channel. Red lines indicate increasing concentrations from 25–80 µM. Purple lines indicate increasing concentrations from 90–120 µM. Blue lines indicate increasing concentrations from 130–150 ng/µl. Green lines indicate no inhibitor present.

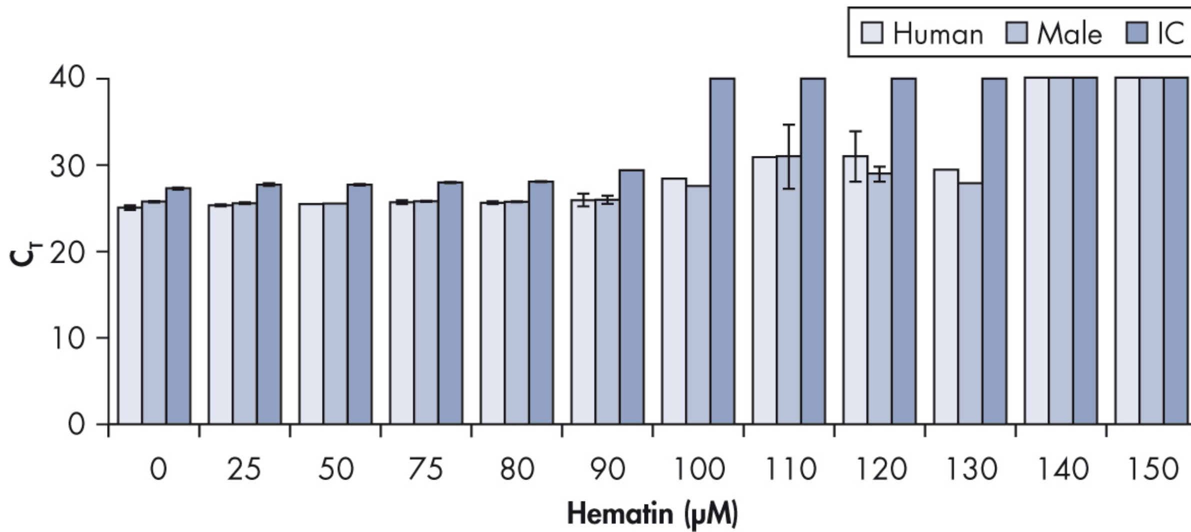

Figure 23. Performance of the Investigator Quantiplex HYres Kit with simulated hematin inhibition on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 90 *µ*M. The figure shows the average C_T ± standard deviation.

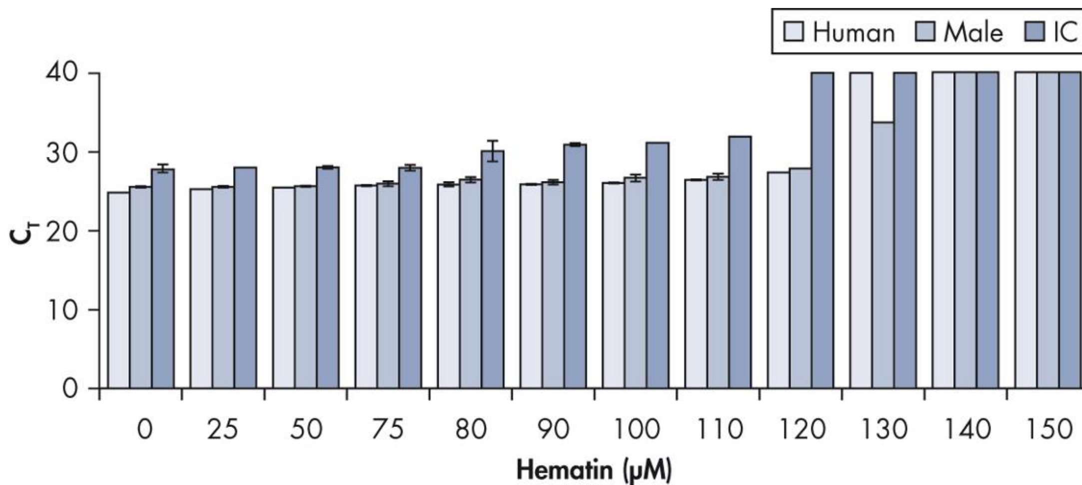

Figure 24. Performance of the Investigator Quantiplex HYres Kit with simulated hematin inhibition on the Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 80 *µ*M. The figure 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 remains reliable up to a final hematin concentration of 80–90 µM (final concentration in the reaction; this value is instrument-dependent and must be validated). This corresponds to a concentration in the DNA sample of 900 µM (using 2 µl DNA sample in the assay, as recommended). Approximately the same inhibitor resistance was confirmed for all 3 validated instruments (data not shown).

The Investigator ESSplex Plus Kit shows resistance to hematin at 1 mM (final concentration in the reaction). Therefore, it is possible to use up to 15 µl of an inhibited DNA sample (containing 900 µM hematin) and still obtain a full DNA profile, without inhibition, using the Investigator ESSplex Plus Kit.

See the *Developmental Validation Report for the Investigator ESSplex Plus Kit* for more information.

Indigo carmine

Indigo is a dye used in certain types of fabrics, such as denim or other dyed materials. Indigo carmine is used to mimic the possible interference of dyes in PCR.

To test the robustness of the kit, the assay was run in the presence of 0, 0.125, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 1.7 mM indigo carmine (Alfa Aesar. cat. no. A16052) under standard conditions as described in the *Investigator Quantiplex HYres Handbook* (1 ng Control DNA Z1). The results are shown in Figure 25 and

Figure **26**.

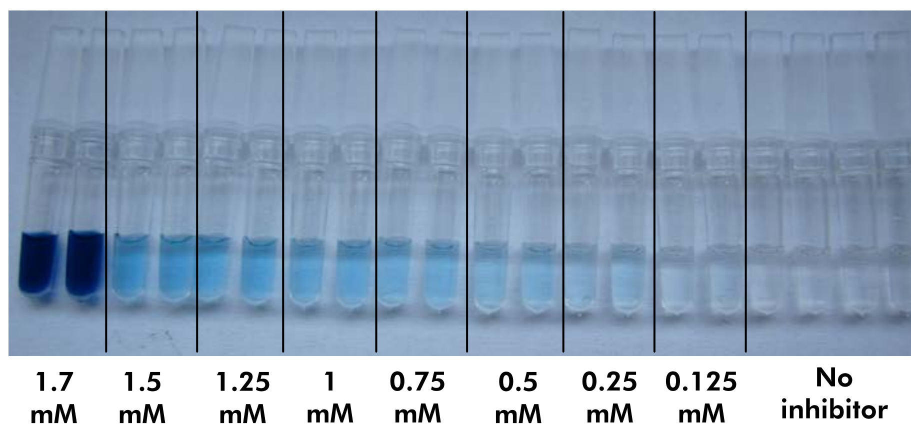

Figure 25. Rotor-Gene Q tubes containing reactions with 0 to 1.7 mM indigo carmine.

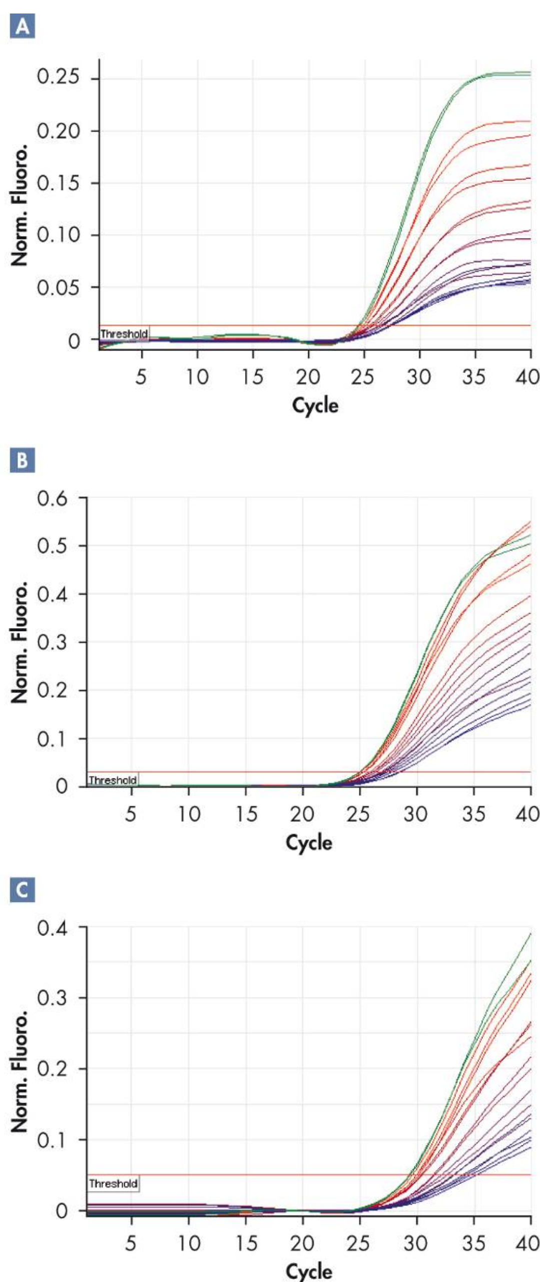

Figure 26. Response to increasing concentrations of indigo carmine. Amplification plots were produced using the Rotor-Gene Q and the Investigator Quantiplex HYres Kit with increasing concentrations of indigo carmine (from 0 to 1.7 mM (final concentration in the reaction). **A** Green channel **B** Red channel **C** Yellow channel. Red lines indicate increasing concentrations from 0.125–0.75 mM. Purple lines indicate increasing concentrations from 1–1.7 *µ*M. Green lines indicate no inhibitor present.

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 remains reliable up to a final indigo carmine concentration of 0.75 mM (final concentration in the reaction) on the Rotor-Gene Q.

However, amplification could not be stably detected by the Applied Biosystems 7500 Real-Time PCR System for Human Identification or the Applied Biosystems 7500 Real-Time PCR System, due to interference by the dark blue color of dye in the reaction mixture (as reported by Opel et al., 2010 [4]). Only the Rotor-Gene Q demonstrated reliable results with this inhibitor.

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 (4).

To test the robustness of the kit, the assay was run in the presence of 0, 1, 2, 3, 4, 5, 6, 7, and 8 mM calcium hydrogen phosphate (VWR; cat. no. 83524.290) under standard conditions as described in the *Investigator Quantiplex HYres Handbook* (1 ng Control DNA Z1). The results are shown in Figures 27–29.

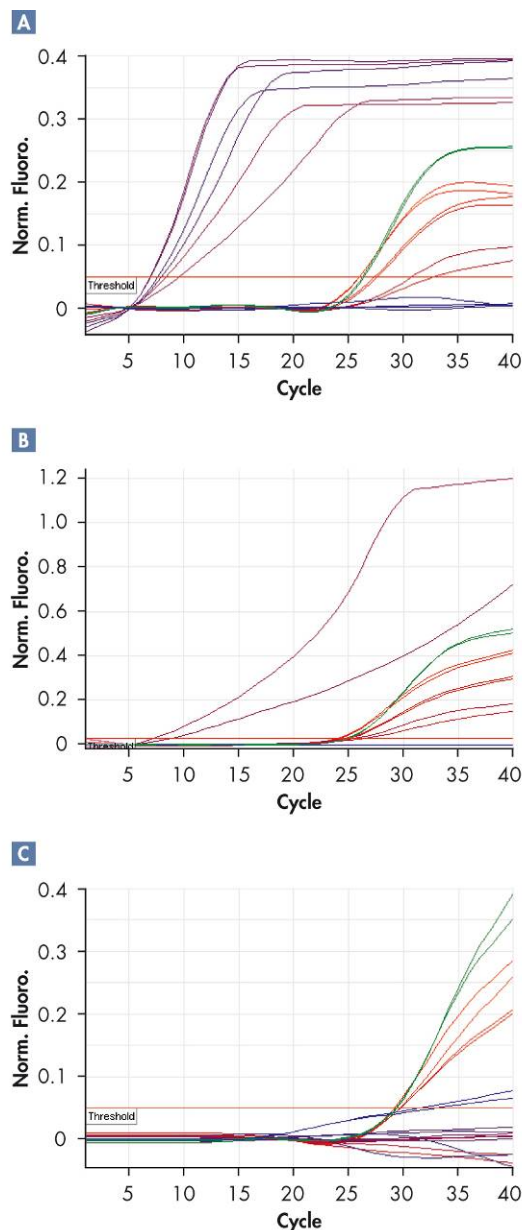

Figure 27. Response to increasing concentrations of calcium hydrogen phosphate. Amplification plots were produced using the Rotor-Gene Q and the Investigator Quantiplex HYres Kit with increasing concentrations of calcium hydrogen phosphate (from 0 to 8 mM (final concentration in the reaction). **A** Green channel **B** Red channel **C** Yellow channel. Red lines indicate increasing concentrations from 1–4 mM. Purple and blue lines indicate increasing concentrations from 5–8 mM. Green lines indicate no inhibitor present.

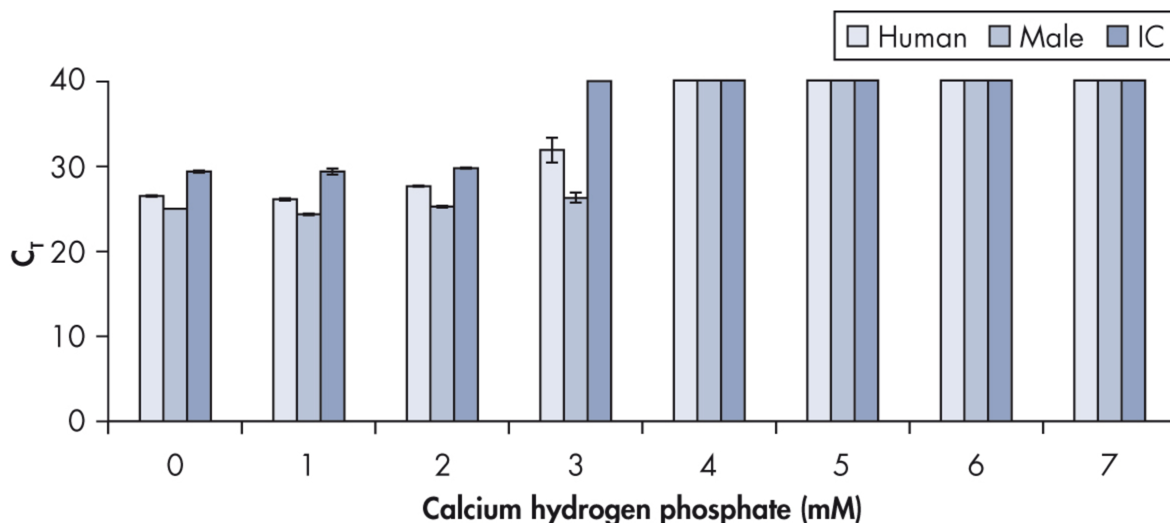

Figure 28. Performance of Investigator Quantiplex HYres Kit with simulated inhibition effect of calcium hydrogen phosphate on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 1 mM. The figure shows average ± standard deviation.

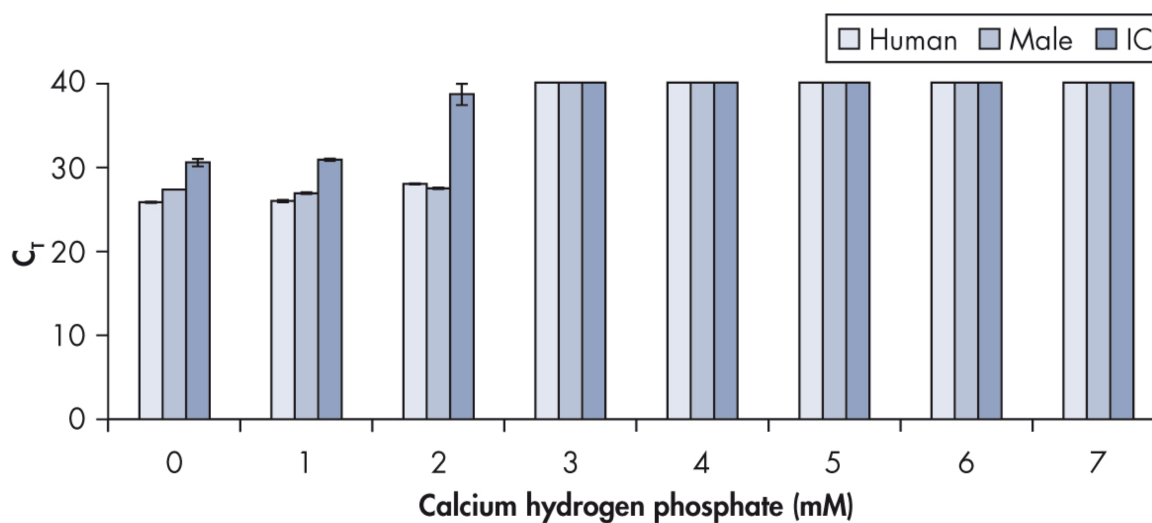

Figure 29. Performance of Investigator Quantiplex HYres Kit with simulated inhibition effect of calcium hydrogen phosphate on the Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 1 mM. The figure shows average ± 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 (4).

To test the robustness of the kit, the assay was run in the presence of 0, 50, 100, 150, 175, 200, 225, 250, 275, 300, 325, and 350 ng/µl tannic acid (Sigma-Aldrich; cat. no. 403040) under standard conditions as described in the Investigator Quantiplex HYres Handbook (1 ng Control DNA Z1). The results are shown in Figure 30.

The effect of tannic acid is clearly visible in the shape of the amplification plot.

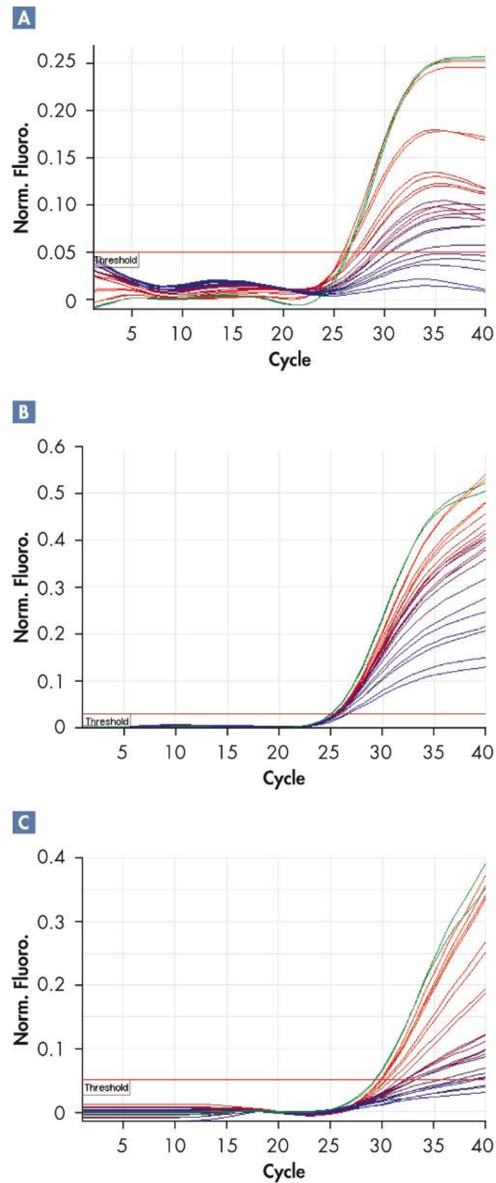

Figure 30. Response to increasing concentrations of tannic acid. Amplification plots were produced using the Rotor-Gene Q and the Investigator Quantiplex HYres Kit with increasing concentrations of tannic acid from 0 to 350 ng/µl (final concentration in the reaction). **A** Green channel **B** Red channel **C** Yellow channel. Red lines indicate increasing concentrations from 50–175 ng/µl. Purple and blue lines indicate increasing concentrations from 200–350 ng/µl. Green lines indicate no inhibitor present.

Contamination of reagents

Laboratory contamination of one of the reagents contained in the Investigator Quantiplex HYres Kit may cause a false positive result in the quantification reaction. Contamination studies were performed to exclude reagent contamination. Duplicates of 95 no-template controls and 1 positive control (Control DNA Z1; 20 ng/μl) were analyzed.

One run is shown as an example (Figure 31). No DNA was detected in any no-template control samples.

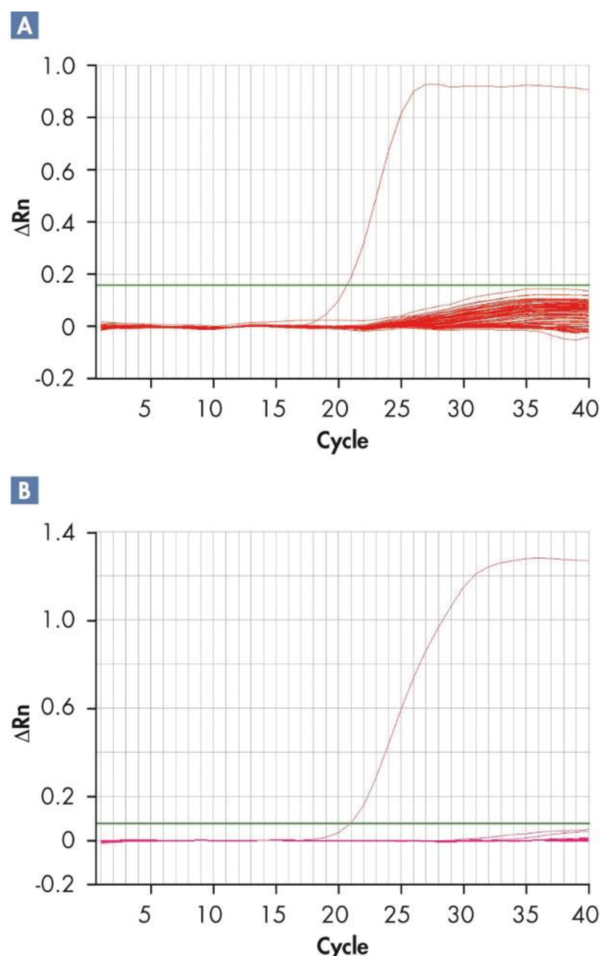

Figure 31. Results of the first run. No detectable contamination was observed in either channel. The human channel is shown above and male channel below. **A** FAM channel **B** Cy5 channel.

The absence of DNA in 190 of 190 NTC samples demonstrated that the Investigator Quantiplex HYres assay is able to accurately quantify samples containing no DNA.

Stability

Stability of the Control DNA Z1 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 Control DNA Z1 for a whole week is a real advantage. Therefore, the stability of the serial dilutions of the Control DNA Z1 at 4°C was tested.

The dilutions were performed using both TE buffer and QuantiTect Nucleic Acid Dilution buffer in low-DNA–binding or untreated plastic 1.5 ml tubes. The dilutions were tested directly after dilution (Day 0) and after 1, 2, 3, 5, and 7 days storage at 4°C. The tests were run on a Rotor-Gene Q following the standard reaction protocol. 3 replicates for each dilution point were tested (Figures 32 and 33; not all data shown; i.e., the stability of the dilutions in TE-buffer using untreated tubes).

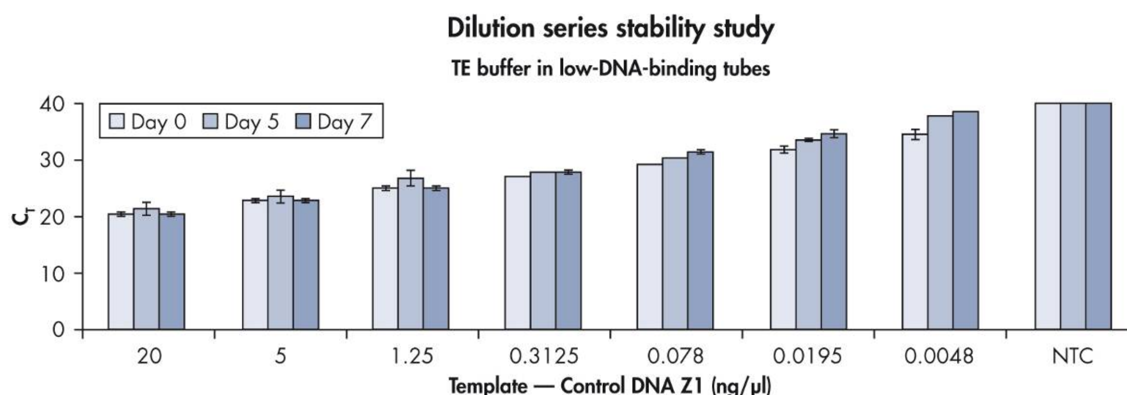

Figure 32. Control DNA Z1 dilution series using TE buffer in low-DNA–binding tubes before storage and after storage for 5 and 7 days at 4°C. The results show C_T shifts after 5 days in the low DNA range. The figure shows average ± standard deviation. **NTC**: No-template control.

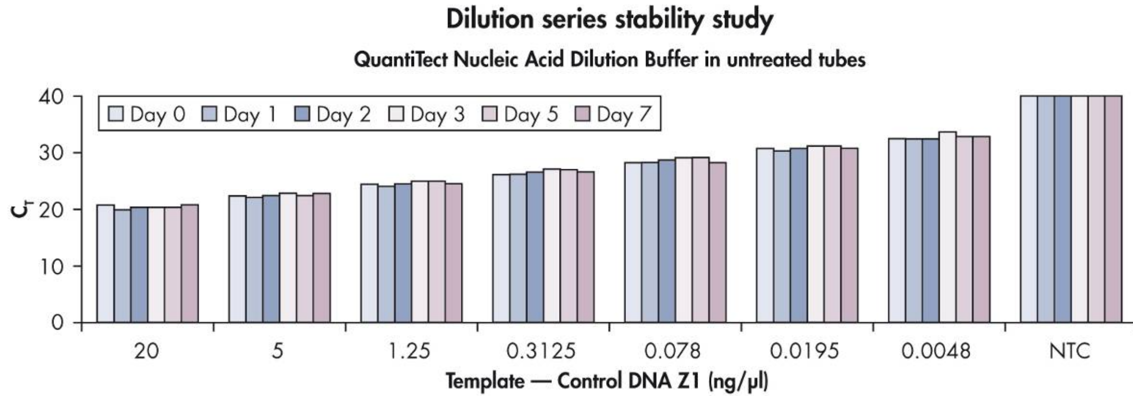

Figure 33. Control DNA Z1 dilution series using the QuantiTect Nucleic Acid Dilution buffer in untreated tubes before storage and after storage for 1, 2, 3, 5, and 7 days at 4°C. The results show no relevant differences, even in the low DNA range. The figure shows average ± standard deviation. **NTC**: No-template control.

The results show that only 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.

Samples were created by mixing a male and a female DNA in ratios of 1:0, 1:50, 1:100, 1:200, 1:500, 1:800, 1:1000, and 0:1. The total amount of male DNA used in this study was 20 pg/µl; a 1:1000 mixture therefore contained 20 pg/µl of the male component DNA and 20 ng/µl of the female component (Table 10). Highly accurate quantification results were obtained in all cases for both targets, the total human and the male on all validated cyclers (Figures 34–36).

Table 10. Amounts of DNA template in the mixtures

Male:female ratio	Male component	Female component
1:0	~20 pg/µl	0
1:50	~20 pg/µl	~1 ng/µl
1:100	~20 pg/µl	~2 ng/µl
1:200	~20 pg/µl	~4 ng/µl
1:500	~20 pg/µl	~10 ng/µl
1:800	~20 pg/µl	~16 ng/µl
1:1000	~20 pg/µl	~20 ng/µl
0:1	0	~20 pg/µl

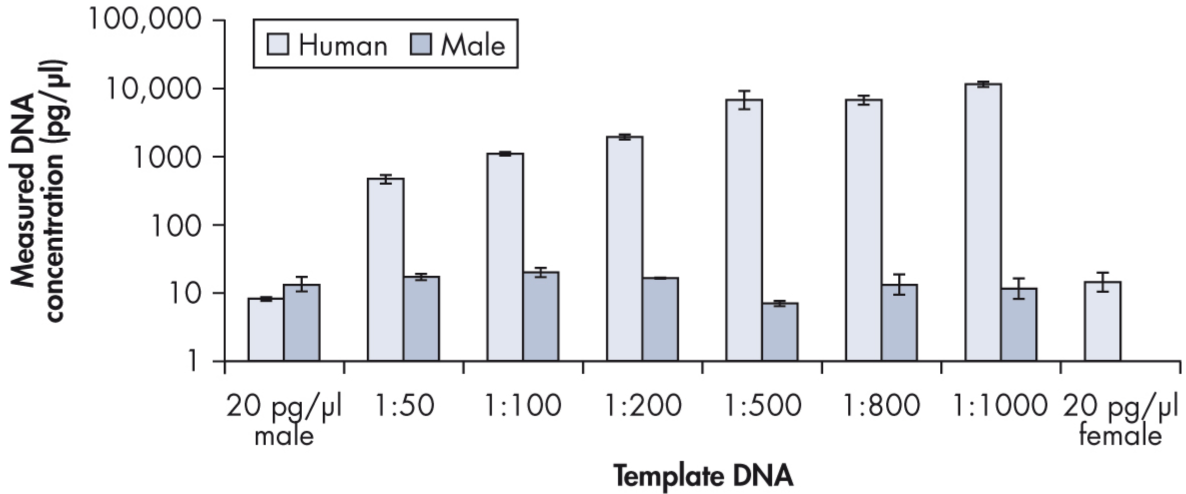

Figure 34. Detection of mixtures using the Investigator Quantiplex HYres Kit on the Rotor-Gene Q. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

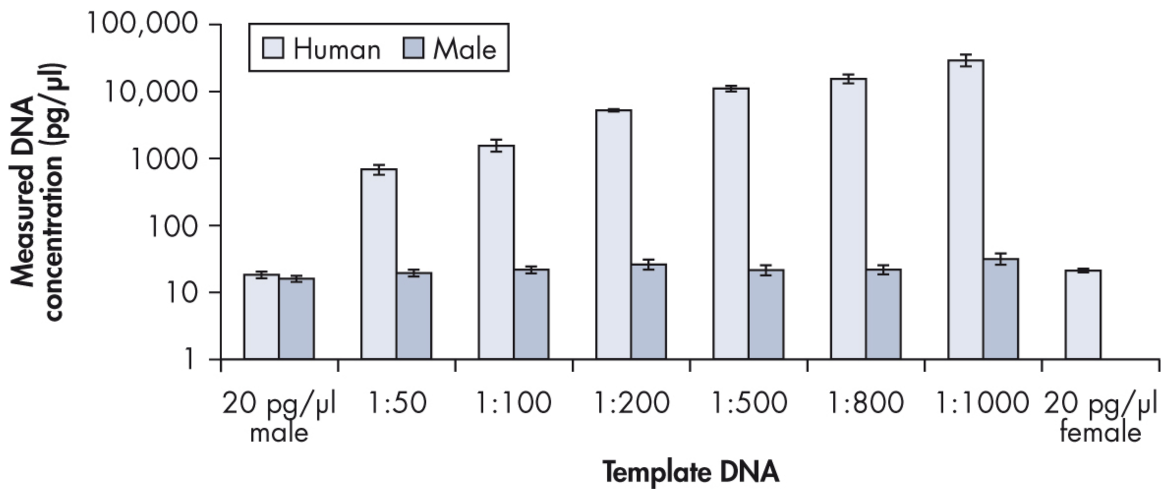

Figure 35. Detection of mixtures using the Investigator Quantiplex HYres Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

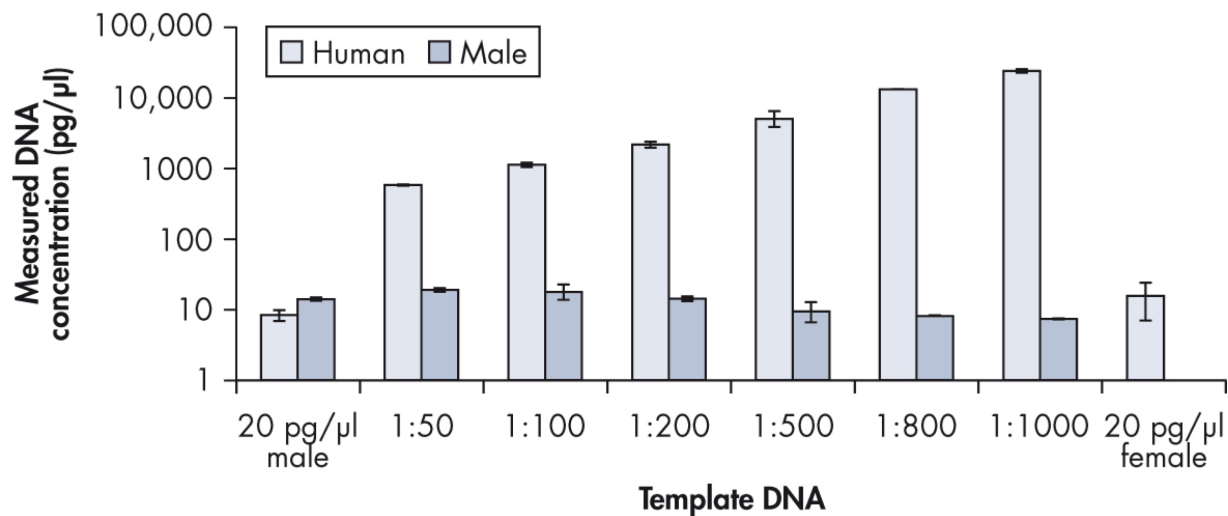

Figure 36. Detection of mixtures using the Investigator Quantiplex HYres Kit on the Applied Biosystems 7500 Real-Time PCR System. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

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 Investigator Quantiplex HYres is in the case of 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 a male and a female DNA in a ratio of 1:5. This mixed DNA was then diluted to 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 further samples (Table 11).

Highly accurate quantification results were obtained for both the total human and the male component (Table 11). STR reactions were setup using the Investigator Argus Y-12 QS Kit (cat. nos. 383615 or 383617) according to the quantification of the male component.

Table 11. Amounts of DNA template in the mixtures

DNA Sample	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	300.00	282.91	50.00	46.76	500
Sample 2	18.75	15.82	3.13	3.32	53
Sample 3	4.69	4.21	0.78	0.35	13.2
Sample 4	2.34	2.53	0.39	0.14	6.6
Sample 5	1.17	2.31	0.20	0.11	3.3
Sample 6	0.59	1.05	0.10	0.41	1.7
Sample 7	0.29	0	0.05	0.19	0.8
Sample 8	0.15	0	0.02	0.29	0.4
Sample 9	0.07	0.44	0.01	0	0.2

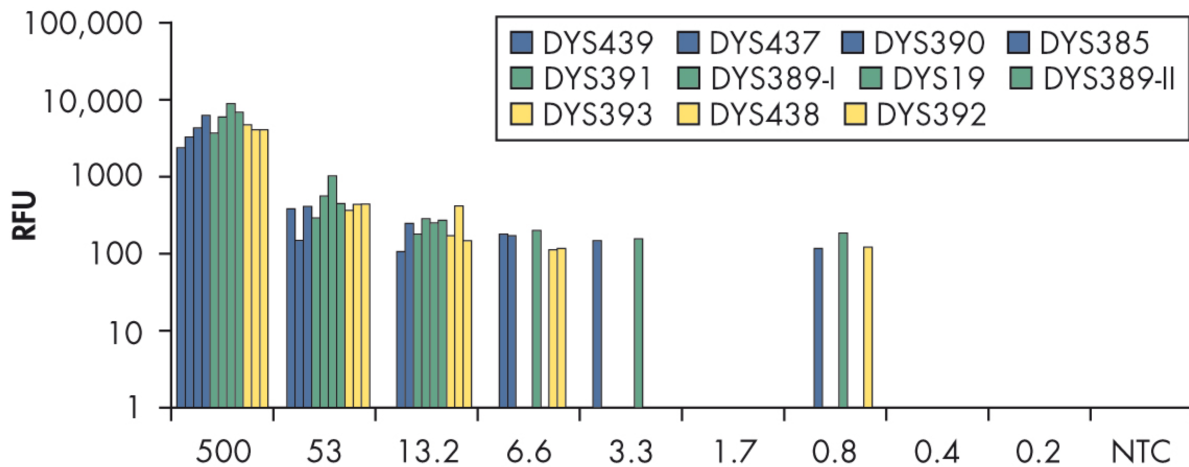

Figure 37. STR results showing the mean peak height in RFU. The results show the correlation of the quantification using the Quantiplex HYres Kit to the STR results using the Investigator Argus Y-12 QS Kit. The figure shows average ± standard deviation. **NTC**: No-template control.

With decreasing amounts of DNA, the average peak heights decreased. It was not possible to detect a full profile using DNA amounts lower than ~50 pg (samples 2–9). Sporadic alleles could be detected using an input DNA amount between 13.2 pg and 0.4 pg (samples 4 to 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

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3. Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., and Little, S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat. Biotechnol. **17**, 804.
4. 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 HYres Kit (200)	Reaction Mix FQ, Primer Mix IC YQ, Control DNA Z1, QuantiTect Nucleic Acid Dilution Buffer	387116
Rotor-Gene Q 5plex	Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories	Inquire
Rotor-Gene Q 5plex HRM	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels, plus HRM channel, laptop, software, accessories	Inquire
Rotor-Gene Q 6plex	Real-time PCR cycler with 6 channels, including laptop, software, accessories	Inquire

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