QIAGEN Validation Report

Developmental validation of the Investigator® Quantiplex Kit

The Investigator Quantiplex 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 Kit was developed for the quantification of human genomic 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 optimum amplification conditions for the Investigator Quantiplex Kit are given on page 4. A target validation was performed in an external study (page 7). The kit was validated for reproducibility (page 10), sensitivity (page 16), inter- and intra-run precision (page 18). It was tested for cross-reactivity with other species (page 22), and its performance with inhibitors (page 25) and contamination (page 33) was assessed. Component performance after simulated transport was also tested (page 34).

The validation of Investigator Quantiplex 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 Kit is a ready-to-use system for the detection of human DNA using real-time PCR. It provides fast and accurate quantification of human 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 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 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.

In addition, the Investigator Quantiplex 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 Scorpion primers and a novel, fast chemistry. Scorpion 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.

Scorpion primers are well known for their rapid hybridization to the target sequence, via the intramolecular reaction (Whitcombe, 1999). The reaction chemistry was carefully optimized to further support the rapid mechanism.

Instrumentation for validation

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

- Rotor-Gene Q
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7500 Fast Real-Time PCR System

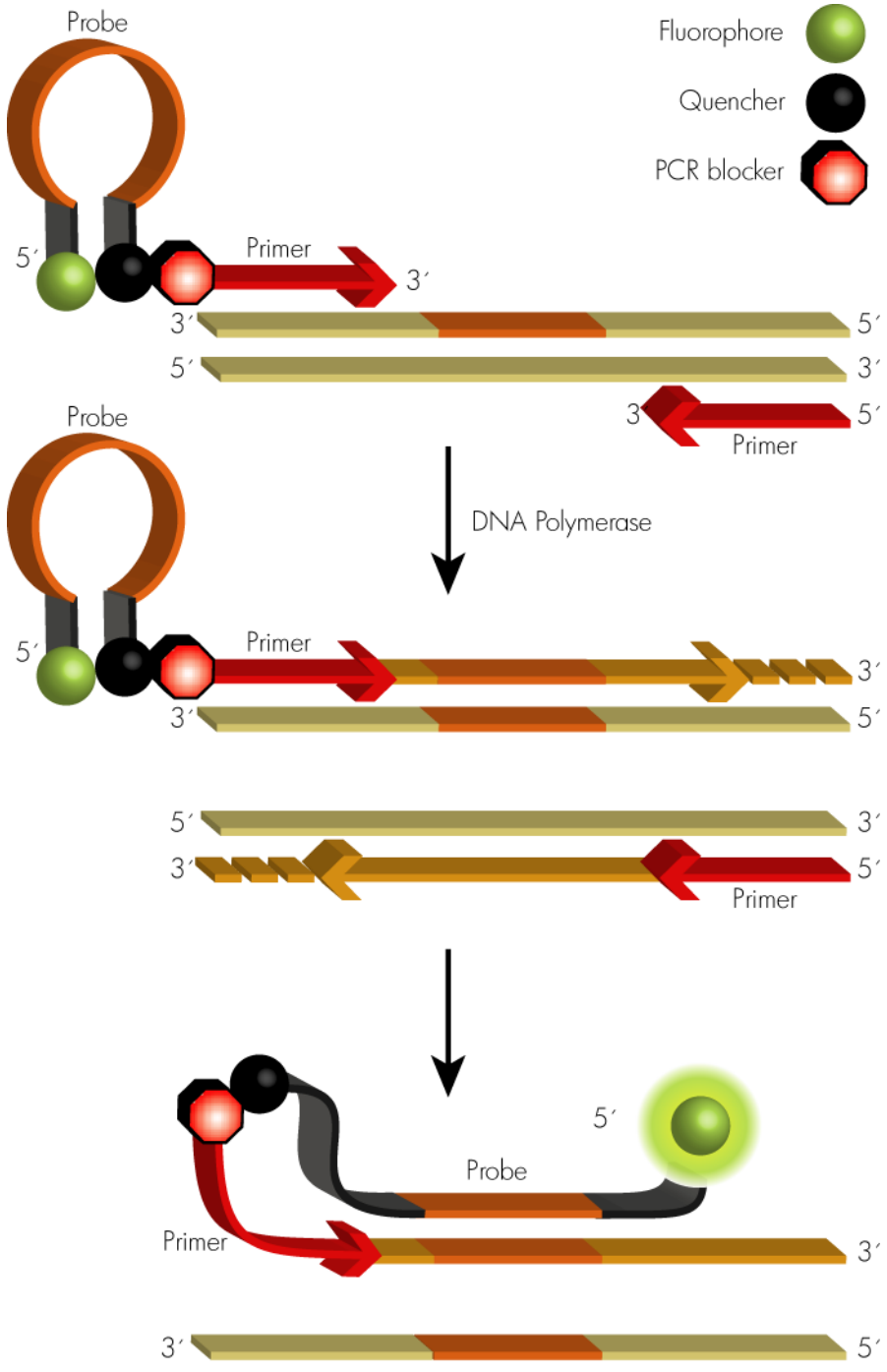

Figure 1. Scorpion primers and their function.

Amplification conditions

The amplification conditions developed during validation are shown in Tables 1–4 (pages 4–6). 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 the 0.1 ml Strip Tubes on the 72-well rotor (cat. no. 981103 or 981106). The Rotor-Gene Q Software version 2.0.2 (Build 4) was used.

For the Applied Biosystems 7500 Real-Time PCR System, all the data presented in this validation report were obtained using optical plates with cat. no. N801-0560. For the Applied Biosystems 7500 Fast Real-Time PCR System, all the data presented were obtained using optical plates with cat. no. 4346906. For both instruments, the 7500 Fast System SDS Software version 1.4.0.25 was used.

Table 1. Master mix for DNA quantification

Component	Volume per 25 µl reaction	Final concentration
Reaction Mix FQ, 2.18x	11.5 µl	1x
Primer Mix IC FQ, 2.18x	11.5 µl	1x
Total volume of master mix	23 µl	

Table 2. Cycling conditions for the Rotor-Gene Q

Step	Temp	Time		Additional Comments
Initial PCR activation step	95°C	1 min		PCR requires an initial incubation at 95°C for 1 min to activate the DNA polymerase
Two-step cycling:				
Denaturation	95°C	1 s	40 cycles	
Combined annealing/ extension	60°C	10 s		Perform fluorescence data collection using the green and the yellow channels with auto-gain optimization

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

Step	Temp	Time		Additional comments
Initial PCR activation step	95°C	1 min		PCR requires an initial incubation at 95°C for 1 min to activate the DNA polymerase
Two-step cycling:				
Denaturation	95°C	5 s	40 cycles	
Combined annealing/ extension	60°C	32 s		Perform fluorescence data collection using the FAM and VIC channels

Table 4. Cycling conditions for the Applied Biosystems 7500 Fast Real-Time PCR System

Step	Temp	Time		Additional comments
Initial PCR activation step	95°C	1 min		PCR requires an initial incubation at 95°C for 1 min to activate the DNA polymerase
Two-step cycling:				
Denaturation	95°C	5 s	40 cycles	
Combined annealing/ extension	60°C	25 s		Perform fluorescence data collection using the FAM and VIC channels

Results of developmental validation

Target validation study

For a precise quantification of human 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 a real-time PCR addressing 2 different targets in the same reaction vessel. The first target addressed was a non-variable 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 addressed was the target used in the Investigator Quantiplex Kit (4NS1C). The ΔC_T values, the difference between the C_T value of the first and the second target, 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 8). 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 Scorpion Oligo Mix containing primers and scorpion primers were provided by QIAGEN. The reactions were performed on the Rotor-Gene Q and the Applied Biosystems 7500 Real-Time PCR System.

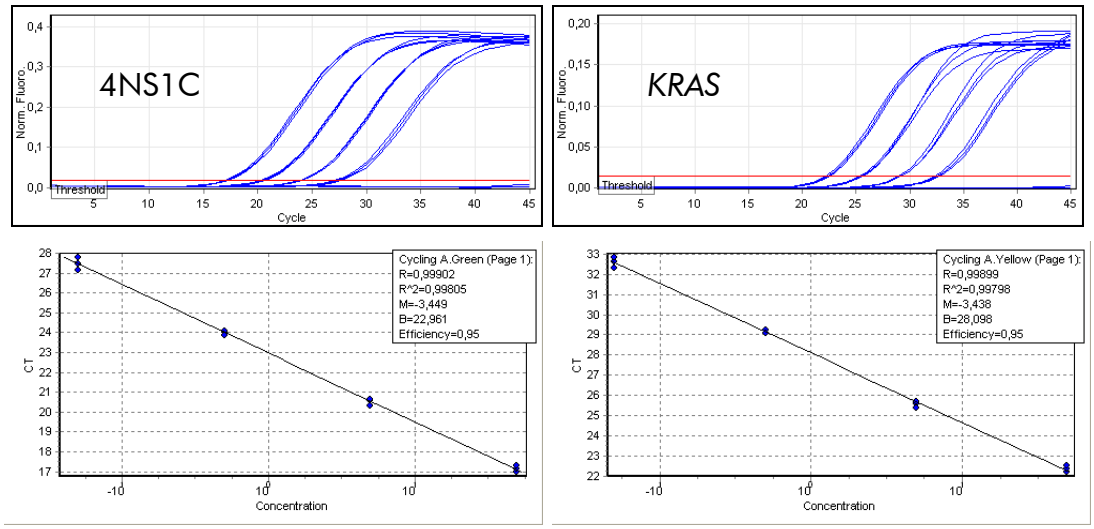

Figure 2. PCR efficiency and linearity of the target validation duplex qPCR. These parameters are comparable for both green and yellow channels using DNA concentrations between 50 and 0.05 ng/µl.

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, showing that there is a consistent copy number between males and females. The mean ΔC_T value and standard deviation were calculated for each population.

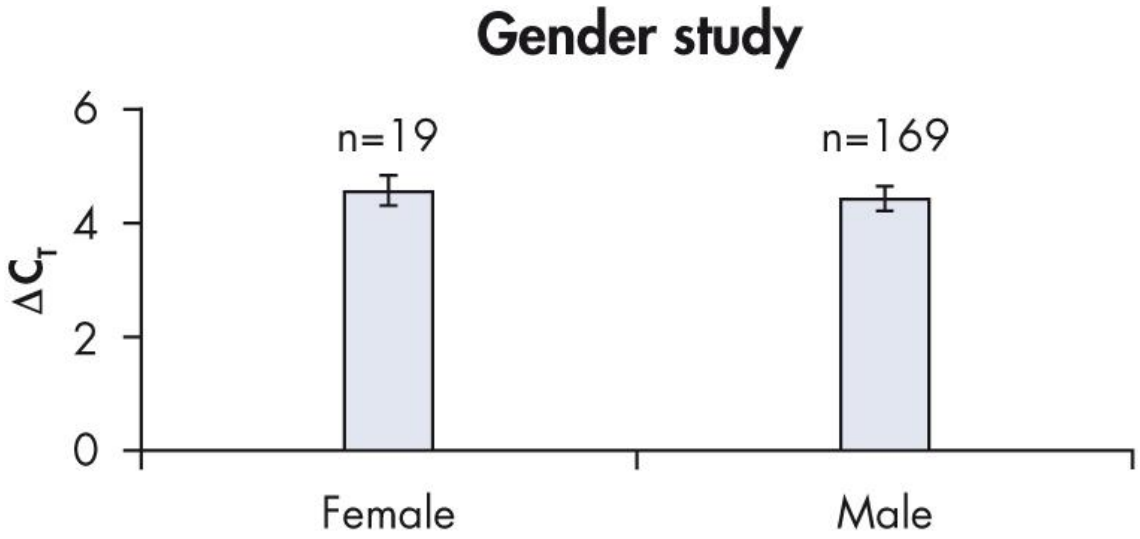

Figure 3. Highly comparable ΔC_T values (highly stable copy number) were detected for both male and female subjects. The figure shows the average ΔC_T ± standard deviation.

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.

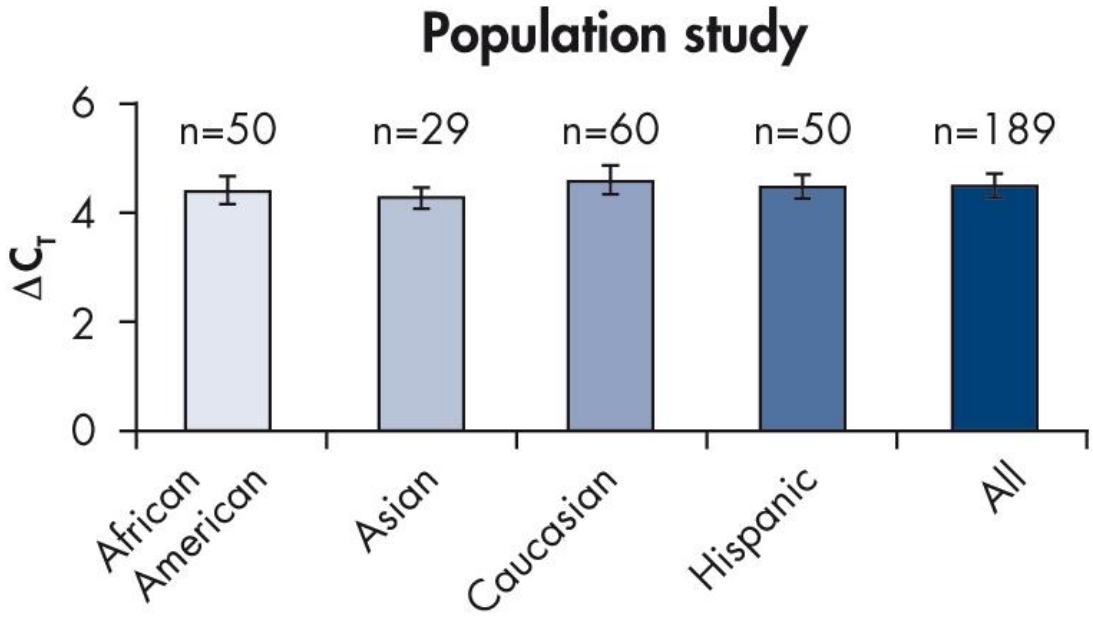

Figure 4. Comparable ΔC_T values (highly stable copy number) were detected for the 4 main human population groups. The figure shows the average ΔC_T ± standard deviation.

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 human target of 20±1, considering a haploid genome (40: diploid genome).

Reproducibility

Reproducibility is critical in forensic analysis to ensure consistency of results. This was tested to ensure sample-to-sample reproducibility.

Sample-to-sample reproducibility was tested on the Rotor-Gene Q and on the Applied Biosystems 7500 and Fast 7500 Real-Time PCR Systems by taking 3 male and 2 female DNA samples at 5 different concentrations (~25 ng/µl, ~12.5 ng/µl, ~1 ng/µl, ~0.5 ng/µl, and ~0.05 ng/µl). Dilutions were made using the QuantiTect Nucleic Acid Dilution buffer. Each sample was quantified 3 times using the same instrument on different days. The 3 runs were set up independently. Tables 5–7 (pages 10–15) show the data from the study. The mean quantity and coefficient of variation (%) were calculated for each sample dilution.

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

Table 5. Highly reproducible results comparing 3 different runs performed on 3 different days using the same Rotor-Gene Q

DNA sample	Run 1 Concentration (ng/µl) ±CV (%)	Run 2 Concentration (ng/µl) ±CV (%)	Run 3 Concentration (ng/µl) ±CV (%)
Male 1 (~25 ng/µl)	19.74±7.11	17.08±4.55	20.50±18.89
Male 1(~12.5 ng/µl)	10.43±3.73	8.92±8.98	11.21±10.76
Male 1 (~1 ng/µl)	1.06±16.22	1.10±8.02	1.04±17.26
Male 1 (~0.1 ng/µl)	0.12±0.19	0.10±8.14	0.10±3.48
Male 1 (0.05 ng/µl)	0.05±22.78	0.05±5.73	0.06±8.62
Male 2 (~25 ng/µl)	20.17±12.64	19.42±7.34	19.50±1.39
Male 2 (~12.5 ng/µl)	9.73±9.38	9.70±11.43	9.46±11.15
Male 2 (~1 ng/µl)	0.95±6.12	1.04±15.90	1.02±1.29
Male 2 (~0.1 ng/µl)	0.11±15.96	0.11±16.62	0.12±5.13
Male 2 (0.05 ng/µl)	0.05±8.01	0.05±2.04	0.05±25.21

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DNA sample	Run 1 Concentration (ng/µl) ±CV (%)	Run 2 Concentration (ng/µl) ±CV (%)	Run 3 Concentration (ng/µl) ±CV (%)
Male 3 (~25 ng/µl)	18.21±10.59	17.94±7.68	19.97±2.65
Male 3 (~12.5 ng/µl)	10.78±7.40	9.23±3.92	9.79±4.53
Male 3 (~1 ng/µl)	1.06±13.60	0.94±2.32	1.06±1.18
Male 3 (~0.1 ng/µl)	0.10±0.20	0.11±2.01	0.11±10.12
Male 3 (0.05 ng/µl)	0.06±4.55	0.06±9.50	0.07±15.21
Female 1 (~25 ng/µl)	19.65±1.44	20.15±9.45	17.84±10.17
Female 1 (~12.5 ng/µl)	9.48±10.96	9.05±7.56	9.67±0.31
Female 1 (~1 ng/µl)	1.01±13.24	0.96±3.7	1.08±5.86
Female 1 (~0.1 ng/µl)	0.10±16.49	0.11±10.36	0.12±16.81
Female 1 (0.05 ng/µl)	0.05±12.76	0.06±7.22	0.05±3.10
Female 2 (~25 ng/µl)	19.49±11.95	19.88±9.45	18.42±11.22
Female 2 (~12.5 ng/µl)	11.03±9.29	9.44±2.02	9.55±9.33
Female 2 (~1 ng/µl)	0.93±7.76	0.98±11.61	1.00±3.62
Female 2 (~0.1 ng/µl)	0.10±11.27	0.13±2.42	0.12±4.09
Female 2 (~0.05 ng/µl)	0.05±6.59	0.05±16.50	0.05±9.94

CV: Coefficient of variation.

Table 6. Highly reproducible results comparing 3 different runs performed on 3 different days on the same Applied Biosystems 7500 Real-Time PCR System

DNA sample	Run 1 Concentration (ng/µl) ±CV (%)	Run 2 Concentration (ng/µl) ±CV (%)	Run 3 Concentration (ng/µl) ±CV (%)
Male 1 (~25 ng/µl)	25.60±5.53	24.69±1.22	24.94±1.13
Male 1 (~12.5 ng/µl)	13.08±0.92	12.01±4.88	12.57±1.39
Male 1 (~1 ng/µl)	1.12±1.34	1.12±0.55	1.20±4.55
Male 1 (~0.1 ng/µl)	0.12±12.03	0.12±4.40	0.11±15.95
Male 1 (0.05 ng/µl)	0.07±9.88	0.07±5.59	0.07±5.18
Male 2 (~25 ng/µl)	25.62±3.93	24.74±7.44	24.65±8.81
Male 2 (~12.5 ng/µl)	12.88±2.69	12.21±4.68	12.48±3.48
Male 2 (~1 ng/µl)	1.25±3.29	1.21±12.17	1.32±0.31
Male 2 (~0.1 ng/µl)	0.13±7.59	0.13±4.52	0.11±17.77
Male 2 (0.05 ng/µl)	0.07±1.01	0.08±7.00	0.07±2.72
Male 3 (~25 ng/µl)	25.61±7.68	24.17±3.81	24.39±5.94
Male 3 (~12.5 ng/µl)	12.99±11.26	11.78±1.69	13.11±2.63
Male 3 (~1 ng/µl)	1.18±5.35	1.21±0.21	1.33±0.48
Male 3 (~0.1 ng/µl)	0.14±0.91	0.13±3.30	0.12±6.03
Male 3 (0.05 ng/µl)	0.07±4.21	0.07±6.71	0.07±3.11

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DNA sample	Run 1 Concentration (ng/µl) ±CV (%)	Run 2 Concentration (ng/µl) ±CV (%)	Run 3 Concentration (ng/µl) ±CV (%)
Female 1 (~25 ng/µl)	25.93±8.56	23.02±2.29	24.08±0.38
Female 1 (~12.5 ng/µl)	12.92±0.59	12.30±0.86	12.71±1.92
Female 1 (~1 ng/µl)	1.21±1.83	1.19±3.77	1.29±9.56
Female 1 (~0.1 ng/µl)	0.14± 3.01	0.13±9.22	0.12±0.42
Female 1 (0.05 ng/µl)	0.06±15.85	0.07±10.20	0.07±2.49
Female 2 (~25 ng/µl)	25.80±8.74	23.00±4.53	25.99±1.43
Female 2 (~12.5 ng/µl)	14.57±18.85	12.70±12.34	13.96±20.69
Female 2 (~1 ng/µl)	1.24±0.38	1.18±1.50	1.24±5.68
Female 2 (~0.1 ng/µl)	0.13±8.41	0.12±11.54	0.12±1.88
Female 2 (~0.05 ng/µl)	0.07±2.38	0.08±11.08	0.07±0.74

CV: Coefficient of variation.

Table 7. Highly reproducible results comparing 3 different runs performed on 3 different days on the same Applied Biosystems 7500 Fast Real-Time PCR System

DNA sample	Run 1 Concentration (ng/µl) ±CV (%)	Run 2 Concentration (ng/µl) ±CV (%)	Run 3 Concentration (ng/µl) ±CV (%)
Male 1 (~25 ng/µl)	31.34±4.61	30.74±6.93	33.15±0.48
Male 1 (~12.5 ng/µl)	15.91±6.85	15.75±2.49	15.81±5.17
Male 1 (~1 ng/µl)	1.13±6.02	1.05±5.30	1.14±8.82
Male 1 (~0.1 ng/µl)	0.11±9.65	0.11±14.56	0.11±16.31
Male 1 (0.05 ng/µl)	0.08±26.14	0.08±21.90	0.08±3.71
Male 2 (~25 ng/µl)	33.86±5.61	33.1±1.50	32.26±1.11
Male 2 (~12.5 ng/µl)	17.25±4.62	16.45±2.96	15.5±2.31
Male 2 (~1 ng/µl)	1.23±6.00	1.27±10.32	1.22±8.87
Male 2 (~0.1 ng/µl)	0.12±4.14	0.12±3.88	0.13±19.89
Male 2 (0.05 ng/µl)	0.09±8.14	0.07±5.34	0.09±1.99
Male 3 (~25 ng/µl)	32.12±1.17	32.19±4.48	33.21±1.14
Male 3 (~12.5 ng/µl)	16.89±0.64	16.79±8.97	16.38±9.16
Male 3 (~1 ng/µl)	1.32±8.75	1.29±1.66	1.29±1.99
Male 3 (~0.1 ng/µl)	0.12±6.48	0.12±1.21	0.13± 10.60
Male 3 (0.05 ng/µl)	0.07±1.76	0.07±18.10	0.08±19.31

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DNA sample	Run 1 Concentration (ng/µl) ±CV (%)	Run 2 Concentration (ng/µl) ±CV (%)	Run 3 Concentration (ng/µl) ±CV (%)
Female 1 (~25 ng/µl)	33.29±0.71	32.06±2.68	33.01±2.72
Female 1 (~12.5 ng/µl)	16.49±1.61	15.87±3.91	16.21±1.06
Female 1 (~1 ng/µl)	1.23±17.09	1.23±11.81	1.18±0.63
Female 1 (~0.1 ng/µl)	0.12±16.54	0.12±5.90	0.13±20.93
Female 1 (0.05 ng/µl)	0.07±3.54	0.07±4.97	0.08±11.05
Female 2 (~25 ng/µl)	32.29±9.40	32.74±7.31	32.95±1.63
Female 2 (~12.5 ng/µl)	18.12±10.01	17.46±8.29	20.06±28.97
Female 2 (~1 ng/µl)	1.15±0.35	1.19±1.51	1.22±4.03
Female 2 (~0.1 ng/µl)	0.11±13.04	0.11±10.93	0.13±23.39
Female 2 (~0.05 ng/µl)	0.08±2.79	0.07±1.33	0.08±5.89

CV: Coefficient of variation.

Sensitivity

The Investigator Quantiplex Kit is designed to detect a broad range of DNA quantities. Figures 5–7 (pages 16 and 17) show a serial dilution of Control DNA Z1 from 20 ng/µl to 0.3 pg/µl. The optimal linear dynamic range of the assay is in the range of 20 ng/µl to 4.9 pg/µl. DNA could be detected down to 0.3 pg/µl using the standard conditions specified in the *Investigator Quantiplex Handbook* using all 3 validated instruments (Rotor-Gene Q and Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems).

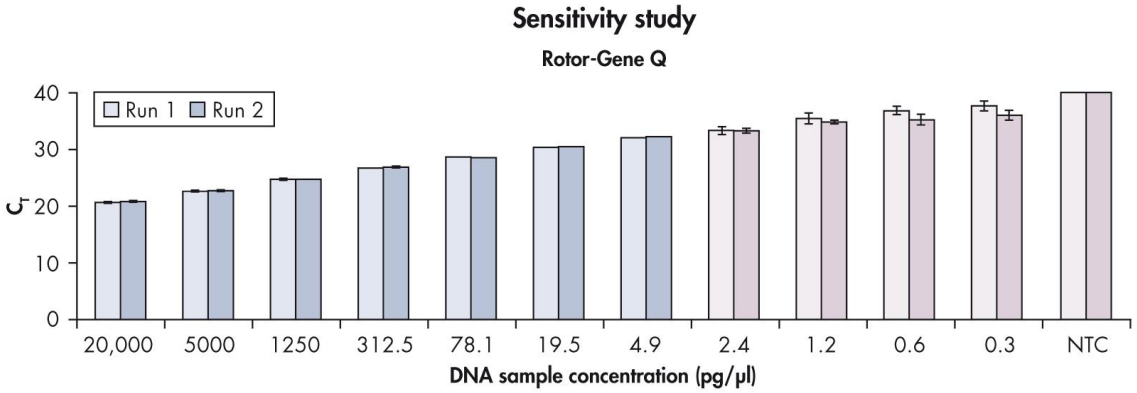

Figure 5. Detection of human DNA down to 0.3 pg/µl using the Investigator Quantiplex Kit on the Rotor-Gene Q. The figure shows the average C_T ± standard deviation. NTC: No-template control.

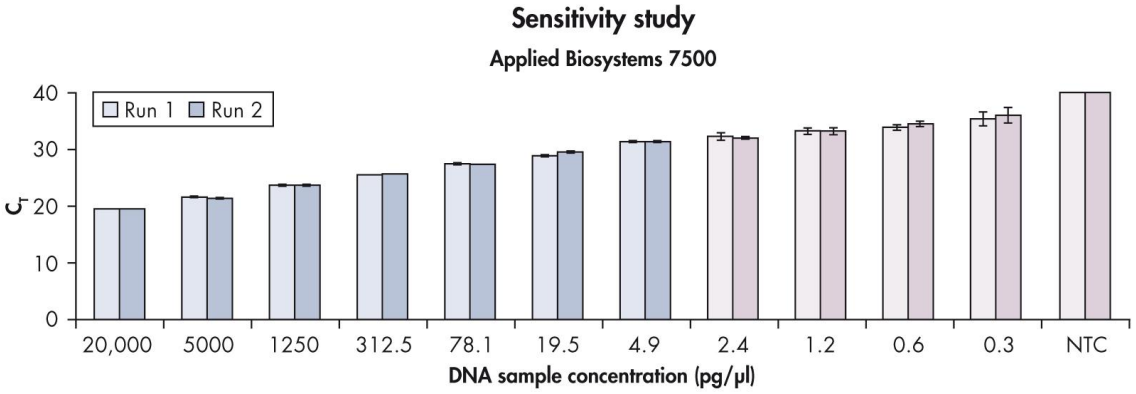

Figure 6. Detection of human DNA down to 0.3 pg/µl using the Investigator Quantiplex Kit on the Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation. NTC: No-template control.

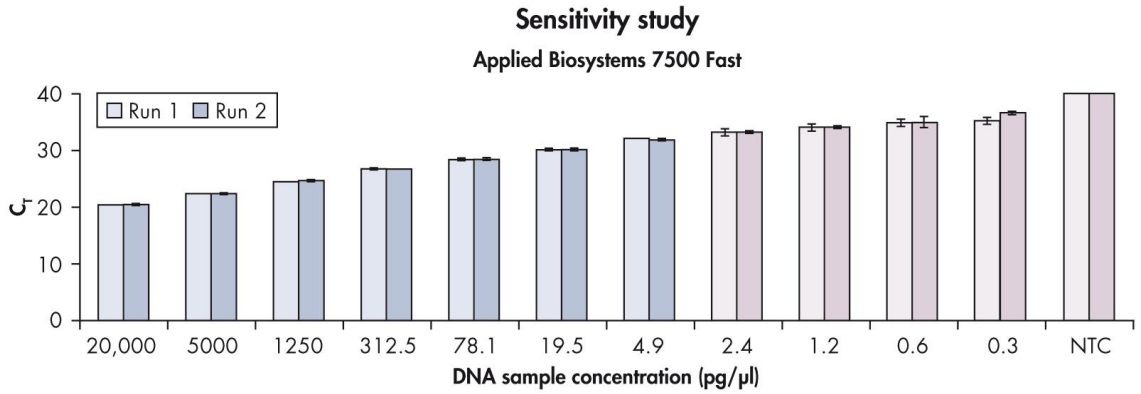

Figure 7. Detection of human DNA down to 0.3 pg/µl using the Investigator Quantiplex Kit on the Applied Biosystems 7500 Fast Real-Time PCR System. The figure shows the average C_T ± standard deviation. NTC: No-template control.

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 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 reaction. Figure 8 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.

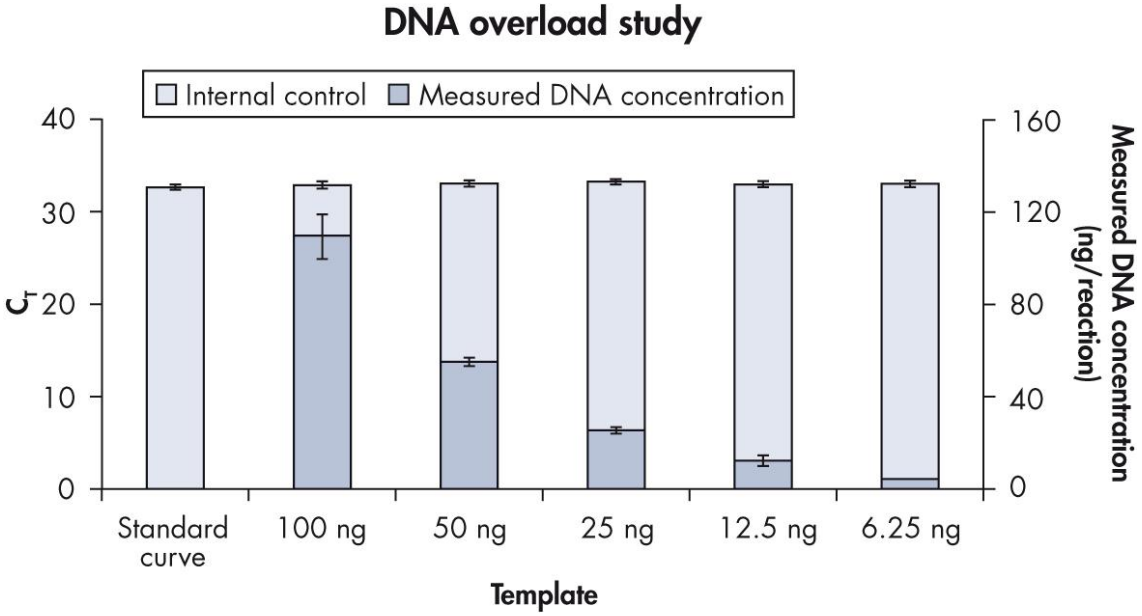

Figure 8. DNA overload test on the Rotor-Gene Q. The figure shows the average C_T ± standard deviation. The C_T value for the internal control is stable even at high DNA concentrations.

Inter- and intra-run precision

The inter-run precision of the Investigator Quantiplex Kit was tested by performing 3 runs on different instruments using the same reagents on the same day. Control DNA Z1 was diluted to concentrations ranging from 20 ng/µl to 4.9 pg/µl. Each dilution was measured using 9 replicates. The dilutions were quantified using the Rotor-Gene Q following the recommended cycling protocol (Figure 9).

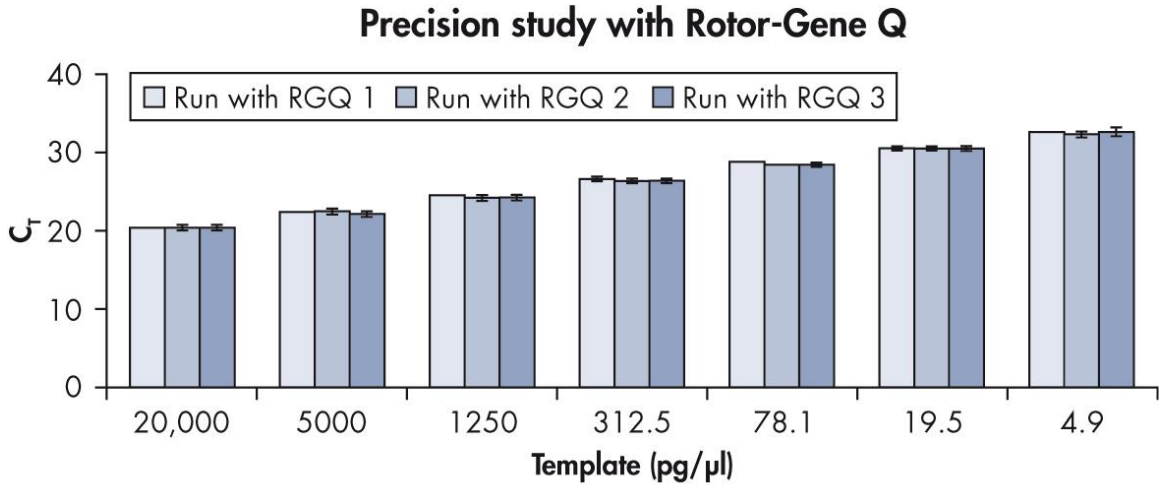

Figure 9. High inter-run precision determined using 3 different Rotor-Gene Q instruments (RGQ 1, RGQ 2, RGQ 3) on the same day. The figure shows the average C_T ± standard deviation.

The intra-run precision was determined by setting up 2 different runs on the same instruments on different days. Control DNA Z1 was diluted to concentrations ranging from 20 ng/µl to 4.9 pg/µl. Each dilution was measured in 9 replicates and dilutions were quantified using the Rotor-Gene Q and the Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems (Figures 10–12, pages 20-21).

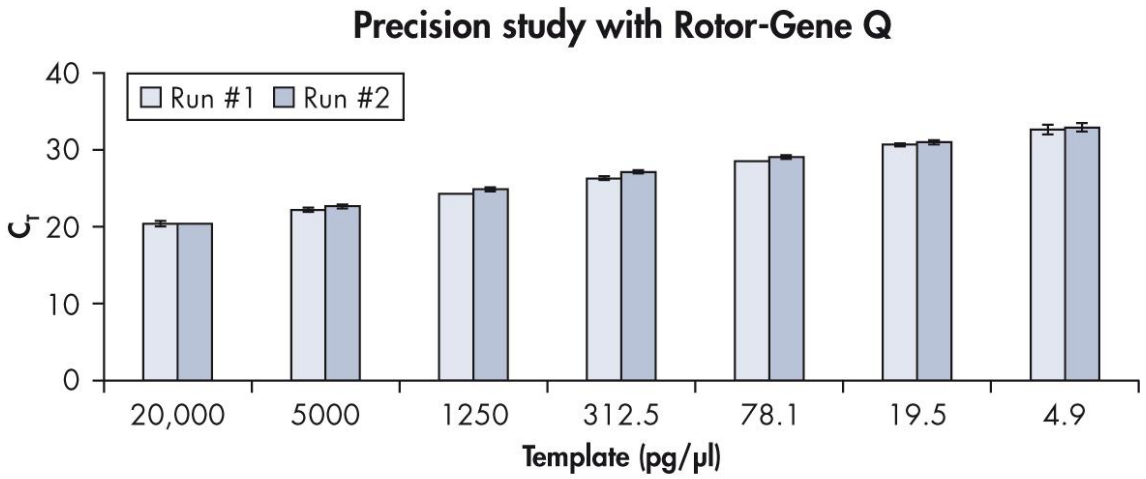

Figure 10. High intra-run precision determined in duplicate runs using a Rotor-Gene Q. The figure shows the average C_T ± standard deviation.

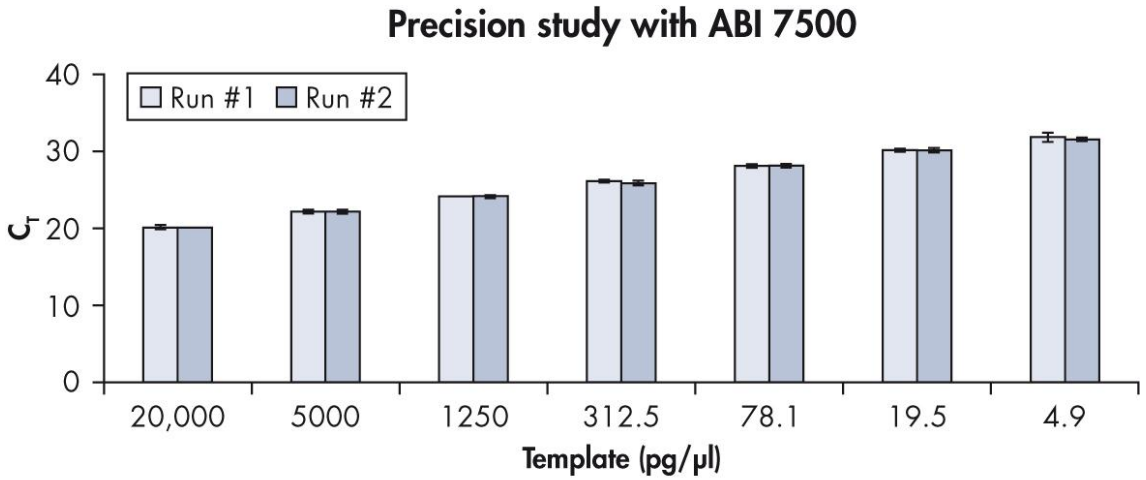

Figure 11. High intra-run precision determined in duplicate runs using an Applied Biosystems 7500 Real-Time PCR System. The figure shows the average C_T ± standard deviation.

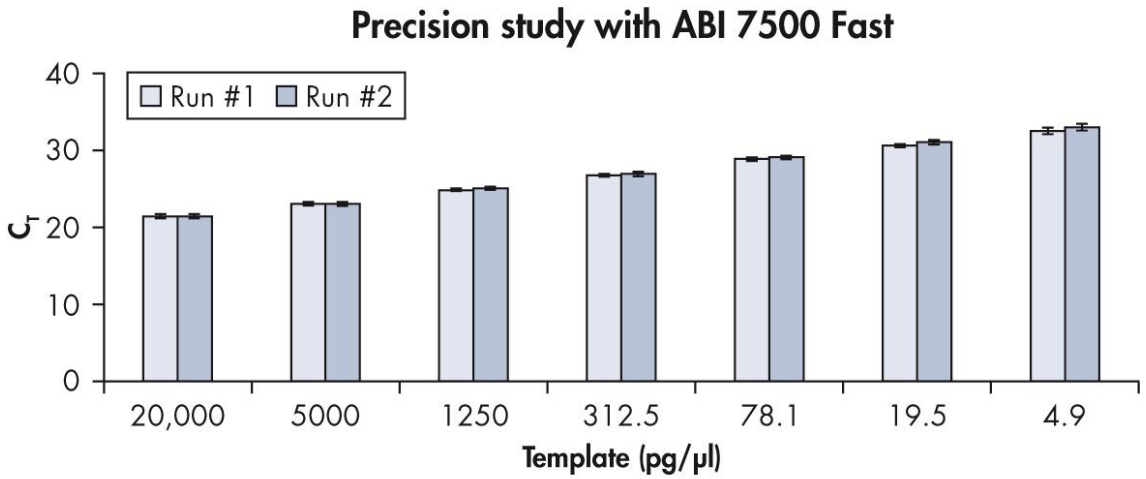

Figure 12. High intra-run precision determined in duplicate runs using an Applied Biosystems 7500 Fast Real-Time PCR System. The figure shows the average C_T ± standard deviation.

Systematic differences between instruments are not expected to affect final sample quantification results. When samples and quantification standards are run on the same plate and instrument, the C_T values are affected proportionately.

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 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 could be shown for the DNA from the tested common vertebrates under standard conditions, as shown in Figure 13.

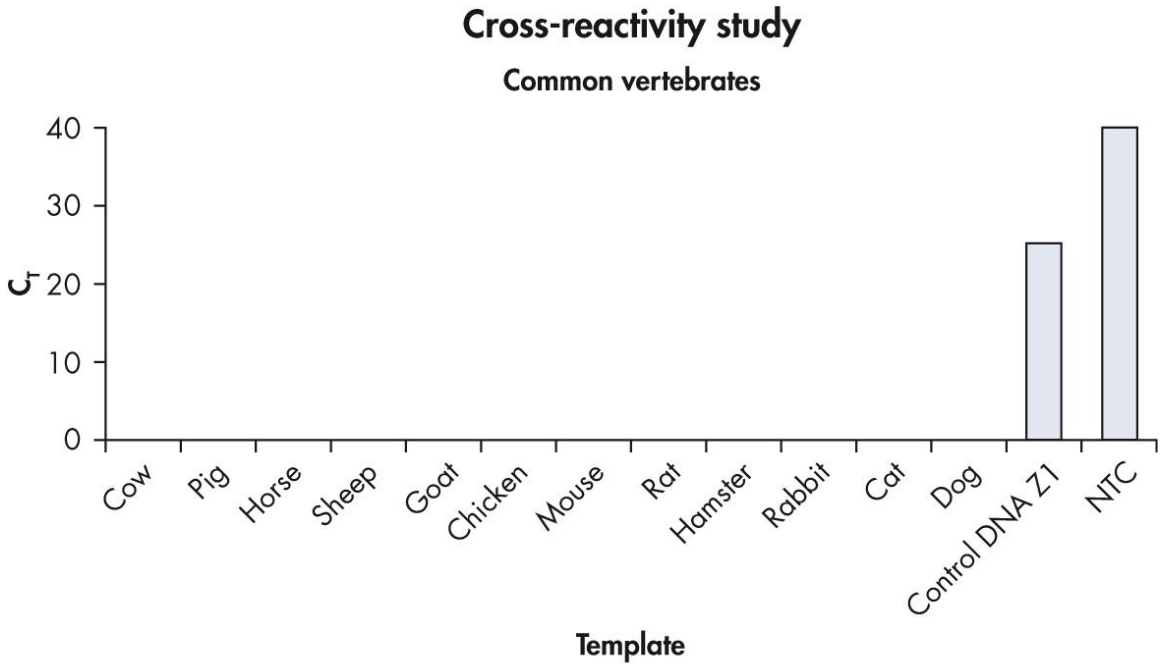

Figure 13. Results of a cross-reactivity study on common vertebrate species. The figure shows the average C_T ± standard deviation. NTC: No-template control.

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 14).

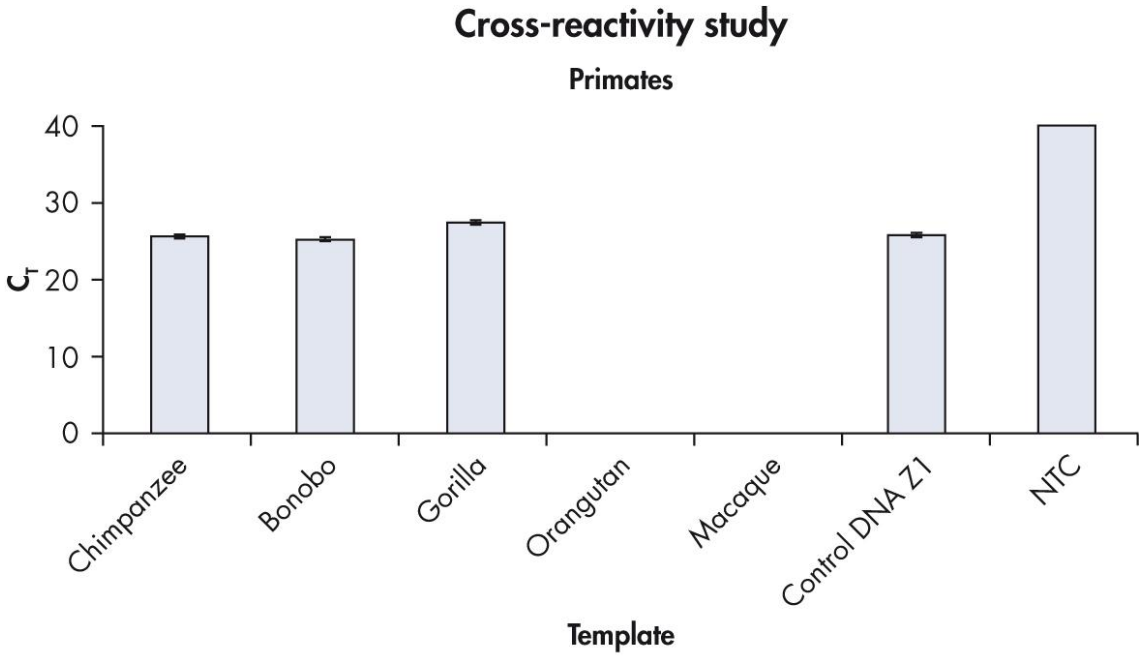

Figure 14. Results of a cross-reactivity study on primates. The figure shows the average C_T ± standard deviation. NTC: No-template control.

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 15.

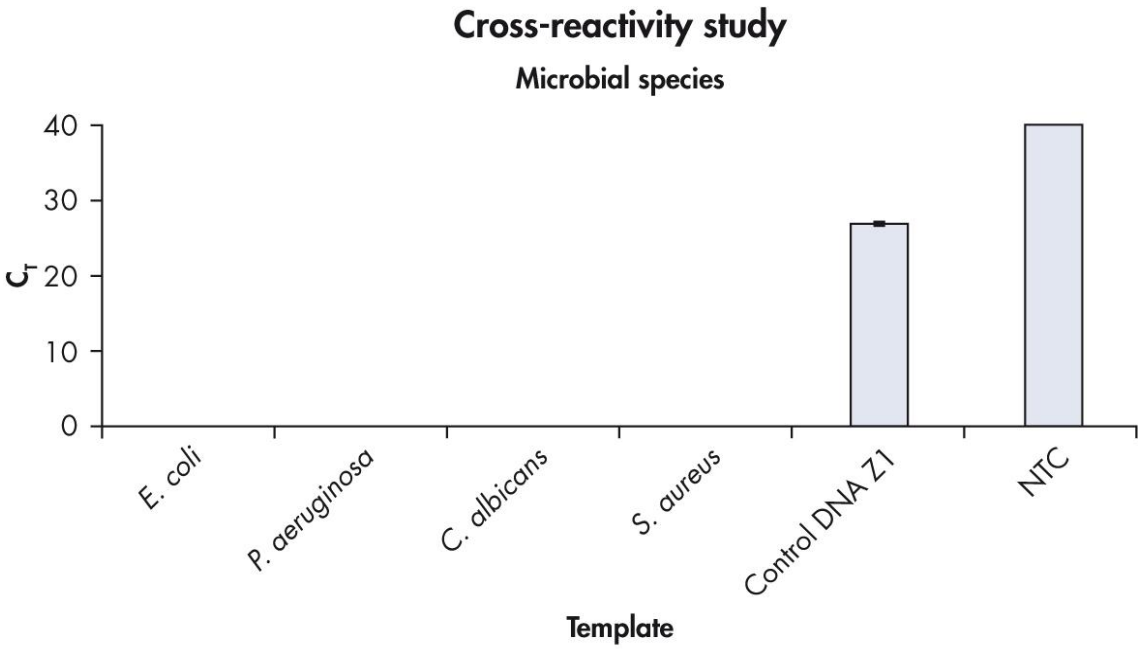

Figure 15. Results of a cross-reactivity study on microbial species. No cross-reactivity could be shown for the tested microbes. The figure shows the average C_T ± standard deviation. NTC: No-template control.

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

In conclusion, these experiments show that the Investigator Quantiplex 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 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 Handbook* (1 ng Control DNA Z1). The results are shown in Figures 17 and 18 (page 26).

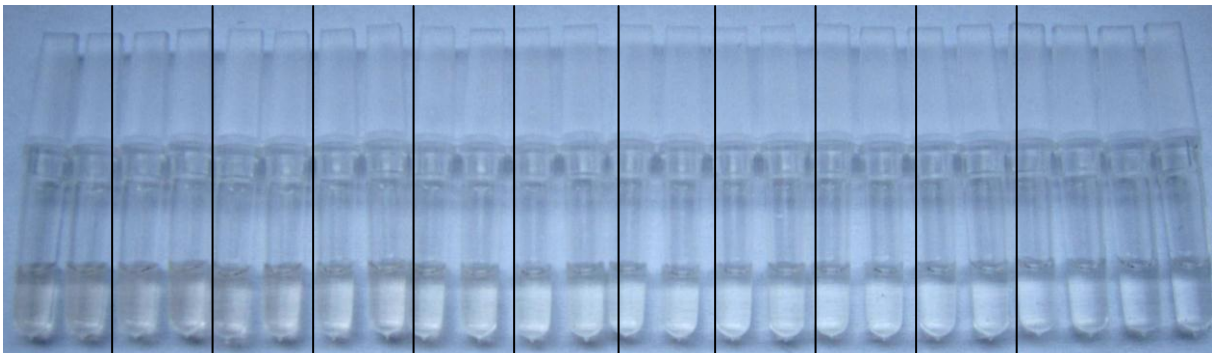

Figure 16. Rotor-Gene Q tubes containing reactions with 0 to 50 ng/µl humic acid.

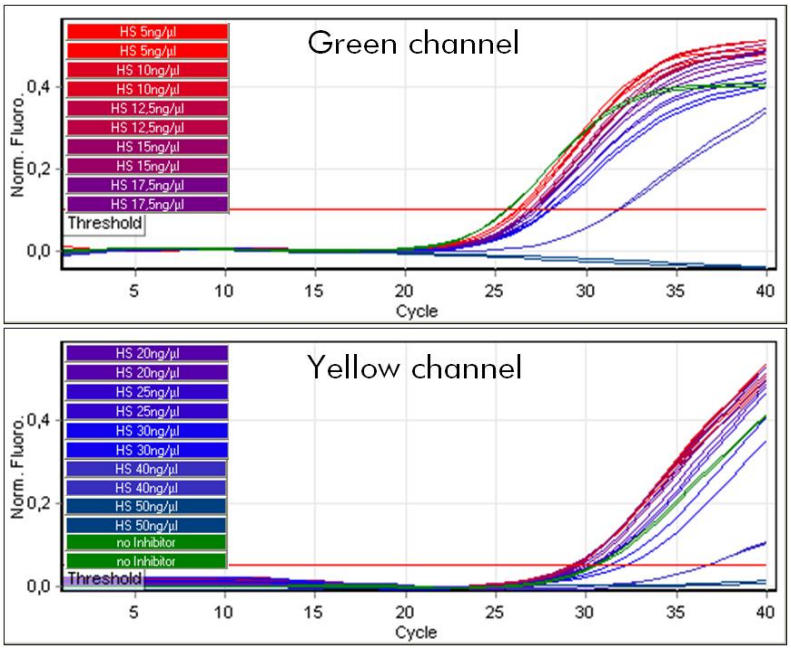

Figure 17. Response to increasing concentrations of humic acid. Amplification plots were produced using the Rotor-Gene Q green and yellow channels and the Investigator Quantiplex Kit with increasing concentrations of humic acid from 0 to 50 ng/µl (final concentration in the reaction).

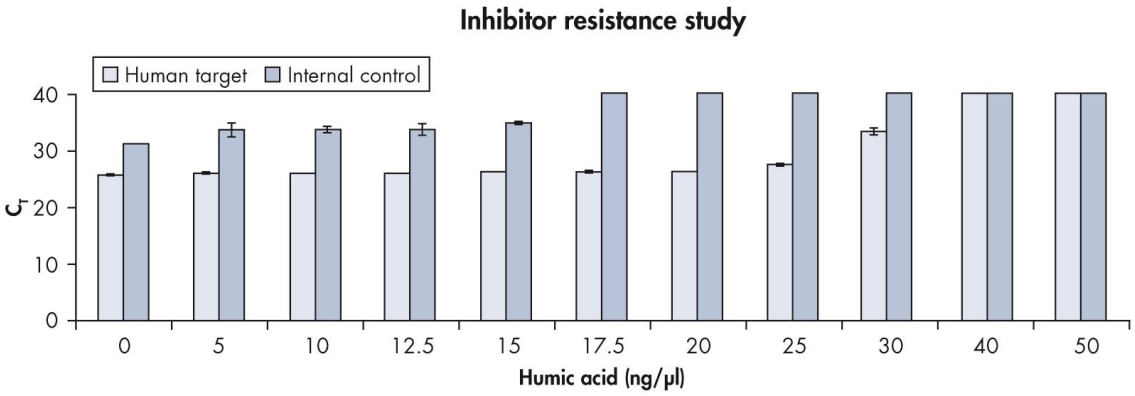

Figure 18. Performance of the Investigator Quantiplex Kit with simulated humic acid inhibition on an Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 20 ng/µl. The figure 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 20 ng/µl in the PCR. This corresponds to a concentration in the DNA sample of 250 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. Investigator PCR Kits are very flexible with regard to reaction setup, as a very broad range of DNA sample volumes may be added to the reaction. Please check the respective developmental validation report for more information about the inhibitor tolerance of each Investigator PCR Kit.

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 Handbook* (1 ng Control DNA Z1). The results are shown in Figures 20 and 21 (page 28).

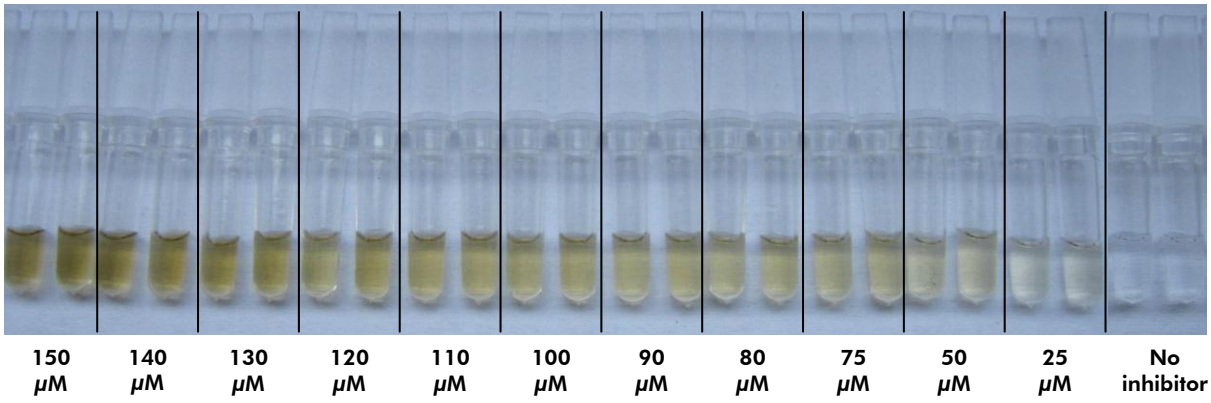

Figure 19. Rotor-Gene Q tubes containing reactions with 0–150 µM hematin.

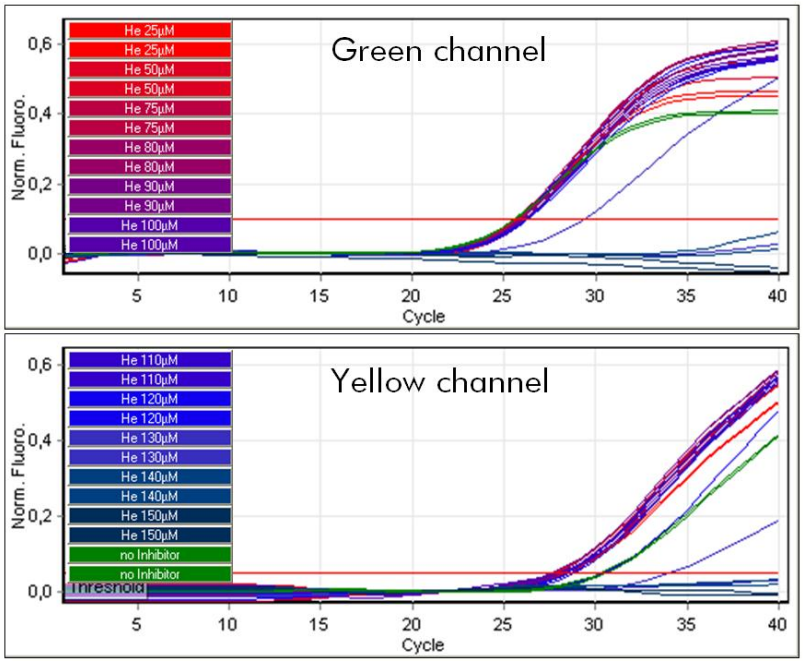

Figure 20. Response to increasing concentrations of humic acid. Amplification plots were produced using the Rotor-Gene Q green and yellow channels and the Investigator Quantiplex Kit with increasing concentrations of hematin from 0 to 150 µM (final concentration in the reaction).

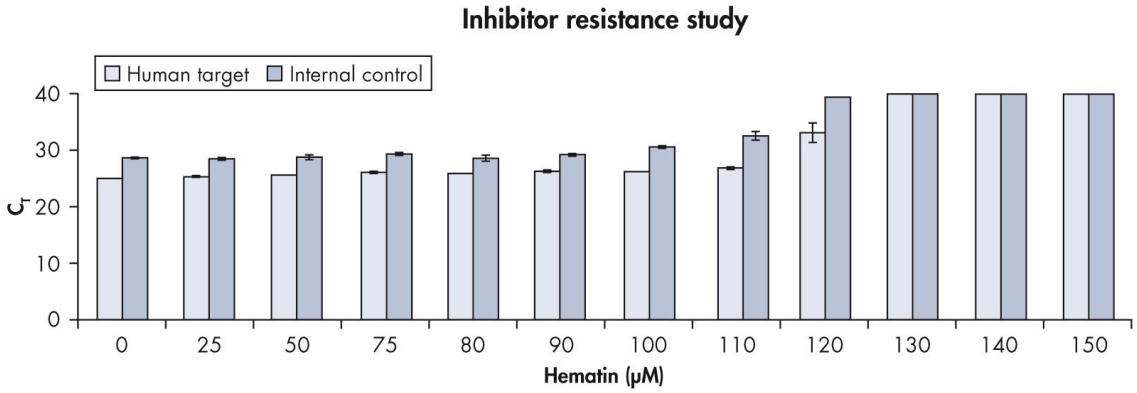

Figure 21. Performance of the Investigator Quantiplex Kit with simulated hematin inhibition on an Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 110 µM. The figure shows 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 remains reliable up to a final hematin concentration of 110 µM (final concentration in the reaction). This corresponds to a concentration in the DNA sample of 1.375 mM (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).

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 Handbook* (1 ng Control DNA Z1). The results are shown in Figure 23 (page 30).

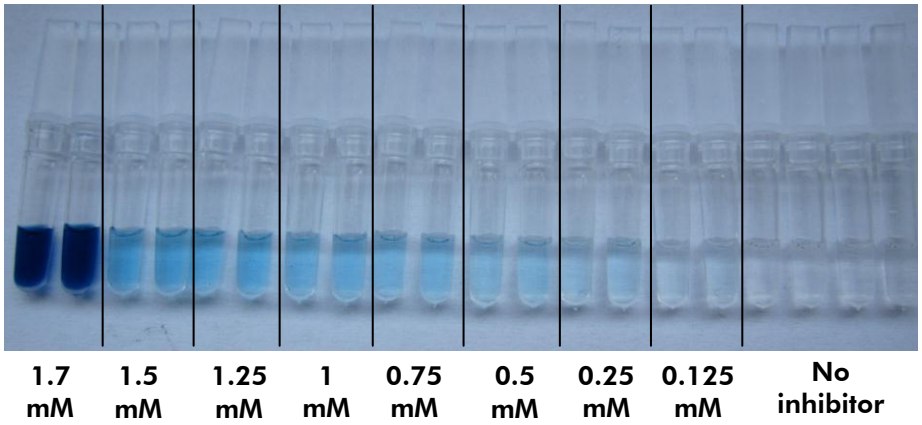

Figure 22. Rotor-Gene Q tubes containing reactions with 0–1.7 mM indigo carmine.

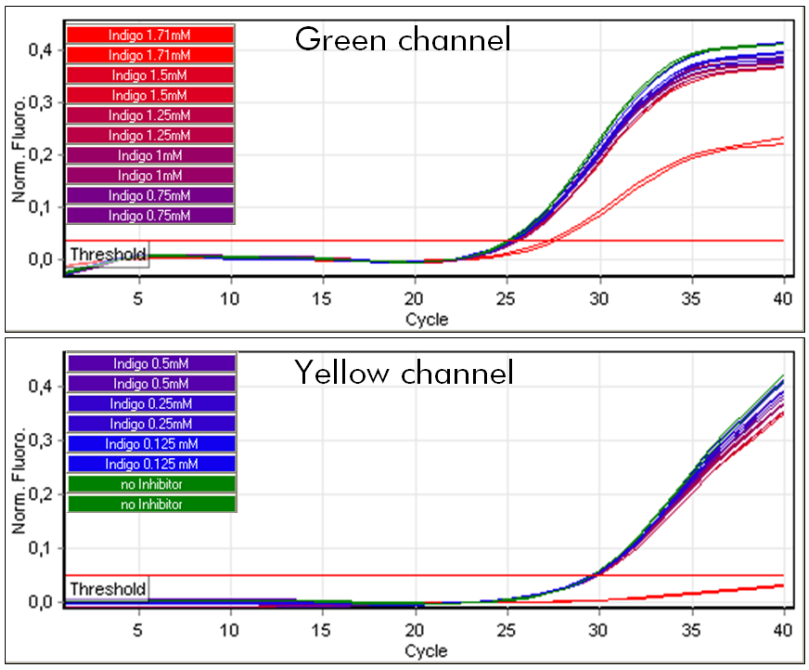

Figure 23. Response to increasing concentrations of indigo carmine. Amplification plots were produced using the Rotor-Gene Q green and yellow channels and the Investigator Quantiplex Kit with increasing concentrations of indigo carmine.

It was shown that indigo carmine does not have a high inhibitory effect on the Investigator Quantiplex Kit when run on the Rotor-Gene Q. The quantification reaction remains uncompromised, even at concentrations where the color shifts to light blue.

However, amplification could not be stably detected by the Applied Biosystems 7500 Real-Time PCR System or the Applied Biosystems 7500 Fast 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). Only the Rotor-Gene Q showed 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 (Opel et al., 2010).

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 Handbook* (1 ng Control DNA Z1). The results are shown in Figures 24 and 25 (page 31).

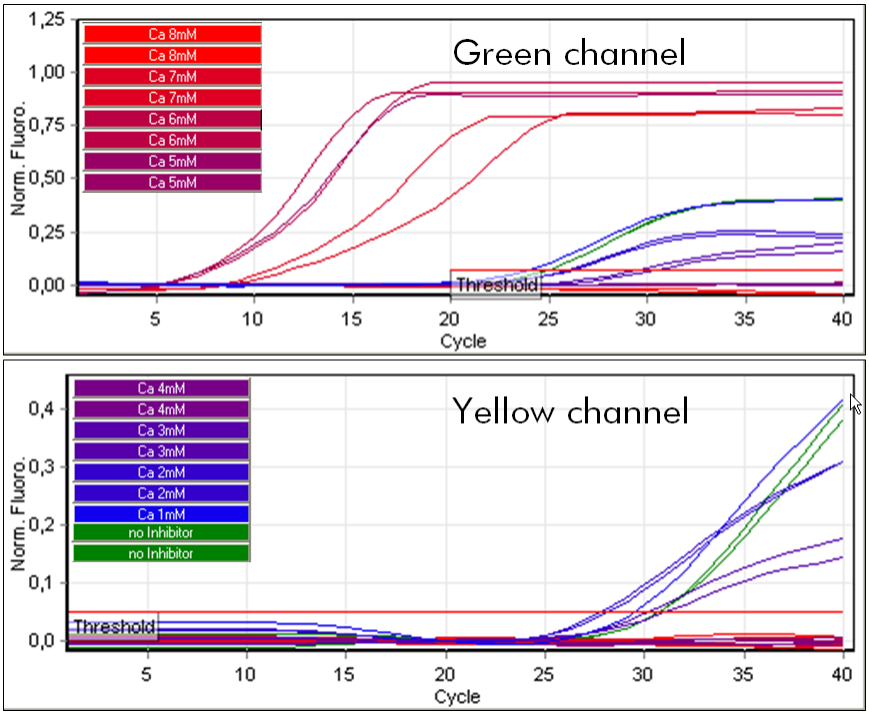

Figure 24. Response to increasing concentrations of calcium hydrogen phosphate. Amplification plots were produced using the Rotor-Gene Q green and yellow channels and the Investigator Quantiplex Kit with increasing concentrations of calcium hydrogen phosphate.

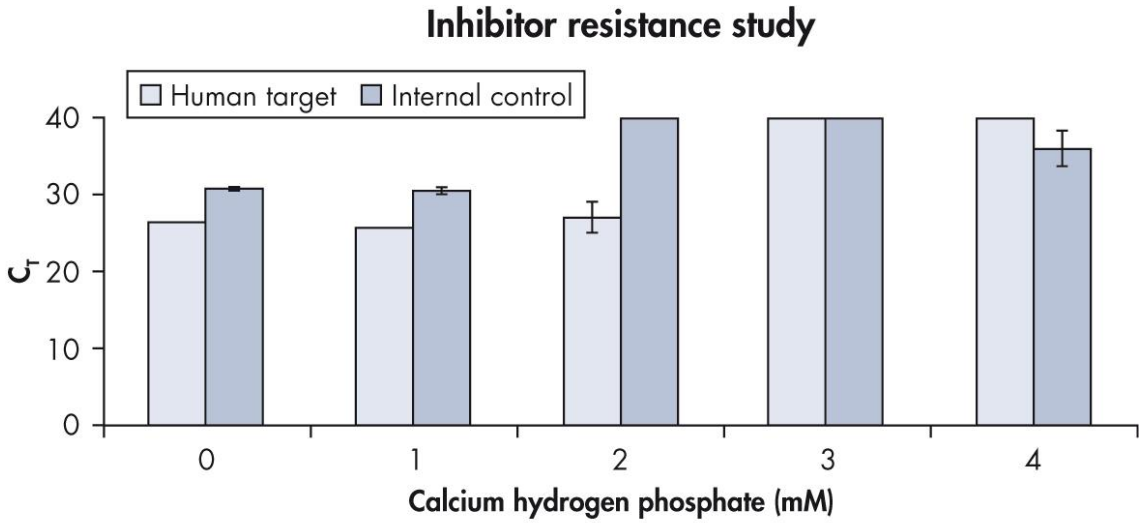

Figure 25. Performance of Investigator Quantiplex Kit with simulated inhibition effect of calcium hydrogen phosphate on an Applied Biosystems 7500 Real-Time PCR System. The internal control reports the presence of the inhibitor (C_T shift) while the quantification is reliable up to a concentration of 1mM. 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 supposed to be a DNA polymerase inhibitor that also affects availability of the DNA template (Opel et al., 2010).

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 Handbook* (1 ng Control DNA Z1). The results are shown in Figure 26.

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

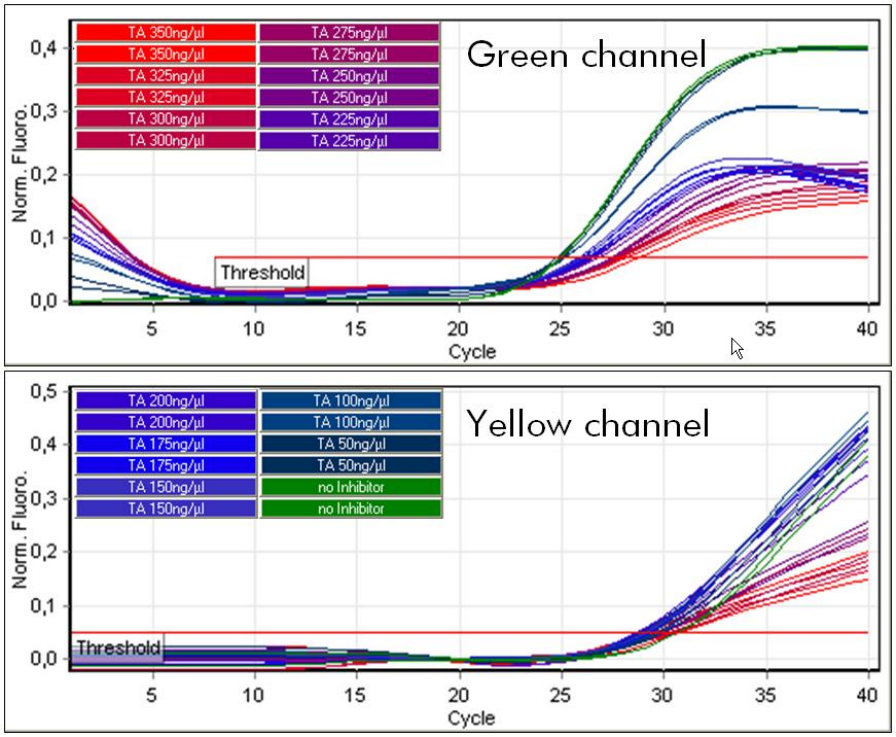

Figure 26. Response to increasing concentrations of tannic acid. Amplification plots were produced using the Rotor-Gene Q green and yellow channels and the Investigator Quantiplex Kit with increasing concentrations of tannic acid.

Contamination of reagents

Laboratory contamination of one of the reagents contained in the Investigator Quantiplex Kit may cause a false positive result in the quantification reaction. Contamination studies were performed to exclude reagent contamination. Duplicates of 94 no-template Controls (NTC) and 2 positive controls (Control DNA Z1; 20 ng/µl) were analyzed.

Run 1 is shown as an example (Figure 27). No DNA was detected in any NTC samples of either plate.

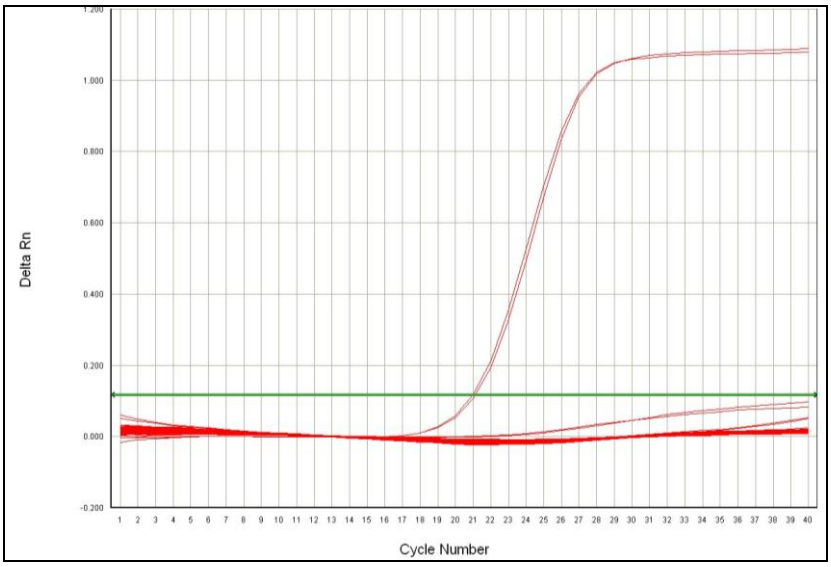

Figure 27. Results of the first run. No detectable contamination could be observed.

The absence of DNA in 188 of 188 NTC samples demonstrated that the Investigator Quantiplex 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 1.5ml 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 28 and 29; not all data shown).

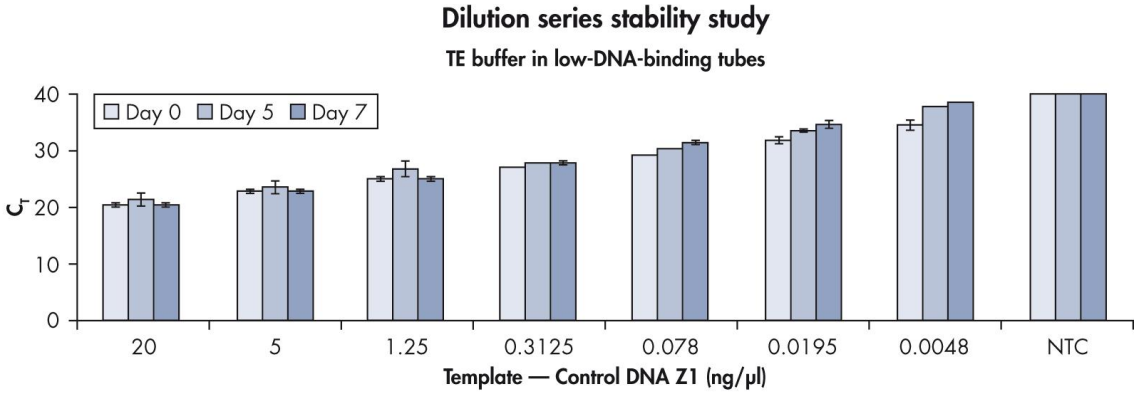

Figure 28. Control DNA Z1 dilution series using TE buffer in low-DNA-binding tubes before storage and after storage for 5 and 7 days at 4°C. The results show C_T shifts after 5 days in the low DNA range. The figure shows average ± standard deviation. NTC: No-template control.

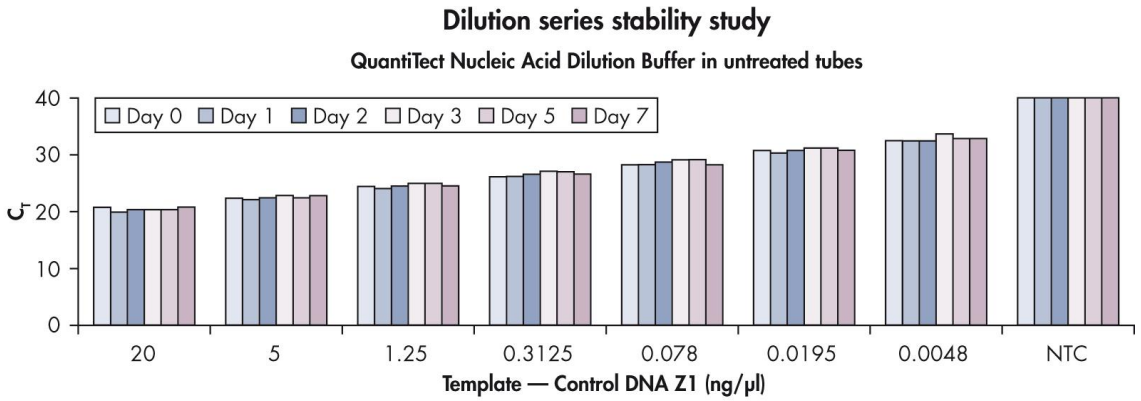

Figure 29. Control DNA Z1 dilution series using the QuantiTect Nucleic Acid Dilution buffer in untreated tubes before storage and after storage for 1, 2, 3, 5, and 7 days at 4°C. The results show no relevant differences even in the low DNA range. The figure shows average ± standard deviation. NTC: No-template 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.

Stability after simulated long-distance transport

The performance of the Investigator Quantiplex Kit was assessed after simulated transportation. Kits were stored overnight on dry ice and then thawed over the day to simulate the variation in temperature conditions that can occur during long-distance transport. This cycle was repeated for 3 days. After the 3 days, components (i.e., Reaction Mix FQ, Primer IC Mix FQ, and Control DNA Z1) from these kits were used to quantify standard dilutions of Control DNA Z1. The reaction was performed on an Applied Biosystems 7500 Real-Time PCR instrument.

The results indicate that the performance before and after storage on dry ice was comparable for all of the components (Figures 30–32, pages 36 and 37).

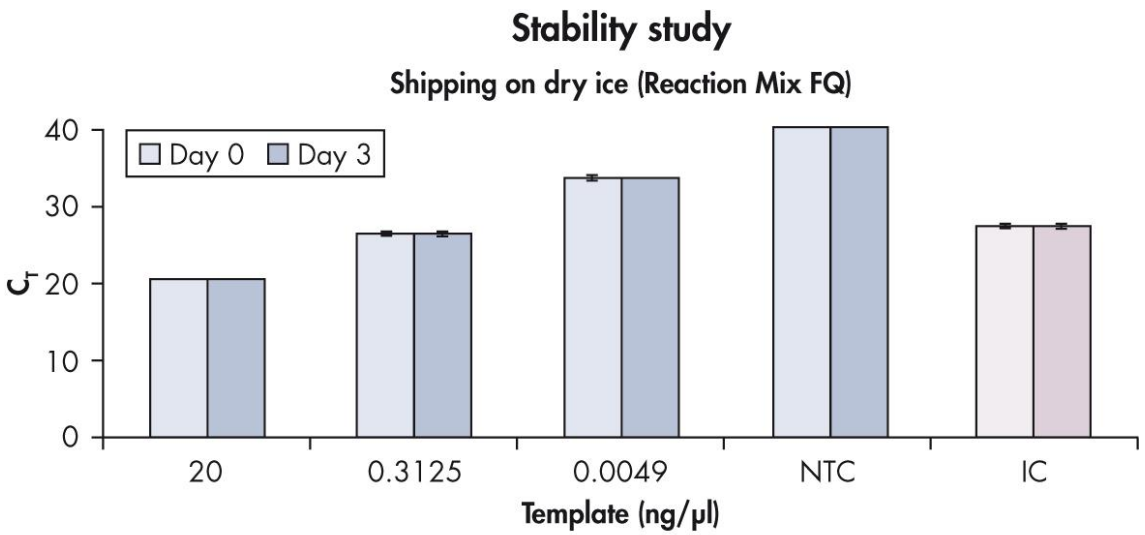

Figure 30. Stability of Reaction Mix FQ after simulated shipping. The results indicate that the performances before and after simulated shipping were comparable with a C_T >40. The figure shows average ± standard deviation. IC: Internal control; NTC: No-template control.

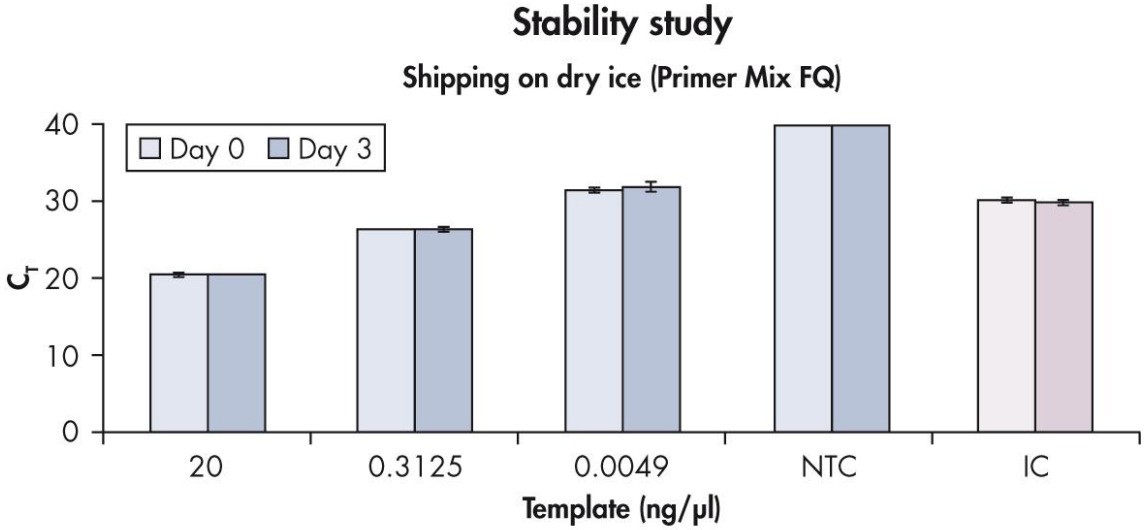

Figure 31. Stability of Primer IC Mix FQ after simulated shipping. The results indicate that the performances before and after simulated shipping were comparable. The figure shows average ± standard deviation. IC: Internal control; NTC: No-template control.

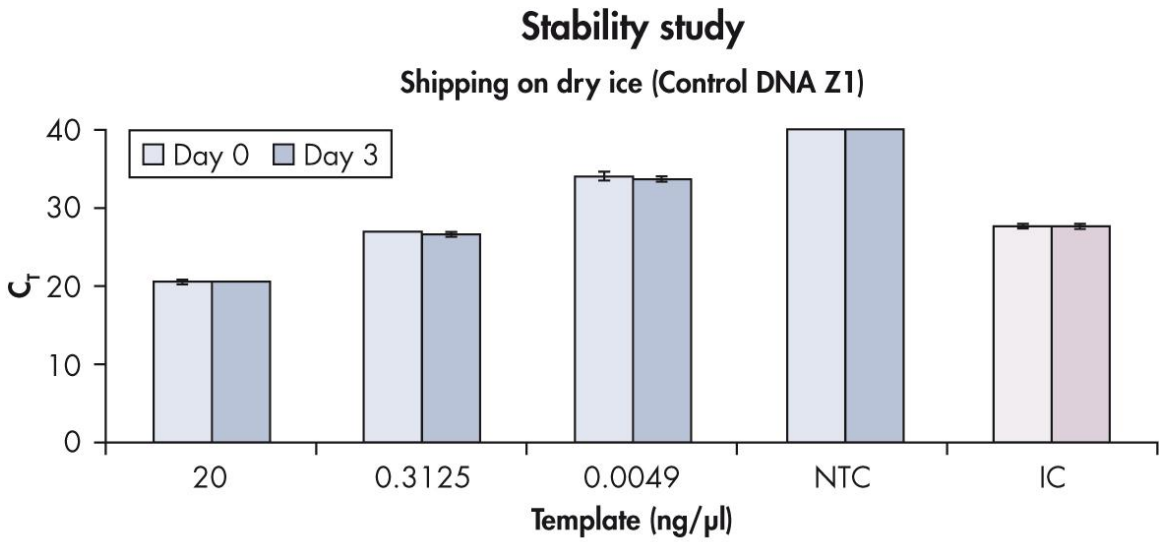

Figure 32. Stability of Control DNA Z1 after simulated shipping. The results indicate that the performances before and after simulated shipping were comparable. The figure shows average ± standard deviation. IC: Internal control; NTC: No-template control.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

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Cited reference

Opel, K.L., Chung, D., and McCord, BR. (2010) A study of PCR inhibition mechanisms using real time PCR. J. Forensic Sci. **55(1)**, 25.

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.

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Kit (200)	Primer mix, reaction mix, Control DNA, and Dilution Buffer	387016

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.

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