Validation Report August 2018

Developmental validation of the Investigator® Quantiplex® Pro RGQ Kit

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

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

The optimum amplification conditions for the Investigator Quantiplex Pro RGQ Kit are given on pages 4 and 5. A target validation was performed in an internal and external study (page 6). The kit was validated for reproducibility, repeatability (page 12) and sensitivity (page 21). It was tested for cross-reactivity with other species (page 28), and its performance with inhibitors (page 31) and contamination (page 40) was assessed. The quantification of male:female mixtures was also tested (page 42).

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

Principle and procedure

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

The kit contains reagents and a DNA polymerase for specific amplification of 4NS1C®, which is a 91 bp proprietary multicopy region present on several autosomes of the human genome. It was selected to give high sensitivity with high reliability within different individuals and populations. The target region was validated in an internal and external study. The human target is detected using the FAM™ dye channel on Applied Biosystems® 7500 Real-Time PCR Systems or the yellow channel on the Rotor-Gene® Q.

The target region for male DNA quantification was selected in order to reliably give the same high sensitivity within different individuals and populations, and in the presence of mixed DNA samples. The male quantification target region is detected as an 81 bp fragment using the Cy5® dye channel on Applied Biosystems instruments or the green channel on the Rotor-Gene Q.

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

Furthermore, the kit detects a longer autosomal amplification product (353 bp) targeting the same locus (4NS1C) as the 91 bp autosomal target. Due to the differently sized autosomal targets, the longer autosomal target is more susceptible to DNA degradation, allowing for a precise assessment of the degradation status of the DNA. The larger autosomal quantification target region is detected as a 353 bp fragment using the TAMRA dye channel on Applied Biosystems instruments or the red channel on the Rotor-Gene Q.

A unique feature of the kit is that it detects a longer gonosomal amplification product (359 bp) targeting the same locus as the smaller 81 bp gonosomal male target. Due to the differently sized gonosomal targets, the longer gonosomal target is more susceptible to DNA degradation, allowing for a precise assessment of the degradation status of male DNA. The larger gonosomal quantification target region is detected as a 359 bp fragment using the orange channel on the Rotor-Gene Q.

Detection of amplification is performed using TaqMan® probes and a novel, fast PCR chemistry. Dual-labeled probes, such as TaqMan probes, contain a fluorescent reporter and a quencher at their 5' and 3' ends, respectively. During the extension phase of PCR, the 5' and 3' exonuclease activity of QuantiNova® DNA Polymerase cleaves the fluorophore from

the quencher. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

Instrumentation for validation

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

- Rotor-Gene Q
- Applied Biosystems 7500 Real-Time PCR System for Human Identification (only for stability data of the Male Control DNA M1 dilution series)

Amplification conditions

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

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

For the Rotor-Gene Q, all the data for assay validation presented in this validation report were obtained using Q-Rex software 1.0 or higher.

Table 1. Master mix for DNA quantification

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

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

Temperature	Temperature	Time	Number of cycles	Additional comments
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C
Two-step cycling:			40	
Denaturation	95°C	5 s		
Combined annealing / extension	60°C	20 s		Perform fluorescence data collection

Table 3. Cycling conditions for the Applied Biosystems 7500 Real-Time PCR Systems (only for stability data of the Male Control DNA M1 dilution series)

Temperature	Temperature	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:			40	
Denaturation	95°C	5 s		
Combined annealing / extension	60°C	35 s		Perform fluorescence data collection

Table 4. Cycling conditions for the Rotor-Gene Q (for assay validation)

Temperature	Temperature	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:			40	
Denaturation	95°C	5 s		
Combined annealing / extension	60°C	10 s		Perform fluorescence data collection

Results of developmental validation

Human target validation study

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

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

Reaction efficiency and linearity using different template amounts are important parameters to be able to compare the simultaneous amplification of all targets. The amplification of all systems was confirmed to be in the linear range with efficiency >90% and an R^2 value >0.99 using DNA concentrations between 50 and 0.08 ng/µl. An example obtained on the Rotor-Gene Q is shown in Figure 1 (page 7).

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

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

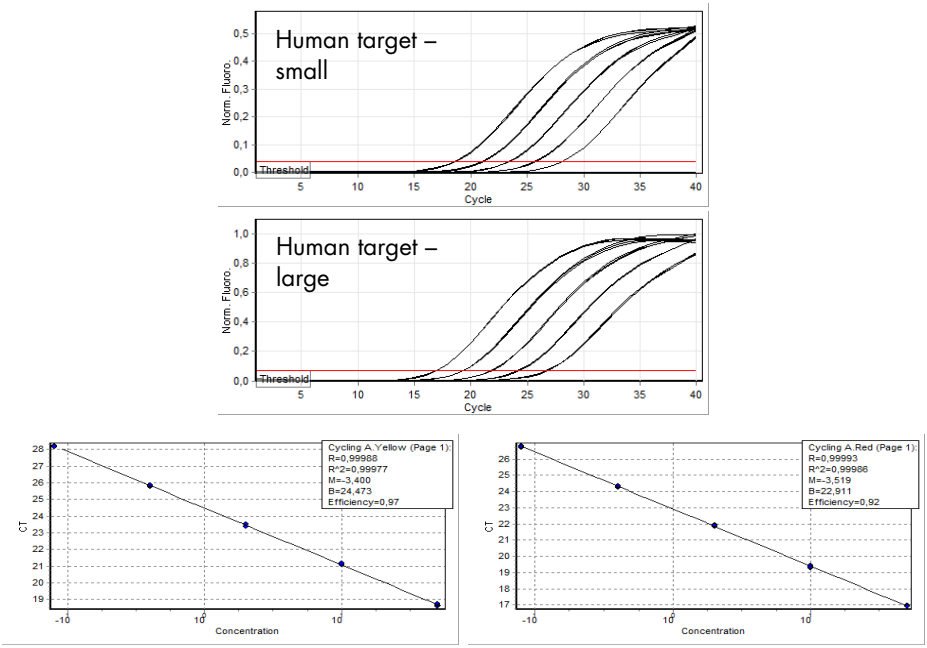

Figure 1. PCR efficiency and linearity of the target validation qPCR for the small and large human autosomal targets. These parameters are comparable for both yellow (human small) and red (human large) channels using DNA concentrations between 50 and 0.08 ng/µl.

In the study, DNA from 317 individuals was examined to ensure reproducibility across the four main human population groups: African-American, Asian, Caucasian and Hispanic. Figure 2 shows the ratio of the DNA quantification values for the small human autosomal target/large human autosomal target and represents the human degradation index (HDI). The average value for the HDI for all populations in this study is 1.10±0.14. The theoretical ideal ratio for the HDI is 1. These results demonstrate that there is a consistent copy number across the four population groups studied.

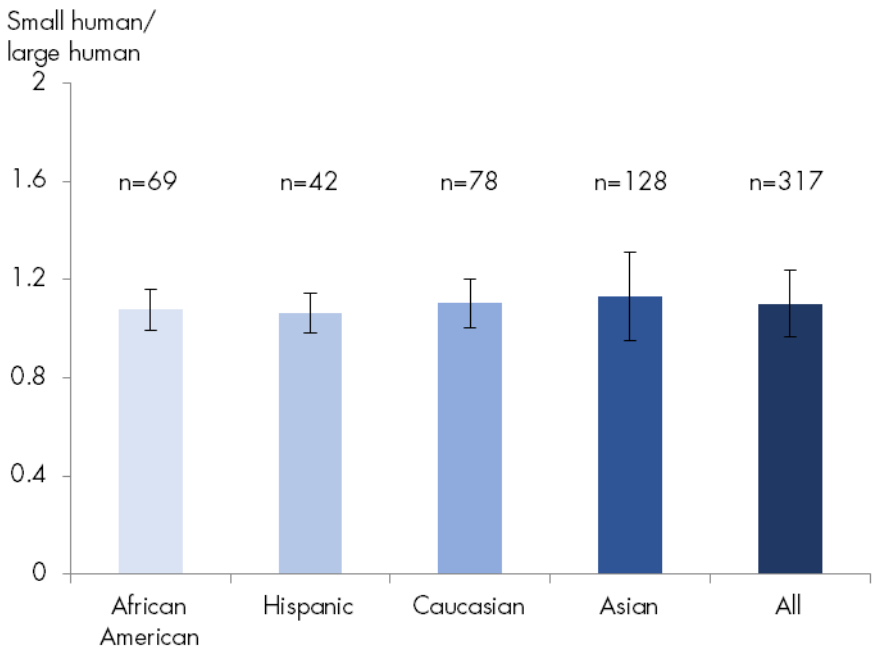

Figure 2. Comparable DI values for the human targets were detected for the 4 main human population groups. The figure shows the average DI ± standard deviation.

Male target validation study

In an internal and external study, the copy number of the male target sequence was analyzed. The first target analyzed was the small human autosomal target in the Investigator Quantiplex Pro RGQ Kit; the second and third target analyzed were the small male gonosomal and large male gonosomal target in the Investigator Quantiplex Pro RGQ Kit. The sequences detected by the primers used in this study for all three targets refer to multi-copy targets in the human genome. Reaction efficiency and linearity using different template amounts are important parameters to be able to compare the simultaneous amplification of all targets. The amplification of all systems was confirmed to be in the linear range with efficiency >90% and an R^2 value >0.99 using DNA concentrations between 50 and

0.08 ng/µl. An example obtained on the Rotor-Gene Q is shown in Figure 3 (page 9). Participants were asked to use between 1 and 10 ng human DNA per 20 µl reaction, which lies within the linear reaction range. The reaction was performed using the QIAGEN Quantiplex Pro Reaction Mix. The ready-to-use Primer Oligo Mix containing primers and TaqMan probes was provided by QIAGEN. The reactions were performed using the Rotor-Gene Q.

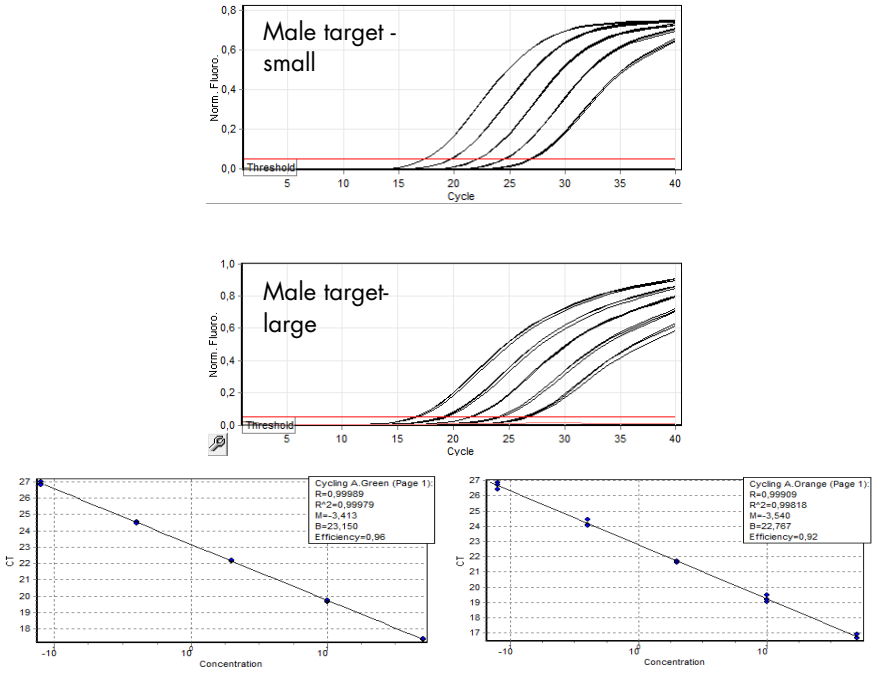

Figure 3. PCR efficiency and linearity of the target validation qPCR for the small and large male gonosomal targets. These parameters are comparable to the parameters obtained for the small human autosomal and large human autosomal targets in the red and yellow channel between 50 and 0.08 ng/µl.

In the study, DNA from 317 individuals was examined to ensure reproducibility across the four main human population groups: African-American, Asian, Caucasian and Hispanic. Figure 4 shows the human/male ratio of the DNA quantification values for the small human autosomal target/small male gonosomal target. The average value for the ratio across all populations in this study is 1.04±0.16. The theoretical ideal ratio is 1. These results demonstrate that there is a consistent copy number across the four population groups studied. Figure 5 shows the ratio of the DNA quantification values for the small male gonosomal target/large male gonosomal target and represents the male degradation index (MDI). The average value for the MDI for all populations in this study is 1.07±0.13. The theoretical ideal ratio for the DI is 1. These results demonstrate that there is a consistent copy number across the four population groups studied.

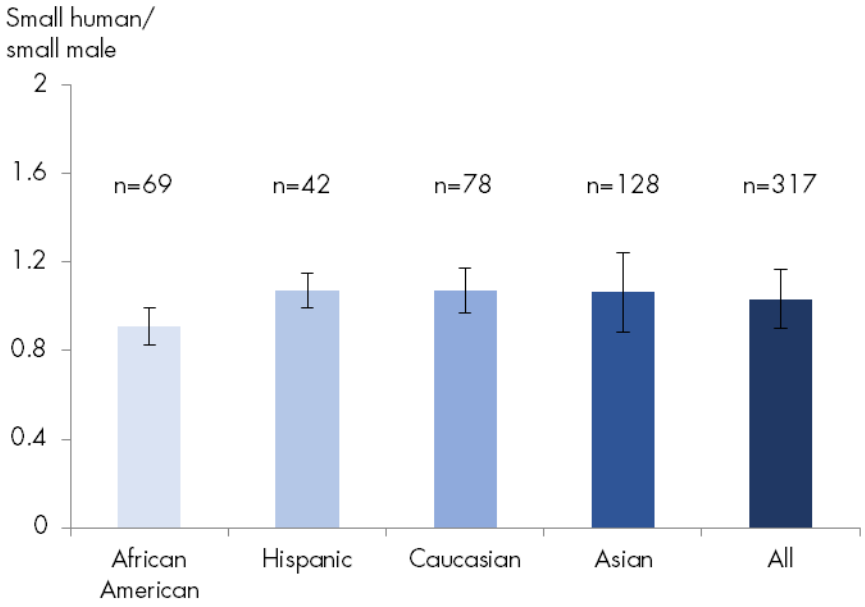

Figure 4. Comparable small human/small male ratios were detected for the four main human population groups. The figure shows the average ratios ± standard deviation.

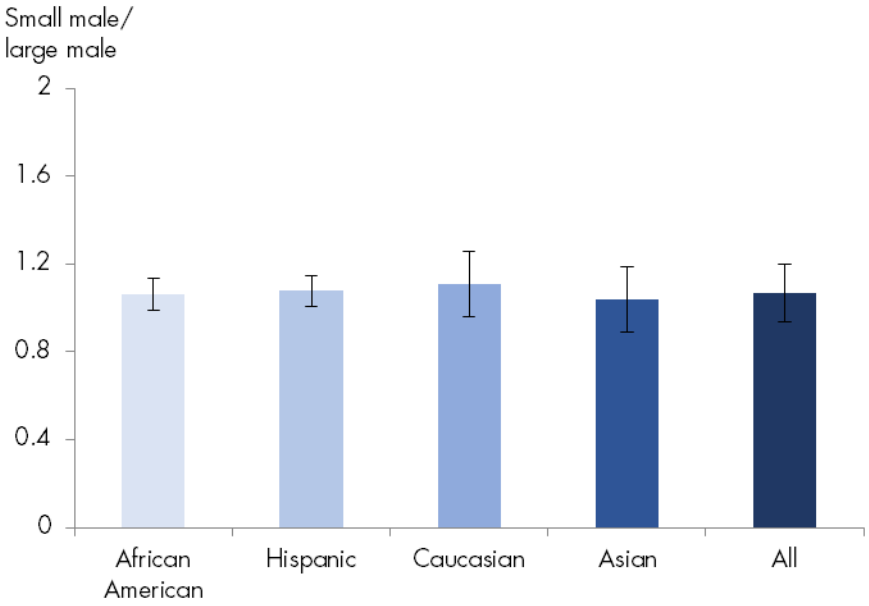

Figure 5. Comparable small male/large male ratios were detected for the four main human population groups. The figure shows the average ratios ± standard deviation.

Reproducibility and repeatability

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

Following the ENFSI guidelines, we tested reproducibility (the variation in average measurements obtained when two or more people measure the same parts or items, using the same measuring technique) and repeatability (the variation in measurements obtained when one person measures the same unit, with the same measuring equipment). All analysis was set up using the QIAgility® system for automated liquid handling.

Reproducibility and repeatability were tested on the Rotor-Gene Q, by taking 5 replicates of the 4 standard dilutions and the no-template control (NTC), and 5 replicates of 3 male and 3 female DNAs.

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

The runs were set up independently. Tables 5–12 (pages 13–20) show the data from the study.
The mean quantity and standard variation were calculated for each sample dilution.
The reproducibility and repeatability of the DNA quantification using the Investigator Quantiplex Pro RGQ Kit was demonstrated for the Rotor-Gene Q.

Table 5. Highly reproducible results comparing 2 different runs performed by 2 different operators on the same Rotor-Gene Q (instrument 1) using the Rotor-Disc® 100

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 2 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.53 ± 0.03	5%	0.56 ± 0.06	11%
	Male DNA 2	8.33 ± 0.85	10%	7.88 ± 0.62	8%
	Male DNA 3	2.59 ± 0.2	8%	2.46 ± 0.17	7%
	Female DNA 1 (NIST 2372)	0.56 ± 0.06	10%	0.61 ± 0.06	10%
	Female DNA 2	2.47 ± 0.16	7%	2.43 ± 0.19	8%
	Female DNA 3	1.26 ± 0.06	5%	1.37 ± 0.14	10%
Human degradation	Male DNA 1 (NIST 2372)	0.56 ± 0.03	5%	0.64 ± 0.09	14%
	Male DNA 2	9.7 ± 0.62	6%	7.86 ± 0.5	6%
	Male DNA 3	3.1 ± 0.09	3%	2.65 ± 0.1	4%
	Female DNA 1 (NIST 2372)	0.58 ± 0.09	15%	0.67 ± 0.04	7%
	Female DNA 2	2.92 ± 0.05	2%	2.54 ± 0.15	6%
	Female DNA 3	1.61 ± 0.06	4%	1.6 ± 0.16	10%
Male	Male DNA 1 (NIST 2372)	0.53 ± 0.07	13%	0.54 ± 0.06	11%
	Male DNA 2	7.37 ± 1.13	15%	6.83 ± 0.78	11%
	Male DNA 3	2.28 ± 0.39	17%	2.06 ± 0.17	8%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.55 ± 0.04	8%	0.56 ± 0.02	4%
	Male DNA 2	7.56 ± 1.05	14%	6.58 ± 0.27	4%
	Male DNA 3	2.47 ± 0.11	4%	2.3 ± 0.27	12%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 6. Highly reproducible results comparing 2 different runs performed by 2 different operators on the same Rotor-Gene Q (instrument 2) using the Rotor-Disc 100

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 2 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.57 ± 0.03	6%	0.61 ± 0.02	3%
	Male DNA 2	8.77 ± 0.58	7%	9.4 ± 0.63	7%
	Male DNA 3	2.51 ± 0.16	7%	2.79 ± 0.22	8%
	Female DNA 1 (NIST 2372)	0.56 ± 0.03	6%	0.65 ± 0.06	9%
	Female DNA 2	2.76 ± 0.26	10%	3.02 ± 0.33	11%
	Female DNA 3	1.53 ± 0.22	15%	1.82 ± 0.25	14%
Human degradation	Male DNA 1 (NIST 2372)	0.61 ± 0.03	5%	0.72 ± 0.02	3%
	Male DNA 2	9.27 ± 0.57	6%	9.28 ± 0.39	4%
	Male DNA 3	2.67 ± 0.17	6%	3.04 ± 0.39	13%
	Female DNA 1 (NIST 2372)	0.57 ± 0.03	6%	0.72 ± 0.04	6%
	Female DNA 2	2.75 ± 0.09	3%	3.35 ± 0.42	13%
	Female DNA 3	1.42 ± 0.11	8%	2.02 ± 0.1	5%
Male	Male DNA 1 (NIST 2372)	0.6 ± 0.08	14%	0.67 ± 0.1	15%
	Male DNA 2	7.65 ± 0.33	4%	8.71 ± 1.15	13%
	Male DNA 3	2.47 ± 0.21	8%	2.68 ± 0.23	9%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.58 ± 0.05	9%	0.7 ± 0.07	10%
	Male DNA 2	7.07 ± 0.23	3%	8.07 ± 0.59	7%
	Male DNA 3	2.14 ± 0.09	4%	2.62 ± 0.32	12%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 7. Highly reproducible results comparing 2 different runs performed by 2 different operators on the same Rotor Gene Q (instrument 1) using the Rotor-Disc 72

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 2 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.66 ± 0.07	10%	0.56 ± 0.02	4%
	Male DNA 2	10.97 ± 1.23	11%	9.08 ± 0.67	7%
	Male DNA 3	3.02 ± 0.2	7%	2.55 ± 0.23	9%
	Female DNA 1 (NIST 2372)	0.7 ± 0.04	5%	0.76 ± 0.07	10%
	Female DNA 2	3.47 ± 0.46	13%	3.62 ± 0.19	5%
	Female DNA 3	1.85 ± 0.24	13%	2.03 ± 0.11	6%
Human degradation	Male DNA 1 (NIST 2372)	0.7 ± 0.13	19%	0.63 ± 0.04	6%
	Male DNA 2	11.97 ± 2.1	18%	9.21 ± 0.24	3%
	Male DNA 3	3.2 ± 0.56	17%	2.74 ± 0.16	6%
	Female DNA 1 (NIST 2372)	0.7 ± 0.04	5%	0.73 ± 0.03	4%
	Female DNA 2	3.9 ± 0.58	15%	3.67 ± 0.22	6%
	Female DNA 3	1.76 ± 0.24	14%	2.15 ± 0.1	5%
Male	Male DNA 1 (NIST 2372)	0.75 ± 0.13	18%	0.61 ± 0.11	17%
	Male DNA 2	11.36 ± 3.27	29%	8.52 ± 0.93	11%
	Male DNA 3	2.98 ± 0.56	19%	2.36 ± 0.39	17%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.6 ± 0.05	8%	0.66 ± 0.05	7%
	Male DNA 2	8.81 ± 0.74	8%	8.22 ± 0.24	3%
	Male DNA 3	2.54 ± 0.3	12%	2.54 ± 0.16	6%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 8. Highly reproducible results comparing 2 different runs performed by 2 different operators on the same Rotor-Gene Q (instrument 1) using strip tubes

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 2 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.62 ± 0.04	6%	0.61 ± 0.04	6%
	Male DNA 2	9.49 ± 0.54	6%	8.88 ± 0.34	4%
	Male DNA 3	3.06 ± 0.36	12%	3 ± 0.34	11%
	Female DNA 1 (NIST 2372)	0.52 ± 0.01	2%	0.49 ± 0.01	2%
	Female DNA 2	2.19 ± 0.19	9%	2.15 ± 0.04	2%
	Female DNA 3	2.3 ± 0.14	6%	1.08 ± 0.08	7%
Human degradation	Male DNA 1 (NIST 2372)	0.79 ± 0.02	2%	0.69 ± 0.04	5%
	Male DNA 2	10.4 ± 0.73	7%	10.28 ± 0.41	4%
	Male DNA 3	3.31 ± 0.15	5%	3.41 ± 0.15	4%
	Female DNA 1 (NIST 2372)	0.52 ± 0.04	8%	0.45 ± 0.02	3%
	Female DNA 2	2.56 ± 0.18	7%	2.51 ± 0.17	7%
	Female DNA 3	2.73 ± 0.22	8%	1.23 ± 0.07	6%
Male	Male DNA 1 (NIST 2372)	0.63 ± 0.06	9%	0.65 ± 0.05	8%
	Male DNA 2	8.76 ± 0.67	8%	8.48 ± 0.82	10%
	Male DNA 3	2.58 ± 0.18	7%	2.84 ± 0.46	16%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.67 ± 0.07	11%	0.67 ± 0.08	12%
	Male DNA 2	9.33 ± 1.03	11%	10.01 ± 0.41	4%
	Male DNA 3	2.93 ± 0.16	6%	3.08 ± 0.04	1%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 9. Highly repeatable results comparing 2 different runs performed by the same operator on the same Rotor-Gene Q (instrument 1) using the Rotor-Disc 100

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 1 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.53 ± 0.03	5%	0.67 ± 0.24	36%
	Male DNA 2	8.33 ± 0.85	10%	8.92 ± 0.77	9%
	Male DNA 3	2.59 ± 0.2	8%	2.49 ± 0.33	13%
	Female DNA 1 (NIST 2372)	0.56 ± 0.06	10%	0.61 ± 0.13	20%
	Female DNA 2	2.47 ± 0.16	7%	2.62 ± 0.2	8%
	Female DNA 3	1.26 ± 0.06	5%	1.28 ± 0.11	8%
Human degradation	Male DNA 1 (NIST 2372)	0.56 ± 0.03	5%	0.99 ± 0.15	15%
	Male DNA 2	9.7 ± 0.62	6%	18.49 ± 1.06	6%
	Male DNA 3	3.1 ± 0.09	3%	5 ± 0.27	5%
	Female DNA 1 (NIST 2372)	0.58 ± 0.09	15%	0.98 ± 0.15	15%
	Female DNA 2	2.92 ± 0.05	2%	5.15 ± 0.29	6%
	Female DNA 3	1.61 ± 0.06	4%	2.34 ± 0.24	10%
Male	Male DNA 1 (NIST 2372)	0.53 ± 0.07	13%	0.53 ± 0.07	14%
	Male DNA 2	7.37 ± 1.13	15%	8.2 ± 0.68	8%
	Male DNA 3	2.28 ± 0.39	17%	2 ± 0.24	12%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.55 ± 0.04	8%	0.56 ± 0.05	8%
	Male DNA 2	7.56 ± 1.05	14%	7.68 ± 0.22	3%
	Male DNA 3	2.47 ± 0.11	4%	2.17 ± 0.16	7%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 10. Highly repeatable results comparing 2 different runs performed by the same operator on the same Rotor Gene Q (instrument 2) using the Rotor-Disc 100

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 1 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.57 ± 0.03	6%	0.62 ± 0.07	11%
	Male DNA 2	8.77 ± 0.58	7%	9.63 ± 1.01	10%
	Male DNA 3	2.51 ± 0.16	7%	2.98 ± 0.1	3%
	Female DNA 1 (NIST 2372)	0.56 ± 0.03	6%	0.68 ± 0.05	8%
	Female DNA 2	2.76 ± 0.26	10%	3.08 ± 0.31	10%
	Female DNA 3	1.53 ± 0.22	15%	1.83 ± 0.25	14%
Human degradation	Male DNA 1 (NIST 2372)	0.61 ± 0.03	5%	0.69 ± 0.04	5%
	Male DNA 2	9.27 ± 0.57	6%	9.79 ± 0.86	9%
	Male DNA 3	2.67 ± 0.17	6%	3.22 ± 0.18	6%
	Female DNA 1 (NIST 2372)	0.57 ± 0.03	6%	0.76 ± 0.08	10%
	Female DNA 2	2.75 ± 0.09	3%	3.29 ± 0.2	6%
	Female DNA 3	1.42 ± 0.11	8%	1.79 ± 0.11	6%
Male	Male DNA 1 (NIST 2372)	0.6 ± 0.08	14%	0.58 ± 0.04	7%
	Male DNA 2	7.65 ± 0.33	4%	8.34 ± 1.69	20%
	Male DNA 3	2.47 ± 0.21	8%	2.58 ± 0.44	17%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.58 ± 0.05	9%	0.58 ± 0.05	8%
	Male DNA 2	7.07 ± 0.23	3%	7.81 ± 0.81	10%
	Male DNA 3	2.14 ± 0.09	4%	2.39 ± 0.11	4%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 11. Highly repeatable results comparing 2 different runs performed by the same operator on the same Rotor-Gene Q (instrument 1) using the Rotor-Disc 72

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 1 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.66 ± 0.07	10%	0.61 ± 0.03	4%
	Male DNA 2	10.97 ± 1.23	11%	8.97 ± 0.6	7%
	Male DNA 3	3.02 ± 0.2	7%	2.77 ± 0.19	7%
	Female DNA 1 (NIST 2372)	0.7 ± 0.04	5%	0.7 ± 0.05	8%
	Female DNA 2	3.47 ± 0.46	13%	3.45 ± 0.51	15%
	Female DNA 3	1.85 ± 0.24	13%	1.97 ± 0.13	7%
Human degradation	Male DNA 1 (NIST 2372)	0.7 ± 0.13	19%	0.65 ± 0.07	11%
	Male DNA 2	11.97 ± 2.1	18%	9.62 ± 0.55	6%
	Male DNA 3	3.2 ± 0.56	17%	3.17 ± 0.15	5%
	Female DNA 1 (NIST 2372)	0.7 ± 0.04	5%	0.76 ± 0.02	2%
	Female DNA 2	3.9 ± 0.58	15%	3.82 ± 0.56	15%
	Female DNA 3	1.76 ± 0.24	14%	2.1 ± 0.13	6%
Male	Male DNA 1 (NIST 2372)	0.75 ± 0.13	18%	0.54 ± 0.06	11%
	Male DNA 2	11.36 ± 3.27	29%	8.32 ± 0.78	9%
	Male DNA 3	2.98 ± 0.56	19%	2.49 ± 0.29	12%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.6 ± 0.05	8%	0.67 ± 0.1	14%
	Male DNA 2	8.81 ± 0.74	8%	9.28 ± 0.93	10%
	Male DNA 3	2.54 ± 0.3	12%	2.77 ± 0.32	12%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Table 12. Highly repeatable results comparing 2 different runs performed by the same operator on the same Rotor Gene Q (instrument 1) using strip tubes

Target	DNA sample	Operator 1 Concentration (ng/µl) ± standard deviation	CV	Operator 1 Concentration (ng/µl) ± standard deviation	CV
Human	Male DNA 1 (NIST 2372)	0.62 ± 0.04	6%	0.64 ± 0.02	4%
	Male DNA 2	9.49 ± 0.54	6%	9.58 ± 0.41	4%
	Male DNA 3	3.06 ± 0.36	12%	2.95 ± 0.25	8%
	Female DNA 1 (NIST 2372)	0.52 ± 0.01	2%	0.52 ± 0.03	6%
	Female DNA 2	2.19 ± 0.19	9%	2.23 ± 0.19	8%
	Female DNA 3	2.3 ± 0.14	6%	2.37 ± 0.17	7%
Human degradation	Male DNA 1 (NIST 2372)	0.79 ± 0.02	2%	0.77 ± 0.03	3%
	Male DNA 2	10.4 ± 0.73	7%	10.59 ± 0.67	6%
	Male DNA 3	3.31 ± 0.15	5%	3.4 ± 0.16	5%
	Female DNA 1 (NIST 2372)	0.52 ± 0.04	8%	0.57 ± 0.08	15%
	Female DNA 2	2.56 ± 0.18	7%	2.47 ± 0.1	4%
	Female DNA 3	2.73 ± 0.22	8%	2.79 ± 0.08	3%
Male	Male DNA 1 (NIST 2372)	0.63 ± 0.06	9%	0.62 ± 0.01	2%
	Male DNA 2	8.76 ± 0.67	8%	8.29 ± 0.44	5%
	Male DNA 3	2.58 ± 0.18	7%	2.68 ± 0.49	18%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA
Male degradation	Male DNA 1 (NIST 2372)	0.67 ± 0.07	11%	0.69 ± 0.09	13%
	Male DNA 2	9.33 ± 1.03	11%	9.71 ± 0.46	5%
	Male DNA 3	2.93 ± 0.16	6%	2.92 ± 0.09	3%
	Female DNA 1 (NIST 2372)	NA	NA	NA	NA
	Female DNA 2	NA	NA	NA	NA
	Female DNA 3	NA	NA	NA	NA

NA: not available.

Sensitivity

The Investigator Quantiplex Pro RGQ Kit is designed to detect a broad range of DNA quantities. A serial dilution of Male Control DNA M1 from 50 ng/µl down to 0.015625 pg/µl has been prepared to test the sensitivity of Investigator Quantiplex Pro RGQ Kit. The optimal linear dynamic range of the assay is in the range of 50 ng/µl to 0.5 pg/µl total DNA. DNA could be detected with the human and human degradation target down to 0.015625 pg/µl, using triplicates for the range 50 ng/µl to 0.005 ng/µl and 6 replicates for DNA concentration below 0.005 ng/µl (see Figures 6, 7, 10, 11, 14 and 15). Furthermore, the standard conditions specified in the *Investigator Quantiplex Pro RGQ Kit Handbook* have been used for the Rotor-Gene Q.

The sensitivity of the Investigator Quantiplex Pro RGQ Kit has been tested for the male DNA component. Figures 8, 9, 12, 13, 16 and 17 show the quantification of the male component of a serial dilution of Male Control DNA M1 from 50 ng/µl to 0.015625 pg/µl male DNA. The optimal linear dynamic range of the assay is in the range of 50 ng/µl to 0.5 pg/µl of male DNA. DNA could be detected down to 0.015625 pg/µl, using triplicates for DNA concentration above 0.005 ng/µl and six replicates for DNA concentrations below 0.005 ng/µl. Furthermore, standard conditions specified in the *Investigator Quantiplex Pro RGQ Kit Handbook* have been used for the Rotor-Gene Q.

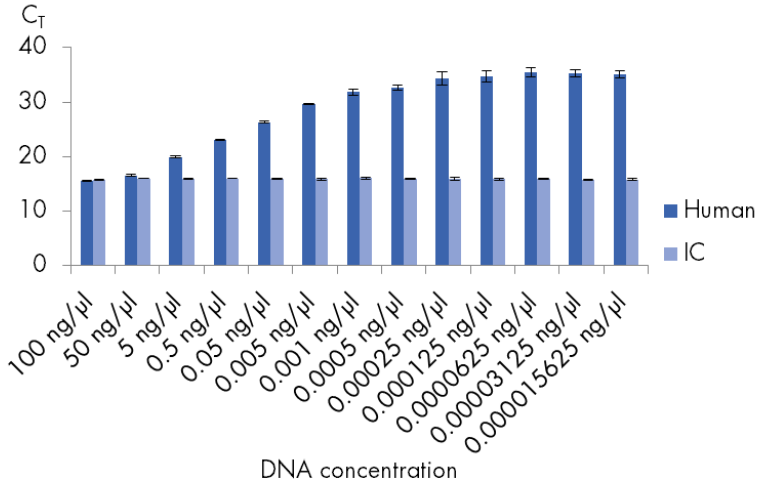

Figure 6. Detection of the human target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro RGQ Kit on the Rotor-Gene Q using strip tubes. The figure shows the average C_T ± standard deviation.

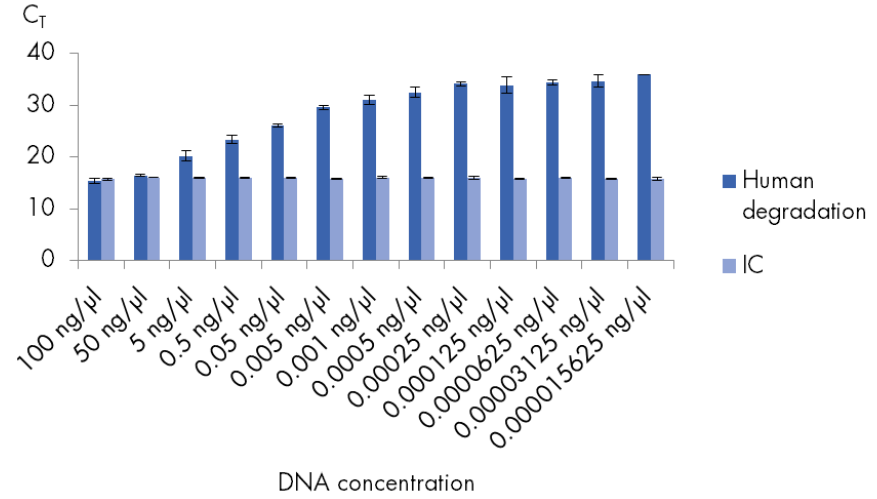

Figure 7. Detection of the human degradation target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro RGQ Kit on the Rotor-Gene Q using strip tubes. The figure shows the average C_T ± standard deviation.

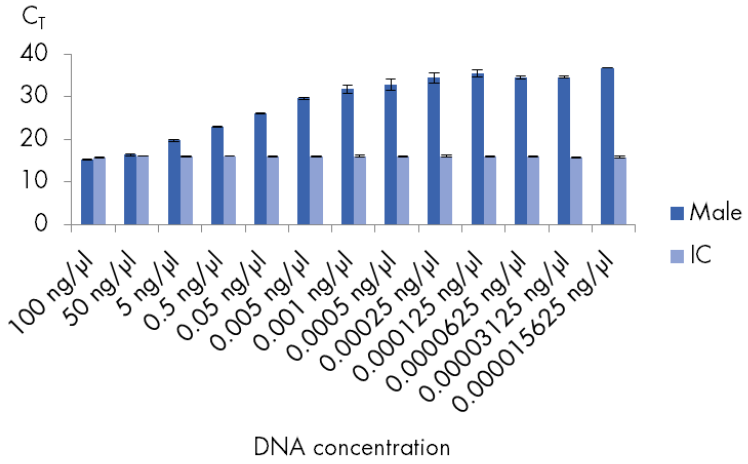

Figure 8. Detection of the male target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro RGQ Kit on the Rotor-Gene Q using strip tubes. The figure shows the average C_T ± standard deviation.

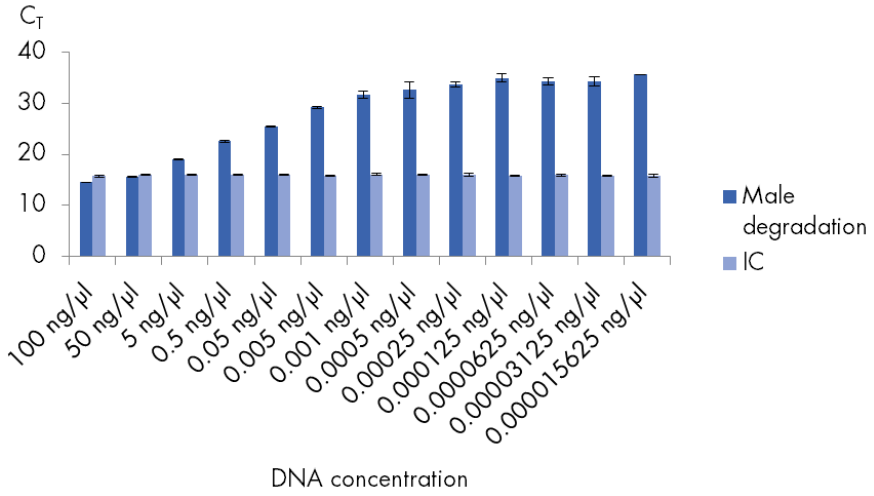

Figure 9. Detection of the male degradation target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using strip tubes. The figure shows the average C_T ± standard deviation.

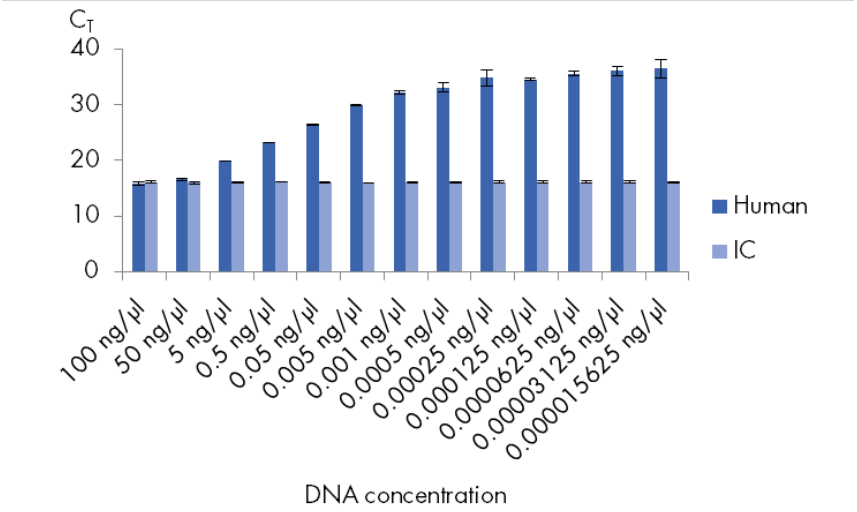

Figure 10. Detection of the human target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 72. The figure shows the average C_T ± standard deviation.

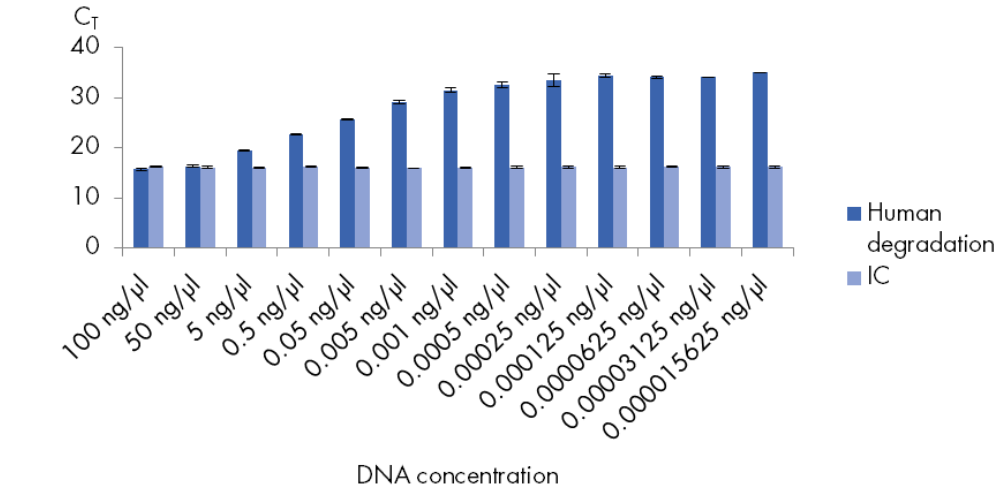

Figure 11. Detection of the human degradation target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 72. The figure shows the average C_T ± standard deviation.

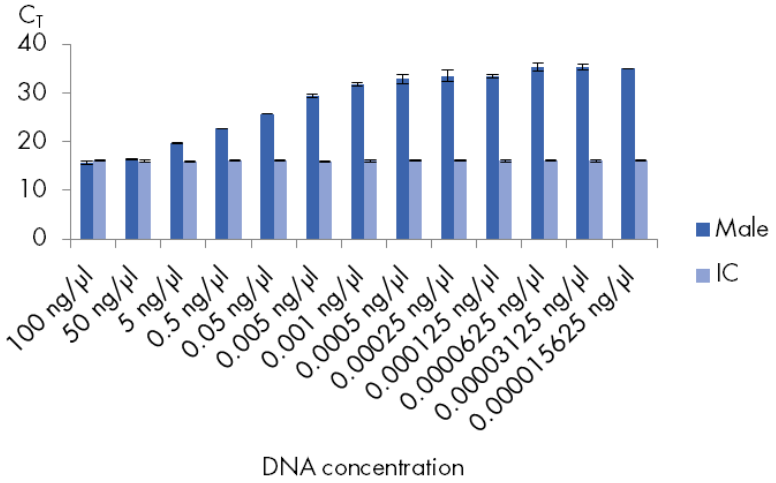

Figure 12. Detection of the male target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 72. The figure shows the average C_T ± standard deviation.

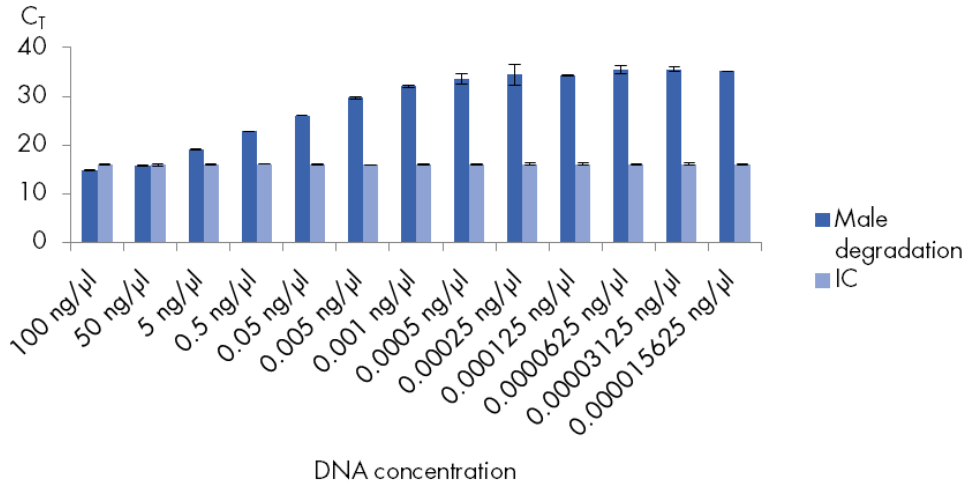

Figure 13. Detection of the male degradation target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 72. The figure shows the average C_T ± standard deviation.

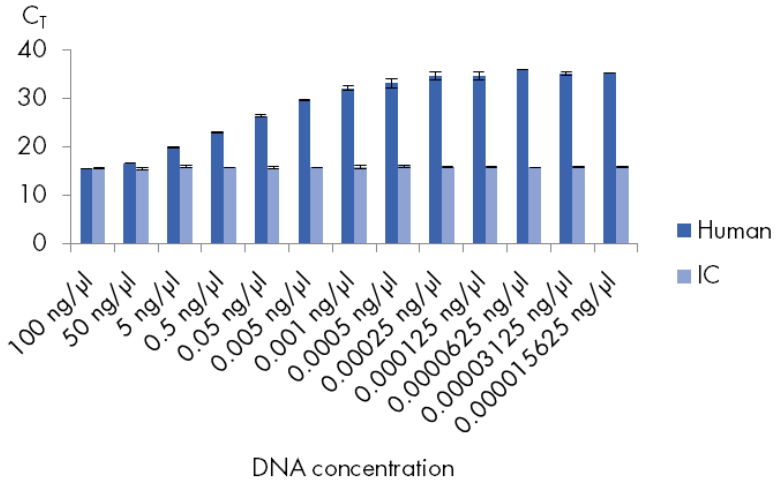

Figure 14. Detection of the human target in male DNA down to 0.015625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 100. The figure shows the average C_T ± standard deviation.

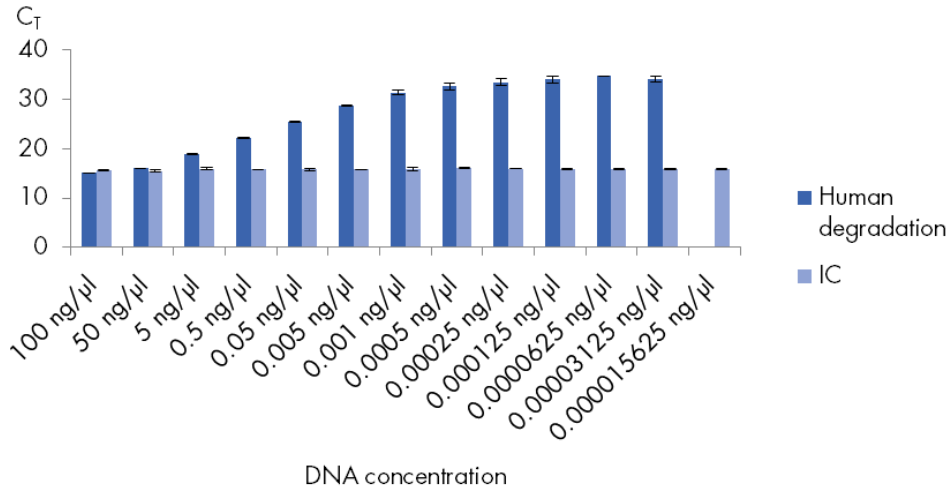

Figure 15. Detection of the human degradation target in male DNA down to 0.03125 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 100. The figure shows the average C_T ± standard deviation.

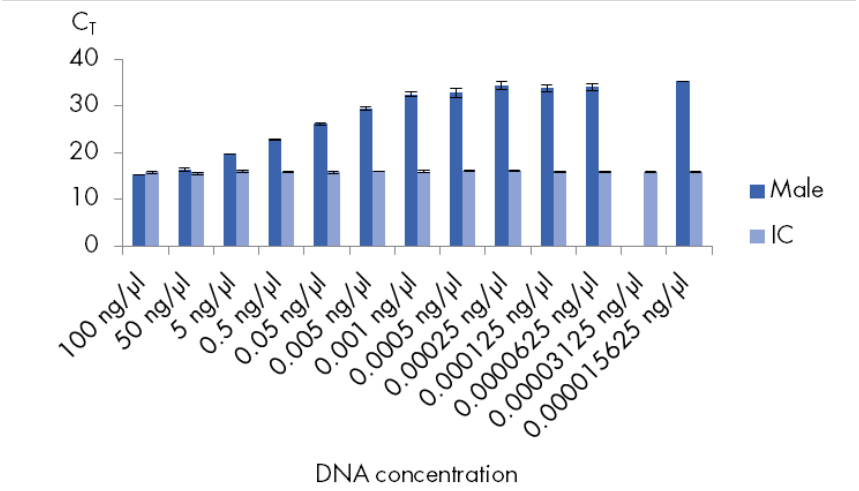

Figure 16. Detection of the male target in male DNA down to 0.0625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 100. The figure shows the average C_T ± standard deviation.

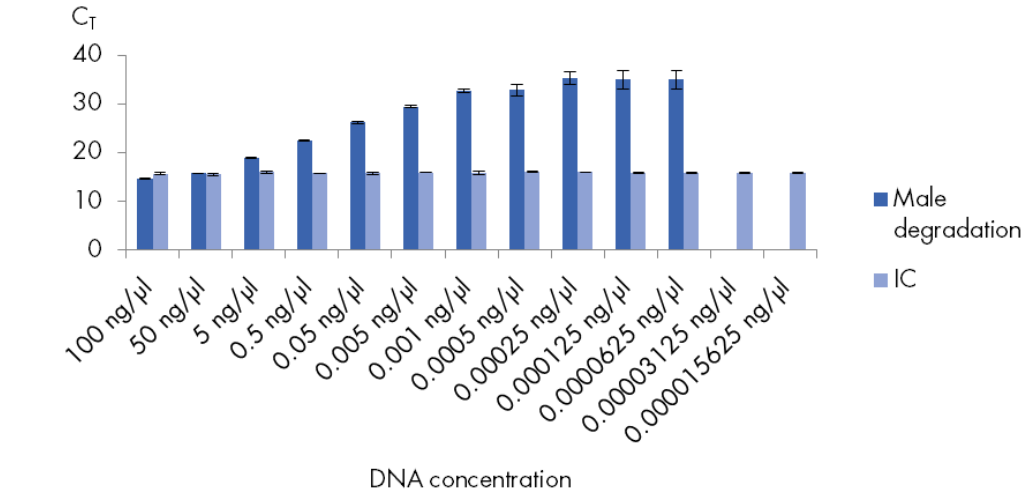

Figure 17. Detection of the male degradation target in male DNA down to 0.0625 pg/µl using the Investigator Quantiplex Pro Kit on the Rotor-Gene Q using the Rotor-Disc 100. The figure shows the average C_T ± standard deviation.

Species specificity

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

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

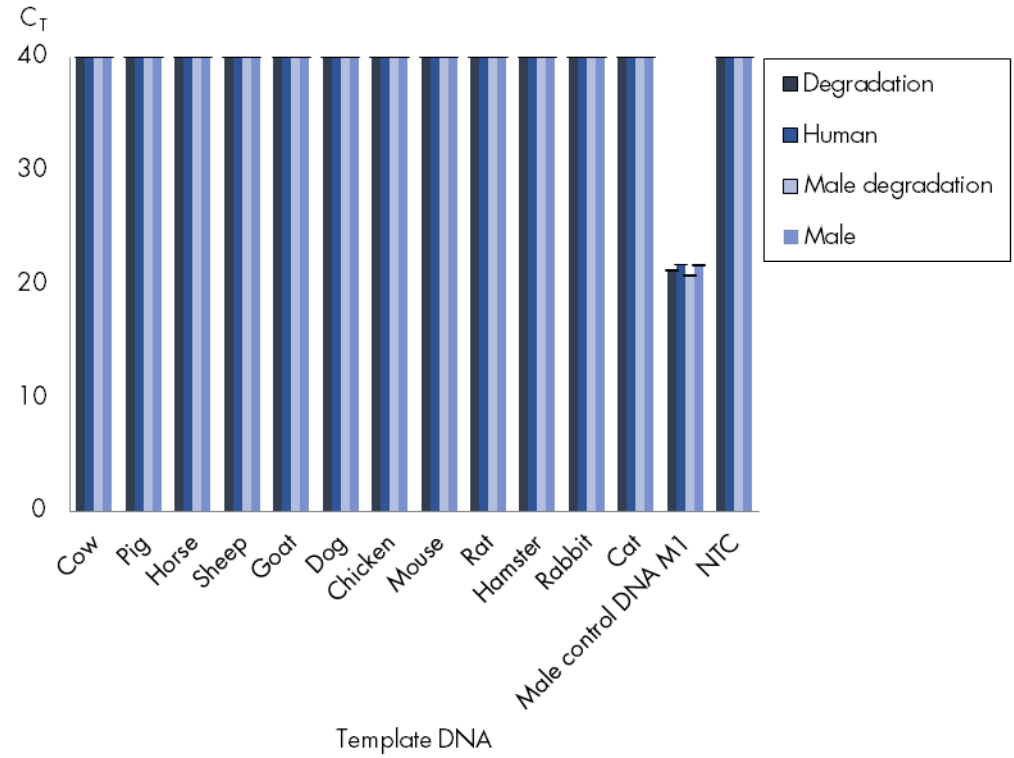

Figure 18. Results of a cross-reactivity study on common vertebrate species. The figure shows the average C_T ± standard deviation. **NTC**: No-template control.

No cross-reactivity was shown for DNA, from the tested common vertebrates, under standard conditions, as shown in Figure 18.

Some primates, including gorillas, chimpanzees, bonobo, orangutans and macaque were also examined, as described above. Due to the evolutionary proximity of the chimpanzee, bonobo, orangutan, macaque and gorilla to humans, positive results were observed for these species DNA (Figure 19).

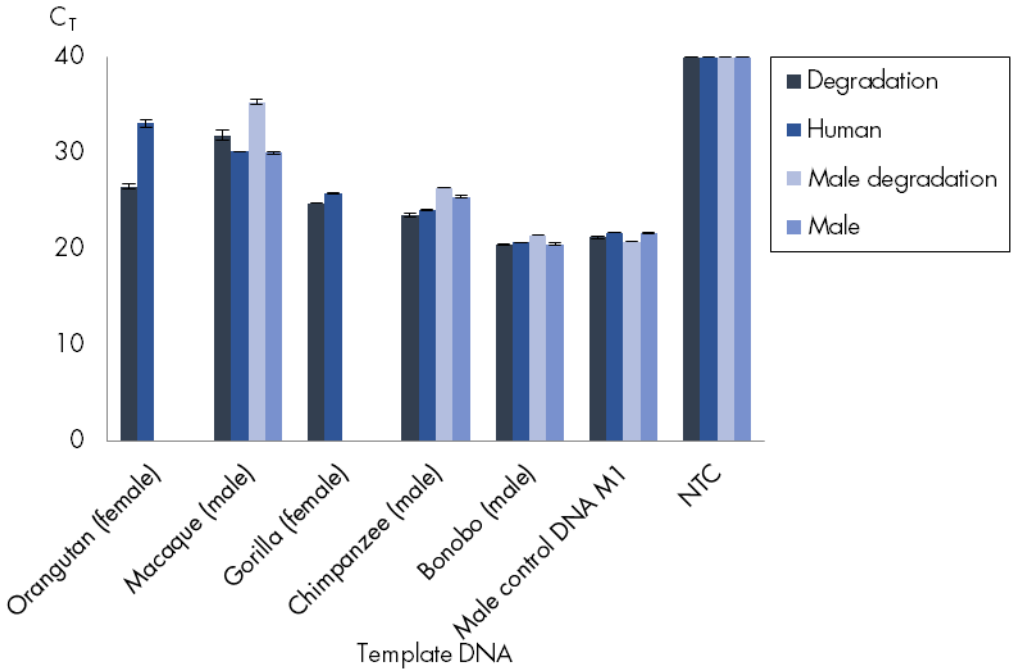

Figure 19. Results of a cross-reactivity study on primates. The figure shows the average C_T ± standard deviation. **NTC:** No-template control.

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 each) was tested following the standard assay protocol, with 2.5 ng Male Control DNA M1 as a positive control. None of the tested microbial species yielded detectable DNA under standard conditions, as shown in Figure 20.

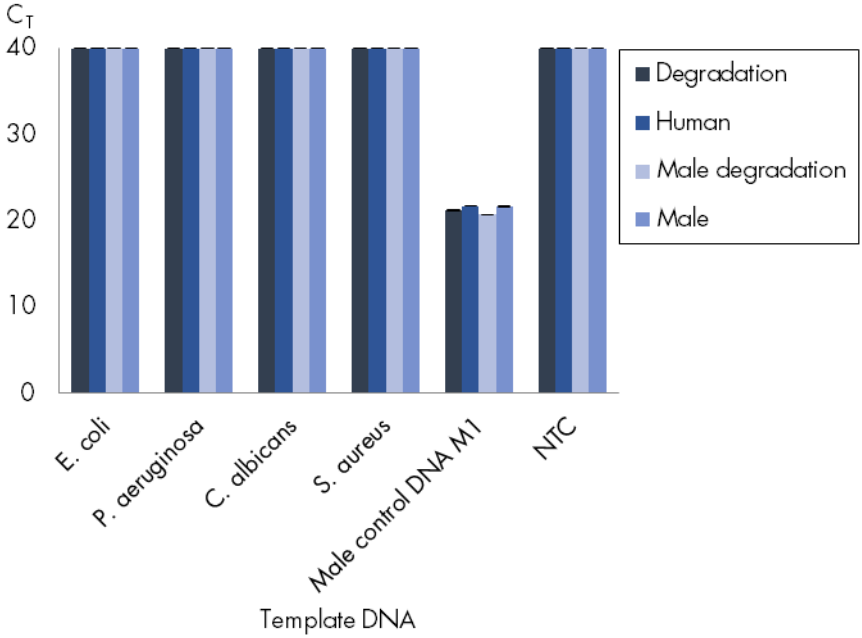

Figure 20. Results of a cross-reactivity study on microbial species. No cross-reactivity could be shown for the tested microbes. The figure shows the average C_T ± standard deviation. **NTC:** No-template control.

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

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

Performance with simulated inhibition

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

The Investigator Quantiplex Pro RGQ Kit contains a 434 bp internal control that was developed to provide information about the presence of inhibitors within a sample. The change in C_T value of the internal control in comparison to non-inhibited samples, such as standard curve samples, provides the user with information regarding the likelihood of successful STR amplification.

Humic acid

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

To test the robustness of the kit, the assay was run in the presence of 0, 25, 33.33, 41.67, 50 and 58.33 ng/µl humic acid (Acros®; cat. no. 120860050) under standard conditions as described in the *Investigator Quantiplex Pro RGQ Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figure 21.

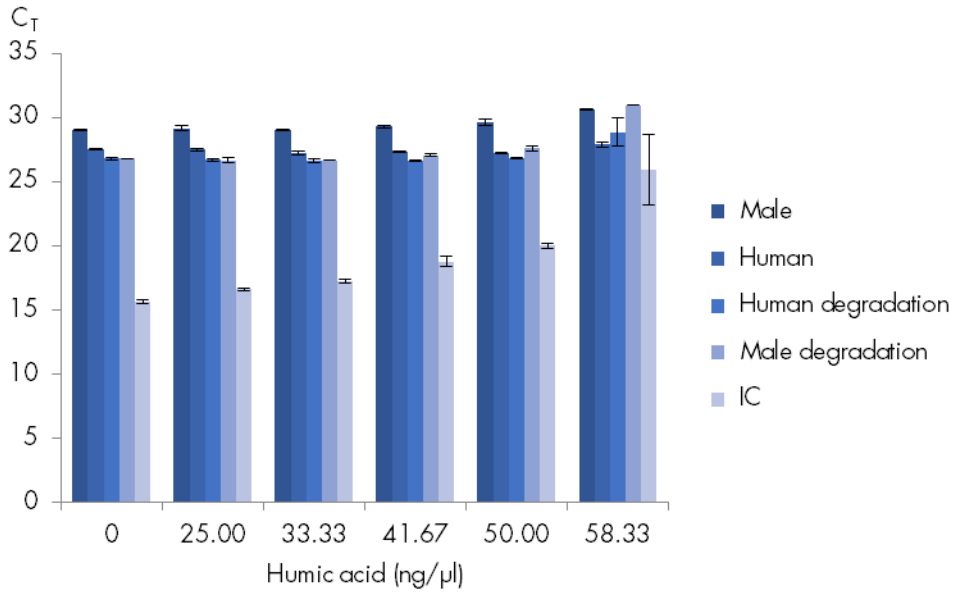

Figure 21. Performance of the Investigator Quantiplex Pro RGQ Kit with simulated humic acid inhibition on the Rotor-Gene Q. The internal control (IC) reports the presence of the inhibitor (C_T shift) while the quantification for the human, male, human degradation and male degradation target are reliable up to a concentration of 50 ng/µl. The figure shows the average C_T ± standard deviation.

It was shown that the internal control acts as a quality sensor and reports the presence of the inhibitor with a C_T shift, while quantification for the human, male, human degradation and male degradation target remains reliable up to a final humic acid concentration of 50 ng/µl in the PCR. This corresponds to a concentration in the DNA sample of 500 ng/µl (using 2 µl DNA sample in the assay, as recommended). Humic acid can have an impact on the large human autosomal and large male gonosomal target due to the large amplicon size, but the presence of humic acid was reliably reported by the IC.

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

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

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

Hematin

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

To test the robustness of the kit, the assay was run in the presence of 0, 125, 166.67, 208.33, 250 and 291.67 µM hematin (ICN Biomedicals Inc.; cat. no. 198969) under the standard conditions described in the *Investigator Quantiplex Pro RGQ Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figure 22.

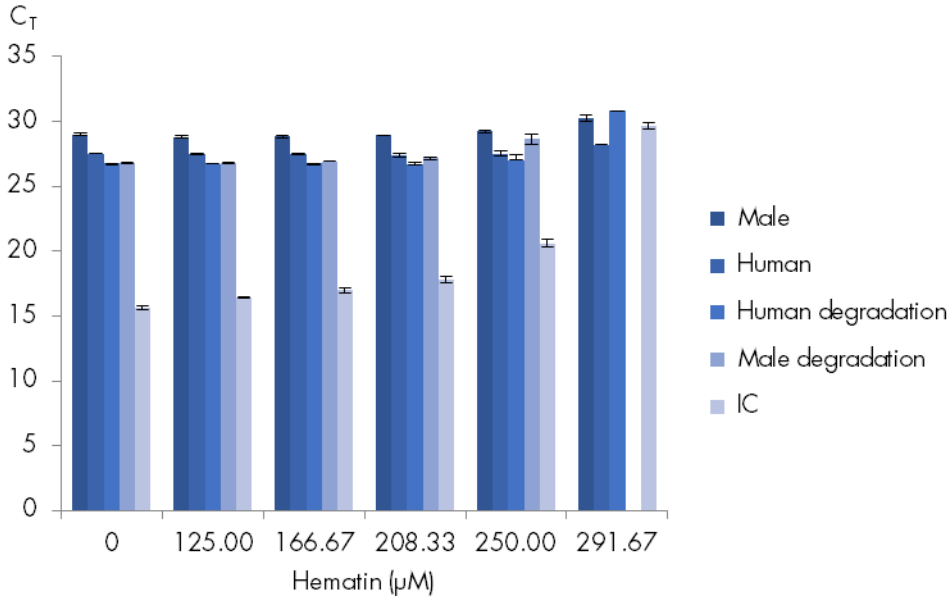

Figure 22. Performance of the Investigator Quantiplex Pro RGQ Kit with simulated hematin inhibition on the Rotor-Gene Q. The internal control reports the presence of the inhibitor (C_T shift) while the quantification for the human, male, human degradation and male degradation target are reliable up to a concentration of 250 µM. The degradation targets are susceptible at higher concentrations than 250 µM. The figure shows the average C_T ± standard deviation.

It was shown that the internal control acts as quality sensor and reports the presence of the inhibitor with a C_T shift, while quantification for the human, male, human degradation and male degradation target remain reliable up to a final hematin concentration of 250 µM (final concentration in the reaction). This corresponds to a concentration in the DNA sample of 2500 µM (using 2 µl DNA sample in the assay, as recommended). Hematin can have an impact on the large human autosomal and large male gonosomal target due to the large amplicon size, but the presence of hematin was reliably reported by the IC.

The internal control of the Investigator Quantiplex Pro RGQ Kit acts as a quality sensor and reports the presence of a broad range of hematin with a broad C_T shift. Therefore, further internal laboratory validation should be performed to determine criteria for obtaining a full

DNA profile without inhibition, using the Investigator ESSplex SE QS and the Investigator 24plex QS kits.

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

Calcium

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

To test the robustness of the kit, the assay was run in the presence of 0, 1.5, 2, 2.5, 3 and 3.5 mM calcium hydrogen phosphate (VWR®; cat. no. 83524.290) under standard conditions, as described in the *Investigator Quantiplex Pro RGQ Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figure 23. The highest concentration of calcium phosphate tested, 3.5 mM, corresponds to a concentration in the DNA sample of 35 mM (using 2 µl DNA sample in the assay, as recommended).

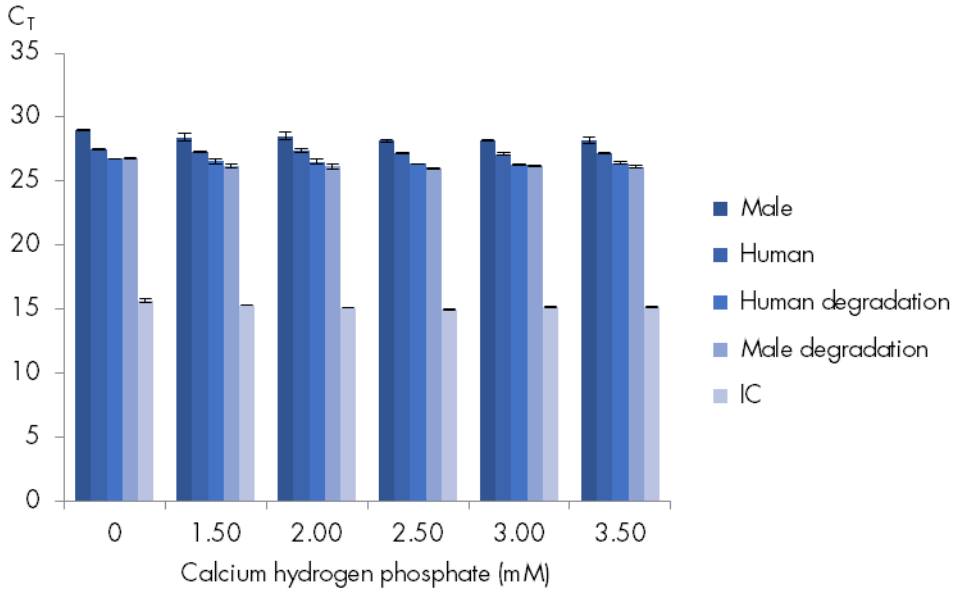

Figure 23. Performance of the Investigator Quantiplex Pro Kit with simulated inhibition effect of calcium hydrogen phosphate on the Rotor-Gene Q. The quantification is reliable up to a concentration of 3.5 mM. The figure shows average C_T ± standard deviation.

Tannic acid

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

To test the robustness of the kit, the assay was run in the presence of 0, 333.33, 500, 666.67, 833.33 and 1000 ng/µl tannic acid (Sigma-Aldrich®; cat. no. 403040), under standard conditions, as described in the *Investigator Quantiplex Pro RGQ Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figure 24.

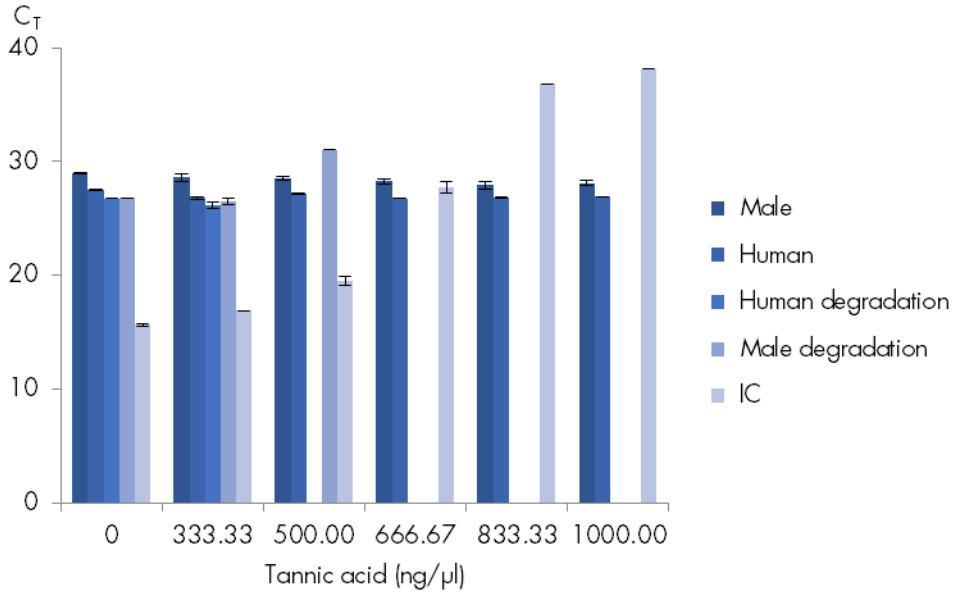

Figure 24. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of tannic acid on the Rotor-Gene Q. The internal control reports the presence of the inhibitor (C_T shift), while the quantification for the human and male target are reliable up to a concentration of 1000 ng/µl. The degradation targets are susceptible to tannic acid at concentrations higher than 333.33 ng/µl. The figure shows average C_T ± standard deviation.

It was shown that the internal control acts as a quality sensor and reports the presence of the inhibitor with a C_T shift, while quantification for the human and male target remain reliable up to a final tannic acid concentration of 1000 ng/µl. This corresponds to a concentration in the DNA sample of 10 µg/µl (using 2 µl DNA sample in the assay, as recommended) Tannic acid can have an impact on the large human autosomal and large male gonosomal target due to the large amplicon size, but the presence of tannic acid was reliably reported by the IC.

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

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

Collagen

Collagen is the main protein compound of many tissues. Collagen is proposed to inhibit DNA polymerase activity. To test the robustness of the kit, the assay was run in the presence of 0, 40, 55, 70, 85 and 100 ng/µl collagen (Sigma-Aldrich; cat. no. 403040) under standard conditions, as described in the *Investigator Quantiplex Pro RGQ Kit Handbook* (66 pg Male Control DNA M1). The highest concentration of collagen tested, 100 ng/µl, corresponds to a concentration in the DNA sample of 1 µg/µl (using 2 µl DNA sample in the assay, as recommended). The effect of collagen is shown in Figure 25.

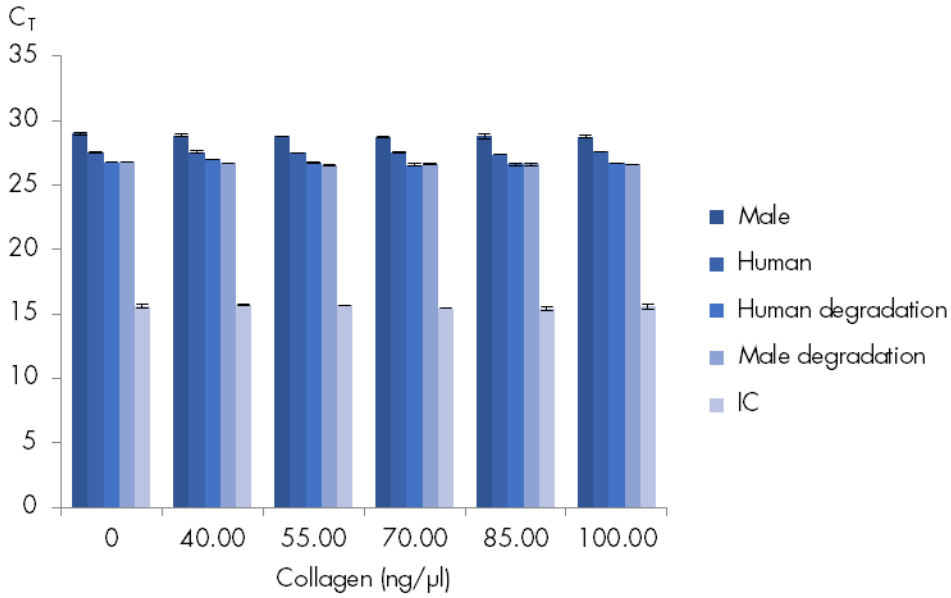

Figure 25. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of collagen on the Rotor-Gene Q. The quantification is reliable up to a concentration of 100 ng/µl in the assay. The figure shows average C_T ± standard deviation.

Ethanol

Ethanol is a potential carryover of the DNA extraction methods. To test the robustness of the kit, the assay was run in the presence of 0, 0.5, 1, 1.5, 2 and 2.5% ethanol, under standard conditions, as described in the *Investigator Quantiplex Pro RGQ Kit Handbook* (66 pg Male Control DNA M1). The results are shown in Figure 26. The highest concentration of ethanol tested, 2.5%, corresponds to a concentration in the DNA sample of 25% (using 2 µl DNA sample in the assay, as recommended).

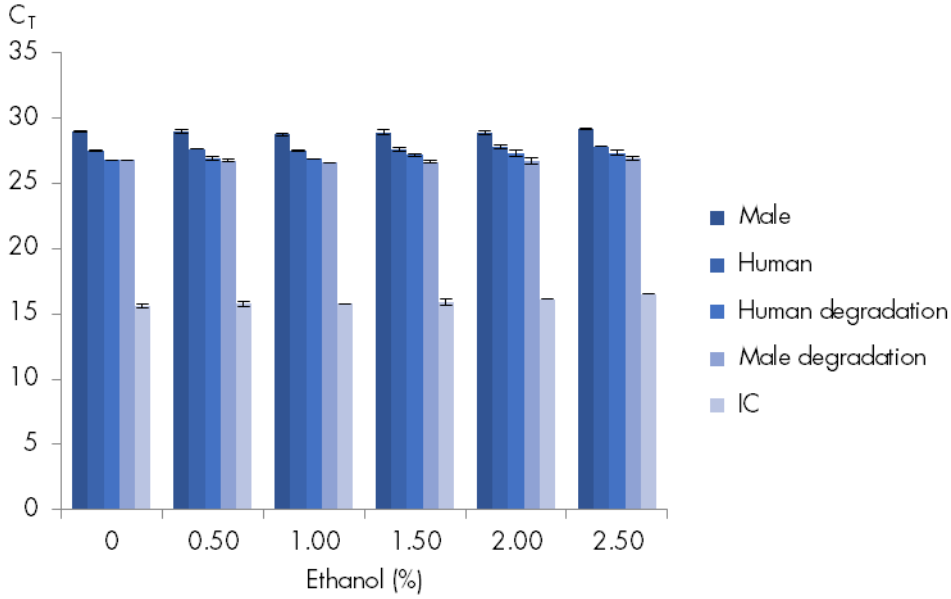

Figure 26. Performance of Investigator Quantiplex Pro Kit with simulated inhibition effect of ethanol on the Rotor-Gene Q. The quantification is reliable up to a concentration of 2.5% ethanol in the assay The figure shows average C_T ± standard deviation.

Contamination of reagents

Laboratory contamination of one of the reagents contained in the Investigator Quantiplex Pro RGQ Kit may result in a false positive in the quantification reaction. Contamination studies were performed to exclude reagent contamination. One run is shown as an example (Table 13). In total, 48 no-template controls and 2 positive controls (Male Control DNA M1; 50 ng/µl) were analyzed.

Table 13. Results of the NTC run (in total no-template controls and 2 positive controls (Male Control DNA M1; 50 ng/µl) were analyzed)

	Male	Human	Human degradation	Male degradation
Positive control 1 (C_T value)	22.44	22.63	21.66	21.75
Positive control 2 (C_T value)	22.53	22.82	21.78	21.83
Number of positive NTCs	0	1	0	0
Positive NTC (C_T value)	NA	35.15	NA	NA

NA: not available.

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

Stability

Stability of the Male Control DNA M1 dilution series

In a forensic laboratory, the maximum number of reactions of a kit may not be performed in a single day. The possibility to set up the dilution series for the Male Control DNA M1 for a

whole week is a real advantage. Therefore, the stability of the serial dilutions of the Male Control DNA M1 at 4°C was tested.

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

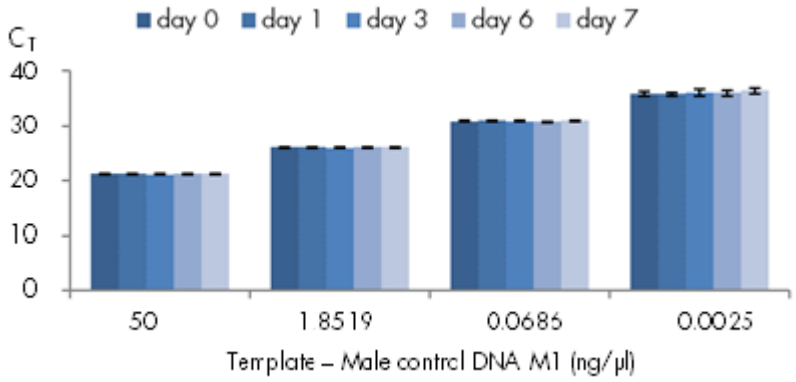

Figure 27. Detection of the degradation target on Male Control DNA M1 dilution series, using the QuantiTect Nucleic Acid Dilution Buffer in untreated tubes, before storage and after storage for 1, 3, 6 and 7 days at 4°C. The results show no relevant differences, even in the low DNA range. The figure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

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

Mixture studies

Evidence samples are frequently composed of more than one individual's DNA. For the correct setup of the downstream STR analysis, it is important to detect even low amounts of male DNA in the presence of high amounts of female background. Samples were created by mixing male and female DNA in ratios of 1:0, 1:10, 1:1000, 1:20000, 1:200000, 1:400000, 1:1000000, 1:2000000 and 1:2500000. The amount of male DNA and female DNA for the mixtures used in this study are listed in Table 14. Highly accurate quantification results were obtained in all cases for the human and male targets on the Rotor-Gene Q (Figure 28).

Table 14. Amounts of DNA template in the mixtures

Male:Female ratio	Male component	Female component
1:0	0.5 pg/µl	0
1:0	2.5 pg/µl	0
1:10	2.5 pg/µl	25 pg/µl
1:1000	2.5 pg/µl	2.5 ng/µl
1:20.000	2.5 pg/µl	50 ng/µl
1:200.000	0.5 pg/µl	100 ng/µl
1:400.000	0.25 pg/µl	100 ng/µl
1:1.000.000	0.2 pg/µl	200 ng/µl
1:2.000.000	0.1 pg/µl	200 ng/µl
1:2.500.000	0.1 pg/µl	250 ng/µl
1:1.000*	0.1 ng/µl	100 ng/µl

* Male DNA degraded to 150 bp.

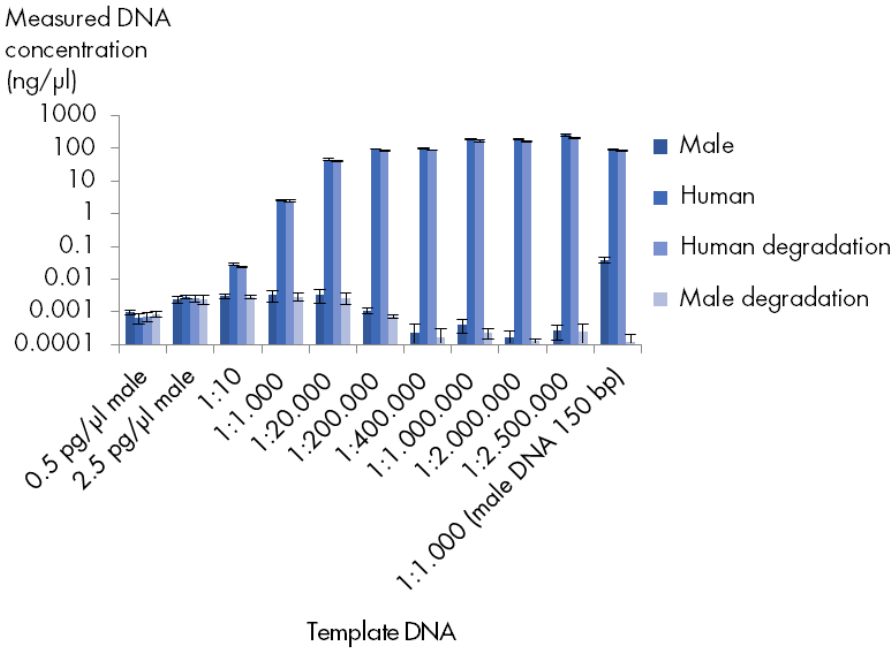

Figure 28. Detection of mixtures using the Investigator Quantiplex Pro RGQ Kit on the Rotor-Gene Q. The results show an accurate quantification of low amounts of male DNA even in the presence of high amounts of background female DNA. The figure shows average ± standard deviation.

Degraded DNA Samples

Environmental degradation may occur with forensic casework samples and is a classic challenge in routine genetic fingerprinting. The kit detects a longer autosomal amplification product (353 bp) targeting the same locus (4NS1C) as the 91 bp autosomal target. A unique feature of the Investigator Quantiplex Pro RGQ kit is that it detects also a longer gonosomal amplification product (359 bp) targeting the same locus as the smaller 81 bp gonosomal male target. Due to the differently sized autosomal and gonosomal targets, the longer targets are more susceptible to DNA degradation, allowing for a precise independent assessment of the degradation status of the human and male component in DNA samples.

The Investigator Quantiplex Pro RGQ Kit was tested for performance on degraded DNA samples on the Rotor-Gene Q. Male genomic DNA was sheared with a Covaris® S220 Focused-ultrasonicator to average fragment sizes of 500, 300 and 150 bp. Each fragment size (0.46 ng) was tested with the Investigator Quantiplex Pro RGQ Kit, according to the kit handbook instructions. The degradation indices (DIs) were calculated using the QIAGEN Quantification Assay Data Handling and STR Setup Tool v3.0. Reliable detection of the degradation status of the DNA was obtained (Figure 29). The calculated degradation index (HDI=quantification value for small human autosomal/quantification value for large human autosomal and the MDI=quantification value for small male gonosomal/quantification value for large male gonosomal target) are depicted in Table 15.

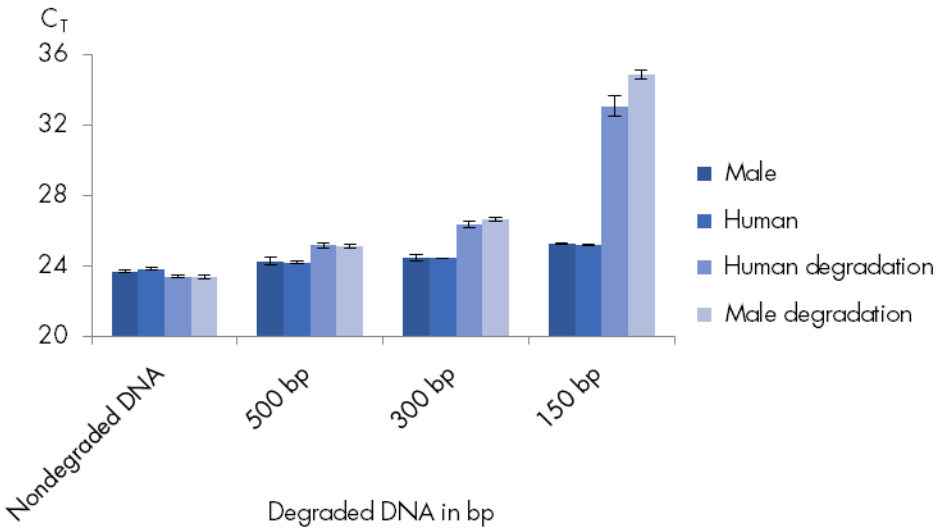

Figure 29. Detection of the degraded DNA using the Investigator Quantiplex Pro RGQ Kit on the Rotor-Gene Q. The results show the detection of DNA degradation indicated by the increase in C_T values for both degradation targets. The figure shows average C_T ± standard deviation.

Table 15. Calculated degradation indices (HDI and MDI)

	Human Degradation Index	Male Degradation Index
Nondegraded DNA	1.01	1.17
500 bp	2.77	2.54
300 bp	5.51	6.07
150 bp	390.65	846.93

Link between quantification results and genetic profile

The quantification reaction is performed in order to enhance the rate of first-time success in the STR reaction. Therefore, it is imperative that the quantification result correlates with the downstream application.

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

In order to test the link between the male quantification and the results using gonosomal markers, different samples were created by mixing male and female DNA in a ratio of 1:5. This mixed DNA was then diluted into a series of 9 samples. The total amount of DNA contained in sample 1 was 300 pg/µl, and different dilution factors were used for the remaining samples (Table 16).

Highly accurate quantification results were obtained for both the total human and the male component (Table 16). STR reactions were setup using the Investigator Argus Y-12 QS Kit (cat. no. 383615), according to the quantification of the male component.

Table 16. Amounts of DNA template in the mixtures

	Theoretical human DNA (pg/µl)	Measured human DNA (pg/µl)	Theoretical male DNA (pg/µl)	Measured male DNA (pg/µl)	DNA in Y-STR (pg)
Sample 1	300.00	304.06	50.00	49.29	500.00
Sample 2	18.75	16.43	3.13	2.24	37.83
Sample 3	4.69	4.07	0.78	0.56	9.41
Sample 4	2.34	2.57	0.39	0.33	5.66
Sample 5	1.17	1.00	0.20	0.13	2.16
Sample 6	0.59	0.58	0.10	0.11	1.82
Sample 7	0.29	0.33	0.05	0.00	0.00
Sample 8	0.15	0.15	0.02	0.00	0.00
Sample 9	0.07	0.20	0.01	0.05	0.85

With decreasing amounts of DNA, the average peak heights decreased (Figure 30). It was not possible to detect a full profile using DNA amounts lower than approximately 38 pg (samples 3–9). Sporadic alleles could be detected using an input DNA amount between 9.4 pg and 0.9 pg due to stochastic effects. These results demonstrated the correlation between DNA quantification and STR profile quality.

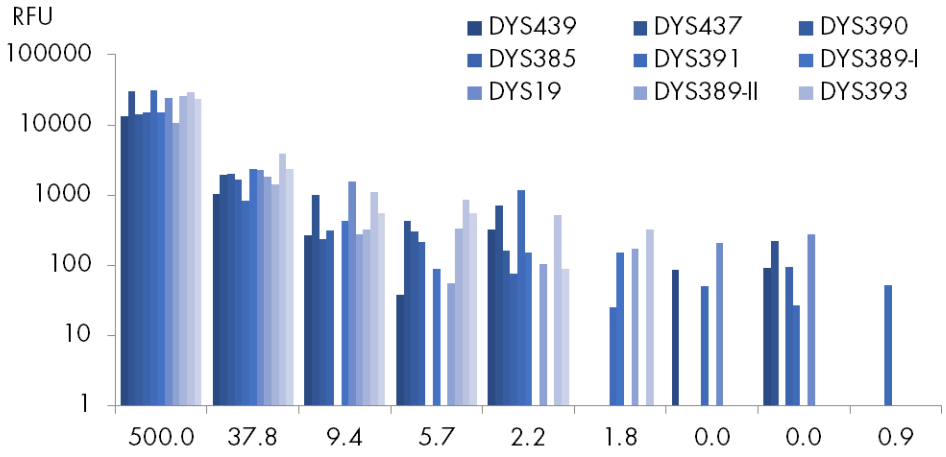

Figure 30. STR results showing the mean peak height in RFU. The results show the correlation of the quantification using the Investigator Quantiplex Pro RGQ Kit to the STR results using the Investigator Argus Y-12 QS Kit. The figure shows average RFU ± standard deviation.

The Investigator Quantiplex Pro RGQ kit enables the assessment of the degradation status of the male component independently of the human component in a DNA sample. This is particularly beneficial when analyzing sexual assault samples, where low levels of the male perpetrator's DNA could be highly degraded in a background of non-degraded female DNA. Information about the degradation status of the male component allows for a precise determination of subsequent processing steps (for instance, increased DNA input for Y-STRs to compensate the degradation of the male DNA or even Y-SNPs for highly degraded male DNA).

In order to test the correlation between the male degradation result with a useful Y-STR profile a female:male mixture sample was created by mixing 100 pg/µl of degraded male DNA (150 bp fragment size) and 100 ng/µl of non-degraded female DNA (ratio 1:1000). The degradation index of the male component was measured (Table 17).

Table 17. Calculated degradation indices (HDI and MDI)

	Human Degradation Index	Male Degradation Index
1:1.000 (male DNA degraded to 150 bp)	1.06	323.37

STR reactions were setup using the Investigator Argus Y-12 QS Kit (cat. no. 383615), with an increasing DNA input (Figure 31). These data demonstrate that male DNA degradation can be compensated by increasing the sample input to obtain a useful Y-STR profile.

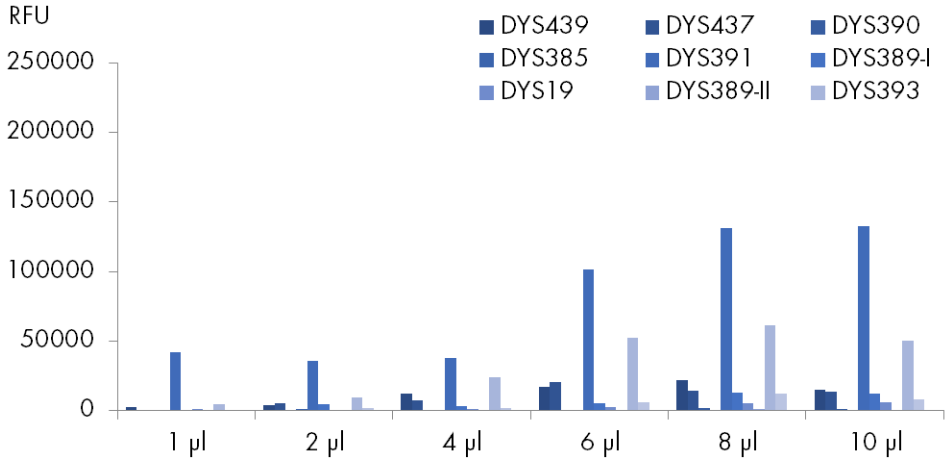

Figure 31. STR results showing the mean peak height in RFU. The results show the correlation between the male degradation result and Y-STR profile using the Investigator Argus Y-12 QS Kit. Samples contain 100 pg/µl of degraded male DNA (150 bp fragment size) and 100 ng/µl of non-degraded female DNA. Increasing the sample input can compensate the male DNA degradation. The figure shows average RFU ± standard deviation.

Cited references

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2. Revised Validation Guidelines of Scientific Working Group on DNA Analysis Methods (SWGDAM) Forensic Science Communications, July 2004, Volume 6, Number 3. **www.cstl.nist.gov/strbase/validation/SWGDAM_Validation.doc**.
3. Opel, K.L., Chung, D., and McCord, B.R. (2010) A study of PCR inhibition mechanisms using real time PCR. J. Forensic Sci. **55**, 25.

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Pro RGQ Kit (200)	Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316
Rotor-Gene Q 6plex System	Real-time PCR instrument with 6 channels (blue, green, yellow, orange, red, crimson), including laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9001660

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.