Application Note

Evaluation of the Investigator® Quantiplex® Pro RGQ Kit for DNA quantification and quality assessment of forensic casework samples

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Introduction

DNA profiling of forensic casework samples using short tandem repeat (STR) analysis is established as one of the most powerful tool for human identification. However, most samples submitted for DNA profiling typically contain limited or poor quality DNA, or are collected on substrates that include PCR inhibitors such as hematin. Because of the narrow dynamic range of an STR-PCR reaction, these factors frequently cause the PCR reaction to fail. It is of note that, with an accurate assessment of the quantity and quality of DNA in a sample and careful optimization of the input DNA levels, the success rate of STR amplification can be increased significantly.

The new Investigator Quantiplex Pro RGQ Kit is designed to provide the maximum possible information on forensic casework samples, including levels of input DNA, sample inhibitors, degradation and mixtures, thereby enabling actionable insights from a subsequent analysis of the sample. In addition to male and total human DNA quantification as well as assessment of potential PCR inhibitors, the kit comprises both male and total human DNA degradation markers. The unique male DNA degradation feature, combined with the ability to detect a single picogram of male DNA in a background of 400 ng of non-degraded female DNA, allows for an accurate assessment of the male perpetrator DNA in sexual assault samples. This also empowers investigators to make well-informed decisions about the downstream analysis (for example, to conduct Y-STR typing if successful autosomal STR analysis is considered unlikely).

Here we describe an evaluation of the Investigator Quantiplex Pro RGQ Kit, considering DNA mixtures, inhibition detection and assessment of both male and human DNA degradation.

Materials and methods

Samples

Fourteen samples were analyzed in this study, as described in Tables 1 and 3.

Table 1. Samples analyzed in this study

Sample	Description	Volume	Sample	Description	Volume
Tube 1	DNA 1 (Male DNA not degraded)	100 µl	Tube 8	DNA 8 (Mixture DNA Female/degraded Male 1:4)	40 µl
Tube 2	DNA 2 (Male DNA 300 bp degraded)	20 µl	Tube 9	Inhibitor 1 (Humic acid 500 ng/µl)	40 µl
Tube 3	DNA 3 (Male DNA 150 bp degraded)	20 µl	Tube 10	Inhibitor 2 (Humic acid 660 ng/µl)	40 µl
Tube 4	DNA 4 (Mixture DNA 1:200,000)	20 µl	Tube 11	Inhibitor 3 (Humic acid 800 ng/µl)	40 µl
Tube 5	DNA 5 (Mixture DNA 1:400,000)	20 µl	Tube 12	Inhibitor 4 (Hematin 2500 µM)	40 µl
Tube 6	DNA 6 (Mixture DNA 1:1,000,000)	20 µl	Tube 13	Inhibitor 5 (Hematin 3000 µM)	40 µl
Tube 7	DNA 7 (Mixture DNA Female/degraded Male 1:5,000)	20 µl	Tube 14	Inhibitor 6 (Hematin 4000 µM)	40 µl

Sample preparation

All samples and solutions were mixed thoroughly before use to avoid localized concentrations of salt. Fresh serial dilutions of the Male Control DNA M1 were prepared according to Table 2. Each sample was thoroughly mixed by vortexing before centrifugation and an aliquot was removed for the next dilution.

Table 2. Preparation of male control DNA for the standard curve

Standard name	Standard	Dilution step	Concentration (ng/µl)
Standard 1	Control DNA M1 (Std. 1)	–	50
Standard 2	Standard DNA 2 (Std. 2)	130 µl Nucleic Acid Dilution Buffer + 5 µl Control DNA (Stock)	1.8519
Standard 3	Standard DNA 3 (Std. 3)	130 µl Nucleic Acid Dilution Buffer + 5 µl Standard 2	0.0686
Standard 4	Standard DNA 4 (Std. 4)	130 µl Nucleic Acid Dilution Buffer + 5 µl Standard 3	0.0025
NTC	Nucleic Acid Dilution Buffer (NTC)	–	–

In addition, inhibitory samples were prepared by mixing DNA 1 with inhibitors in a ratio of 1:1 as shown in Table 3.

Table 3. Preparation of inhibitory samples

Sample	Component
Sample 1	10 µl DNA 1 + 10 µl Humic acid (500 ng/µl)
Sample 2	10 µl DNA 1 + 10 µl Humic acid (660 ng/µl)
Sample 3	10 µl DNA 1 + 10 µl Humic acid (800 ng/µl)
Sample 4	10 µl DNA 1 + 10 µl Hematin 2500 µM
Sample 5	10 µl DNA 1 + 10 µl Hematin 3000 µM
Sample 6	10 µl DNA 1 + 10 µl Hematin 4000 µM
Sample 7	10 µl DNA 1 + 10 µl Nucleic Acid Dilution Buffer

Master mix containing all components for the PCR except template (sample) DNA and nuclease-free water was prepared according to Table 4 and mixed thoroughly, before dispensing 18 µl per sample into Rotor-Gene® Q Tubes.

Table 4. Master mix for DNA quantification using the Investigator Quantiplex Pro RGQ Kit

Component	Volume per 20 µl reaction
Quantiplex Pro RGQ Reaction Mix	9 µl
Quantiplex Pro RGQ Primer Mix	9 µl
Total volume of master mix	18 µl

For quantification, 2 µl of QuantiTect® Nucleic Acid Dilution Buffer was added to the NTC tubes and 2 µl of control DNA dilutions was added to standard curve tubes. 2 µl of unknown sample DNA was added to the individual sample tubes. All tubes were mixed thoroughly. PCR tubes were placed inside the appropriate rotor in the Rotor-Gene Q cycler, and locking ring was attached. Empty positions in the rotor were filled with empty PCR tubes.

Quantification

The run profile was setup using the Q-Rex Software 1.0 according to manufacturer's instructions. The cycling conditions (Table 5), sample layout and sample details necessary for the run were provided in the Investigator Quantiplex Pro RGQ Q-Rex Template Files.

Table 5. Cycling conditions for the Rotor-Gene Q

Step	Time	Temperature	Number of cycles	Comment
Initial activation step	3 min	95°C	–	PCR requires an initial incubation at 95°C to activate the DNA polymerase
Two-step cycling:			40	
Denaturation	5 s	95°C		
Combined annealing/extension	10 s	60°C		Perform fluorescence data collection using the green, yellow, orange, red and crimson channels with auto-gain optimization

Analysis

After the run, standard curves for all target markers were created and samples were analyzed using the Absolute Quantification HID method according to the handbook. Values generated from this analysis were then exported and further interpretation of the run data was done using the QIAGEN Quantification Assay Data Handling Tool. In general, the standard curve is the best fit for a linear regression to standard dilution series data. The equation is written in the form $y = mx + b$, where x = log concentration and y = Cq (Cq stands for quantification cycle). The slope (m) and R^2 value of the standard curve were checked for all targets to determine whether they fall within the typical range. The R^2 value is a measure of how close the data points are to the fitted regression line. A typical standard curve has an R^2 value of ≥ 0.990. The slope (m) indicates the PCR efficiency. A slope of –3.3 indicates a PCR reaction with 100% efficiency (i.e., the number of target copies doubles each cycle). The y-intercept (b) is defined as the y value (Cq) when x (log concentration) equals 0, and hence corresponds to the Cq value for a sample with a concentration of 1 ng/µl.

Results and discussion

The standard curve for all targets are shown in Figures 1–4 and Table 6.

Table 6. Standard curve parameter

Target	R^2	Slope (m)	Efficiency (Eff.)	y-intercept (b)
Typical range	**≥ 0.990**	**–3.0 to –3.6**	**100%**	**–**
Human	0.99988	–3.301	101%	22.65
Male	0.99849	–3.302	101%	22.43
Human degradation	0.99946	–3.295	101%	21.97
Male degradation	0.99903	–3.48	94%	21.52

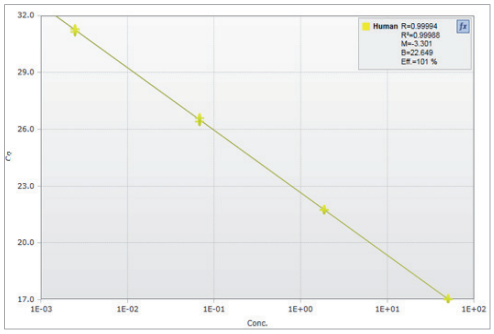

Figure 1. Absolute quantification standard for human target.

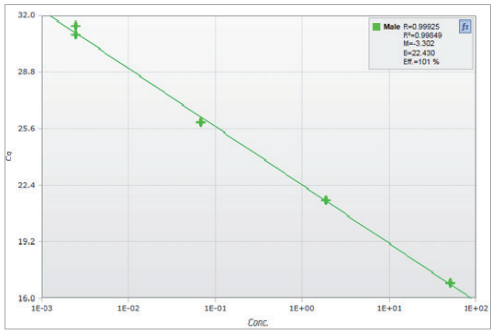

Figure 2. Absolute quantification standard for male target.

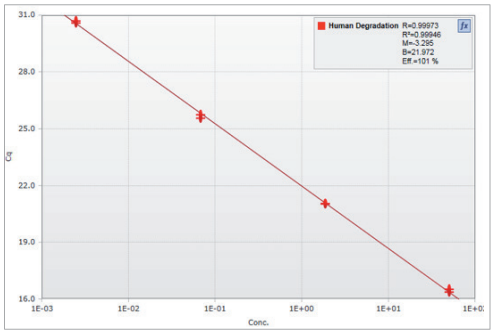

Figure 3. Absolute quantification standard for human degradation target.

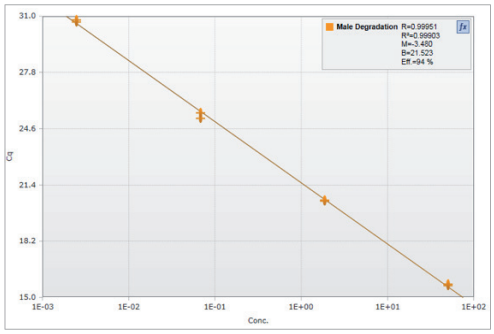

Figure 4. Absolute quantification standard for male degradation target.

Human, male, human degradation and male degradation markers were amplified efficiently and fitted very well to the standard curve with R^2 values of 0.99988, 0.99849, 0.99946 and 0.99903, respectively. All samples showed amplification in the Internal Control (IC) channel, indicating absence of external inhibitors during the PCR run. Moreover, none of the channels showed any amplification for NTC, indicating there was no contamination during this run. Analysis of the field test samples using the Quant Assay Data Handling Tool gave expected results (Table 7). The

Quantiplex Pro RGQ detected even the lowest amounts of male DNA in a high background of female DNA (DNA 4, DNA 5 and DNA 6) and these samples were correctly flagged as possible mixture. Degraded samples below 150 bp flagged possible degradation under both degradation and male degradation category. Sample DNA 7, which was a female/male mixture 1:5,000, where the male component was degraded, was flagged under both degradation and male degradation category. This demonstrates that the kit serves as an excellent tool to detect trace amounts of male DNA in a female DNA background, even when the DNA is highly degraded.

Table 7. Quant data import results for field test samples

Target	Human (ng/µl)	Human degraded (ng/µl)	Male (ng/µl)	Male degraded (ng/µl)	IC (C_T)	Mixture threshold	Degradation threshold	Male degradation threshold	Inhibition threshold
DNA 1: Non degraded	0.282	0.3114	0.2859	0.3141	15.834				
	0.0066	0.0109	0.0091	0.0095	16.18				
DNA 2: 300 bp degraded	0.1942	0.0319	0.1847	0.0309	15.93				
	0.2021	0.0351	0.166	0.034	15.891				
DNA 3: 150 bp degraded	0.1713	0.0008	0.1275	0.0002	15.874		possible degradation	possible degradation	
	0.1419	0.0008	0.1288	0.0004	16.066		possible degradation	possible degradation	
DNA 4: Mixture 1:200,000	74.5317	64.6238	0.0008	0.0005	15.821	possible mixture			
	98.9195	95.6961	0.0009	0.0012	15.59	possible mixture			
DNA 5: Mixture 1:400,000	82.5707	76.6409	0.0002	Undetermined	15.642	possible mixture		possible degradation	
	85.3221	74.0819	0.0001	Undetermined	15.841	possible mixture		possible degradation	
DNA 6: Mixture 1:1,000,000	5.3999	6.6658	Undetermined	Undetermined	16.386				
	172.6313	175.2331	0.0003	0.0003	15.643	possible mixture			
DNA 7: Female/ male 1:5,000; male DNA degraded	188.0726	203.9765	0.0187	Undetermined	15.457	possible mixture		possible degradation	
	177.4558	166.997	0.0163	Undetermined	15.664	possible mixture		possible degradation	
DNA 8: Female/ male 1:4; male DNA degraded	0.3955	0.344	0.0299	Undetermined	15.856	possible mixture		possible degradation	
	0.3891	0.3267	0.0346	0.0005	15.821	possible mixture		possible degradation	
Sample 1: Humic acid 500 ng/µl	0.1715	0.1798	0.154	0.1602	17.25				possible inhibition
	0.1839	0.1854	0.1502	0.1839	16.872				possible inhibition
Sample 2: Humic acid 660 ng/µl	0.1907	0.2203	0.15	0.1748	18.096				possible inhibition
	0.1791	0.2104	0.1376	0.2059	18.061				possible inhibition
Sample 3: Humic acid 800 ng/µl	0.1702	0.1721	0.1281	0.1547	18.837				possible inhibition
	0.1827	0.192	0.1226	0.1515	19.345				possible inhibition

Target	Human (ng/µl)	Human degraded (ng/µl)	Male (ng/µl)	Male degraded (ng/µl)	IC (C_T)	Mixture threshold	Degradation threshold	Male degradation threshold	Inhibition threshold
Sample 4: Hematin 2500 µM	0.1357	0.1537	0.1417	0.1423	16.778				
	0.154	0.1541	0.1554	0.1735	17.131				possible inhibition
Sample 5: Hematin 3000 µM	0.18	0.233	0.1355	0.141	17.294				possible inhibition
	0.1801	0.2025	0.1738	0.1325	17.626				possible inhibition
Sample 6: Hematin 4000 µM	0.1574	0.1533	0.1451	0.1261	18.963				possible inhibition
	0.2267	0.2047	0.1889	0.1472	19.288				possible inhibition
Sample 7: Male not degraded + Nucleic Acid Dilution Buffer	0.1404	0.1612	0.1858	0.1668	16.145				
	0.1621	0.188	0.155	0.1704	15.725				

Correlation between theoretical input and measured DNA concentration

The linear correlation between theoretical input and measured DNA concentration for control DNA is shown in Table 8 (for more details, refer to the 'Analysis' section). Figures 5 and 6 demonstrate the correlation between theoretical and measured concentrations of total human and male DNA, respectively. From these data, we can demonstrate a good linear correlation between theoretical and measured concentrations for both human and male targets (R^2 = 0.9992 and 0.9998, respectively).

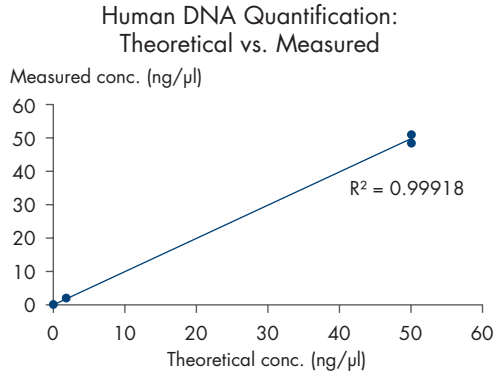

Figure 5. Correlation between theoretical input and measured concentration of total human DNA.

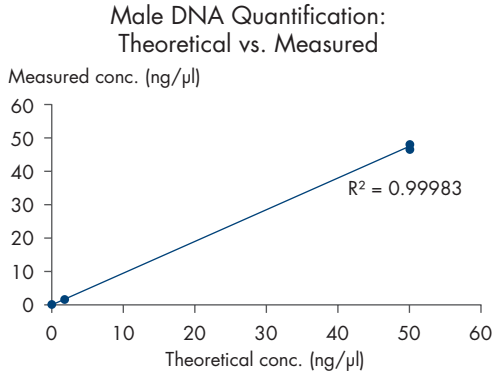

Figure 6. Correlation between theoretical input and measured concentration of total male DNA.

Table 8. Correlation between theoretical and measured concentrations for serial dilutions of M1 control DNA

Dilution factor	Given concentration (ng/µl)	Concentration (ng/µl)
Neat	50	47.01235
	50	48.07478
1:27	1.8519	1.821393
	1.8519	1.807875
1:729	0.0686	0.082392
	0.0686	0.083754
1:19,683	0.0025	0.002618
	0.0025	0.001875

Test for accuracy and precision

The Investigator Quantiplex Pro RGQ Kit was tested for its accuracy and precision on the Rotor-Gene Q System by choosing 5 replicates of male NIST A (2372) and female NIST B (2372) DNA, diluted 1:100 respectively. This experiment was conducted independently by QIAGEN using the QIAgility® system for automated liquid handling. All dilutions were made using the QuantiTect Nucleic Acid Dilution Buffer. The mean quantity and standard deviation were calculated for each NIST DNA dilution, suggesting high accuracy and precision of the assay.

Table 9. Precision and accuracy testing of NIST samples prepared using the QIAgility

Target	DNA sample	Concentration (ng/µl) ± standard deviation	CV
Human	NIST A (2372) Male DNA 1:100	0.58 ± 0.05	8.5%
	NIST B (2372) Female DNA 1:100	0.56 ± 0.03	5.2%
Human degradation	NIST A (2372) Male DNA 1:100	0.65 ± 0.09	13.2%
	NIST B (2372) Female DNA 1:100	0.61 ± 0.04	7.0%
Male	NIST A (2372) Male DNA 1:100	0.59 ± 0.05	7.8%
	NIST B (2372) Female DNA 1:100	NA	NA
Male degradation	NIST A (2372) Male DNA 1:100	0.58 ± 0.04	6.0%
	NIST B (2372) Female DNA 1:100	NA	NA
Expected	NIST A (2372) Male DNA 1:100	0.57	
	NIST B (2372) Female DNA 1:100	0.61	

Conclusion

The Rotor-Gene Q and the Investigator Quantiplex Pro RGQ Kit demonstrated excellent sensitivity for male DNA even in a high background of female DNA. In addition, the Internal Control provided accurate and informative feedback on the presence of inhibitors in the samples. The unique male degradation marker represents a significant benefit of using the kit for sexual assault sample analysis, where low levels of poor quality male perpetrator DNA are frequently masked by high levels of female victim DNA.

Summary

- The Investigator Quantiplex Pro RGQ Kit accurately quantifies both human and male DNA.
- Quality control markers included in the kit enable reliable identification of inhibition and degradation, enabling confident decision making regarding sample processing for downstream analysis.
- The male degradation marker offers unique advantages for forensic casework, for example, when processing sexual assault samples.
- The QIAgility instrument facilitates reliable sample preparation for quantification using the Investigator Quantiplex Pro RGQ Kit. Concordance with the NIST sample sets was demonstrated in regard to accuracy and precision of the assay.

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Pro RGQ Kit (200)	For use on QIAGEN RotorGene Q Real-Time Systems: Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316
QIAgility System HEPA/UV (incl. PC)	Robotic workstation for automated PCR setup (with UV light and HEPA filter), notebook computer, and QIAgility Setup Manager Software: includes installation and training, 1-year warranty on parts and labor	9001532
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

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