Application Note

Comparison and optimization of two qPCR DNA quantification assays to improve STR analysis workflow efficiency

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Introduction

Analysts in forensic labs routinely perform DNA profiling analyses, including STR analyses, as part of their casework investigations. In many labs, including the Forensic Science Laboratory of the French Gendarmerie (IRCGN), the STR analysis workflow consists of DNA extraction and purification, followed by STR amplification, for all samples that are received.

In cases where amplification fails, an appropriate rework strategy must be determined based on the resulting STR profile data. This review is time-consuming and reduces the efficiency and throughput of DNA labs. However, forensic labs can save time and resources by performing a DNA quantification step between the purification and STR amplification. This quick additional step allows analysts to pre-select which samples to amplify and optimize the PCR amplification with a normalized amount of input DNA. To determine the optimal protocol for DNA quantification prior to STR analysis, we compared two commercially available qPCR-based quantification assays: QIAGEN's Investigator® Quantiplex® Pro Kit and Thermo Fisher Scientific's Quantifiler™ Trio DNA Quantification Kit. Working with both full and half volume reactions, we evaluated these kits for their reliability, sensitivity and robustness against different buffers in the STR analysis workflow.

Materials and methods

DNA samples and experimental conditions

- Reaction volume sample group: 84 distinct DNA samples were used for the full and half volume reaction tests. These 84 samples were randomly selected from the Forensic Science Laboratory of the French Gendarmerie's routine workflow.
- Threshold determination sample group: 1047 known DNA samples, representing every sample analyzed in the Forensic Science Laboratory of the French Gendarmerie that had previously been categorized by an expert into "Negative profile", "Low DNA profile" or "High DNA profile" groups.
- Population sample group: 1008 unknown DNA samples, representative of the samples that are routinely analyzed in the lab. They were quantified using the below methods and an STR profile ▷

was obtained in order to assess the efficiency of each threshold.

- Sensitivity to buffers: 4 different routinely used buffers or water were used as negative controls to assess the sensitivity of the two quantification assay kits to buffer type. The buffers were: elution buffer, Quantifiler THP DNA Dilution Buffer (Thermo Fisher Scientific), the QuantiTect® Nucleic Acid Dilution Buffer (QIAGEN) and deionized water (DI) water (Milli-Q®).

DNA quantification and assessment

The two quantification assays compared were the Investigator Quantiplex Pro Kit (QIAGEN) and the Quantifiler Trio DNA Quantification Kit (Thermo Fisher Scientific). Both assays use four specific targets to provide information about the quantity of human DNA (short fragment), the degradation status of the DNA (longer fragment of human DNA), the presence of male DNA (specific male DNA fragment) and an internal PCR control to assess PCR inhibition.

The assay characteristics are listed in Table 1.

The Investigator Quantiplex Pro reagents were dispensed using the QIAgility® instrument[3], reducing hands-on time and normalizing the process across the different samples.

DNA quantification was carried out on an Applied Biosystems® 7500 Real-Time PCR System and the results were analyzed using HID Real-Time PCR Analysis Software v.1.3.1 from Thermo Fisher Scientific.

Table 1. Characteristics of the Investigator Quantiplex Pro Kit and the Quantifiler Trio DNA Quantification Kit

	Large target	Small target	Male target	Internal PCR control (IPC)	Limit of detection
Investigator Quantiplex Pro Kit [1]	353 bp	91 bp	81 bp	434 bp	0.5 pg/µl* – 200 ng/µl
Quantifiler Trio DNA Quantification Kit [2]	214 bp	80 bp	75 bp	130 bp	< 5 pg/µl*

* Stochastic effects may influence the results for lower quantities.

Results and discussion

Full and half volume reactions

We first assessed the reproducibility of the two quantification kits using full and half volume reaction conditions for 84 different samples (see Materials and methods, "Reaction volume sample group"). The samples were quantified in triplicate using both kits under both conditions. Three different parameters were examined:

1. The reproducibility
2. The repeatability
3. The homogeneity of the variance

A first statistical test indicated that the data were not normally distributed, and a non-parametric test was then used to assess the homogeneity of the variance. This second test revealed a homogeneity of group variance, indicating that the results could be compared between the three replicates of a given kit under a given condition. This test confirmed the repeatability of the results for each quantification method (data not shown).

Considering the non-normality of the data and the homogeneity of the variance, a third statistical test was

applied to compare the different kits and conditions. The null hypothesis proposed that there was no difference between the groups and was accepted for a P value > 0.05. Below this value, the groups were considered statistically different. The results of this analysis are summarized in Table 2.

We found no statistical difference between the groups, reflecting the reproducibility of the different quantification methods. Together, these results confirm that the quantification results are reproducible and comparable between the full and half volume conditions, as well as between the two kits.

Table 2. P value results for each kit and condition using the third statistical test

Kit	Investigator Quantiplex Pro Kit		Quantifiler Trio DNA Quantification Kit	
Condition	Full volume	Half volume	Full volume	Half volume
P value	0.91	0.76	0.57	0.31

Sensitivity to buffer

As buffer and lab-grade water typically contain only trace amounts of contaminating DNA (between 10^{-2} and 10^{-3} pg/µl), they typically have little impact on DNA quantification results. However, if a DNA quantification assay produces misleading measurements of DNA concentration due to sensitivity to a buffer, the amount of DNA contained in a sample may be overestimated. This could result in too little sample being used in a subsequent PCR amplification, reducing the chances of producing a usable STR profile. We assessed the sensitivity of the two quantification kits under different buffer conditions by amplifying the reactions in the absence of added DNA; these tests were performed in triplicate. The buffer conditions used were:

1. DI water (Milli-Q)
2. Elution buffer
3. QuantiTect Nucleic Acid Dilution Buffer (QIAGEN)
4. Quantifiler THP DNA Dilution Buffer (Thermo Fisher Scientific)

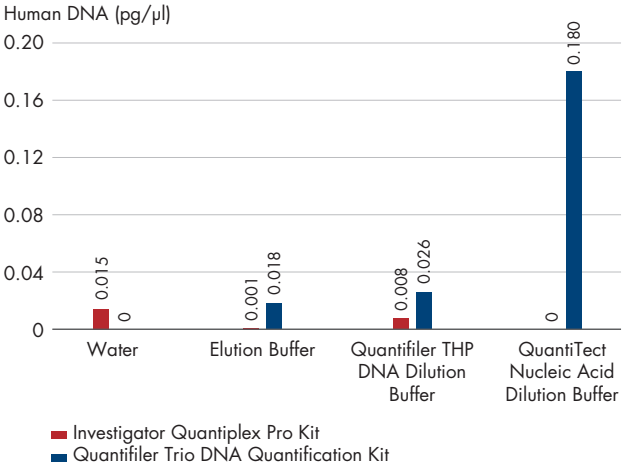

Figure 1. Negative control measurements of DNA concentrations for different buffers using the Investigator Quantiplex Pro Kit (red) and the Quantifiler Trio DNA Quantification Kit (blue).

As shown in Figure 1, the concentration measurements determined following amplification of elution buffer or Quantifiler THP DNA Dilution Buffer were around ten times lower using the Investigator Quantiplex Pro Kit than the Quantifiler Trio DNA Quantification Kit. Further, while no DNA was detected using the QuantiTect Nucleic Acid Dilution Buffer with the Investigator Quantiplex Pro Kit, a notably high measurement was obtained using the Quantifiler Trio DNA Quantification Kit. Finally, the Quantifiler Trio DNA Quantification Kit gave a negative result for water, while an extremely low measurement was obtained using the Investigator Quantiplex Pro Kit.

These results indicate that the Investigator Quantiplex Pro Kit provides comparable results regardless of the buffer used, with minimal amplification across different buffers and water (0 to 0.015 [pg/µl]). The Quantifiler Trio DNA Quantification Kit produced more variable results, with measurements ranging from ▷

0 to 0.026 [pg/µl], and a peak at 0.180 [pg/µl] when using the QuantiTect Nucleic Acid Dilution Buffer.

Defining a DNA concentration threshold for STR amplification

The goal of the DNA quantification step is to determine the suitability of a DNA sample for STR amplification. To this end, we next determined an appropriate DNA concentration threshold that could be used to determine if a sample should be amplified or discarded.

To define the appropriate threshold, 1047 known DNA samples (see Materials and methods, "Threshold determination sample group") were quantified at half-volume using both quantification kits. The goal was for the chosen threshold to fall between samples that had already been qualified by an expert to give a negative result or a low DNA profile.

Figure 2 shows the quantifications of the 1047 samples, using both kits, on a logarithmic scale. Each sample has been categorized by an expert into one of three categories: "Negative profile", "Low DNA profile" or "High DNA profile."

As shown in Figure 2, the shape and distribution of the samples are similar between the two kits. However, the measured concentrations differ, with the Quantifiler Trio DNA Quantification Kit consistently giving higher concentration results than the Investigator Quantiplex Pro Kit. The minimum and maximum concentrations for each kit are summarized in Table 3.

Table 3. Minimum and maximum concentrations observed using both quantification kits in (ng/µl)

	Minimum concentration	Maximum concentration
Investigator Quantiplex Pro Kit	$3x10^{-6}$	22.5
Quantifiler Trio DNA Quantification Kit	$1x10^{-4}$	34.8

Based on these results, we defined threshold concentration values (Table 4). These thresholds correspond to the regions of the graph where the datapoints overlap between negative and low DNA profiles.

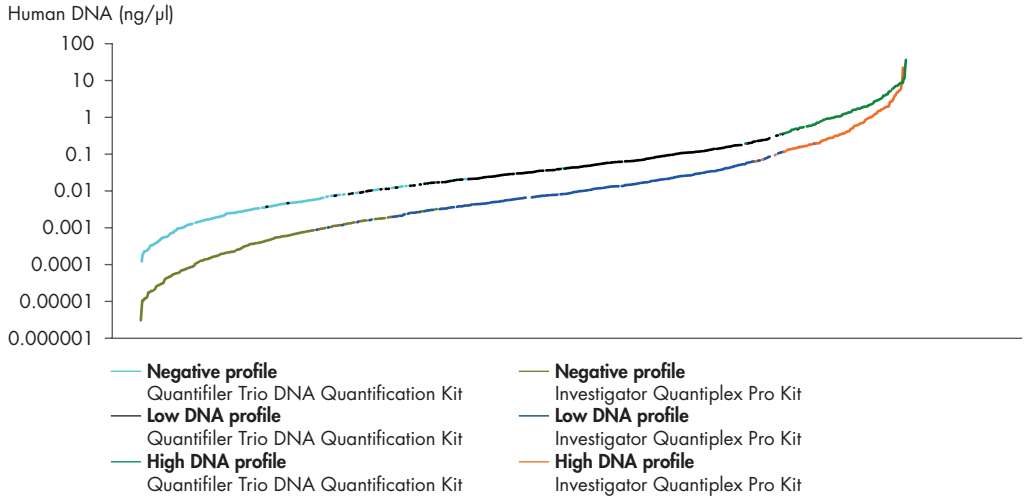

Figure 2. DNA concentration, presented on a logarithmic scale, for all samples in the "Threshold determination sample group" analyzed using the two quantification kits. Colored regions of the graph represent expert classifications, as indicated.

Table 4. Summary of the determined thresholds in [ng/µl]

	Threshold 1	Threshold 2	Threshold 3	Threshold 4	Threshold 5
Investigator Quantiplex Pro Kit	$3x10^{-3}$	$4x10^{-3}$	$5x10^{-3}$	$6x10^{-3}$	
Quantifiler Trio DNA Quantification Kit	$1x10^{-3}$	$7x10^{-3}$	$11x10^{-3}$	$14x10^{-3}$	$37x10^{-3}$

Test of the DNA concentration thresholds

Finally, we assessed the thresholds defined in Table 4 for each kit using 1008 unknown samples (see Materials and methods, "Population sample group"). We performed DNA quantifications using the two kits and produced STR profiles for each sample, allowing us to compare the thresholds with expert classifications. Table 5 summarizes the results for the two quantification assays using the defined thresholds for each kit.

The number of samples that produced successful STR amplifications ("positive") and unsuccessful amplifications ("negative") are indicated, as well as the percentages of false negatives and false positives. The last column provides information regarding the expert's classifications based on the STR profile, made independently of the quantification results.

Table 5. Success of STR amplifications after either expert review or pre-selecting samples using DNA quantification with the two kits at different thresholds

		Investigator Quantiplex Pro Kit				Quantifiler Trio DNA Quantification Kit					Expert review
L	Threshold concentration ($x10^{-3}$ ng/µl)	3	4	5	6	1	7	11	14	37	N/A
1	No.of negative samples	418	473	522	552	136	395	461	506	659	654
2	No.true negatives	418	472	518	544	136	390	450	488	596	654
3	No.of false negatives	0	1	4	8	0	5	11	18	63	N/A
4	Percentage of false negatives	0	0.21%	0.77%	1.45%	0	1.27%	2.39%	3.56%	9.56%	N/A
5	Percentage of false negatives in the total population	0%	0.10%	0.40%	0.79%	0%	0.50%	1.09%	1.79%	6.25%	N/A
6	No.of positive samples	590	535	486	456	872	613	547	502	349	354
7	No.of true positives	267	266	263	259	267	262	256	249	204	267
8	No.of false positives	323	269	223	197	605	351	291	253	145	87
9	Percentage of false positives	54.75%	50.28%	45.88%	45.20%	69.38%	57.26%	53.20%	50.40%	41.55%	24.58%
10	Selection efficiency	41.47%	46.83%	51.39%	53.97%	13.49%	38.69%	44.64%	48.41%	59.13%	64.88%
11	Agreement between the quantification and expert screening	100%	99.63%	98.50%	97.00%	100%	98.13%	95.88%	93.26%	76.40%	N/A

In this assay, false negatives (Table 5, rows L4 and L5) represent samples that could have produced useable STR profiles but would be discarded because the DNA concentration falls below the chosen threshold. It is therefore desirable to minimize the percentage of false negatives. The Investigator Quantiplex Pro Kit performed better in this regard, producing a lower percentage of false negatives (0–0.79%) compared to the Quantifiler Trio DNA Quantification Kit (0–6.25%), regardless of the threshold used. The Investigator Quantiplex Pro Kit therefore provides a high level of confidence that very few samples capable of producing an STR profile will be erroneously disregarded.

To reduce costs and increase efficiency, it is also important to minimize the percentage of false positives (Table 5, row L9), which will lead to amplification of samples that cannot produce a useable STR profile. As shown in Table 5, the threshold for Investigator Quantiplex Pro Kit with the lowest percentage false positives (45.20%) also produced a lower corresponding percentage of false negatives (0.79%) compared to the Quantifiler Trio DNA Quantification Kit (lowest false positive percentage: 41.55%; false negative percentage: 6.25%). In addition, the Investigator Quantiplex Pro Kit gave a lower percentage of false positives across the different thresholds compared to the Quantifiler Trio DNA Quantification Kit. These results indicate that the Investigator Quantiplex Pro Kit can better support forensic labs in optimizing their STR amplifications while reducing costs. However, regardless of the quantification assay used, fewer negative samples were correctly discarded using the threshold methods compared to the expert screening (Table 5, row L1), contributing to the percentage of false positives.

The optimal threshold and assay kit can also be determined by assessing the selection efficiency (Table 5, row L10) and the agreement between the quantification and expert screening (Table 5, row L11). First, the selection efficiency describes the ratio of true negative samples to the total number of samples. For the threshold method to be useful, this percentage should be as close as possible to the efficiency of the expert's review. Although the Quantifiler Trio DNA Quantification Kit with a threshold of $37x10^{-3}$ gave the highest selection efficiency, it also produced the highest percentage of false negatives, taking it out of consideration. Second, the agreement between the quantification and expert screening describes the ratio between the number of true positive samples determined using the threshold method and the number observed by the expert.

For the Quantifiler Trio DNA Quantification Kit, a threshold of 1 or $7x10^{-3}$ limited the percentage of false negative samples (0 and 0.5%, respectively). However, these thresholds also gave a lower selection efficiency (Table 5, row L10) compared to the Investigator Quantiplex Pro Kit and a higher percentage of false positives (Table 5, row L9).

Although the expert selection efficiency was higher than for either quantification assay, the Investigator Quantiplex Pro Kit at thresholds of 4 or $5x10^{-3}$ produced low percentages of false positives (50.28% and 45.88%, respectively) and false negatives (0.10% and 0.40%, respectively), with good agreement between the quantification and the expert screening (99.63% and 98.5%, respectively).

Conclusion

We first demonstrated the reproducibility and repeatability of DNA quantification results when using full or half volume reactions. Our results indicate that half volume reactions using either the Investigator Quantiplex Pro Kit or the Quantifiler Trio DNA Quantification Kit produce reliable quantification measurements.

We then assessed the sensitivity of the two assays to different buffers used for DNA purification and quantification. While the Investigator Quantiplex Pro Kit showed minimal spurious amplification for the different buffers, the Quantifiler Trio DNA Quantification Kit showed a tendency to overestimate the amount of DNA in the reactions. This overestimation could lead to an increase of negative STR amplifications, as the input volume of DNA for the amplification will be underestimated.

Finally, we determined an appropriate DNA concentration threshold to decide if a sample should be amplified or discarded for each kit. A comparison of the thresholds determined for the two kits demonstrated that the Investigator Quantiplex Pro Kit produced lower percentages of false positives and false negatives compared to the Quantifiler Trio DNA Quantification Kit, allowing for a more precise selection of samples for STR amplification. In addition, the thresholds determined for the Investigator Quantiplex Pro Kit gave lower percentages of false negatives when compared with the current French Gendarmerie workflow.

The French Gendarmerie's current STR analysis workflow requires STR amplification of every sample received, followed by a review of each profile by an expert to assess the best rework strategy. While this approach is advantageous in producing an extremely low percentage of false negatives, as none of the samples are discarded, it is time consuming and expensive.

The new strategy described in this application note, which uses reliable quantification data produced with an optimized DNA concentration threshold and quality controls present in the qPCR-based quantification assays, allows fewer samples to be analyzed without sacrificing the quality of the results. Negative samples can be identified and discarded at the beginning of the workflow, providing more resources for positive samples. In addition, dilutions can be made before the first amplification for inhibited or high concentration DNA samples, leading to an increase in usable STR profiles and a reduction in rework.

Summary

The addition of a short quantification step reduces the cost and rework associated with the STR analysis workflow, while simultaneously increasing sample throughput and reducing turnaround time. As shown in this study, the Investigator Quantiplex Pro Kit is particularly well-positioned to significantly increase the number of samples that will consistently generate useable STR profiles in forensic labs.

References

1. *Investigator Quantiplex Pro Handbook*, March 2018, QIAGEN
2. Quantifiler HP and Trio DNA Quantification Kits, October 2018, Thermo Fisher Scientific
3. *QIAgility User Manual*, May 2018, QIAGEN

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Pro Kit (200)	For use on Applied Biosystems 7500 Real-Time Systems: Quantiplex Pro Reaction Mix, Quantiplex Pro Primer Mix, Quantiplex Pro Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387216
Investigator Quantiplex Pro Calibration Kit	For use on Applied Biosystems 7500 Real-Time Systems: Calibration Standard FAM (60 µl), Calibration Standard JOE (60 µl), Calibration Standard ATTO 550 (60 µl), Calibration Standard ROX (60 µl), Calibration Standard ATTO 647N (60 µl), Quantiplex Pro Calibration Buffer (30 ml)	387416
QIAgility System HEPA/UV (incl. PC)	Instrument and service agreement package: robotic workstation for automated PCR setup (with UV light and HEPA filter), notebook computer and QIAgility software; includes installation, application training and one-year warranty on labor, travel and parts	9001532

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