Application Note

Verification of virtual standards with the Investigator® Quantiplex® Pro Kit on the QuantStudio™ 5 Real-Time PCR System

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Introduction

Forensic laboratories worldwide require reliable options for streamlining their workflows to maximize sample throughput while maintaining quality standards and success rates. Standard curves, required for quantitative real-time PCR applications, are laborious to prepare and analyze, and therefore represent a good target for increasing laboratory efficiency. In some cases, standards may be left out of real-time PCR runs if virtual standards, representing previously determined standard curves, are instead implemented. The use of virtual standards can save researchers' time and increase sample throughput, as more samples may be run in place of new standards. However, whether virtual standards are compatible with samples prepared under different circumstances, such as with distinct reagent kit lots, must be confirmed.

In this application note, we examined the use of virtual standards with the Applied Biosystems® QuantStudio™ 5 Real-Time PCR System using the QIAGEN® Investigator® Quantiplex® Pro Kit chemistry, and determined the effects on the reliability and precision of DNA quantification.

Methods

Samples of Quantiplex Pro Control DNA M1 from the Investigator Quantiplex Pro Kit were quantified using full reaction volumes (20 µl) on a QuantStudio 5 Real-Time PCR System, calibrated with the Investigator Quantiplex Pro Calibration Kit. All sample processing was conducted in accordance with the relevant manufacturers' manuals or the laboratory's standard operating procedures. ▷

Figure 1. The Quantiplex Pro Kit.

The amplification conditions used for the validation of the virtual standards are shown in Tables 1 and 2. An input volume of 2 µl of sample, control DNA, or standard was used per reaction.

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 Applied Biosystems QuantStudio 5 Real-Time PCR System

Function	Temperature	Time	No. of cycles	Additional Comments
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C for 3 min
Denaturation	95°C	5 s	40	Perform fluorescence data collection (in the 60°C step only)
Combined annealing/ extension	60°C	35 s		

The quantification results from the QuantStudio Design and Analysis Software (v1.5) were imported into the QIAGEN Quantification Assay Data Handling and STR Setup Tool v3.3.1 for analysis (note that this method also works with v3.4.5, the latest version of the software at the time of publication). The QIAGEN Quantification Assay Data Handling and STR Setup Tool is free-to-use software that can be downloaded from the QIAGEN website. This tool is compatible with the analysis of quantification data derived from all commonly used RT-PCR platforms, including the QuantStudio 5.

Users implementing virtual standards can benefit from the quality assessment offered by the Quantification Assay Data Handling tool by incorporating a fixed internal PCR control (IPC) value into their data analysis. This fixed IPC value is used by the tool in the absence of quantification standards within a run to assess the levels of inhibition for each sample. In this case, QIAGEN recommends that an IPC value is determined from a statistically significant number of samples (at least two standard dilution sample sets). Once this value has been determined, users may enter the value by pressing the "Fixed IPC" button found on the "Quant Result Import" sheet in the latest version of the Quantification Assay Data Handling tool.

It should be noted that the import of virtual standards is unique to the QuantStudio 5 instrument in conjunction with the QuantStudio Design and Analysis Software for human identification and forensic applications. This feature is not available when using the Applied Biosystems HID Real-Time PCR Analysis Software to operate the QuantStudio 5 instrument with custom dye sets.

Results

A 27-fold dilution series with four concentration points was manually prepared using the Control DNA M1 (50 ng/µl) supplied in the Investigator Quantiplex Pro Kit, as recommended in the *Investigator Quantiplex Pro Handbook*. The samples were added to the PCR plate, leaving the task assignment set to "Unknown" in the QuantStudio Design and Analysis Software for all targets. No standards were prepared or run at the same time as the samples.

Sample analysis was then performed on the QuantStudio instrument using imported standard curves (virtual standards) that were previously prepared using the same lot (160052754) of the Investigator Quantiplex Pro Kit. Three independent sets of virtual standards were applied to the dataset to enable a comparison of their suitability and reliability for use in the DNA quantification assay.

Figure 2 demonstrates that the data produced using the imported virtual standards was highly reproducible and independent of the individual virtual standard used for non-trace samples. However, samples of lower concentration were subject to higher variability. As indicated in the figure, the lowest concentration sample labeled "Prepared Sample 4" exhibited a coefficient of variation of 12% between the three virtual standards. This level of variability would have a significant impact on downstream STR amplification results, and could lead to the exclusion of a sample from

downstream processing if a minimum quantification threshold was required for sample progression. As a result, caution should be used when applying virtual standards where low-template samples are anticipated.

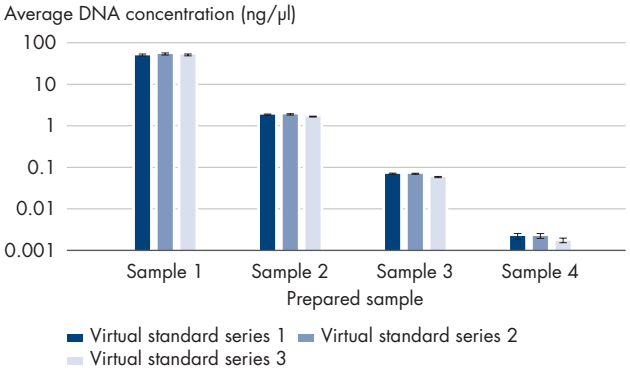

Figure 2. Analysis of Control DNA M1 dilution series samples with three different virtual standards.

The coefficient of variation ranged from 1% to 12% across the four sample groups, with variability increasing as the sample concentration decreased (Table 3). The impact of this variability should be validated within individual laboratories to determine the practical impact on sample progression to STR amplification. Each laboratory should carefully consider if samples with concentrations slightly below the cut-off threshold for STR amplification may in fact exhibit concentrations above the cut-off when alternative standards are used. In such circumstances, these samples could still provide STR data that would otherwise have been disregarded.

Table 3. Quantification results (ng/µl) using 3 different virtual standard curves (VS = virtual standard)

Virtual standards	Sample 1	Sample 2	Sample 3	Sample 4
VS1	48.6	1.793	0.0690	0.0023
VS2	50.3	1.858	0.0718	0.0024
VS3	49.9	1.678	0.0589	0.0018
Average	49.6	1.776	0.0666	0.0021
Standard deviation	0.69	0.075	0.0055	0.0003
Coefficient of variation (%)	1%	4%	8%	12%

Use of virtual standards from different Investigator Quantiplex Pro Kit lots

We next performed the quantification assay using imported virtual standards that were prepared using different Investigator Quantiplex Pro Kit lots (2, 3 and 4) on the QuantStudio instrument. The average quantification values produced using kit lot 1, shown in Table 3 and reproduced in Table 4, were used as benchmarks for this analysis.

As shown in Figure 3, we observed high variability in the DNA quantification results using virtual standards prepared with the different kit lots. We found that the concentrations determined using kit lots 2–4 deviated by up to 51.4% from the lot 1 benchmarks (Table 4). This would have a statistically significant impact on the calculated sample concentrations.

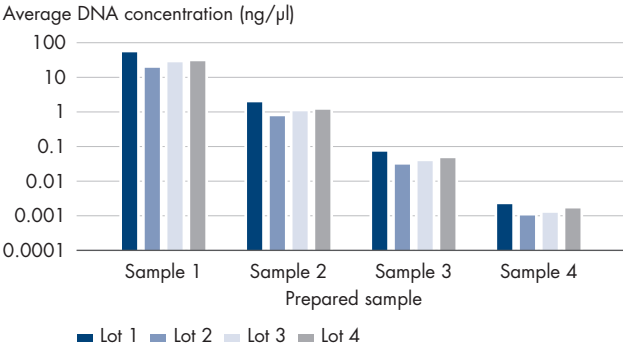

Figure 3. Analysis of Control DNA M1 dilution series samples with four different kit lots.

Table 4. Analysis of Control DNA M1 dilution series samples (ng/µl) with four different kit lots (VS = virtual standard)

	Sample 1	Sample 2	Sample 3	Sample 4
Lot 1 VS 1	48.6	1.793	0.0690	0.0023
Lot 1 VS 2	50.3	1.858	0.0718	0.0024
Lot 1 VS 3	49.9	1.678	0.0589	0.0018
Average Lot 1	49.6	1.776	0.0666	0.0021
Lot 2	18.0	0.713	0.0294	0.0010
Lot 3	26.6	0.973	0.0372	0.0012
Lot 4	27.7	1.080	0.0440	0.0015
Average variance of Lots 2 – 4 from Lot 1 benchmark (%)	51.4%	48.1%	44.6%	41.3%

These results indicate that to maintain reliable and accurate results when implementing virtual standards, it is critical that the imported standard curves were prepared using the same Investigator Quantiplex Pro Kit lot. When a new Investigator Quantiplex Pro Kit lot is received by a laboratory, standards from that kit should be prepared, run and analyzed to mitigate slight variations between lots.

Verification using mock casework samples

Finally, we analyzed mock casework samples to determine the impact of using standards prepared with either the same or different Investigator Quantiplex Pro Kit lots on the quantification assay.

The mock casework samples consisted of neat blood that was diluted using deionized water and extracted using the laboratory's standard operating procedure. These samples were evenly split into three sets (n=8) and amplified using the Investigator Quantiplex Pro Kit. Standards were then amplified in triplicate using either the same kit lot (1) or one of the three alternative kit lots (2, 3 or 4). These standards were then used for the analysis of the blood samples.

As shown in Figure 4, although three different virtual standards were used, all three analyses produced similar quantification results when the standards were prepared with the same kit lot. The average DNA concentration of the mock casework sample determined with the three different

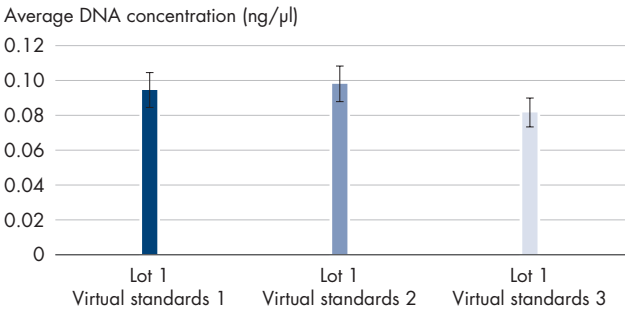

Figure 4. Mock casework sample analysis with 3 virtual standards prepared with the same kit lots (n=8).

kit lot 1 virtual standards was 0.09 ng/µl. The coefficient of variation between the virtual standards was 7.9%, comparable to that calculated for the Control DNA M1 of a similar concentration analyzed using kit lot 1 (Table 3, Sample 3).

In contrast, samples that were prepared with kit lot 1 and subsequently analyzed using virtual standards prepared with the alternative kit lots (Figure 5, depicted in grey) exhibited variable and reduced DNA concentrations. The concentrations of these samples differed by up to 56.2% from the concentration of the samples analyzed with the kit lot 1 virtual standards (Table 5).

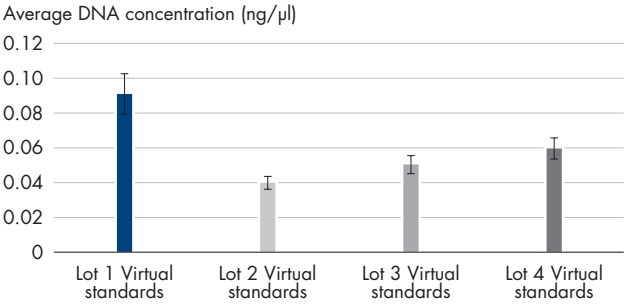

Figure 5. Mock casework sample analysis with virtual standards from alternative kit Lots (n=8).

Table 5. Mock casework sample analysis with virtual standards from alternative kit lots

	Lot 1	Lot 2	Lot 3	Lot 4
Average concentration (ng/µl)	0.0911	0.0399	0.0508	0.0597
Variance from Lot 1 benchmark (%)	–	56.2%	44.3%	34.5%

These results demonstrate that virtual standards can be used to accurately quantify samples when the samples and corresponding standards were amplified with the same reagent lot components. QIAGEN therefore recommends that when laboratories receive new kit lots, a new standard curve is prepared for use as the virtual standard.

Conclusion

With increasing laboratory submissions and demand on finite resources, there is a growing need for streamlined laboratory workflows. The preparation and analysis of standard curves is an obvious target for increasing laboratory efficiency. However, excluding standard curve samples from real-time PCR runs can lead to inaccurate quantification of precious casework samples, especially when dealing with low-template samples.

Here we describe a technique for implementing virtual standards in the quantification of casework samples using the Investigator Quantiplex Pro Kit. Our results indicate that the corresponding standards should be prepared using the same lot of reagents as that used for amplifying the casework samples. Using this method, laboratories can avoid including standards in their real-time PCR runs, thereby increasing efficiency and decreasing processing costs.

References

1. *QIAGEN Investigator Quantiplex Pro Kit Handbook*, March 2018
2. ThermoFisher Scientific, QuantStudioTM real-Time PCT Instrument user guide, June 2017

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

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