IntelliQube® Real-Time Quantitative PCR Performance
performed in collaboration with TATAA Biocenter

ABSTRACT

The IntelliQube is a fully integrated liquid handling and real-time quantitative PCR instrument optimized for use with miniaturized reactions in 768-well Array Tape®. The performance characteristics of this innovative instrument were determined by running a series of PCR-based experiments with human genomic DNA calibrated against the National Institute of Standards and Technology (NIST) standard reference material (SRM) and measured with the ValidPrime® human genome specific assay, and a commercially available alien DNA spike, which is a commonly used internal amplification control. Our analysis concluded that the IntelliQube performed exceedingly well for common measures of instrument performance such as Cq uniformity, dynamic range, and resolution. Deviation between replicates was minimal, dynamic range was sufficiently wide, and the ability to confidently resolve small changes between sample populations was high.

IntelliQube

INTRODUCTION

Quantitative real-time PCR (qPCR) has developed into the central platform and gold standard for molecular research and molecular diagnostics. Behind this success is the development of routines for quality control, validation and performance assessment. A group of opinion leaders, coordinated by Professor Stephen Bustin, drafted the minimum information for publication of quantitative real-time PCR experiments "MIQE" guidelines that summarize what information about assays and test performance shall be reported when submitting a scientific report for publication. The European Commission funded the project SPIDIA to generate results and tools on the preanalytical processes in molecular diagnostics, based on which the European Commission for Standardization (CEN) drafted nine new technical specifications, and earlier this year the International Organization for Standardization (ISO) launched eight new projects within "Clinical laboratory testing and in vitro diagnostic test systems." The National Institute of Standards and Technology (NIST), has made standard reference materials (SRMs) available for genetic analyses and the Clinical Laboratory and Standards Institute (CLSI) offers guidelines and protocols to validate tests' performance. These tests have been implemented for qPCR applications in the GenEx PCR Analysis Software from MultiD Analyses.

In this study, we continued our evaluation of the performance characteristics of the new IntelliQube instrument using a set of qPCR experiments that interrogated common measures of qPCR instrument performance such as uniformity/repeatability, resolution, and dynamic range of the instrument. Our testing was performed with the highly characterized ValidPrime assay (TATAA Biocenter) for the quantification of human genomic DNA (hgDNA) that has been calibrated against the Human DNA Quantitation Standard (SRM 3072) from NIST and a commercially available DNA spike (TATAA Biocenter) typically used to measure PCR efficiency and assess inhibition. Previously, we estimated the PCR efficiency to be 99.7% (95% confidence range: 97.7% – 101.6%), the limit of detection (LoD) to 3 molecules, and the limit of quantification (LoQ) to 32 molecules (at a relative standard deviation of 35%). In this application note we continue the assessment of the IntelliQube, estimating the repeatability/uniformity of the instrument, the dynamic range of a representative test system, and the resolution of small fold changes.

MATERIALS AND METHODS

The test systems are based on using human genomic DNA (hgDNA, Cat#: BIO-35025, TATAA Biocenter) and a calibrated DNA spike template (Universal DNA spike, Cat# DS10PFI, TATAA Biocenter) with the associated ValidPrime and DNA Spike probe-based PCR assays, respectively. TATAA Probe GrandMaster® Mix was used in all experiments and the IntelliQube was used for the automated assembly of PCR reactions for all tests performed. The Dispense Pipette and Dispense Jet on the IntelliQube were used for sample and assay dispensing, respectively. The thermal cycling conditions for all experiments consisted of an initial activation time of one minute at 93.5 °C, followed by 50 cycles consisting of 93.5 °C for 10 seconds and 57 °C for 30 seconds. Following qPCR, Cq data was exported from the IntelliScore® instrument software and analyzed with GenEx PCR analysis software (MultiD) for all advanced analyses.

PCR uniformity was assessed using 768 replicates within a 768-well array. Each PCR reaction consisted of 800 nL containing 2,048 copies of hgDNA and 800 nL of 2X TATAA Probe GrandMaster Mix and ValidPrime assay for a total reaction volume of 1.6 µL.

The dynamic range of the IntelliQube was assessed with a standard curve for a 10-fold dilution series of the DNA Spike template, which was prepared to correspond to $4x10^7$ to 0.4 copies per reaction. The Spike template was diluted in a background of 1 ng/µL of hgDNA. Each 1.6 µL reaction consisted of 800 nL of Spike template/background and 800 nL of 2X TATAA Probe GrandMaster Mix and Spike assay. Each dilution point was represented with a total of 36 replicates.

Lastly, to assess the resolution of the system, a dilution series of hgDNA was prepared to correspond to 2048, 1781, 1707, 1638, 1575, and 1365 copies per reaction. Each PCR reaction consisted of 800 nL of hgDNA and 800 nL of 2X TATAA Probe GrandMaster Mix and ValidPrime assay for a total reaction volume of 1.6 µL. Each dilution point was represented with a total of 128 replicates.

RESULTS AND CONCLUSIONS

A high degree of repeatability/uniformity is critical in qPCR. It is typically assessed through intra-plate variation, which is the reciprocal of repeatability, and confounds the measurements of DNA. The intra-plate variation should be as low as possible and is measured by performing technical replicates on a single plate using a standard or calibrator sample. A large batch of calibrator is made and subsequently distributed across the reaction wells to eliminate pipetting errors. The template concentration must

Figure 1. Amplification curves demonstrating Cq uniformity across 768-wells

be high enough to make sampling ambiguity negligible (refer to the Real-Time qPCR Sensitivity Application Note). Figure 1 shows results obtained with the IntelliQube using the ValidPrime assay and 2,048 copies/reaction of hgDNA (TATAA Biocenter). The data were measured across all 768-wells and were found to be exceedingly congruent.
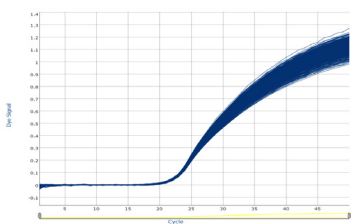
The spread of the extracted Cq values is presented in a frequency diagram (Figure 2) and was analyzed with the QC tool in the GenEx software (Table 1). The tool fits the data to a normal distribution and performs a normality test. The data are expected to pass the normality test and a failed test would indicate some asymmetry or bias across the plate, such as a temperature difference. The replicates are then tested for outliers, using the Grubb's statistical test, which would identify any deviation. All of the IntelliQube replicates passed the Grubb's outlier test. Finally the standard deviation (SD) of the replicate Cq values was calculated. The SD on the IntelliQube for this study was 0.087 cycles, which is far below 0.20 - 0.25 cycles claimed as performance criteria by the majority of qPCR instrument manufacturers.
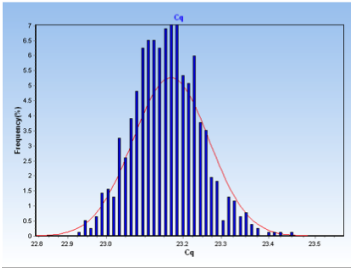

Figure 2. Normal distribution of Cq values across 768-wells.

	Cq
Average	23.15695223
SD	0.08717
Count	768
CV	0.376434618
Normality test	Passed
P-Value	>0.1
KS value	0.018019164
No of outliers	0
Missing data	Ignored
Valid(%)	100
Settings	
Normalize	Yes
Step	0.01
Check for outlier	Yes
Confidence level(%)	95
Exclude outliers	Yes
Validation rate(%)	95

Table 1. GenEx test statistics for Cq values across 768-wells of hgDNA amplified with the IntelliQube.

The DNA spike was also used to assess the dynamic range and precision of the IntelliQube. A standard curve is shown from 40 (log10 = 1.5) to 40 million copies (log10 = 7.5), which is an impressive range, only limited at low concentra-
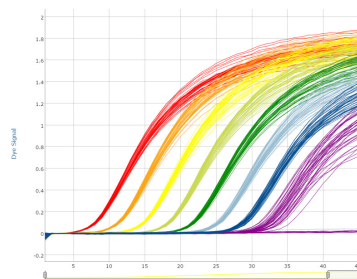
Figure 3. Amplification curves demonstrating a linear dynamic range of quantification from 4×10^7 to 40 copies/reaction.
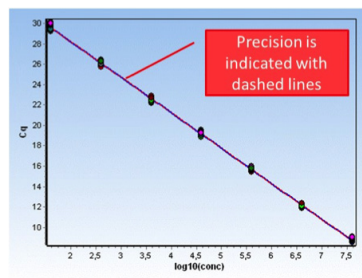

Figure 4. Standard curve demonstrating a linear dynamic range of quantification from 4×10^7 to 40 copies/reaction.

tion by sampling ambiguity (refer to the Real-Time qPCR Sensitivity Application Note) and at high concentration by the DNA amount that can be handled/dissolved in the small reaction volumes used in the IntelliQube (Figure 3 and 4). The data are fitted to a straight line as well as to second and third order polynomials according to the linearity test by the CLSI using the GenEx software. The fits are compared. If the second or third order polynomials improve the fit significantly, there is deviation from linearity and the range would be exceeding the linear dynamic range. The IntelliQube data passed the CLSI linearity test. The precision of the standard curve is assessed by calculating the Working-Hoteling confidence band, which is indicated in the graph by two dashed lines in red on each side of the best linear fit shown in blue. Without enlarging the graph it is hardly possible to discern the uncertainty in the fit indicated by the confidence band. This leads to highly exact estimates of PCR efficiencies not attainable with other instruments. Using the DNA spike, for example, the PCR efficiency under the running conditions tested was estimated at: 93.1% < 93.6% < 94.1%, where the outer numbers indicate the 95% confidence range.

Finally, the resolution of the IntelliQube was assessed using ValidPrime (TATAA Biocenter), which is a calibrated assay for quantification of residual genomic DNA. The resolution of a qPCR instrument is the ability to measure a concentration difference and can be determined experimentally by loading 1.0 template concentration in half of the wells and X template in the other half reflecting fold changes of 1.5, 1.3, 1.25, 1.2, and 1.15 (Figure 5). If the two sets of replicates separate, then the resolution is at least X-fold. This is evaluated using a Power test strategy explained in Figure 6. Each set of replicates is expected to be normally distributed around a mean, which are separated by $\log_2 X$ cycles, where X is the fold change. A decision line is set between the two means and the number of replicates from each set that fall on the wrong side of the decision line. Working at 95% confidence, no more than 5% of the replicates shall fall on the wrong side at a valid resolution. The Cq values from the amplification curves in Figure 5 are compared pair-wise in

1.5

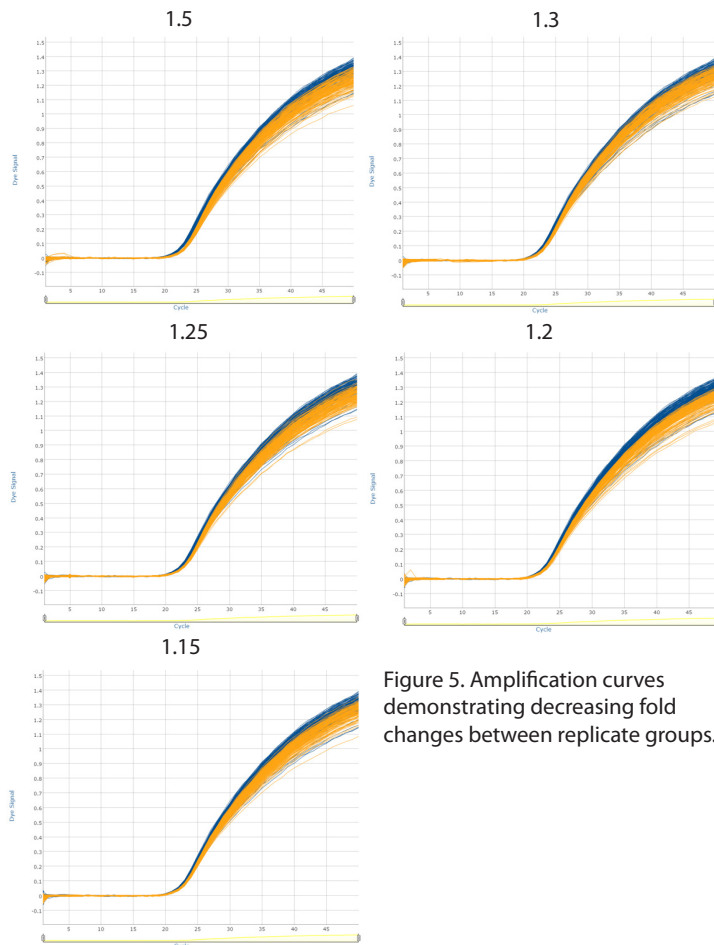
1.3

1.25

1.2

1.15

Figure 5. Amplification curves demonstrating decreasing fold changes between replicate groups.
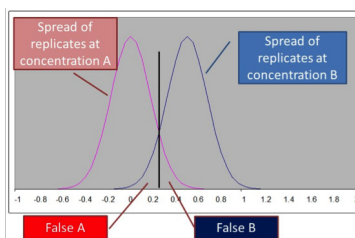

Figure 6. Power test principle for assessing sensitivity and specificity of normally distributed sample sets.

Figure 7. Paired measurements reflecting fold changes of 1.5, 1.3, 1.25, and 1.2 all separated at 95% confidence. At a fold change of 1.15 the number of falsely classified samples exceeded 5%. Hence, the resolution quantifying human genomic DNA with the ValidPrime assay using the IntelliQube is sufficient to measure a fold difference of 1.2 or greater.

In summary, these experiments demonstrate that the IntelliQube performs at an industry leading level, facilitating outstanding qPCR results with Array Tape and the added benefits of inline automation. Seamless integration of liquid handling, thermal cycling, and detection systems within the IntelliQube enables users to achieve efficient and economical high throughput sample processing in Array Tape without compromising important parameters of qPCR performance.
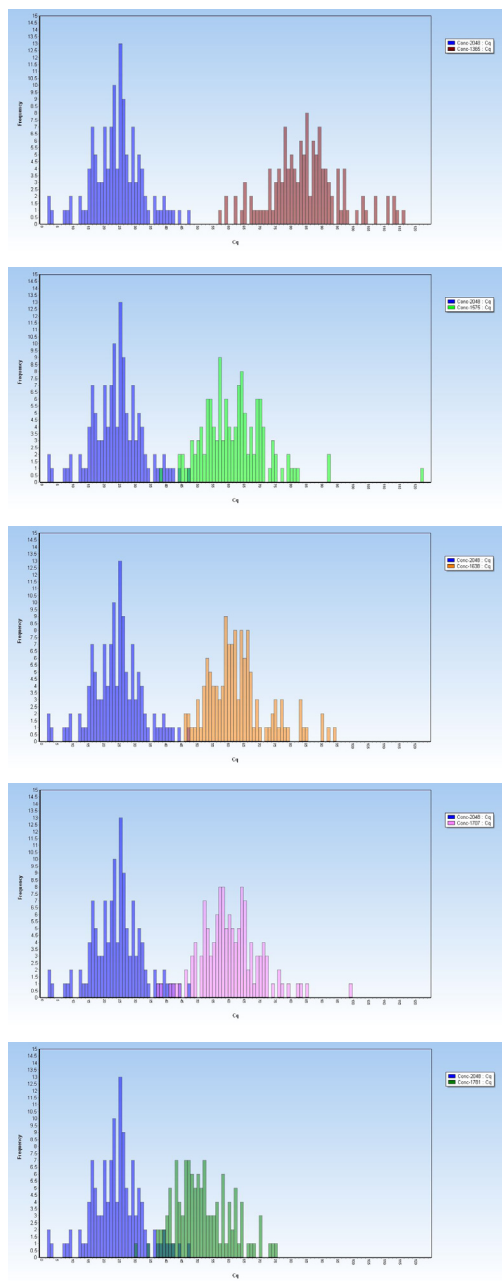
Figure 7. Frequency diagrams demonstrating separation between replicate groups at decreasing fold changes.

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