Characterisation of real-time qPCR sensitivity on the IntelliQube

performed in collaboration with TATAA Biocenter

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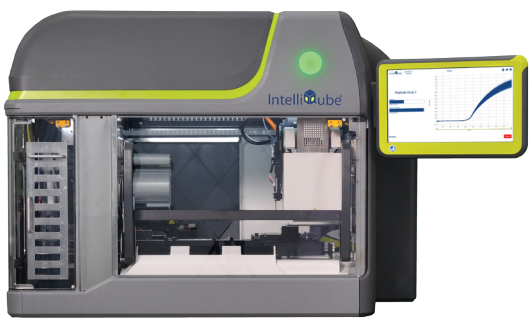
Abstract

The IntelliQube® is a fully integrated liquid handling and real-time quantitative PCR (qPCR) instrument optimised for use with miniaturised reactions in 768-well Array Tape®. We evaluated the technical performance of the IntelliQube 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. In this study, we estimated the limit of detection (LoD), limit of quantification (LoQ) and PCR efficiency. The LoD was estimated between 2 and 3.7 molecules, which corresponds to the theoretical sensitivity limited by sampling ambiguity only. LoQ, corresponding to a relative standard deviation of 35%, was between 8 and 16 molecules, and PCR efficiency was 99.6% (95% confidence range: 97.8-101.4%). The sensitivity of the instrument was tested using a digital PCR setup. When loading an average of two molecules per well, we recovered 1.96 (95% confidence range: 1.80-2.15) molecules. A small loss was observed when loading an average of one molecule per well. Taken together, these data are evidence of excellent performance with a technical error negligible relative to the sampling error, virtually perfect PCR under optimum conditions, and a sensitivity to detect one to two template molecules per well.



IntelliQube

Introduction

Quantitative real-time PCR (qPCR) is maturing as a technology, becoming the preferred central platform for most of the molecular research and molecular diagnostics performed today. Behind this success is the field's increasing awareness of the importance of quality control, validation, and performance assessment. The MIQE guidelines, drafted by a group of opinion leaders coordinated by Professor Stephen Bustin, details the information about assays and test performance that 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 test performance.

In this study we evaluate the new high-throughput qPCR instrument with integrated liquid handling: the IntelliQube. Our testing was performed with the highly characterised 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.

Performance parameters including PCR efficiency, limit of detection (LoD), and limit of quantification (LoQ), allow for simple and direct comparison with other qPCR platforms. We also ran the IntelliQube in a digital PCR setup, whereby in separate experiments we distributed one or two single molecules of the calibrated hgDNA sample across all the 768 wells of the IntelliQube Array Tape such

that we could determine the total number of molecules loaded from the fraction of PCR positive wells. Although the IntelliQube is not a digital PCR platform; rather a platform optimised for high-throughput qPCR, such an experiment provided a comparative baseline by which to establish the sensitivity, in terms of the minimum number of target molecules per well the instrument can detect.

Materials and methods

The test system is based on calibrated human genomic DNA (hgDNA, (CAT# CHG50, TATAA Biocenter) with a stock concentration of 188 ng/µl. Calibration samples were prepared by two-fold serial dilutions with multiple replicates at each concentration (Table 1) and analysed with the ValidPrime assay, which is a highly optimised FAM™ labeled probed-based PCR assay specific for a single loci in haploid hgDNA. PCR mix was prepared using the TATAA Probe GrandMaster® Mix L-Rox™ (Table 2). A 2-step PCR protocol was used (Table 3). The IntelliQube qPCR instrument was used for all reaction setup and qPCR analysis in Array Tape (LGC Douglas Scientific). All experiments were performed with 768-well Array Tape, with reactions composed of 800 nL of sample material and 800 nL of 2X master mix for a total reaction volume of 1.6 µL. Following qPCR, Cq data was exported from the IntelliScore® instrument software and analysed with GenEx PCR analysis software, ver. 7 (MultiD) for all advanced analyses. Outliers were identified using the Grubb's test.

Stock	Starting conc. (copies/µL)	Dilution factor	Final conc. (copies/rxn)	Replicates in Array Tape
Std 1	56969.70	1:22.25	2048	32
Std 2	2560	1:2	1024	32
Std 3	1280	1:2	512	64
Std 4	640	1:2	256	64
Std 5	320	1:2	128	64
Std 6	160	1:2	64	64
Std 7	80	1:2	32	64
Std 8	40	1:2	16	64
Std 9	20	1:2	8	64
Std 10	10	1:2	4	64
Std 11	5	1:2	2	64
Std 12	2.50	1:2	1	128

Table 1: Serial dilution of calibrated hgDNA

Reagents	Stock conc.	Volume (µL)	Final conc.
Primers (Fw & Rv)	10 µM each (Fw/Rv)	0.064	400 nM
Probe	10 µM	0.032	200 nM
TATAA Probe GrandMaster Mix L-Rox	2X	0.8	1X
Total volume (of which 0.8 µL is used):		**0.9**	

Table 2: Reaction mix for qPCR

Step	Time	Temperature	Cycles
Enzyme activation	1 min	95 °C	1
Denaturation	10 sec	95 °C	50
Annealing/extension	30 sec	60 °C (FAM acqusition)	

Table 3: PCR protocol

Results and conclusions

Figure 1 shows a qPCR standard curve plotted as Cq versus log10 concentration of the calibration samples. The spread of replicates is seen to increase with decreasing number of target molecules. This is due to a phenomenon known as sampling ambiguity. As the number of target molecules per reaction volume gets low, the reproducibility is compromised. For example, if the average concentration is four molecules per microliter and we analyse replicate 1 microliter samples, many of them will contain four molecules. However, due to chance, some will contain three, perhaps only two molecules, while others will have five or even six. Under ideal conditions, the frequency of samplings containing different numbers of molecules is given by the Poisson distribution (Figure 2). Hence, when there is no measurement error, only about 20% of the samplings will actually contain four molecules. 20% of the samplings will contain three molecules, 15% will contain two, 16% will contain five, etc. This sampling variation is insignificant when the average number of molecules per sampling is large, but is often the dominant contribution at very low concentrations.

LoD is defined as "The lowest amount of analyte in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value." In molecular diagnostics we commonly report results and make conclusions at a confidence of 95%. Using this as criteria, we can calculate the theoretical limit of detection (LoD) by qPCR due to sampling ambiguity, i.e., when analysing a subset of the total sample. This is the lowest concentration that leads to at least 95% of the sample replicates being positive; hence, at this concentration the risk for a false negative result shall be less than 5%. Figure 3 analyses the data in Figure 1 for LoD, showing the frequency of positive calls as function log2 concentration of the hgDNA. Intersecting the graph at 95% we read the LoD as 2.5 molecules with a 95% confidence range of 2 to 3.7. This range comprises the theoretical LoD of 3 molecules that is determined by sampling ambiguity. For other real-time PCR systems the LoD is usually higher, because of sampling and measurement errors.

The limit of quantification is defined as "The concentration of analyte leading to a signal which cannot be confounded with the blank (background) and that can be quantified." LoQ can be determined from the spread of replicates at each concentration of the standard curve. Criteria have to

be established, which depends on the particular test and its requirements.

A commonly used criteria is that the LoQ corresponds to the lowest concentration that gives a relative standard deviation, also known as coefficient of variation (CV), of no more than 35% on back calculated concentrations. Figure 4 analyses the data in Figure 1 for LoQ showing the CV of the estimated concentrations versus log10 of the concentration of the hgDNA. A red-dashed horizontal line indicates CV=35%, identifying LoQ between 8 and 16 molecules, with 16 being the lowest concentration with a CV≤35%. The dilution factor for the standard curve was 2-fold in these experiments. This becomes important when estimating the LoQ. We know LoQ in this case is below 16, but above the next lowest dilution which happens to be 8 molecules. The theoretical limit for the LoQ at a CV of 35% due to sampling ambiguity equals 10 molecules, which falls within the range estimated on the IntelliQube.

The standard curve in Figure 1 can now be truncated at 16 molecules and PCR efficiency can be analysed over the concentration range above the LoQ (Figure 5). For the analysis of hgDNA with the ValidPrime assay on the IntelliQube, we estimate PCR efficiency to be 99.6% with the 95% confidence interval of 97.8 - 101.4%.

The IntelliQube is optimised for high-throughput qPCR, which includes individual loading of samples and assays into Array Tape reaction wells, providing total reaction volumes of 1.6 µl to reduce sampling ambiguity of manual pipetting methods. In comparison, digital PCR (dPCR) platforms have optimised processes to distribute a single sample across all reaction partitions and use much smaller reaction volumes to distribute on average around one molecule per volume. Still, the large number of wells provided by Array Tape on the IntelliQube and the robust integrated liquid handling for the loading of the samples and assays allows designing of atypical dPCR experiments. Loading hgDNA of known concentration and analysing with ValidPrime, we register the numbers of positive reads from which we can estimate the hgDNA concentration. Comparing the measured estimate with the known concentration, we establish the sensitivity of the instrument. When loading an average of two molecules per well in 64 wells we registered 55 positive reads, which corresponds to an average of 1.96 template molecules per well (95% confidence range: 1.80-2.15, Table 4). With an average of two molecules per well, 27% of the 64 wells (n=17) should contain just a single molecule (Figure 2). Hence, this excellent agreement with theoretical values is evidence the IntelliQube, under these conditions, detects the single target molecule in those wells. Loading an average of one hgDNA molecule per well, 54.7% positive reads were observed. This corresponds to an average of 0.79 template molecules per well, which is somewhat less than expected. Hence, with this lower loading, there are potential losses of about 21% of the loaded material, which is possibly attributable to the dispensing step. However, with the detection of the majority of the loaded template molecules, we can conclude that the sensitivity of the IntelliQube should be in the range of 1 - 2 molecules per reaction.

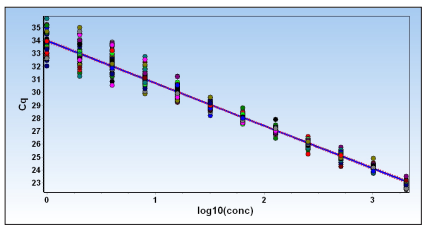


Figure 1: Standard curve with all data

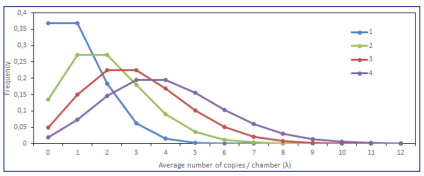


Figure 2: Poisson distribution

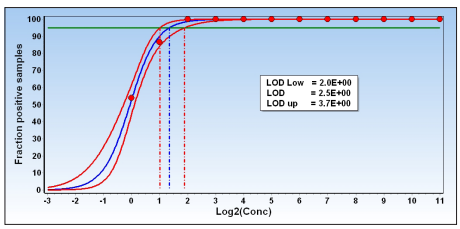


Figure 3: Limit of detection

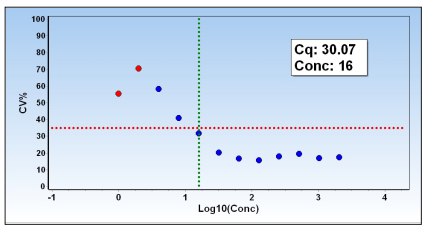


Figure 4: Limit of quantification

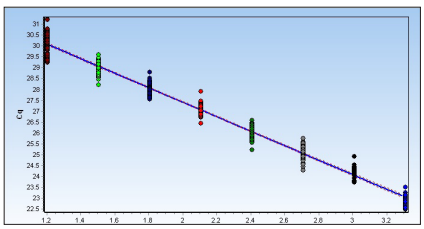


Figure 5: Standard curve at conc. above LoQ

Poisson statistics	1 copy	2 copies
# Positives	70	55
Total wells	128	64
% Positive	54.7%	85.9%
λ (copies/reaction)	0.79	1.96
λ min (95% CI)	0.72	1.80
λ max (95% CI)	0.87	2.15

Table 4: dPCR

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