IntelliQube® Real-Time Quantitative PCR Performance Characterization

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 a series of PCR-based experiments that interrogated PCR uniformity, resolution, and dynamic range. As this is a fully integrated instrument, sample and master mix dispensing, thermal cycling block performance, and optical detection uniformity were all represented in the results. These experiments demonstrate that the IntelliQube uniformity is excellent. The IntelliQube demonstrated the ability to discriminate between a two-fold dilution sample set with statistical significance, and the dynamic range was shown to be linear across an eight-log dilution series. These experimental results demonstrate that the IntelliQube is a robust system that combines quantitative real-time PCR, liquid handling, and reagent cost savings in one convenient and easy to use laboratory instrument.

INTRODUCTION

The IntelliQube from Douglas Scientific is a fully integrated laboratory instrument that combines liquid handling with real-time quantitative PCR (qPCR) analysis in miniaturized reaction volumes. The IntelliQube utilizes Array Tape in a unique and innovative 768-well format in place of standard 384-well microplates. Array Tape is a thin and flexible polypropylene consumable that, in combination with miniature reaction volumes (1.6µL), enables both outstanding PCR performance and profound reagent savings. The performance characteristics of the IntelliQube were determined using a set of qPCR experiments that interrogated the uniformity, resolution, and dynamic range of the instrument.

The PCR uniformity across an array is an important measure of instrument performance, as it indicates consistent dispensing, thermal cycling, and fluorescence detection for each well. The IntelliQube uniformity was determined by qPCR amplification of 768 identical replicates. Purified salmon genomic DNA (10,000 estimated copies per reaction) served as the nucleic acid template and the SClkF2R2 gene was amplified using sequence-specific primers coupled with a FAM-labeled hydrolysis probe.

The resolution of a qPCR instrument indicates its ability to distinguish between small differences in nucleic acid template concentration. A two-fold resolution was demonstrated in the IntelliQube by qPCR analysis of 768 reactions containing either 10,000 or 5,000 estimated copies of salmon genomic DNA, and the same SClkF2R2 assay described above. The two groups were expected to have Cq values separated by one cycle.

Dynamic range is also an important measure of qPCR instrument performance, as it indicates the versatility and robustness of the system across a wide range of nucleic acid template concentrations. The dynamic range of the IntelliQube was demonstrated across an eight-log dilution series of a plasmid containing sequence from the soybean lectin gene. The lectin sequence was amplified using sequence-specific primers coupled with a FAM-labeled hydrolysis probe.

Additional experiments were performed to demonstrate the utilization of all five fluorescence channels, as well as a multiplex reaction with FAM and HEX reporters. Taken together, these characterization experiments demonstrate that the IntelliQube performs at an industry leading level, enabling outstandingq PCR results in miniaturized reactions with the use of Array Tape.

MATERIALS AND METHODS

Salmon Genomic DNA: Salmon sperm DNA was purchased from Sigma-Aldrich (Cat #: D1626) and dissolved in TE buffer, pH 8.0 (IDT). The final concentration of DNA was determined using a NanoPhotometer® (IMPLEN). An estimated genome size of 2.58 Gb for Chum salmon *(Oncerhynchus keta)* was used to calculate the number of genome copies used in each PCR reaction.

Soybean Lectin Plasmid: A 500 bp fragment of the soybean *(Glycine max)* lectin gene was synthesized and inserted into a vector (pUC57) by GenScript. The lyophilized plasmid was dissolved in TE buffer, pH 8.0 (IDT). The number of plasmid copies used in characterization experiments was calculated using the size of the pUC57 vector containing the 500 bp lectin sequence (3,210 bp).

Master Mix and Assays: SensiMix™ II Probe Lo-ROX (Bioline) was used for all PCR reactions. The master mix was provided at 2X concentration and used according to the manufacturer's instructions. All primers and probes were obtained from Biosearch Technologies. Sequence information for the oligos used in these studies can be found in Table 1. The primers and probe targeting the soybean lectin gene were described previously *(R. Alary, 2002)*. A set a primers and probe was designed to target a sequence from the SClkF2R2 gene of salmon. Oligos were added at 2X concentration to the 2X master mix to achieve a final concentration in the PCR reaction of 200nM probes, 900nM primers, and 1X master mix. Table 2 lists the suppliers for the components of these experiments.

Instrumentation: The IntelliQube was used for sample and master mix dispensing, thermal cycling, and real-time fluorescence detection for all characterization experiments.

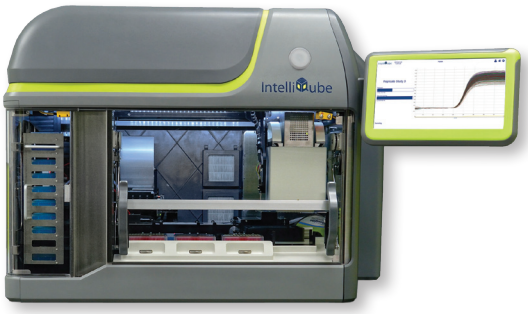

Figure 1: IntelliQube: The IntelliQube is a fully integrated liquid handling and real-time quantitative PCR instrument optimized for use with miniaturized reactions in 768-well Array Tape.

The IntelliQube is shown in Figure 1. Array Tape for IntelliQube consists of 768 reaction wells per array. All of the qPCR reactions described below contained 800nL of DNA sample dispensed in two consecutive dispensing actions using the multichannel, 384 tip CyBio Dispense Pipette head. 800nL of 2X master mix containing 2X assay was dispensed with the non-contact Dispense Jet to create 1.6µL total volume reactions. The thermal cycling protocol for all experiments performed in the IntelliQube had an initial ten minute activation step at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Gene/Assay (GenBank Accession #)	Oligo Description	Dye Label	Sequence
Soybean lectin	Forward Primer	Unlabeled	AACCGGTAGCGTTGCCAG
(K00821.1)	Reverse Primer	Unlabeled	AGCCCATCTGCAAGCCTTT
	Probe	FAM/HEX/TAMRA/ Quasar 670	TTCGCCGCTTCCTTCAACTTCACCT
Salmon SClkF2R2	Forward Primer	Unlabeled	CATGCTCCGAGGGACGATC
(AF494062.1)	Reverse Primer	Unlabeled	GGGACTTGAAGTTTCCGATGAAC
	Probe	FAM	CCCAAAGAGCCTCCTGTGTATGAGTA

Table 1: Primers and Probes

Reagent	Source
SensiMix™ II Probe Lo-ROX	Bioline
Salmon genomic DNA	Sigma-Aldrich
pUC57 vector containing soybean lectin sequence	GenScript
Unlabeled primers and BHQ® probes	Biosearch Technologies

Table 2: Reagents and assays

PCR Uniformity: Samples from a plate containing 384 replicates of salmon genomic DNA were dispensed into 768 wells of a single array at a concentration of 12,500 genomic copies per microliter (uL), resulting in 10,000 copies per reaction. Master mix containing 2X assay was dispensed into each well. The mean Cq and standard deviation of all 768 replicates was analyzed to determine PCR uniformity.

Two-Fold Resolution: A sample plate was prepared with 192 replicates of 12,500 copies per microliter (10,000 copies/reaction) and 192 replicates of 6,250 copies per microliter (5,000 copies/reaction) of salmon

genomic DNA. The 768-well array contained a total of 384 replicates at each concentration. Master mix containing 2X SClkF2R2 assay was dispensed into each well. The ability to distinguish between a two-fold dilution of starting DNA template was determined by comparing the mean Cq and standard deviation of each group. A one Cq difference between a two-fold dilution is expected, assuming 100% PCR efficiency. To discriminate between a two-fold dilution in 95% of cases, the Cq standard deviation at each concentration must be ≤ 0.250.

Dynamic Range: An eight-log dilution series of lectin plasmid was created with 10-fold dilutions to contain a final concentration of 10^8 to 10^1 copies per reaction. There were a total of 96 replicates at each concentration. Master mix containing 2X lectin assay with a FAM labeled probe was dispensed into each well. The slope of the standard curve and PCR efficiency were calculated.

Fluorescence Channel Verification: Samples from a plate containing 384 replicates of lectin plasmid at a concentration of 1,250 copies per microliter (1,000 copies/reaction) were dispensed into 768 wells. The probe targeting the lectin sequence was designed with either a FAM, HEX, TAMRA, or Quasar 670 fluorophore in order to demonstrate performance of each fluorescence channel in the IntelliQube. A fifth channel detects ROX and was used for normalization in all experiments. The same forward and reverse primers flanking the lectin probe were used for each assay. All four tips on the Dispense Jet were used to dispense 800nL of each lectin assay into the array, one probe assay per tip, for a total of 192 replicates of each probe.

Duplex PCR: A DNA sample containing lectin plasmid (1,000 copies/reaction) and Salmon genomic DNA (500 copies/reaction) was dispensed into each well of a 768-well array. In one 384-well offset, 2X master mix containing a duplex lectin-HEX and SClkF2R2-FAM assay was dispensed. For the second 384-well offset, 2X master mix containing only the lectin-HEX assay was dispensed.

Results and Conclusions

A series of five experiments was completed to characterize the real time PCR capabilities of the IntelliQube. PCR uniformity was demonstrated across 768 replicates in a single array. The mean Cq was 22.17 with a standard deviation of 0.181, as shown in Figure 2. The two-fold resolution experiment consisting of 10,000 or 5,000 copies per reaction produced mean Cq values of 22.18 and 23.33 for each group, respectively. Figure 3 shows the amplification curves for each group. The standard deviations for the Cq values of 10,000 copies and 5,000 copies were 0.174 and 0.213, respectively. The standard deviation of each group was ≤ 0.250, allowing for discrimination between this two-fold difference in starting template in 95% of cases.

The IntelliQube was demonstrated to have a linear dynamic range across eight logs using a dilution series of lectin plasmid (10^8-10^1 copies/reaction), as shown in Figure 4. The slope of the standard

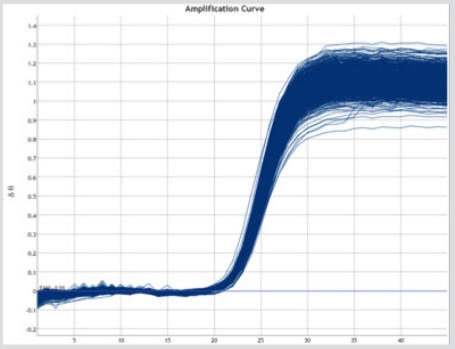

Figure 2: Uniformity. PCR amplification curves demonstrating uniformity across a 768-well array. Each well contained 10,000 copies of salmon genomic DNA amplified using the SClkF2R2 assay (FAM) with SensiMix™ II Probe Lo-ROX master mix in a 1.6uL reaction. The mean Cq was 22.17 with a standard deviation of 0.181. ΔRn values on the y-axis are plotted against PCR cycle on the x-axis.

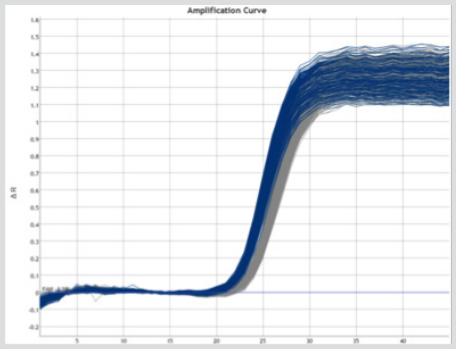

Figure 3: Two-Fold Resolution. PCR amplification curves demonstrating one cycle difference between mean Cq values for 10,000 and 5,000 copies of salmon genomic DNA. The SClkF2R2 target (FAM) was amplified using SensiMix™ II Probe Lo-ROX master mix in 1.6uL reactions. The mean Cq for 10,000 and 5,000 copies was 22.18 and 23.33, respectively, with standard deviations of 0.174 and 0.213. There were 384 replicates of each dilution. ΔRn values on the y-axis are plotted against PCR cycle on the x-axis.

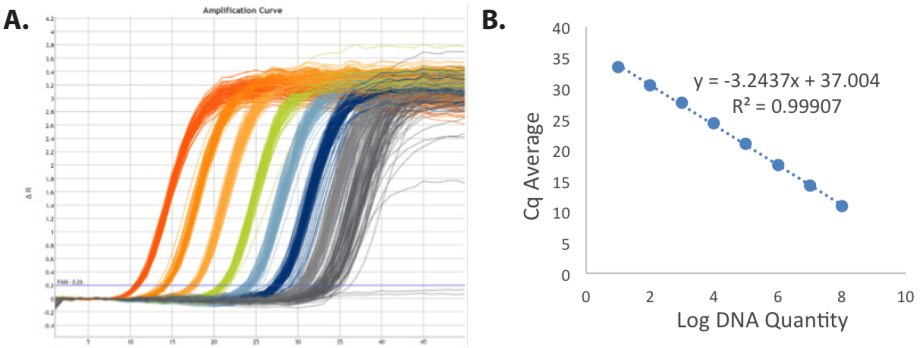

Figure 4: Dynamic Range. A) PCR amplification curves of a ten-fold dilution series of lectin plasmid DNA (10^8-10^1 copies/reaction) amplified using a lectin assay (FAM) with SensiMix™ II Probe Lo-ROX master mix in 1.6uL reactions. The graph shows 96 replicates of each dilution. ΔRn values on the y-axis are plotted against PCR cycle on the x-axis. B) The standard curve generated from the dilution series data showing a slope of -3.24 and a calculated PCR efficiency of 103.4%.

curve was -3.24 with a calculated PCR efficiency of 103.4%. Under ideal conditions with 100% PCR efficiency, a slope of -3.32 is expected. A large dynamic range allows for detection of samples with high and low copy number in the same run. To provide additional fluorescent dye flexibility and multiplexing options, the IntelliQube offers five fluorescence detection channels. To evaluate the performance of these channels, an experiment was completed using a singleplex lectin assay with probes labeled with either FAM, HEX, TAMRA, or Quasar 670. Figure 5 shows ROX-normalized PCR amplification curves using four fluorescence channels in the same array. The mean Cq values for all four fluorescent dye-labeled probes were within one cycle, demonstrating consistency across the dye channels.

The IntelliQube offers users the ability to multiplex PCR reactions for improved throughput, data quality, and reagent savings. To demonstrate duplex PCR detection, both salmon genomic DNA and lectin plasmid were used with HEX (lectin) and FAM (salmon) reporters in the same reaction. The mean Cq for the lectin-HEX assay in a duplex reaction was 21.40 with a standard deviation of 0.27. The mean Cq for the lectin-HEX assay in a singleplex reaction was 21.56 with a standard deviation of 0.28. Detection of the lectin plasmid using the HEX channel was comparable in both duplex and singleplex reactions, as shown in Figure 6.

In summary, these characterization experiments demonstrate that the IntelliQube performs at an industry leading level, enabling outstanding qPCR results with the benefits of inline automation. By seamlessly integrating liquid handling, thermal cycling, and detection systems, the IntelliQube enables users to achieve efficient and economical high throughput sample processing in Array Tape for both quantitative and endpoint PCR applications.

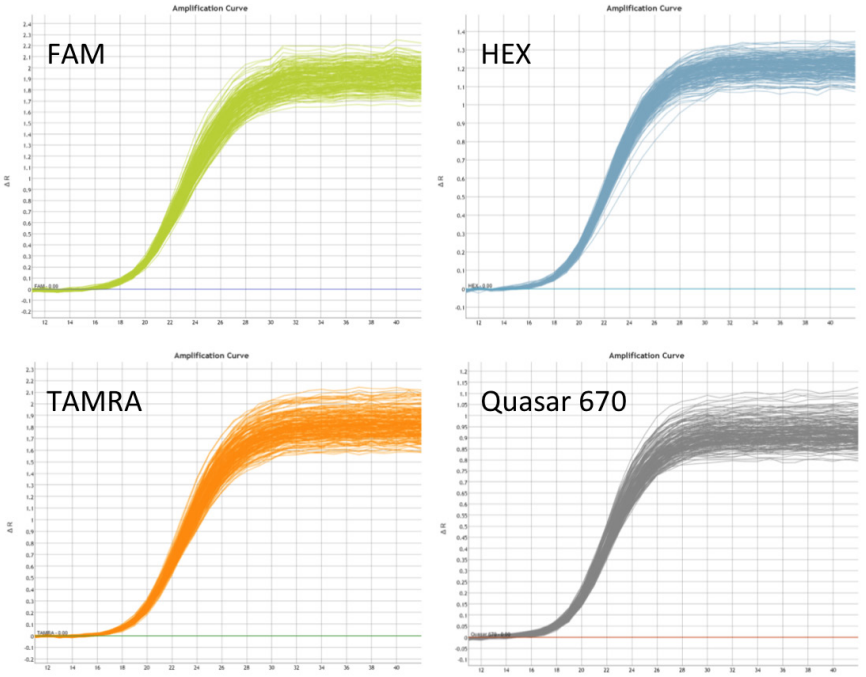

Figure 5: Fluorescence Channel Verification. Detection of PCR amplification using all five fluorescence channels with singleplex reactions. Each graph shows 192 replicates of 1,000 copies/reaction of lectin plasmid amplified using a lectin probe labeled with either FAM, HEX, TAMRA, or Quasar 670. The ROX channel was used for normalization. ΔRn values on the y-axis are plotted against PCR cycle on the x-axis. Two wells were omitted from analysis for HEX.

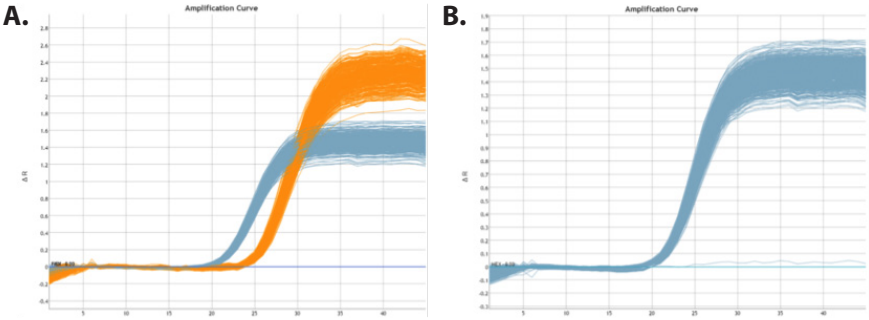

Figure 6: Duplex PCR. A) PCR amplification curves demonstrating duplex PCR detection of 500 copies of salmon genomic DNA (orange) and 1,000 copies of lectin plasmid (blue) in the same well. The salmon DNA was detected with a FAM-labeled probe and the lectin plasmid was detected with a HEX-labeled probe. The graph shows one 384-well offset. B) PCR amplification of 1,000 copies of lectin plasmid across the whole 768-well array. One 384-well offset contained the singleplex lectin assay (HEX) and the other 384-well offset contained the duplex salmon and lectin assay as described in (A). The graph in (B) demonstrates that the detection of lectin plasmid is equivalent in the duplex and singleplex reactions. ΔRn values on the y-axis are plotted against PCR cycle on the x-axis.

References

Alary R., Serin, A., Maury D., Jouira H.B., Sirven, J.P., Gautier M.F., Joudrier, P. Comparison of simplex and duplex real-time PCR for the quantification of GMO in maize and soybean. Food Control 2002; 13:235-244.