Application note
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Multiplex One-step RT-qPCR for Gene Expression Analysis using the IntelliQube® and BHQ® probes

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Abstract

One-step reverse transcription-PCR (RT-PCR) is widely used for gene expression analysis, RNA virus detection, and routine RNA quantification experiments. In this study, we evaluated the IntelliQube real-time PCR instrument for multiplex one-step RT-PCR performance using commercially available RNA, one-step RT-PCR master mix, and BHQ probe-based assays from LGC Biosearch Technologies. The IntelliQube combines liquid handling with real-time qPCR analysis in miniaturized reaction volumes, providing laboratories a flexible platform to scale their RT-PCR workflows. In these experiments, RT-PCR performance was consistent run-to-run and across singleplex and multiplex assay formats, enabling the analysis of multiple targets in a single reaction. Combined with the automated process and economic benefits of Array Tape®, the IntelliQube and associated BHQ probe chemistry can be an effective platform for multiplex one-step RT-PCR applications.

Introduction

One-step reverse transcription-PCR (RT-PCR) is a common technique used to directly amplify RNA samples. In a one-step workflow, the reverse transcription (RT) and quantitative PCR (qPCR) steps are performed in the same reaction, reducing the number of pipetting steps and decreasing process time. However, unlike two-step strategies where complementary DNA (cDNA) is generated in a separate reaction, all of the cDNA is used up in the PCR process and unavailable for future testing. One-step RT-PCR workflows benefit users wanting to process many samples at a time against one or a few markers. Some of the more popular research applications for one-step RT-PCR include virus detection and quantification, gene expression analysis, and routine RNA quantification experiments.

While there are several PCR instruments on the market upon which these tests can be performed, there remains an unmet need for a fully-automated solution that provides laboratories the flexibility and scalability necessary to economically expand their RT-PCR workflows. The IntelliQube from LGC Douglas Scientific®, shown in Figure 1, is designed to address automation and throughput needs by producing accurate and reliable results with walk-away automation. The IntelliQube is a fully integrated laboratory instrument that combines liquid handling with real-time qPCR analysis in miniaturized reaction volumes. The system supports a five channel fluorescence detection well suited for the variety of fluorophores available for BHQ probe assay designs. The system utilizes Array Tape in a unique 768-well format in place of standard 384-well microplates. Array Tape is a thin and flexible polypropylene consumable that supports 1.6 µL reaction volumes.

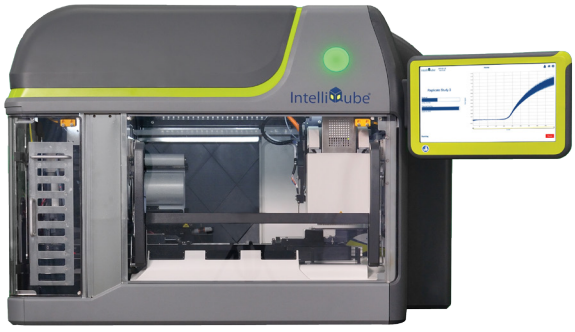

Figure 1: The IntelliQube is a fully integrated liquid handling and real-time PCR instrument optimized for use with miniaturized reactions in 384- or 768-well Array Tape.

In this study, we evaluated the IntelliQube for one-step RT-PCR performance using BHQ probe-based assays, commercially available RNA, and one-step RT-PCR master mix. Expression of the Mucin 1 (Muc1) gene was tested across three tissues types including lung, liver, and kidney. To demonstrate multiplexing capabilities, Muc1 gene expression was measured in singleplex, duplex and triplex formats incorporating two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA-box binding protein (TBP). Assay performance and gene expression analysis was compared across the plex formats and run-to-run consistency was evaluated.

Materials and Methods

Instrumentation: The IntelliQube was used for reaction set-up, thermal cycling, and real-time fluorescence detection. DNA or RNA samples (800 nL) were dispensed into Array Tape with the Felix 384-channel pipette head from CyBio® Product Line. Commercially available one-step master mix containing 2X assay (800 nL) was dispensed with the non-contact Dispense Jet to create 1.6 µL reactions. Fluorescence values were obtained at the end of each cycle over the course of 45 cycles to generate real-time amplification curves.

Synthetic DNA Template Preparation: DNA templates (500 base pairs) of the Muc1, GAPDH, and TBP genes were synthesized. The lyophilized stocks were suspended with 1X TE pH 8. A stock concentration of 1.25 x 10^8 copies/µL was prepared by diluting the DNA templates with water. Serial dilutions were prepared in water contained 1 ng/µL salmon sperm DNA as a carrier. The dilutions ranged from 1.25 x 10^7 - 1.21 copies/µL. A representation of the dilution scheme can be found in Figure 2.

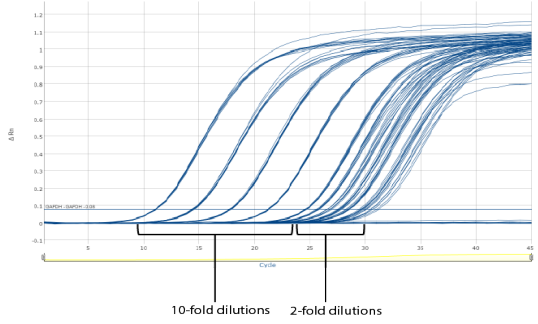

Figure 2. Amplification curves from the GAPDH assay representing the 10-fold and 2-fold DNA dilutions used to determine assay performance. The 10-fold dilutions ranged from1.25x10^7-1.25x10^3 copies/µL. Two-fold dilutions ranged from 1.25x10^3 to 1.21 copies/µL. This dilution scheme was used for all assays. All amplification curves were generated using IntelliScore Software.

Total RNA Preparation: Lung, liver, and kidney purified total RNA was obtained from four different manufacturers (Agilent, Cell Applications, Clonetech, and ThermoFisher). All RNA was thawed upon receipt, serially diluted in 10-fold increments covering a range of 62.5 ng/µL - 6.25 fg/µL, and transferred to multiple sample plates. All sample plates were stored at -20°C until ready to use.

Gene Target Identification: Muc1 gene was selected based on varying expression levels across three tissue types (liver, lung, and kidney) (Song et al., 2013). GAPDH and TBP were selected as reference genes.

Assay Design: Intron-exon junctions for Muc1, GAPDH, and TBP were identified using Ensembl (version 84). Multiple BHQ probe and primer sets were designed using RealTimeDesign™ Software to span exon-exon junctions to mitigate amplification of genomic DNA. NCBI Primer-Blast was used for in silico verification of the specificity of the primers and probes (Ye et al., 2012). Primer-Blast can give a good indication of assay specificity, however PCR testing for specificity against expected off-target sequences should be performed. BHQ probe and primer sets were initially screened with the synthetic DNA dilutions. Successful primer/probe combinations were subsequently used in additional primer optimization work.

Primer Concentration Optimization (Multiplex): Multiple primer concentrations (600, 300, 150, and 75 nM) were tested across all assay combinations in singleplex, duplex, and triplex formats to identify optimum primer concentrations for best assay performance. For each assay, the probe concentration remained fixed at 200 nM. Serial dilutions of the DNA were used for primer optimization. There were four replicates of each concentration and plex combination per dilution. The empirically determined optimum primer concentrations were used for all remaining tests (75 nM Muc1, 150 nM GAPDH, and 150 nM TBP).

Multiplex Assay Performance: The serial dilutions of the DNA template were used to determine assay performance with eight replicates per dilution for each assay. All plex formats were run with the optimized primer concentrations. GenEx Software was used to estimate the Limit of Detection (LoD) and Limit of Quantification (LoQ). The LoD is considered the lowest concentration that leads to at least 95% of the sample replicates being positive. The LoQ is lowest concentration that gives a relative standard deviation, also known as coefficient of variation (CV), of no more than 35% on back calculated concentrations. IntelliScore® Software was used to calculate PCR efficiencies using template dilutions at and above the LoQ.

RNA Assay Performance: Assay performance with RNA samples was characterized by comparing PCR efficiency between the DNA template and RNA. Serial dilutions of the total RNA were used for the comparison. The RNA tissue type that had the highest expression for each respective assay was used to make the standard curves. There were four replicates per dilution for each concentration of RNA and assay. GenEx Software was used to determine what RNA dilutions would be incorporated into the standard curve. The incorporated RNA dilutions had to have all replicates amplify and have a relative standard deviation below 35% between all replicates. IntelliScore® Software was used to generate the standard curves and PCR efficiencies using the dilutions selected.

Gene Expression Analysis: Gene expression for Muc1 was tested across three tissues types obtained from four different vendors. TBP and GAPDH were used as

reference genes. The assays were set up in singleplex, duplex, and triplex formats for plex comparison. Each reaction contained 50 ng of total RNA from each sample. All samples were run in quadruplicate for each array. The experiment was performed three times to assess run-to-run variability. Fold change of Muc1 expression across the three tissues was determined using the Comparative Cq method. Statistical analysis was performed using a three way ANOVA. The model was reduced to evaluate the influence of multiplexing, tissue type, and run-to-run variability on gene expression. Tukey posttest was used for comparison of groups.

Results and Discussion

BHQ probe-based assays, designed to detect GAPDH, TBP, and Muc1 gene targets, were successfully tested in conjunction with the IntelliQube. Initial primer optimization was required prior to testing assay performance in a multiplex format. The results of proper primer optimization is illustrated in Figure 3. Typically, PCR inhibition can be alleviated by reducing primer concentration of particular assays. Once optimized, these primer concentrations were used during all subsequent tests. Multiplex assay performance was characterized by assessing LoD, LoQ, and PCR efficiency in singleplex, duplex, and triplex formats for GAPDH, TBP, and Muc1. The amplification curves generated for each assay using synthetic DNA are shown in Figure 4. Overlaying the curves from each plex format demonstrates a negligible impact on assay performance when combining multiple assays. A shift in Cq value would be expected if PCR inhibition was occurring. Cq values obtained from the IntelliScore Software were used in the GenEx Software to analyze assay performance with respect to LoD, LoQ, and PCR efficiency. All of these parameters remained consistent across the plex formats as shown in Table 2. It is important to note that these synthetic DNA templates are not calibrated standards and the concentrations are estimated. Therefore, it is reasonable to expect some variability at the lower limit due to pipetting differences when creating the initial template stocks.

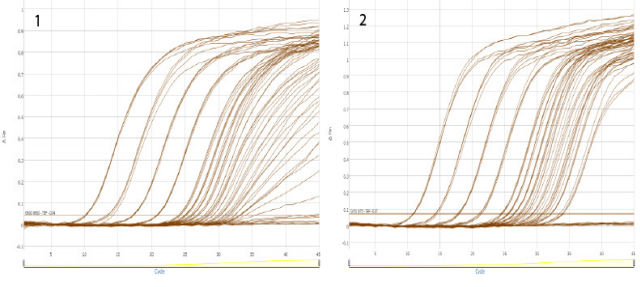

Figure 3. Amplification curves from the TBP assay representing the necessity of optimizing primer concentrations when performing multiplex reactions. The fanning effect observed in the left image represents un-optimized primer concentrations, while the image on the right shows appropriate curve shape after primer optimization. Synthetic template dilutions ranged from $1.25x10^7$ - 1.21 copies/µL. Amplification curves were generated using IntelliScore Software.

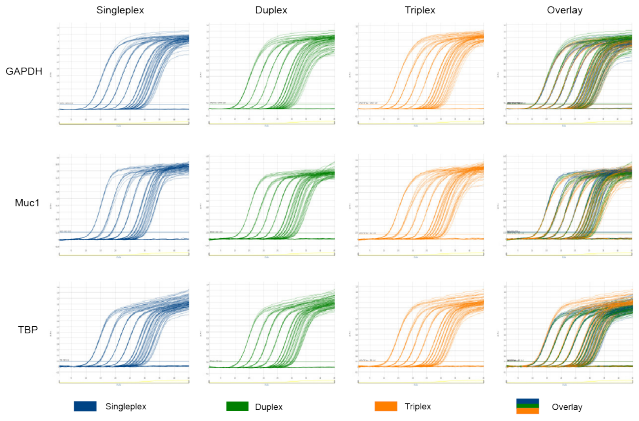

Figure 4. Side-by-side comparison and overlay of the amplification curves generated using DNA in singleplex, duplex, and triplex formats for GAPDH, Muc1, and TBP. The probe fluorophores used for the GAPDH, Muc1, and TBP assays were CAL Fluor Orange 560, FAM, and Quasar 670, respectively. The data was normalized with a ROX passive reference. Amplification curves cover the linear dynamic range of the concentrations tested for each assay using the IntelliQube. All amplification curves were generated using IntelliScore Software.

As a follow up, assay performance was assessed with RNA samples in a one-step RT-qPCR approach to verify performance in the expected sample matrix versus using DNA template only. The RT-qPCR amplification curves generated for each assay are shown in Figure 5. In contrast to the DNA templates with known concentrations, the RNA has varying levels of expression for each target, explaining the difference in the number of dilutions with observable amplification. Nevertheless, the PCR efficiencies for the assays tested were comparable between the two sample types as shown in Table 2.

After evaluating assay performance, gene expression of Muc1 was tested across three tissues types obtained from four different vendors. The assays were set up in singleplex, duplex, and triplex formats for plex comparison. The gene expression of Muc1 across the three tissues was measured in fold change using the comparative Cq method with TBP and GAPDH as reference genes. A three way ANOVA was used to identify the influence of multiplexing, tissue type, and run-to-run variables have on gene expression. The results of the ANOVA can be found in Table 3. Cq values were consistent between runs, regardless of the plex format as represented in Figures 6 and 7. Tissue type was the only factor that showed significant difference on the relative expression of Muc1 (Figure 8). There was no significant difference in expression based on plex format or between runs. Differences in Muc1 expression across tissue types are highlighted in Table 4. Muc1 was found to be upregulated in kidney 143-fold and lung 297-fold in comparison to liver as represented in Figure 8. These relative expression levels in each tissue correlated with published data (Song et al., 2013).

Table 1. Estimated LoD, LoQ, and PCR efficiency for GAPDH, Muc1, and TBP assays based on the amplification curves shown in Figure 4. The DNA concentrations ranged from $1.25x10^7$ down to 1.21 copies/µL. LoD and LoQ values (copies/reaction) were calculated using GenEx Software. IntelliScore Software was used to determine PCR efficiency. *Muc1 duplex reaction data is for the combination of Muc1 and TBP. SP = Singleplex, DP = Duplex, TP = Triplex.

	LoD			LoQ			Efficiency (%)		
Assay	SP	DP (TBP-Muc1)	TP	SP	DP (TBP-Muc1)	TP	SP	DP (TBP-Muc1)	TP
GAPDH	5	N/A	8	16	N/A	16	97.6	N/A	97.2
Muc1	7	3*	10	31	16*	16	98.4	96.7*	96.4
TBP	4	4	8	16	16	16	98.2	99.4	98.9

Table 2. Comparison of PCR efficiencies between DNA and RNA.

	Assay	R^2	PCR Efficiency
DNA	GAPDH	0.999	97.6
	Muc1	0.998	98.4
	TBP	0.999	98.2
RNA	GAPDH	0.998	93.8
	Muc1	0.997	96.2
	TBP	0.996	94.7

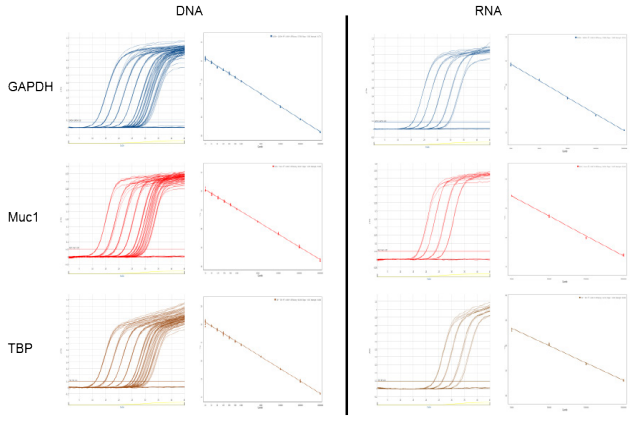

Figure 5. Amplification curves and standard curves comparing assay performance with DNA and RNA for GAPDH, Muc1, and TBP assays using the IntelliQube. DNA dilutions covered a concentration range of $1.25x10^7$- 1.21 copies/µL, while total RNA covered a concentration range of 62.5 ng/µL - 6.25 fg/µL. Only dilutions within the linear region of the standard curve are included in the amplification and standard curve plots. All amplification curves and standard curves were generated using IntelliScore Software.

Table 3. General reduced ANOVA table showing significance of each fixed effect (plex, tissue type, and between runs) on Muc1 expression. A Pr > F value below 0.05 was considered significant.

Type III Test of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
plex	2	6	0.52	0.6195
tissue	2	6	3433.89	<.0001
run	2	6	0.75	0.5102

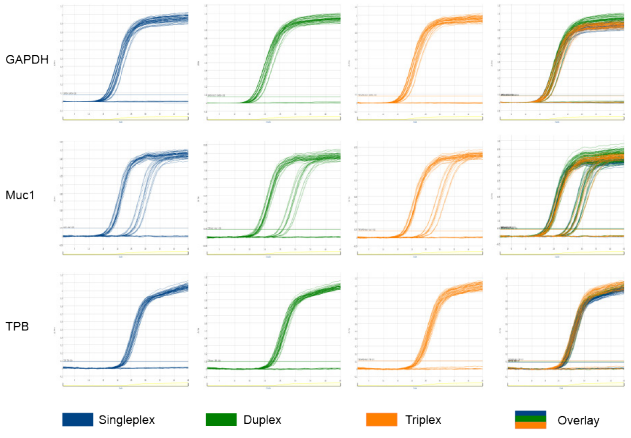

Figure 6. Side-by-side comparison and overlay of the one-step RT-PCR amplification curves in singleplex, duplex, and triplex formats generated during the gene expression testing on the IntelliQube. RNA samples representing three tissue types obtained from four different manufacturers were tested for Muc1 expression at a concentration of 50 ng of total RNA per reaction. The probe fluorophores used for the GAPDH, TBP, and Muc1 assays were CAL Fluor Orange 560, FAM, and Quasar 670, respectively. ROX dye was used as a passive reference. GAPDH and TBP were used as reference genes. All amplification curves were generated using IntelliScore Software.

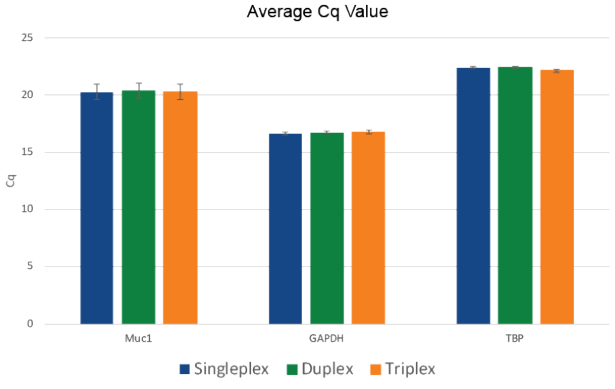

Figure 7. Average Cq values compared across the plex formats. Muc1, GAPDH, and TBP expression was tested at 50 ng of total RNA per reaction across three tissue types from four suppliers. Samples were tested in quadruplicate. Three individual runs were performed. IntelliScore Software was used to obtain Cq values. The error bars represent the standard error of the mean.

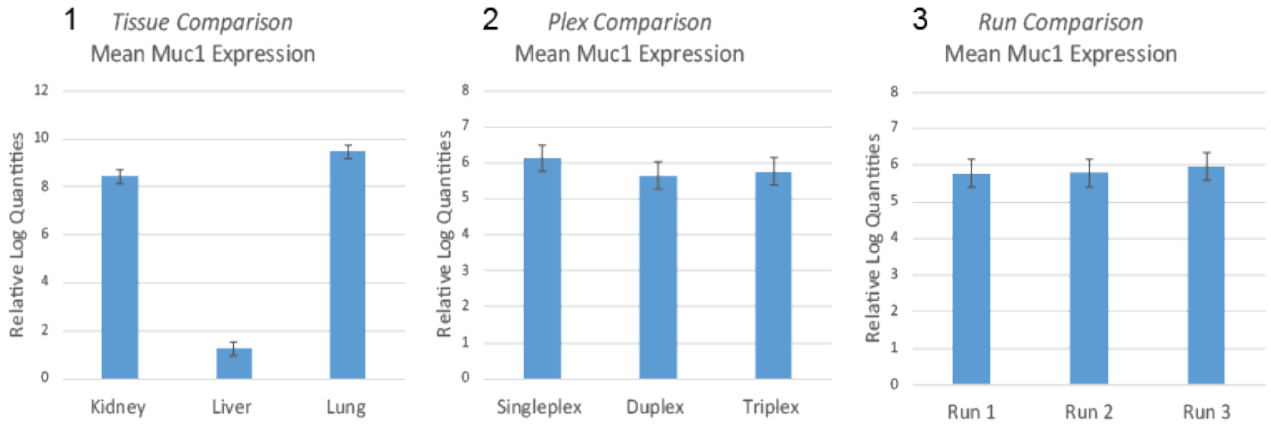

Figure 8. A comparison of the mean relative log quantities of Muc1 expression with respect to 1) tissue type, 2) plex format, and 3) run-to-run. The error bars represent the standard error of the mean.

Table 4. Difference in Muc1 expression between the three tissue types displayed in log scale and fold change including confidence intervals and p-values for comparison. P-values are adjusted for multiple comparison and a value below 0.05 was considered significant.

Tissue	Log2 scale			Fold change			Adj P
	Difference	Lower CI	Upper CI	Defference	Lower CI	Upper CI	
Kidney x Liver	7.166	6.8349	7.4971	143.6088	114.159	180.656	<.0001
Kidney x Lung	-1.0499	-1.381	-0.7188	0.483002	0.38395	0.6076	0.0002
Liver x Lung	-8.2159	-8.547	-7.8848	0.003363	0.00267	0.00423	<.0001

Conclusion

This study demonstrates the ability of the IntelliQube and associated Array Tape technology to successfully multiplex BHQ qPCR assays in a one-step RT-PCR format for gene expression studies, without compromising data quality. This particular study utilized three probe dyes and a passive reference dye for multiplexing. Future study is planned to add an additional target in order to take advantage of all fluorescence channels on the IntelliQube. It is important to note that in order to obtain the benefits of multiplexing, upfront assay optimization work will be required in most cases, regardless of the instrumentation used.

In addition to miniaturizing reactions in Array Tape, the ability to screen multiple targets in a single reaction further reduces the amount of time and reagents required. The IntelliQube integrates liquid handling, thermal cycling, and detection within a single instrument, reducing the number of manual liquid handling steps and subsequently minimizing pipetting errors. Furthermore, the ability to process samples in a walk-away system enables laboratories to improve overall efficiency. In addition to gene expression analysis, the ability to process RNA samples in a one-step RT-PCR process opens up the door to other RNA-based applications such a virus detection and other research. Therefore, with the use of BHQ probes, the IntelliQube is a great addition to laboratories seeking to increase throughput and efficiency, while maintaining high quality one-step RT-PCR results.

References

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