Application note
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BHQ® Probe Assay Development for Animal Health Screening on the IntelliQube®

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Abstract

Polymerase chain reaction (PCR) is a popular method for detection and quantification of microorganisms. Real-time quantitative PCR (qPCR) methods are becoming increasingly sensitive, fast, and economical, making them a critical aspect of routine animal health diagnostics. In this study, we demonstrated the performance of dual-labeled BHQ probe-based assays for the detection and quantification of microbial pathogens on the IntelliQube real-time PCR instrument. Primer and probe sequences were designed using RealTimeDesign™ software. Assay performance was assessed following recommendations from the MIQE guidelines using a synthetic DNA template to measure PCR efficiency, linear dynamic range, Limit of Detection (LoD), and Limit of Quantification (LoQ) (Bustin et al., 2009). With the automated workflow and economic benefits of Array Tape®, the IntelliQube in conjunction with BHQ probes may prove to be an effective platform in animal health screening and animal diagnostics.

Introduction

Nucleic acid testing is a powerful tool in animal health diagnostics for the detection and quantification of microorganisms. In particular, quantitative PCR (qPCR) is becoming increasingly popular as it offers accurate and sensitive detection of pathogens with less time and effort compared to conventional culture and ELISA based methods. Early detection of pathogens such as bacteria, viruses, and parasites, in animals, can assist in the identification of appropriate solutions to manage diseases that threaten public health, endanger food supplies, or jeopardize the economic security of the animal industries. Timely and accurate pathogen detection can direct the treatment of livestock in an efficient and cost effective manner, reducing the extent of morbidity and mortality associated with microbial infections. Therefore, development of accurate, fast, and economical methods for microbial detection and quantification is of the utmost importance for ensuring the safety and productivity of our animal industries. While several methods and instruments for animal health screening currently exist, there remains an unmet need for a fully-automated qPCR method that gives laboratories the flexibility and scalability necessary to expand the availability of these tests to farmers, veterinarians, and researchers.

The IntelliQube real-time PCR instrument (Figure 1) used in conjunction with dual-labeled BHQ probes and primers from LGC Biosearch Technologies is well suited to address this need 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 quantitative PCR (qPCR) analysis in miniaturized reaction volumes (1.6 µL). In this study, RealTimeDesign software was used to design primers and BHQ probes targeting *Escherichia coli* O157, *Salmonella enterica*, *Pasteurella multocida*, *Mycobacterium avium* subsp. *paratuberculosis*, *Tritrichomonas foetus*, *Cryptosporidium parvum*, and chicken anemia virus. These targets were selected to represent a range of pathogens impacting a variety of animal species and to characterize assay performance on the IntelliQube system.

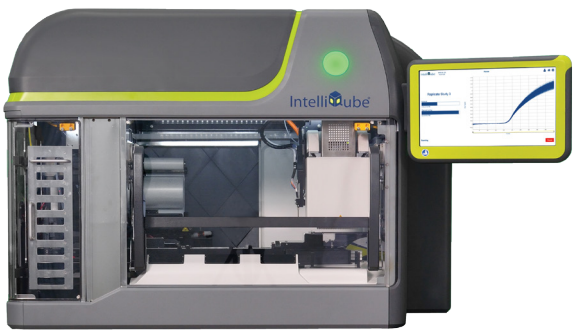

Figure 1: The IntelliQube is a fully integrated liquid handling and real-time quantitative PCR instrument optimized for use with miniaturized reactions in 384- or 768-well Array Tape.

Materials and Methods

Gene Target Identification: Relevant genes for pathogen detection were identified, and the specificity of the sequences to their respective targets was verified by

NCBI BLASTN program version 2.2.29 (Altschul et al., 1990). Gene information can be found in Table 1.

Table 1. Assay target information

Pathogen	Class	Target Gene
Escherichia coli O157	Gram (-) Bacteria	gb|CP015846.1 |:2874415-2875509
Pasteurella multocida	Gram (-) Bacteria	gb|KP660556.1
Salmonella enterica	Gram (-) Bacteria	gb|M90846.1
Mycobacterium avium subsp. *paratuberculosis*	Gram (+) Bacteria	gb|AF503873.1
Cryptosporidium parvum	Protozoan Parasite	gb|FJ379573.1
Tritrichomonas foetus	Protozoan Parasite	gb|AF339736.1
Chicken anemia virus	ssDNA Virus	gb|KF318726.1

Assay Design: BHQ probes and primers were designed using RealTimeDesign software (oligo sequence information availible upon request). The BHQ probes were designed with a FAM fluorophore. NCBI Primer-Blast was used to verify specificity of the primers and probes to their respective targets (Ye et al., 2012). Primer-Blast gives a good indication to the specificity of the assay, however PCR testing for specificity across expected off-target contaminants should be performed.

Synthetic DNA Template Preparation: Synthetic DNA templates (500 base pairs) were synthesized and 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 synthetic DNA templates with water. Serial dilutions were prepared with water containing 1 ng/µL salmon sperm DNA acting as a carrier. Serial dilutions ranged from 1.25 x 10^7 to 1.21 copies/µL. A representation

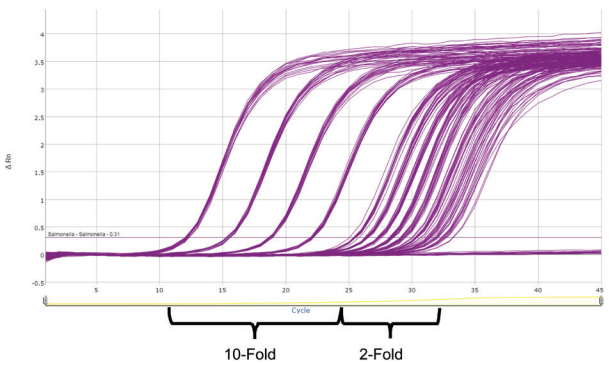

Figure 2. Amplification curves from the *Salmonella enterica* assay representing the DNA dilution series used to assess assay performance. The 10-fold dilutions ranged from 1x10^7- 1x10^3 copies/reaction. Two-fold dilutions ranged from 1x10^3 to 0.98 copies/reaction. This dilution series was used for all assays tested.

of the dilution scheme can be found in Figure 2.

Master Mix: A commercially available PCR master mix was used according to the manufacturer's instructions for all reactions. The assays were added at 2X concentration to the 2X master mix to achieve a final concentration of 1X (200 nM probe/600 nM primer) in the PCR reaction.

Instrumentation and Software: The IntelliQube was used for reaction set-up, thermal cycling, and real-time fluorescence detection. DNA samples (800 nL) were dispensed into Array Tape with the multi-channel CyBio® Dispense Pipette. Master mix containing 2X assay (800 nL) was dispensed with the non-contact Dispense Jet to create 1.6 µL reactions. A total of 16 replicates of each dilution were dispensed. Fluorescence values were obtained at the end of each cycle over the course of 45 cycles to generate real-time amplification curves and calculate Cq values using the IntelliScore® Software. GenEx PCR Analysis Software (MultiD) was used to determine LoD, LoQ, and PCR efficiencies.

Results and Discussion

Seven BHQ probe-based assays, each designed to detect a unique microbial pathogen, were successfully designed and tested in conjunction with the IntelliQube. Synthetic DNA templates for each target region were used to assess assay performance in Array Tape using 1.6 µL reactions. Assay performance was characterized using a dilution series to assess LoD, LoQ, standard curve linearity, and overall PCR efficiency. The amplification curves generated for each assay are shown in Figure 3. Cq values obtained from the IntelliScore Software were used in GenEx software to further analyze performance.

LoD is defined as the lowest amount of analyte in a sample that can be detected within a stated probability (Tholen et al., 2004). A probability of 95% is commonly practiced (Kubista, 2014). Using this criteria, we can calculate the theoretical LoD by qPCR due to sampling ambiguity. This is the lowest concentration that leads to at least 95% of the sample replicates being positive. All seven assays were tested over a range of seven logs (10^7 - 0.98 DNA copies/reaction). The LoD values calculated for each assay are shown in Table 2. Pathogen detection limits were maintained at or below 9 copies/reaction for all assays. It is important to note that these synthetic templates were not considered calibrated stocks and therefore it is reasonable to expect some variability at the lower limit due to pipetting differences when creating dilutions.

LoQ is referred to as the lowest amount of analyte in a sample that can be quantified with acceptable precision and bias under stated experimental conditions (Tholen et al., 2004). A commonly used criteria is that the LoQ

Table 2. Assay performance showing Limit of Detection (LoD) and Limit of Quantification (LoQ) values based on copies/reaction for each assay. The standard curves including dilutions at and above the LoQ were used to determine the slope, intercept, R^2, and PCR efficiency for each of the assays listed. The assays were tested in 1.6 µL reactions on the IntelliQube. LoD and LoQ data was generated using GenEx Analysis Software from MultiD. IntelliScore Software was used to determine slope, intercept, R^2, and PCR efficiency.

Pathogen	Slope	Intercept	R^2	Efficiency (%)	LoD	LoQ
Escherichia coli O157	-3.37	36.6	.999	98.2	8	16
Pasteurella multocida	-3.41	36.5	.999	96.5	5	16
Salmonella enterica	-3.31	35.3	.999	100.5	4	8
Mycobacterium avium subsp. *paratuberculosis*	-3.31	35.4	.999	100.4	9	16
Cryptosporidium parvum	-3.41	36.2	.999	96.5	9	16
Tritrichomonas foetus	-3.39	36.5	.999	97.3	7	16
Chicken anemia virus	-3.34	35.6	.999	99.1	4	16

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 (Kubista, 2014). The LoQ for each assay can be found in Table 2. All seven assays showed an estimated LoQ of 16 copies/reaction, except for the Salmonella enteric assay which had a LoQ calculated at 8 copies/reaction. The spread in replicates did increase at the lowest concentration of target molecules as can be seen from the standard curves generated with the IntelliScore Software (Figure 4), as is expected due to sampling ambiguity. As the number of target molecules per reaction volume decreases, the reproducibility is compromised, which is best explained by the Poisson distribution. The small concentration difference seen between the LoD and LoQ of each assay highlights the precision allowed by the integrated liquid handling system within the IntelliQube.

After establishing the LoQ for each assay, standard curves were analyzed omitting data points below the LoQ threshold. These standard curves shown in Figure 4 were used to evaluate PCR efficiency and linearity. With the concentrations tested, all seven assays showed a linear dynamic range (LDR) ≥ 5 logs. The LDR represents the highest to the lowest DNA copy number that a given assay can quantify (Bustin et al., 2009). For the purposes of this study, concentrations greater than $1x10^7$ were not used, and it is likely this range could be increased by adding more concentrated samples. The estimated slope, intercept, and PCR efficiency for each assay can be found in Table 2. All assays had efficiencies >96%, which is with well within the acceptable range.

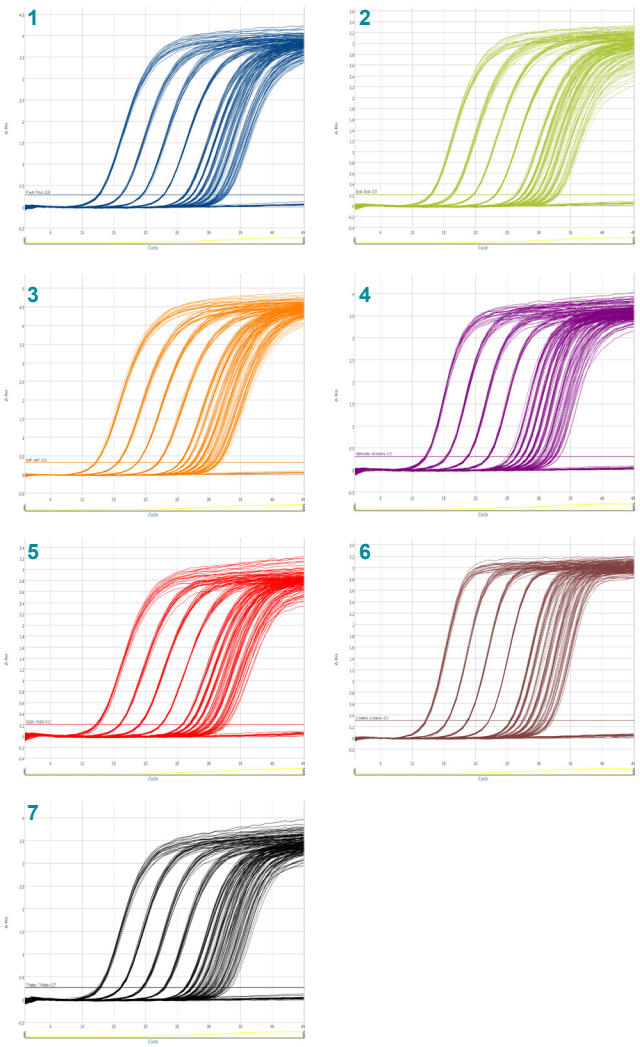

Figure 3. Amplification curves covering the linear dynamic range of the DNA concentrations tested for *Pasteurella multocida* (1), *Escherichia coli* O157 (2), *Mycobacterium avium* subsp. *paratuberculosis* (3), *Salmonella enterica* (4), *Cryptosporidium parvum* (5), Chicken anemia virus (6), and *Tritrichomonas foetus* (7) assays using the IntelliQube. All amplification curves were generated using IntelliScore Software.

Conclusion

These results highlight the successful design and performance of seven microbial dual-labeled BHQ qPCR assays run in a miniaturized format with the IntelliQube. Future testing would require verification of assay target specificity and performance with genomic DNA, although this was outside the scope of this work. In addition to assay miniaturization, the IntelliQube integrates liquid handling, thermal cycling, and detection within a single instrument to reduce the number of manual liquid handling steps and subsequently minimize pipetting errors. Furthermore, the ability to process samples in a walk-away automated instrument system reduces hands-on time and enables laboratories to improve overall efficiency. Taken together, these benefits make the IntelliQube and associated BHQ probe chemistry an attractive system for animal health diagnostics, allowing laboratories to screen samples in a timely, accurate, and economical fashion for better disease management.

References

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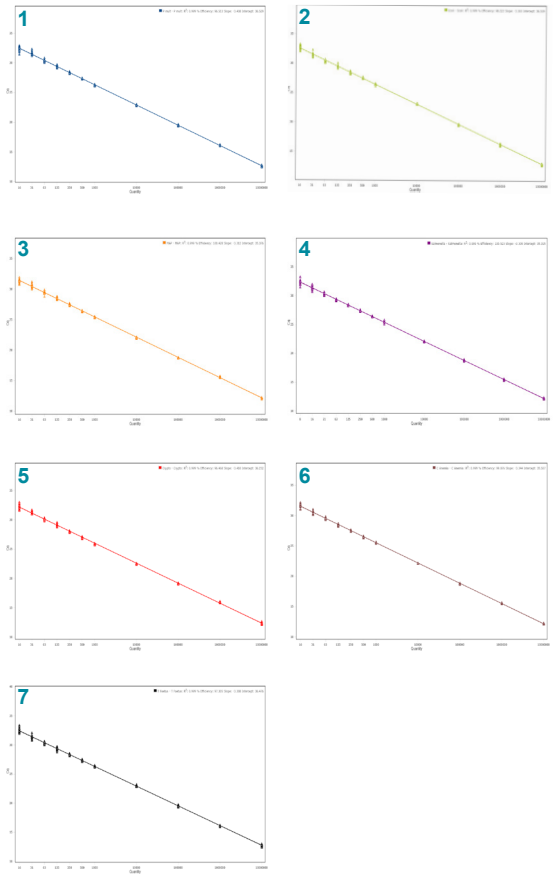

Figure 4: The standard curves using concentrations at and above the LoQ were used to calculate PCR efficiency and linearity for *Pasteurella multocida* (1), *Escherichia coli* O157 (2), *Mycobacterium avium* subsp. *paratuberculosis* (3), *Salmonella enterica* (4), *Cryptosporidium parvum* (5), Chicken anemia virus (6), and *Tritrichomonas foetus* (7). The assays were tested on the IntelliQube and all plots were generated using IntelliScore Software.