Development of a Multiplex Real-time PCR Assay for the Detection of *Campylobacter* Species for use on a Food Security System Platform

P2-30

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ABSTRACT

Introduction
It is estimated that campylobacteriosis affects over 2.4 million people each year. The United States Department of Agriculture's Food Safety and Inspection Service is currently in the process of setting standards for acceptable levels of *Campylobacter* species.

Purpose
In an effort to meet the upcoming *Campylobacter* species testing regulations, Idaho Technology has developed a quantitative, multiplex, real-time PCR assay for the detection of *Campylobacter* species for use on the R.A.P.I.D.® LT Food Security System (FSS) platform.

Methods
A real-time PCR assay using hybridization probes was designed to detect *Campylobacter* species. This multiplexed assay detects a target and an amplification control in separate fluorescent channels. Assay sensitivity was evaluated on serial ten-fold dilutions of purified nucleic acid. Live organism quantification was evaluated using an external standard curve made from PCR amplified *Campylobacter jejuni* subsp. *jejuni* nucleic acid.

Results
The multiplex PCR assay is sensitive to approximately 10 copies of genomic DNA (85% success and 90% confidence). The linear range of the external standard curve was between 100,000 and 1,000 copies. Standard curves specific to relevant sample matrices are being incorporated into the R.A.P.I.D.® LT FSS software.

Significance
The FSS software is flexible and can be adapted to any regulations that will be established. Incorporation of relevant standard curves into the R.A.P.I.D.® LT FSS software minimizes set-up time and provides a tunable method to detect relevant levels of *Campylobacter* species. Final development of this assay will provide customers with an easy, rapid testing method for *Campylobacter* species.

- **E**asy to use PCR instrument
  - Freeze-dried reagents/no proprietary media
  - Designed for minimally trained users
- **A**ccurate and specific pathogen testing
  - Double specificity (primer + probe)
  - Extensive scientific support & expertise
- **T**imely results
  - Shortened enrichment times
  - PCR in under an hour

METHODS

Background
Currently, there are Compliance Guidelines for the testing of *Campylobacter*, but no official regulations. In preparation for the release of these regulations, Idaho Technology, Inc., has developed a multiplexed, semi-quantitative, real-time PCR assay for use on the R.A.P.I.D. LT Food Security System (FSS) platform. To accomplish this, an amplification control (AC) was added to the previously designed *Campylobacter* biothreat assay to maintain the FSS multiplexed assay format.

The R.A.P.I.D. LT FSS is a PCR-based detection method used to detect pathogens from enriched food samples. In general, the method involves enriching a sample for a specified amount of time in commercially available media. Following this, mechanical cell lysis is performed to release the DNA within the pathogen. Freeze-dried PCR reagents are then rehydrated and DNA amplification and melting peak analysis are performed on the R.A.P.I.D. LT instrument using glass capillaries. Data and results are automatically interpreted by the R.A.P.I.D. LT software.

Multiplex PCR
The R.A.P.I.D. LT FSS utilizes multiplexed PCR techniques. Sequence specific, shared primers are used to amplify both the target and the internal AC DNA. Hybridization probes, utilizing fluorescence resonance energy transfer (FRET) chemistry, add specificity to the real-time PCR assay and are used for fluorescent detection (Figure 1A). For the target, a 470 nm wavelength light source excites the donor fluorophore (Figure 1A). That energy is transferred to the acceptor molecule. The acceptor molecule for the target assay then emits light at 640 nm which is detected in Channel 2 (Figure 1B), the target channel. The AC works similarly, however, the acceptor molecule emits light at 705 nm which is detected in Channel 3 (Figure 1C).

Figure 1A. Hybridization Probe Multiplex PCR Schematic

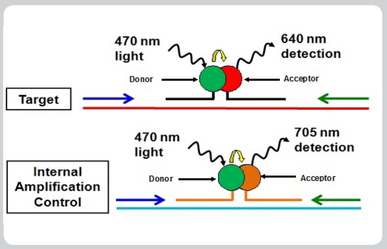


Figure 1B. Multiplexed PCR Amplification Curves (640 nm Detection)

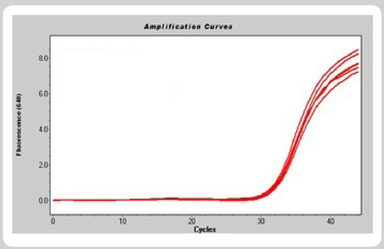
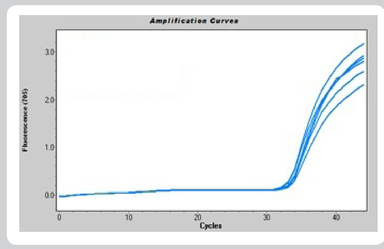


Figure 1C. Multiplexed PCR Amplification Curves (705 nm Detection)



Melting Curve Analysis
Following PCR, a short melting analysis is performed. Melting analysis can be used to both increase assay sensitivity and to determine differences in amplified products. Initially, the sample is cooled to allow the hybridization probes to bind to the available amplified DNA (Figure 2A). Because the hybridization probes are in close proximity, the fluorescence is high. The sample is then heated slowly. During the heating step, the hybridization probes melt off of the DNA strand. This causes the probe pair to separate which in turn leads to a decrease in fluorescence. Melting curves are then generated by plotting fluorescence versus temperature (Figure 2B). Melting peaks are automatically generated by the R.A.P.I.D. LT software by taking the negative derivative of the melting curve (Figure 2C) and plotting it over temperature. If there are sequence variations underneath the hybridization probes, the probes will become less stable and will melt off the DNA at a lower temperature. An example of this can be seen in Figures 2B and 2C with the outlier gray sample marked with an arrow.

Figure 2A. Melt Analysis Schematic

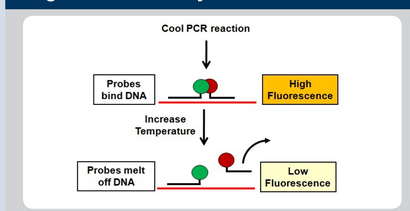


Figure 2B. Melting Curve Analysis

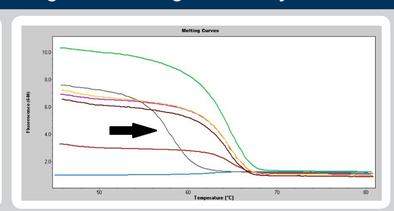
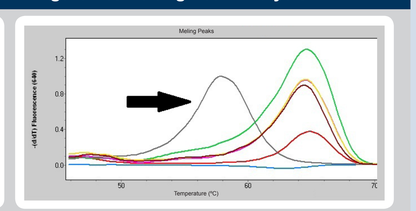


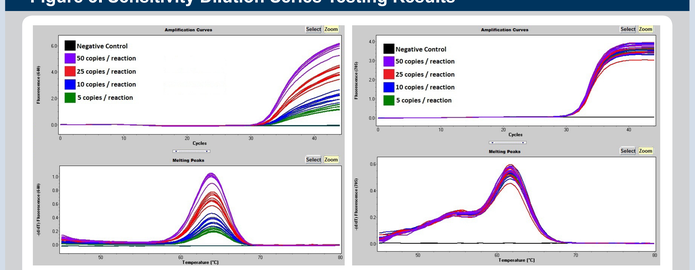
Figure 2C. Melting Peak Analysis



Assay Development
It is estimated that campylobacteriosis affects over 2.4 million people each year. According to the Food and Drug Administration, *Campylobacter jejuni* is the leading cause of bacterial diarrhea in the United States. Most cases of campylobacteriosis are associated with eating raw or undercooked poultry meat or from cross-contamination of other foods by these items. To develop this PCR assay, sensitivity, quantification, and specificity studies were initiated.

Sensitivity
Assay sensitivity in the multiplex format (target + AC) was evaluated using purified DNA isolated from *C. jejuni* (ATCC BAA 1062). The appropriate range for sensitivity was determined by evaluating the multiplexed assay using 10-fold dilutions of purified *C. jejuni* genomic DNA. The AC was held at a constant level in all reactions tested. Sensitivity for the assay was determined to be below 10 copies per reaction when multiplexed (Figure 3).

Figure 3. Sensitivity Dilution Series Testing Results

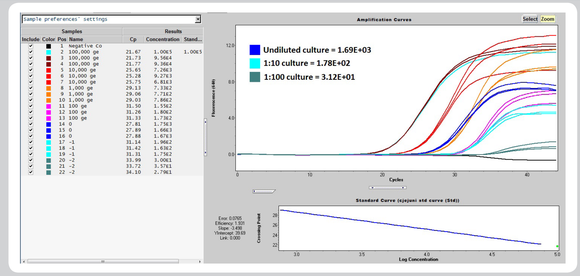


Quantification Feasibility
Using purified DNA isolated from *C. jejuni* subsp. *jejuni* (ATCC 33560), dilution experiments were performed in 10-fold serial dilution steps. Using the R.A.P.I.D. LT software to assign concentration standards, a dilution series of *C. jejuni* genomic DNA was created as an external standard curve.

C. jejuni subsp. *jejuni* (ATCC 33560) was enriched for 46 hours at 37°C in 5 ml of Trypticase Soy Broth™ (TSB) within a microaerophillic atmosphere provided by a large BD Gas-pak™ EZ container system (BD Diagnostic Systems No.:260672) with three Campy Container Sachets (BD Diagnostic Systems No.:260680). Two 10-fold serial dilutions were made in TSB. 5µl of the undiluted culture and each dilution were added to FSS bead beating tubes and disrupted for 5 minutes. From these tubes of lysate, 10µl was combined with 10µl purple reconstitution buffer and freeze-dried *Campylobacter* reagents. Also included in the experiment was a *C. jejuni* subsp. *jejuni* (ATCC 33560) genomic nucleic acid serial dilution.

These organism and nucleic acid dilutions were analyzed using the external standard curve created from the *C. jejuni* subsp. *jejuni* (ATCC 33560) dilution experiment. (Figure 4).

Figure 4. Quantification of *Campylobacter* Using an External Standard Curve



The results of this experiment show that quantifying *Campylobacter* populations in culture is experimentally feasible via the use of standard curves.

Specificity
The *Campylobacter* assay specificity was evaluated against panels of purified nucleic acid and bacterial cultures. Inclusivity testing was performed against *C. jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis* with purified nucleic acid obtained from ATCC (Table 1). Each strain was tested at approximately 2,500 copies per reaction in triplicate reactions. Each of the strains evaluated tested positive on the R.A.P.I.D. LT FSS.

Figure 5. Purified Nucleic Acid *Campylobacter* Inclusivity Testing Results

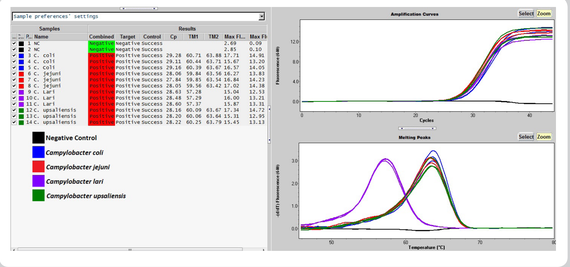


Table 1. Inclusivity Panel of Purified Nucleic Acid *Campylobacter* Isolates

Organism	Strain	Source
Campylobacter coli	BAA-1061	ATCC
Campylobacter jejuni	BAA-1062	ATCC
Campylobacter lari	BAA-1060	ATCC
Campylobacter upsaliensis	BAA-1059	ATCC

The bacterial cultures, grown in-house, were tested for inclusivity. Strains of *C. jejuni*, *C. coli, C. lari, and C. upsaliensis* (Table 2) were enriched for 46 hours at 37°C in 5mL of TSB in a microaerophilic atmosphere as previously described. Five µL of each culture was then added to an FSS bead beating tube and disrupted for 5 minutes. The R.A.P.I.D. LT resulted in positive calls for each of the cultures tested with the exception of the *C. upsaliensis* culture, which showed no visible culture growth. As previously mentioned, testing with *C. upsaliensis* purified genomic DNA resulted in a positive call.

Figure 6. Bacterial Cultures of *Campylobacter* Species Testing Results

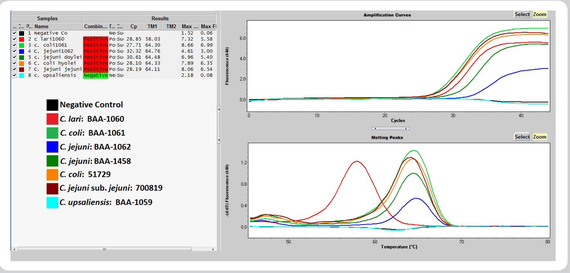


Table 2. Inclusivity Panel of Liquid *Campylobacter* Cultures

Organism	Strain	Source
Campylobacter coli	51729	ATCC
Campylobacter coli	BAA-1061	ATCC
Campylobacter jejuni	BAA-1062	ATCC
Campylobacter jejuni sub. *jejuni*	700819	ATCC
Campylobacter jejuni sub. *doylei*	BAA-1458	ATCC
Campylobacter lari	BAA-1060	ATCC
Campylobacter upsaliensis	BAA-1059	ATCC

Exclusivity testing was performed against 25 *Campylobacter* nearest neighbor purified genomic nucleic acid isolates (Table 3). Exclusivity isolates were tested at 1ng per reaction in duplicate reactions. All isolates tested negative by real-time PCR.

Table 3. Exclusivity Panel *Campylobacter* Nearest Neighbor Purified Nucleic Acid Isolates

Arcobacter butzleri (2)	*Helicobacter spp.* (1)	*Micrococcus luteus* (1)
Arcobacter cryaerophilus (1)	*Helicobacter pylori* (10)	*Morganella morganii* (1)
Bacillus cereus (2)	*Klebsiella pneumoniae* (1)	*Pantoea agglomerans* (1)
Carnobacterium piscicola (1)	*Lactobacillus* (1)	*Pseudomonas fluorescens* (2)
Citrobacter freundii (2)	*Listeria ivanovii* (1)	*Rhodococcus equi* (1)
Escherichia coli (14)	*Listeria monocytogenes* (9)	*Salmonella enterica* (15)
Enterobacter cloacae (2)	*Listeria seeligeri* (2)	*Staphylococcus aureus* (1)
Enterococcus faecium (2)	*Listeria welshimeri* (1)	*Shigella flexneri* (1)
		Yersinia entercolitica (1)

(#) = Number of isolates tested

CONCLUSIONS AND SIGNIFICANCE

- Sensitivity for the *Campylobacter* assay was determined to be 10 copies per reaction at a minimum of 85% success.
- Specificity for the *Campylobacter* assay was 100% for all isolates tested.
- Quantification is feasible using this assay. The incorporation of relevant standard curves minimizes setup time and provides a tunable method to detect relevant levels of *Campylobacter* species.
- The R.A.P.I.D. LT FSS is flexible and can easily be adapted to new or modified regulations.
- When regulations for *Campylobacter* are implemented, this assay will be evaluated as an FSS test kit for production and commercial sale.

ACKNOWLEDGEMENTS
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