Proposal for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Anita Wright
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Proposal Subject:	Alternative analytical method for *Vibrio vulnificus, Vibrio cholerae, Vibrio parahaemolyticus*
Specific NSSP Guide Reference:	Section IV. Guidance Documents Chapter II Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods. (5) Interim Approval by ISSC Executive Board August 2007
Text of Proposal/ Requested Action	Text of proposal: See attached proposal Requested actions: Accept the adoption of DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit as an alternative analytical protocol to determine the levels of *Vibrio vulnificus, V. cholerae, V.parahaemolyticus*
Public Health Significance:	Proposed method will greatly improve the speed of analysis to help the industry to increase the amount of PHP products in the market. For details see attached proposal
Cost Information (if available):	See attached proposal.
Action by 2009 Laboratory Methods Review Committee	Recommended referral of Proposal 09-102 to appropriate committee as determined by Conference Chairman. Rationale: Additional data under development.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-102.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 09-102.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 09-102.

Research Need for Consideration at the Interstate Shellfish Sanitation Conference 2011 Biennial Meeting	
Name of Submitter:	Anita Wright
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Proposed Specific Research Need/Problem to be Addressed:

Improve the speed of analysis to help the industry to increase the amount of PHP products in the market.

How will addressing this research support/improve the mission/role of the ISSC/NSSP/Industry? Support need with literature citations as appropriate.

See attached description

Relative Priority Rank in Terms of Resolving Research Need:

Immediate	☒	**Important**	☐
Required	☐	**Other**	☐
Valuable	☐		

Estimated Cost:

Proposed Sources of Funding/Support:

Time Frame Anticipated: 2009-2010

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**QPCR-MPN Assay using DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for Rapid Detection of *Vibrio* speices in seafood**
Name of the Method Developer	Anita Wright et. al.
Developer Contact Information	Anita Wright 461 AFPL bldg. Newell Dr. Gainesville, FL 32611 352-392-1991 ext. 311

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	An alternative method to confirm vibrio bacteria in shellfish
2. What is the intended purpose of the method?	Y	Replace confirmation step in MPN determination of Vibrios in shellfish
3. Is there an acknowledged need for this method in the NSSP?	Y	End users are requiring faster more economical alternatives to the current approved method
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Quantitative PCR
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	n/a	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	
8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	N/A	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments: See attached application document.		

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** – The fraction or percentage of an analyte or measure and recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** – the range of analyte or measure and concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.

4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Title: QPCR-MPN Assay using DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for Rapid Detection of *Vibrio* species in seafood

JUSTIFICATION FOR NEW METHOD

This protocol is submitted for approval to the Laboratory Methods Review Committee. This proposal was prepared to support the use of a new molecular detection method: **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for** rapid detection of *Vibrio cholerae, V. parahaemolyticus, and V. vulnificus* It will be used in conjunction with current Vibrio MPN assay and will substitute for the use of DNA probe colony hybridization for confirmation of the presence of *Vibrio* species (8). Method was developed by collaborative efforts of Dr. Anita Wright, Dr. Steve Otwell, Victor Garrido, Charlene Burke, and Melissa Evans, University of Florida, Gainesville, Florida and DuPont Qualicon Laboratories. The QPCR method was recently approved for American Organization of Analytical Chemists (AOAC) and has been accepted for publication by the Journal of AOAAC: Morgan Wallace, Anita Wright, Tim Dambaugh, Monica Kingsley, Chris Malota, Bridget Andaloro, Dawn Fallon, Daniel Delduco, George Tice and, DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for the Detection of *Vibrio cholera, parahaemolyticus and vulnificus* from Tuna, Shrimp and Oysters, *AOAC Performance Tested Methods* (15)

The QPCR-MPN method described herein provided increased assay sensitivity and reduced both time and labor costs. Detection of *Vibrio* species was achieved at levels < 30 CFU/g as required for validation protocols (2, 10, 16). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing. The oyster industry's livelihood will be determined by their ability to adapt to FDA demands, and evolving technological breakthroughs. Until this demand has abated, the industry and the scientific community will continue to work in conjunction to learn more and thus protect the public from Vibrio disease.

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Date of Submission
Proposal submission date is June 20, 2009.

Purpose and Intended Use of the Method. *Vibrio* species are responsible for 75% of seafoodborne bacterial infections and 95% of related fatalities (7). *V. vulnificus* the leading cause of death in the US related to seafood consumption and is predominantly associated with consumption uncooked Gulf Coast oysters. *V. parahaemolyticus* is the most common source of outbreaks of infectious disease related to seafood, and *V.*

cholerae contamination threatens the safety of imported seafood products. The proposed method will benefit the seafood industry and the consumer by providing improved, faster, and more accruate deteiction of these pathogens in oysters and other seafood products. This method is being proposed for use in screening potential contamination of seafood products and for validation of Post Harvest Processing (PHP) protocols, as well as for future applications to assure the public of a safer product.

Need for the New Method in the NSSP

QPCR-MPN assay described herein is proposed as an alternative to the standard MPN assay for enumeration of *Vibrio* species using most probable number (MPN) end-point titration of replicate samples in enrichment broth cultures (4, 17). The current standard protocols described in the FDA Bacteriological Analytical Manual (BAM) use growth in enrichment broth, followed by isolation of typical colonies on selective agar medium with subsequent confirmation of each species by DNA probe (16), PCR, or biochemical profiling (8). This method is laborious cost prohibitive, labor intensive, and time consuming (6, 8). Enumeration of multiple *Vibrio* species requires isolation on different selective agars followed by separate confirmation tests that are different for each species. Furthermore, users of this protocol have expresssed difficulty with DNA probe product reliability and plating problems related to "spreading" colonies that interfer with the assay. Total amount of time to perform the traditional MPN method with DNA colony blot hybridization as a confirmatory method is at least 4 days, with numerous steps; additionally, technician requires a great deal of experience in performing this assay for successful quantification to be possible. QPCR-MPN method reduces working time half and offers greater sensitivity for detection of *V. vulnificus*; with detection of 1 bacterium per gram post enrichment in alkaline peptone water (APW) overnight (1, 4, 9, 10, 11, 17).

Although PHP methods are currently employed on < 10% of all domestic raw oyster sales in the United States, the industry continues to examine and employ new technologies and take initiative on expanding acceptance and knowledge regarding these treated oyster products (5). The industry is investing money and resources to ensure a market acceptance by educated oyster public, in addition to mitigating risk potential for the at risk consumers of fresh oysters. ISSC mandated that 25% of oysters havested from the Gulf of Mexico receive some type of validated post havrest processing. Thus, there is an urgent need for improved and more rapid validation methods.

The University of Florida has partnered with several dealers who are using ISSC methods for validation of oyster PHP. Work supporting this proposal was perfomred in 2007-2009 working with mild heat treatment (Panama City), nitrogen freezing (Leavin's seafood) and blast freezing (Buddy Ward's Seafood). Throughout the validation, samples were randomly selected for side-by-side comparisons of standard MPN described by the FDA BAM (8) to MPN using the DuPont Bax QPCR for MPN species-specific identification. Test results support the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster PHP, which was described in a publication by Wright et al., 2007.

Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types

This method is specific to applications testing growth of *V. cholerae, V. parahaemolyticus, and V. vulnificus* in MPN enrichment of oyster homogenates. This QPCR method does not claim to differentiate between pathogenic and nonpathogenic *Vibrio* species. Method was found to be appropriate for up to 1g of oyster tissues. QPCR-MPN provided more sensitive detection than standard MPN, as enriched samples that were PCR positive but negative on selective media were falsely negative on mCPC, as indicated by agreement of positive mCPC and QPCR results in more diluted inocula of the same sample (16). The result is an increase in sensitivity and a reduction in time and labor costs while still permitting detection of *Vibrios* at levels < 30 CFU/g as required for validation protocols (2, 10, 16). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing.

METHOD DOCUMENTATION

Method Title
QPCR-MPN Assay using DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit

Method Scope
This method is designed for MPN analysis of validation trials for oyster PHP and for detection of *Vibrio* species in seafood and monitoring shellfish harvesting waters.

Principle
QPCR-MPN will be substituted as an alternative to the officially recognized NSSP method for MPN analysis of validation trials for oyster PHP (3). Specifically QPCR will be substituted for microbiological/DNA probe confirmation of *V. cholerae, V. parahaemolyticus, and V. vulnificus* following growth in MPN enrichment. Since the FDA and the ISSC have mandated postharvest processing (PHP) of oysters harvested from Gulf Coast states in order to reduce *V. vulnificus* infections validation and verification are necessary in order to ensure that the process will substantially reduce numbers of *V. vulnificus* bacteria to levels to below the predicted threshold for disease. QPCR-MPN is a rapid and reliable method to accomplish agency mandates and industry goals. Validation criteria was recently expanded to include reduction of *V. parahaemolyticus* in PHP oysters. Application to evaluation of other seafood products is also anticipated, especially imported products that may be a greater risk for *V. cholerae* contamination

Proprietary Aspects
Ingredients in **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit** are proprietary information.

Equipment
Applied Biosystems Inc real-time thermocycler 7500S

Reagents
- **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit**
- SYBR green I (Invitrogen)
- Autoclaved molecular grade water

Media (Media are specified in FDA BAM, reference 8)
- Modified colistin polymyxin cellobiose (mCPC) agar
- T1N1 agar
- Alkaline peptone water (APW) enrichment broth
- Phosphate buffered saline (PBS)

Matrix or Matrices of Interest
The validation of post harvest processing for raw gulf coast oysters is performed on oyster homogenate. Thus the matrix is dilutions of oyster homogenate, consisting of oyster meats and PBS.

Sample Collection, Preservation, Preparation, Storage, Cleanup, Test Procedures:
Sample collection will follow procedures described by NSSP for validation of oyster PHP.
Preservation, preparation, storage, cleanup and test procedures follow manufacture's recommendations

Cost of the Method
The cost of the **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit** platform costs approximately $9 per PCR reaction.

Special Technical Skills Required to Perform the Method
Only basic laboratory skills are required.

Special Equipment Required and Associated Cost

Equipment	Approximate Cost
Dupont Bax thermocycler	$45,000 + accessories
Incubator	$3,000 - $6,000
Centrifuge	$2,000
Heat block	$500

Abbreviations and Acronyms

- PHP –post harvest processing
- DNA- deoxyribonucleic acid
- QPCR- quantitative polymerase chain reaction
- APW- alkaline peptone water
- PBS- phosphate buffered saline
- MPN- most probable number

Test Procedures and Quality Control

MEDIA: Dehydrated media is commercially dehydrated. Media must be sterilized according to manufacturer's instructions. Prepared culture media, dehydrated media and media components must be stored in a cool, clean, dry space unless refrigeration is required as per manufacturer instruction. Stored media is labeled with batch number, expiration date and sterilization date. Storage of prepared culture media at room temperature does not exceed 7 days. Refrigerated storage of prepared media with loose fitting closures does not exceed 1 month; screw-cap closures do not exceed 3 months. All prepared media stored under refrigeration are held at room temperature overnight prior to use. To determine the pH of prepared media, a pH meter with a standard accuracy of 0.1 units is used. The pH meter is calibrated with each use and a minimum of two standard buffer solutions (ph 4, 7 and 10) are used to calibrate the pH meter. Standard buffer solutions are used once and discarded.

COLD STORAGE: Refrigerator temperature must be monitored daily; temperature is maintained between 0˚C to 4˚C. Freezer temperature must be monitored at least once daily, freezer temperatures is maintained at -20˚C (DNA storage) and –80˚C (strain storage).

INCUBATOR: Temperature of incubators must be maintained at 30˚C (+/-0.5), 37˚C (+/-0.5), and 40˚C (+/-0.5). Thermometers must be graduated no greater than 0.5˚C increments. Temperatures are taken twice daily.

SUPPLIES: Utensils and containers made of clean borosilicate glass, stainless steel or other non-corroding material. Culture tubes made of a suitable size to accommodate the volume for broth and samples. Sample containers made of glass or other inert material. Dilution bottles and tubes are made of plastic and closed with attached snap-lock lids. Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes. Reusable sample containers must be capable of being properly washed and sterilized. Hardwood applicator transfer sticks, utilized for streaking and picking positive colonies, and Whatman # 3 and #541 filter papers, utilized in colony blot hybridization, are sterilized prior to use and stored in sterile, airtight containers. Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10ml are not used to deliver 1ml; nor, are pipettes larger than 1ml used to deliver 0.1ml. Reagents for DNA extraction and PCR reaction are included in **DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit**

MAINTENANCE: Routine autoclave maintenance must be performed and serviced annually or as needed by a qualified technician and records maintained. Autoclave provides a sterilizing temperature of 121˚C (tolerance 121 +/- 2˚C) as determined daily. Spore suspensions or strips must be used monthly to evaluate the effectiveness of the autoclave sterilization process, with results recorded. Heat sensitive tape must be used with each autoclave batch. Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature must be maintained in an autoclave log.

SHELLSTOCK SAMPLES: A representative sample of shellstock is collected. Shellstock is collected in clean, waterproof, puncture resistant containers. Shellstock labeled with collector's name, type of shellstock, the source, the harvest area, time, date and place of collection. Shellstock are maintained in dry storage between 0 and 10˚C until examined. Examination of the sample is initiated as soon as possible after collection, and does not exceed 24 hours after collection. Shucking knives, scrub brushes and blender jars are sterilized for 35 minutes prior to use. Blades of shucking knives free from debris corrosion. Prior to scrubbing and rinsing debris off shellstock, the hands of the technician are thoroughly washed with soap and water. Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality. Shellstock are allowed to drain in a clean container or on clean towels prior to opening. Prior to opening, the technician washes hands and rinses with 70% alcohol. Shellstock are not shucked directly through the hinge.

FDA-MPN PREPARATION AND METHOD: Contents of shellstock are shucked into a sterile, tared blender jar. At least 12 animals (100 g of meat) are used for analysis. The sample is weighted to the nearest 0.1 gram and an equal amount by weight of sterile PBS diluent is added. Samples are blended at high speed for 90 seconds. Immediately after blending, the homogenized sample is diluted in a multiple dilution series with 3 replicas and inoculated into tubes of APW presumptive media for MPN analysis. Positive and negative controls cultures accompany samples throughout the procedure. Inoculated media are incubated at 37 +/- 0.5˚C. Presumptive tubes are read at 24+/- 2 hours of incubation and transferred if positive. Transfers are made to mCPC plates by sterile hardwood applicator sticks from presumptive positive APW tubes and confirmed by DNA probe.

QPCR-MPN PREPARATION: Prior to DNA extraction and preparing Cepheid© unit for QPCR, all micro-centrifuge tubes and pipette tips are sterilized for 35 minutes. The technician's hands are washed with soap and water. Gloves are worn and rinsed with 70% alcohol. All Pipetteman and Eppendorf pipettes are calibrated semi-annually and prior to use are wiped down with 70% alcohol. All working areas, centrifuge racks, and equipment are wiped down with 70% alcohol. Proper sterile technique is observed throughout the procedure to ensure contamination free samples. 1ml of sample from each positive MPN tube is used for the boil extraction procedure (appendix 1) to extract DNA to be used as template for Sybr green 1 QPCR-MPN assay as described in appendix 2. Cepheid© thermocycler cycle threshold is set at 30 and factory default is utilized for melt curve analysis regarding peak height.

VALIDATION CRITERIA

Ruggedness of Assay

DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for detection of *V. vulnificus, V. parahaemolyticus* and *V. cholerae* was recently accepted for AOAC approval (15). Proposed method will extend applications to MPN analysis of oyster PHP. Validity of MPN assay for detection of *V. vulnificus* has been previously established by ISSC and FDA. The ruggedness of reagents used for PCR is determined by manufacturer and meets specifications. Method uses a bead format that incorporates all reagents on bead to eliminate common pipetting and cross-contamination errors.

Data Comparability and Statistical Analysis

Quantitative PCR was previously applied to most probable number (QPCR-MPN) for validation of PHP and single specie detection of *V. vulnificus in* oysters (17). Published results by Wright et al., 2007 showed that immediately following inoculation of APW (pre-enrichment with either 0.1 or 0.01 g oyster homogenate detection *V. vulnificus* was 100 to 1000 fold more sensitive by QPCR than by growth on selective agar. Following O.N. growth in enrichment, both assays were equally as sensitive. For PHP oysters received nitrogen immersion, side by side comparison of standard MPN vs. QPCR-MPN showed excellent correlation (R^2=0.97 by Pearson's correlation co-efficient) and no significant differences between the two assays (Table 2). Results were comparable for untreated oysters and for PHP oysters at both 1 and 7 days post treatment. In this study results were also examined side by side for both Nitrogen Immersion and Nitrogen Tunnel PHP treatments and statistical comparison of this data, utilizing both JMP from SAS and Minitab, both one way ANOVA and Tukeys post hoc tests show no significant differences ($p < 0.05$) between detection methods.

The AOAC evaluation of the DuPont Bax Vibrio QPCR test kit described application of the assay on five food types; raw shrimp, cooked shrimp, oysters, raw ahi tuna, and raw scallops (See attached draft of publication in appendix). Results supported the applicability of the BAX ® system for detecting *Vibrio* in foods. Samples were analyzed using the BAX ® system method and the FDA-BAM methods for detecting *Vibrio.* One food type, ahi tuna, was tested by an external independent laboratory (the State of Texas Department of Public Health, Consumer Microbiology Division) as a shared matrix. Results were in nearly complete concordance with only two cases where the test kit yielded a result that could not be confirmed by culture. Inclusivity and exclusivity of the assay was determined with all tested isolates (n = 126 target *Vibrio* strains and n = 55 non-*Vibrio* and non-target *Vibrio* species strains) demonstrating expected results and an assessment of test kit stability, lot to lot variability, and assay ruggedness was also performed demonstrating robustness of the assay.

During 2007 summer PHP validation trials were conducted by The University of Florida Aquatic Food Products group in a partnership with the oyster industry in Apalachicola FL. Side by side field trials compared the FDA-MPN to the QPCR-MPN assay are described below (Table 1). Side-by-side sample comparisons of the two assays support application of QPCR technology for validation oyster processing protocols. Samples (n=3), consisting of 12 oysters each, were obtained from untreated oysters (25IS, 29IS); temperature abused (26 TA, 30TA) by incubation O.N. at room temp; PHP heat treated oysters (65.5 for 5 min) after 7 days storage at -20C (26HSD7, 30HSD7); or Blast frozen oyster (-50C) after 42 days storage (26BLD42). The mean MPN/g for the two assay were nearly identical with R^2=0.99.

Table 1: Comparison of MPN Protocols

OYSTER LOT:	Log MPN/g	
	FDA MPN	BAX-QPCR MPN
25IS25,	2.0±0.56	2.0±0.62
29IS	2.0±0.6	2.0±1.03
26TA	4.0±0.64	4.0±0.40
30TA	6.0±0.11	6.0±0.22
26HSD7	<3.0	<3.0
30HSD7	1.0±0.66	1.1±0.58
26BLD42	2.0±0.43	2.1±0.51

Limit of Quantitation and Specificity

The attached AOAC draft manuscript details the limits of quantitation and specificity.
Inclusivity testing (n=50 strains) was performed at ~10^5 cfu/ml, while exclusivity testing (n= 50 strains) was performed at ~10^8 cfu/ml from broth cultures. Additional strains were tested by Wright Lab (see attached Table 2, 3, 4 in appendix)

For AOAC approval for spiked foods, Vibrio strains were inoculated to yield fractional positive results for plus/minus screening, or at levels informative of method performance for MPN-based approaches. Samples were tested with the FDA-BAM culture-based method and by PCR using the BAX® system. Ahi tuna was spiked at three levels with Vc and tested for presence or absence of target in sets of twenty 25g sub-samples and five unspiked sub-samples, with PCR testing from the BAM enrichments. Similarly, scallops were spiked with Vv at a level giving fractional results for the (how many samples?) 1g samples, and each MPN tube was tested by the

BAM method and PCR as were five 25g samples enriched in a comparable manner. Naturally occurring low-level Vc in raw shrimp was also tested using twenty 25g samples with both the BAM method and PCR testing from the same enrichments. All inclusivity/exclusivity testing demonstrated expected results. For effectiveness testing, comparing PCR and culture, results for the spiked ahi tuna (36 positive of 65 samples tested) and shrimp (5 positive of 20 samples tested) were identical with no false negative or false positive results by PCR. Scallop data gave identical MPN results for test and reference methods and 25g enrichments were all positive by PCR.

Additional seeding studies conducted by Wright lab utilized known concentrations of *Vibrio* species to spike APW with or without oyster homogenates. Samples were assayed by QPCR immediately without growth using various combinations of high (10^6), mediun (10^4), low (10^2) concentrations of the three species. All samples were positive for all species with the exception of samples with High Vp and low or medium concentrations of Vv. In these cases, Vv was not detected. However, samples where growth was permitted (O.N. incubation at 37C), all species were detected in all samples.

References

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APPENDIX 1

Table 2 QPCR analysis for *V. cholerae* strains

V. cholerae Strains	Clinical (C) vs. Environmental (E): Description	*Vjp*	*Vv*	*Vc*	CT	CFU/ml
1. Vc598	**C:** classical Inaba O1	-	-	+	23.7	>1.0E+09
2. Vc NRT 36S	C: non O1, O139, NAG-ST (Japan)	-	-	+	23.1	>1.0E+09
3. Vc JVY212		-	-	+	25.7	5.50E+08
4. Vc JVB52		-	-	+	27.2	1.60E+08
5. Vc 5439/62		-	-	+	28.2	7.00E+07
6. Vc 569B	**C:** O1, classical, inaba (India)	-	-	+	24.6	>1.0E+09
7. Vc S171		-	-	+	24.7	>1.0E+09
8. Vc NAG12		-	-	+	25.5	6.50E+08
9. Vc ATCC25874		-	-	+	22.4	>1.0E+09
10. Vc 8		-	-	+	24.8	>1.0E+09
11. Vc B1307 Dacca		-	-	+	24.8	>1.0E+09
12. Vc A5		-	-	+	26.3	3.50E+08
13. Vc I10		-	-	+	25.2	8.20E+08
14. Vc 646	**C:** O1, Ogawa,	-	-	+	24	>1.0E+09
15. Vc 395	**C:** Classical Ogawa O1 (India)	-	-	+	25.6	6.10E+08
16. Vc 6337		-	-	+	35.4	1.90E+05
17. Vc T2001		-	-	+	34.7	3.40E+05
18. Vc T5957		-	-	+	34.7	3.60E+05
19. Vc 2076-79	C: non O1, O139, NAG-ST (oysters, US)	-	-	+	33.7	7.60E+05
20. Vc BA312		-	-	+	34.4	4.30E+05
21. Vc 569B	**C: O1 classical Inaba (India)**	-	-	+	33.9	6.60E+05
22. Vc AI1837		-	-	+	34	6.20E+05
23. Vc Arg-3	**E:**	-	-	+	34.4	4.40E+05
24. Vc C6706	**C: O1, el tor, Inaba (Peru)**	-	-	+	33.9	6.50E+05
25. Vc CA385	**C: O1**	-	-	+	34.6	3.80E+05
26. Vc CO603		-	-	+	34.2	5.00E+05
27. Vc CO845		-	-	+	33.8	7.10E+05
28. Vc N16961	**C: 01 eltor Inaba**	-	-	+	33.9	6.40E+05
29. Vc NG288/36	**C: O139 (Thailand)**	-	-	+	33.7	7.50E+05
30. Vc NRT36S		-	-	+	36.6	7.20E+04
31. Vc PS15		-	-	+	36.2	1.00E+05
32. Vc V5C		-	-	+	34.3	5.00E+05
33. Vc ATCC 25873		-	-	+	0	0
34. Vc SO47W		-	-	+	33.9	6.40E+05
35. Vc RB1		-	-	+	34.9	3.00E+05
36. Vc J31W		-	-	+	39.3	<1.0E+04
37. Vc T96W		-	-	+	36.6	7.00E+04
38. Vc 6358		-	-	+	0	0
39. Vc 5057		-	-	+	34.5	4.20E+05
40. Vc 7261		-	-	+	34	6.40E+05
41. Vc 7165		-	-	+	33.2	1.20E+06
42. Vc 9115		-	-	+	0	0
43. Vc 5145		-	-	+	34.3	4.80E+05
Blank		-	-	-	0	0

Table 3 QPCR analysis for *V. parahaemolyticus* strains

***V. parahaemolyticus* Strains**	**Clinical (C) vs. Environmental (E): Description**	***VP***	***Vv***	***Vc***	**CT**	**CFU/ml**
1. Vp TX2103		+	-	-	25.6	3.50E+08
2. Vp TX3547		+	-	-	26	2.60E+08
3. Vp DAL1094		+	-	-	26.2	2.30E+08
4. Vp 17802		+	-	-	24.6	6.80E+08
5. Vp 43996		+	-	-	25.5	3.70E+08
6. Vp 10290		+	-	-	25.5	3.70E+08
7. Vp Y 9398		+	-	-	23.7	>1.0E+09
8. Vp 5E-3		+	-	-	34.1	1.40E+06
9. Vp 205-757		+	-	-	33.4	2.30E+06
10. Vp AQ3810		+	-	-	33.5	2.10E+06
11. Vp AQ4235		+	-	-	33.1	2.80E+06
12. Vp EDL896		+	-	-	33.4	2.30E+06
13. Vp VP2		+	-	-	33.5	2.20E+06
14. Vp VP250		+	-	-	33	3.00E+06
15. Vp VP331		+	-	-	33.2	2.70E+06
16. Vp VP356		+	-	-	33.3	2.40E+06
17. Vp VP381		+	-	-	33.4	2.30E+06
18. Vp VP53		+	-	-	33.2	2.50E+06
19. Vp VP81		+	-	-	31.7	6.70E+06
20. Vp WP-1		+	-	-	34.2	1.40E+06
21. Vp S162-71		+	-	-	33.5	2.10E+06
22. Vp 3D-38		+	-	-	33.5	2.10E+06
23. Vp EDL 1044		+	-	-	33.1	2.80E+06
24. Vp SN36		+	-	-	33.8	1.80E+06
25. Vp 14d13		+	-	-	33.4	2.30E+06
26. Vp AAG9574		+	-	-	33.3	2.50E+06
27. Vp P125		+	-	-	33.5	2.20E+06
28. Vp HMG38		+	-	-	33.2	2.60E+06
29. Vp P29		+	-	-	34.1	1.40E+06
30. Vp VV104		+	-	-	33.7	1.90E+06
31. Vp VV27-1		+	-	-	33.5	2.10E+06
32. Vp VV27-2		+	-	-	33.9	1.60E+06
33. Vp A602		+	-	-	32.9	3.20E+06
34. Vp FC1011		+	-	-	34.7	9.70E+05
35. Vp B10576		+	-	-	33.4	2.30E+06
36. Vp EDL1041		+	-	-	34	1.60E+06
Blank		-	-	-	0	0

Table 4 QPCR analysis for *V. vulnificus* strains:

vulnificus rains	Clinical (C) vs. Environmental (E): Description	*Vjp*	*Vv*	*Vc*	CT	cfu/ml
Vv MO6-24/O	C	-	+	-	24.3	5.20E+08
Vv FLA 109	C	-	+	-	27	5.90E+07
Vv FLA141	C	-	+	-	27.3	4.80E+07
Vv FLA126	C	-	+	-	28.5	1.90E+07
Vv FLA134	E: oyster	-	+	-	26.5	8.90E+07
Vv FLA129	C	-	+	-	26.8	6.80E+07
Vv FLA127	E: oyster	-	+	-	26.5	9.10E+07
Vv FLA135	E: oyster	-	+	-	26.8	7.00E+07
Vv FLA 115	E: oyster	-	+	-	27.1	5.60E+07
Vv FLA 149	C	-	+	-	28.5	1.90E+07
Vv B3-313/98	E: fish	-	+	-	27.3	4.70E+07
Vv FLA121	E: oyster	-	+	-	26.9	6.20E+07
Vv FLA137	E: oyster	-	+	-	26.4	9.40E+07
Vv B3-302/99	E: fish	-	+	-	26.7	7.60E+07
Vv FLA119	E: oyster	-	+	-	26.7	7.30E+07
Vv FLA116	C	-	+	-	26.5	8.80E+07
Vv FLA102	C	-	+	-	26.7	7.60E+07
Vv B2-2	E: fish	-	??	-	0	0
Vv FLA108	C	-	+	-	26	1.30E+08
ank		-	-	-	0	0

APPENDIX 2: Draft manuscript for AOAC approval:

DuPont Qualicon BAX ® Real Time *Vibrio* Test Kit for the Detection of *Vibrio cholera, parahaemolyticus and vulnificus* from Tuna, Shrimp and Oysters

AOAC Performance Tested Methodsm YYMMXX

ABSTRACT
An evaluation was conducted on five food types; raw shrimp, cooked shrimp, oysters, raw ahi tuna, and raw scallops to demonstrate the applicability of the BAX ® system for detecting *Vibrio* in foods. Samples were analyzed using the BAX ® system method and the FDA-BAM methods for detecting *Vibrio.* One food type, ahi tuna, was tested by an external independent laboratory (the State of Texas Department of Public Health, Consumer Microbiology Division) as a shared matrix. Results were in nearly complete concordance with only two cases where the test kit yielded a result that could not be confirmed by culture. Inclusivity and exclusivity of the assay was determined with all tested isolates (n = 126 target *Vibrio* strains and n = 55 non-*Vibrio* and non-target *Vibrio* species strains) demonstrating expected results and an assessment of test kit stability, lot to lot variability, and assay ruggedness was also performed demonstrating robustness of the assay.

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Scope of method
1.1 Target organisms – Vibrio cholera, parahaemolyticus, and vulnificus. A wide range of *Vibrio* and non-*Vibrio* strains was used for inclusivity/exclusivity testing.
1.2 Matrices – Specific foods tested included shrimp, oysters, tuna, and scallops.
1.3 Performance claims – Sensitivity and specificity equivalent to the official FDA-BAM culture-based method.

Definitions

- From the AOAC International Official Methods of Analysis Program Manual Appendix X [1]: Sensitivity rate (p+) for a food type and inoculation level - The probability that the method, alternative or reference, will classify a test sample as positive, given that a test sample is a known positive. A known positive refers to the confirmation of innoculated analyte.

Sensitivity rate is defined as: Total number of confirmed positive test portions by the method divided by total number of confirmed positive test portions by both the alternative and reference methods.
Specificity rate (p-) for a food type and inoculation level - The probability that the method will classify the test sample as negative, given that the test sample is a known negative. A known negative refers to a confirmed negative test portion.
Specificity rate is defined as: Total number of analyzed negative test portions by the method divided by total number of confirmed negative test portions by both the alternative and reference methods. For microbiological methods involving a confirmation step, a presumptive positive result is taken through the cultural procedure and confirmed to be a positive or determined to be a negative. In other words, the confirmation procedure allows the

sample to be reclassified as a known positive or a known negative. As such, the specificity rate of results after confirmation is always 100%.
False negative rate (pf-) for a food type and inoculation level - The probability that a test sample is a known positive, given that the test sample has been classified as negative by the method. pf- is the number of misclassified known positives divided by the total number of positive test samples (misclassified positives plus the number of correctly classified known positives) obtained with the method. Incidence of false negatives equals 100 minus the sensitivity rate.
False positive rate (pf+) for a food type and inoculation level - The probability that a test sample is a known negative, given that the test sample has been classified as positive by the method. pf+ is the number of misclassified known negatives divided by the total test samples (misclassified positives plus the number of correctly classified known negatives) obtained with the method.
Incidence of false positives equals 100 minus the specificity rate.

Principle

The BAX® system uses the Polymerase Chain Reaction (PCR) to amplify specific DNA fragments, which are stable and unaffected by growth conditions [2]. Each fragment is a genetic sequence that is unique to the targeted organism, thus providing a highly reliable indicator that the organism is present. The BAX® system simplifies the PCR process by combining the requisite PCR reagents into a stable, dry, manufactured tablet already packaged inside the PCR tubes. After hydrating these tablets with prepared samples, the tubes remain sealed to reduce the potential for contamination.

In a typical PCR application, sample DNA is combined with DNA polymerase, nucleotides and primers that are specific for a given nucleotide sequence. The mixture then undergoes a series of timed heating and cooling cycles. Heating denatures the DNA, separating it into single strands. As the mixture cools, the primers recognize and anneal (bind) to the targeted DNA sequence. DNA polymerase then uses nucleotides to extend the primers, thus creating two copies of the targeted fragment (amplification). Repeating cycles of denaturing, annealing and extending produces an exponential increase in the number of target DNA fragments, creating millions of copies in a very short time. If the target sequence is not present, no detectable amplification takes place [2]. Inhibitors to PCR are present in some food matrices. In particular, phenolic compounds found in some spices and other plant-based materials such as high purity cocoa can cause the PCR reaction to shut down. Because of this, each BAX reagent tablet is formulated with a low level control DNA molecule and associated primers. This Internal Positive Control (INPC) must be shown to amplify in the absence of specific pathogen target amplification product for the BAX ® instrument to report a negative result. In the absence of any target or INPC associated product, the instrument reports an indeterminate result.

The BAX® system PCR tablets used in real-time assays also contain multiple dye-labeled probes. Intact probes are short oligonucleotides with quencher dye at one end that absorbs the signal from fluorescent reporter dye at the opposite end. During PCR cooling cycles, probes bind to a specific area within the targeted fragment. During extension, DNA polymerase encounters the probe in its path and breaks the probe apart. This releases the reporter dye, resulting in increased fluorescent signal [3]. In multiplex reactions such as in this test kit, each species specific probe is labeled with a different fluorescent reporter dye, allowing independent detection of the presence or absence of each target. The BAX® system Q7 instrument uses multiple filters to measure specific signal resulting from the presence of each target at the end of each cycle and report results for the presence or absence of *Vibrio cholera*, *vulnificus*, or *parahaemolyticus* in less than 90 minutes.

General information

Vibrio is a gram-negative genera consisting of 65 known species [4]. It can cause seafood and water-borne illnesses and infections in humans. It is most commonly found in marine and freshwater environments and is transmitted to humans mainly through the consumption of raw or undercooked shellfish, particularly oysters, or through contaminated drinking water [5].

The risk of *Vibrio*-caused illness is increased following a natural disaster leading to disruption of water and sanitation systems or massive displacement of a population to inadequate and overcrowded temporary housing. Such an effect was seen in the aftermath of Hurricane Katrina in 2005, where surveillance identified 22 new cases of *Vibrio* illness, including five deaths [5].

The three species of Vibrio that cause the majority of human illness and infection are *Vibrio cholera*, *parahaemolyticus*, and *vulnificus* [6].

Cholera is a major disease that occurs when *Vibrio cholera* colonizes the small intestine and releases enterotoxin(s) leading to a secretory diarrhea that without supportive oral rehydration and replacement of salts can prove fatal. The disease is currently endemic in many countries in South Asia, Africa and the Americas and remains a global threat to public health [6].

Vibrio parahaemolyticus is an invasive organism that primarily affects the colon. It is estimated that up to 4500 cases of *Vibrio parahaemolyticus* infection occur annually in the United States [7]. These illnesses are mainly due to the consumption of undercooked oysters and other seafood.

Vibrio vulnificus is an emerging human pathogen that can cause illnesses such as gastroenteritis and can cause wound infections that can progress to septicemia. Though the total number of cases of *V. vulnificus* infection is small, it is highly pathogenic in certain populations, and thus is responsible for an estimated 1% of all foodborne deaths in the United States [8].

Test Kits Information

5.1 *Test kit name* – BAX® System Real-Time PCR Assay for Screening *Vibrio cholerae*, *parahaemolyticus*, *vulnificus*

5.2 *Test kits catalog numbers* – D12863877

5.3 Ordering information –

5.3.1 DuPont Qualicon, Experimental Station, Bldg. 400, P.O. Box 80400, Rt. 141 & Henry Clay Road, Wilmington, DE 19880-0400, USA, Phone 800-863-6842 or 302-695-5300, Fax 302-695-5301, Internet www.qualicon.com

5.3.2 DuPont Qualicon Europe, Ltd Wedgwood Way, Stevenage Herts SG1 4QN, UK

5.3.3 DuPont Qualicon, Asia/Pacific DuPont Company (Singapore) Pte, Ltd. 1 Harbour Front Place #11-01, Harbour Front Tower One, Singapore 098633

5.4 Test kit components –

5.4.1 PCR tubes with tablets (twelve 8-tube strips, each tube containing 1 PCR tablet)

5.4.2 Flat optical caps for PCR tubes (twelve 8-cap strips)

5.4.3 Lysis buffer (two 12-ml bottles)

5.4.4 Protease (one 400-µl vial)

5.4.5 Package insert (1)

Additional reagents

Protease reagent – Using test kit reagents, pipette 150 µL of protease into one 12-mL bottle of lysis buffer. Label bottle with the date prepared. Reagent will remain stable for up to two weeks if stored at 2-8ºC.

Apparatus

7.1 Incubators – Static incubators at 35 ± 2ºC, 39-40ºC, and a heated water bath capable of maintaining a temperature of 41± 0.2ºC.

7.2 Stomacher, Blender, and Scissors – For sample preparation. Seward model 400 or equivalent stomacher, Blender with blending jars, and autoclavable scissors.

7.3 BAX® system Q7 apparatus (all components listed in this section are included with the *BAX®* Q7 System Start Up package. Components 7.3.3 – Cluster tubes with caps, and 7.3.6 – Pipette tips; after the initial boxes included with the start-up package are used; must be purchased by the test kit user).

7.3.1 BAX® System cycler/detector with computer workstation

7.3.2 BAX® System application software

7.3.3 Cluster tubes with caps and racks for lysis

7.3.4 Capping/de-capping tools – for removing and sealing cluster tube caps and PCR tube caps without jarring the contents

7.3.5 Heating blocks with inserts and thermometers – for maintaining lysis tubes at 37ºC ± 1ºC, 55ºC ± 1ºC and 95ºC ± 1ºC

7.3.6 Pipettes – for transferring reagents; two adjustable mechanical pipettes covering 20-200 µl and 5-50 µl; one repeating pipette; and one multi-channel pipette covering 8 channels and 5-50 µl. Pipettes should be calibrated to deliver required volumes within 10%.

7.3.7 Pipette tips with barriers: 0.5-250 µl, 0.5-100 µl extended barrier; 2.5 ml and 5 ml repeater pipette tips

7.3.8 Cooling block assemblies – for keeping lysate tubes and PCR tubes chilled at 2-8ºC during sample preparation

7.3.9 PCR tube holders – for transferring a rack of tubes from the cooling block to the cycler/detector

7.3.10 Printer

Standard Reference Materials

8.1 DuPont Qualicon culture collection (DD) - proprietary

8.2 *American Type Culture Collection* (ATCC) - *American Type Culture Collection* (ATCC) - www.atcc.org, American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA.

Standard solutions, consumables, and media

Media - where applicable FDA-BAM designations listed in parentheses.

Alkaline peptone water (APW) (M10)
AKI medium (M7)
Arginine glucose slants (AGS) (M16)
Blood agar (5% sheep red blood cells) (M20)
Casamino acids yeast extract (CAYE) broth (M34)
modified Cellobiose polymyxin colistin (mCPC) agar (M98)
Cellobiose colistin (CC) agar (M189)
Motility test medium-1% NaCl (M103)
Oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine.2HCl in dH2O) (R54)
Phosphate buffered saline (PBS) (R59)
Polymyxin B disks, 50 U (Difco or equivalent) (R64)
Saline soln - 0.85% in dH2O (R63)
2% NaCl soln (R71)
Sodium desoxycholate - 0.5% in sterile dH2O (R91)
Thiosulfate citrate bile salts sucrose (TCBS) agar (M147)
T_1N_1 and T_1N_3 agars (1% tryptone and either 1% or 3% NaCl) (M163)
T_1N_0, T_1N_3, T_1N_6, T_1N_8, T_1N_{10} broths (M161)
Tryptic soy agar-magnesium sulfate- 3% NaCl (TSAMS) (32) Trypticase (or tryptic) soy broth (TSB), agar (TSA)(M152) (with added NaCl, 2%)
TSB-1% NaCl-24% glycerol
Urea broth (M171) (or Christensen's urea agar (M4+0) with added NaCl (2%) (R71)
Vibrio parahaemolyticus sucrose agar (VPSA) (M191)
Vibrio vulnificus agar (VVA) (M190)
Chromagar *Vibrio* (DRG International Mountainside, NJ Product number VB912)
API 20E diagnostic strips and reagents (BioMerieux, Hazelwood, Mo.)

All microbiological media was prepared by autoclaving at 121°C at 15 psi for 15 min if preparing $\leq$ 4 L of media and 20 min if preparing > 4 L of media.

Safety Precautions

10.1 *Kits* – The reagents used in the BAX® system should pose no hazards when used as directed. Dispose of lysate, PCR mixture and other waste according to your site practices.

10.2 *Cycler/detector* – Only qualified laboratory personnel should operate the cycler/detector. Do not attempt to repair the instrument. Live power may still be available inside the unit even when a fuse has blown or been removed. Refer to the *User Guide* for maintenance procedures when cleaning the unit or changing a fuse. The heating block can become hot enough during normal operation to cause burns or cause liquids to boil. Wear safety glasses or other eye protection at all times during operation.

10.3 Enrichment Broths- All enrichment broths whether testing positive or negative for this assays targets, may contain enriched pathogens and should be autoclaved following any culture-based confirmatory steps.

General Preparation / Sample preparation and recovery

- 11.1 *Selection of strains for testing*- Strains were taken from the DuPont/Qualicon culture collection (samples tested by Qualicon) (see Table 2), collaborators' culture collections (the University of Florida and the Texas State Department of Public Health), and the American Type Culture Collection (ATCC).

11.2 *Culture preparation for artificially contaminated food – Vibrio* were grown to stationary phase in APW and serially diluted in APW to final concentrations likely to give fractional recovery (based on preparatory studies).

11.3 *Food samples* – Five food types were included in this study; raw ahi tuna, raw shrimp, cooked shrimp, oysters, and raw scallops.

Raw tuna was artificially inoculated with *V. cholera*, cooked shrimp were artificially inoculated with *V. parahaemolyticus*, and raw scallops were artificially inoculated with *V. vulnificus*, while naturally occurring flora was tested in raw shrimp and raw oysters. Reference method enrichment varied according to the sample type examined. Tuna and raw shrimp were tested on a plus/minus basis

according to the FDA-BAM protocols for *V. cholera*. Though much of the FDA-BAM *Vibrio* chapter is MPN-based, and thus the MPN-based methods were used to validate the effectiveness of the assay, it is anticipated that the BAX ® test kit will primarily be used to screen on a presence/absence basis so additional samples were tested to validate this type of screening. That is, samples were tested using the FDA-BAM enrichment conditions and culture confirmation with BAX ® testing from each of the MPN replicates, but with additional unpaired 25g samples enriched in 225 ml of enrichment media before BAX ® testing as a complement. Each 25g sample enrichment was also culture confirmed using the FDA-BAM methodology.

Analysis – BAX® system methodS

12.1 *Prepare equipment* - Turn on heating blocks (37ºC and 95ºC). Check that cooling blocks have been refrigerated overnight. Turn on power to cycler/detector, then to computer. Launch BAX® system application. If instrument diagnostics recommends verification, follow Verification Wizard screen prompts for procedure.

12.2 *Create rack file* – Follow prompts in the Rack Wizard to enter identifying data on the entire rack and on the individual samples.

12.3 *Perform lysis* –Add 5 µL of enrichment from the top of each enrichment to 200 µL of protease reagent in a cluster tube. Place in heating block at 37±1°C for 30 minutes. Transfer tubes to 95°C heating block for 10 minutes. Transfer to cooling block (2–8°C) for 5 minute.

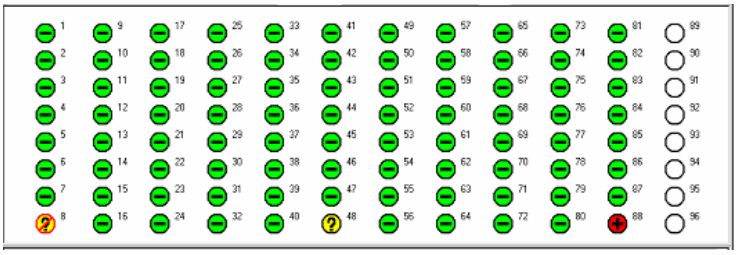
12.4 *Warm up cycler/detector* - Select RUN FULL PROCESS from the menu bar of the application window to heat the instrument to the set temperature (90ºC for the block, 100ºC for the lid).

12.5 *Hydrate PCR tablets with lysate* - Place PCR tube holder over insert of the PCR cooling block (solid side in rear). Place one PCR tube per sample into the holder. Loosen all caps, and remove caps from a row of tubes. Using a multi-channel pipette, transfer 30 µL of lysate to the row of PCR tubes for the *Vibrio* assay. Seal tubes with replacement optical caps. Using new tips, repeat transfer for each row until all samples have been transferred into PCR tubes.

12.6 *Amplify and detect* - Follow screen prompts at the PCR Wizard for loading samples into the cycler/detector and begin the program. The Full Process program takes about 75 min to complete. When finished, the PCR Wizard will prompt you to unload the samples and will automatically display the results.

Interpretation and test result report

Review results on screen as a grid of wells



Negative - Circle with (-) symbol
Positive - Circle with (+) symbol
Indeterminate - Circle with (?) symbol
Error (low signal) - Circle with (?) symbol and slash (/)

Food method comparison studies

Methodology – In accordance with an AOAC-RI approved study design, DuPont Qualicon compared the BAX® system method to the FDA-BAM [9] method for detecting *Vibrio* species in food samples.

Tuna (*V. cholera*) – Internal Qualicon and Independent Laboratory Shared Matrix

For tuna testing, a strain of *V. cholera* was taken from the DuPont Qualicon culture collection and struck for purity on a T_1N_1 agar plate. A single colony was inoculated into a tube containing 10 ml of APW broth, and incubated 18 hrs at 35ºC. The stationary phase culture was enumerated by plating dilutions on T_1N_3 and TSA agar plates. Based on preparatory studies, a dilution factor was established to give inoculation levels appropriate for achieving fractional positive results for the tuna matrix. Samples were inoculated as a master sample of sliced tuna, and mixed well by shaking and hand massaging in a biohazard bag. Samples were divided into analytical size portions into blender jars if they were to be

blended or stomacher bags if they were to be processed by scissors and held at 4°C for 48-72 hours before enrichment (Qualicon tested by scissors processing while the independent laboratory tested by blending). Following this cold stress/acclimation, if processing with scissors, portions of tuna were removed and processed with scissors which were decontaminated with ethanol and allowed to air dry before preparation of another sample. Samples prepared in this way were cut into approximately 1g pieces (~25 pieces per analytical unit). If processing with blending, portions were blended at high speed for 1 min. If processing with blending, portions were blended at high speed for 1 min. Three each samples of 100g, 10g and 1g were also prepared from this mix for MPN analysis.

Tuna portions were mixed as described above in 225 ml of APW and incubated at 35°C for 22 +/- 2 hrs total with reference method plating performed at 6-8 hrs and concurrently with BAX® testing after 16-20 hrs of incubation.

At each reference culture sample point, a 3 mm loop was used to streak for isolation onto dried plates of TCBS, mCPC, and CHROMagar *Vibrio* agar plates. Three or more typical colonies from each agar media when present were struck onto T_1N_3 agar plates and subjected to the initial biochemical screenings specified in the FDA BAM. Colonies which were phenotypically consistent with *Vibrio* (with a preference for *V. cholera* for this spiked study) were subjected to API-20E testing as described in the FDA BAM. If PCR positive samples' culture results had been inconsistent with *V. cholera*, up to 24 additional colonies would have been picked for characterization, but this was not needed for this matrix.

Raw Shrimp (*V. cholera*)

For raw frozen shrimp in an ongoing retail survey, Qualicon found shrimp with a low enough level of naturally occurring *V. cholera* to give fractionally positive results. Twenty samples of 25g each were removed from this batch and blended at high speed for 2 min at high speed in 225 ml of APW and incubated at 35°C overnight (18 +/- 2 hrs) with reference method plating performed at 6-8 hrs and concurrently with BAX® testing after overnight incubation onto TCBS, mCPC, and CHROMagar. Plates were incubated at 35-37°C overnight.

At each reference culture sample point, a 3 mm loop was used to streak for isolation onto dried plates of TCBS, mCPC, and CHROMagar *Vibrio* agar plates. Three or more typical colonies from each agar media were struck onto T_1N_3 agar plates and subjected to the initial biochemical screenings specified in the FDA BAM. Presumptive *V. cholera* was given preference for selection, despite the fact that there were many more colonies consistent with *V. parahaemolyticus,* and most enrichments (11/20) in this study were PCR positive for the presence of this species. Though not part of this study, all *V. parahaemolyticus* PCR positive enrichments did culture confirm for the presence of this species, and none of the PCR negative samples were culture positive. Colonies which were consistent with *Vibrio* in initial screening were subjected to API-20E testing as described in the FDA BAM. In two of the BAX ® positive enrichments, no culture confirmed isolates were initially obtained. Additional isolates were picked (up to 24 per plating media where available) and characterized. In both cases one or more *V. cholera* isolates were recovered. Samples from which one or more confirmed *V. cholera* isolates were obtained were considered reference method positive in this study.

Cooked Shrimp (*V. parahaemolyticus*)

Frozen, cooked shrimp were tested for artificially introduced *V. parahaemolyticus*. Cooked refrigerated shrimp were spiked as master samples at two levels with *V. parahaemolyticus* strain TD3129 in which at least one level was likely to be informative of method performance when compared to the reference MPN method. Shrimp were held at 4°C for 48-72 hrs to acclimate the introduced *Vibrio*. For the FDA BAM method, from the spiked master samples, five replicates of 50g of shrimp were weighed into blender jars and homogenized at high speed for 90 sec and used for analysis. The entire animal was used for blending. PBS (450 ml) was added and blended for 1 min at 8,000 RPM. This constituted the 1:10 dilution. Two further serial dilutions were prepared in PBS for final 1:100 and 1:1000 dilutions (in testing of artificially contaminated product, since very low spike levels were used, no further dilutions

were performed). Since this was a cooked product, 3 x 10 ml portions of the 1:10 dilution were transferred into 3 tubes containing 10 ml of 2X APW. This represented the 1 g portion. Similarly, 3 x 1 ml portions of the 1:100 and 1:1000 dilutions were inoculated into 10 ml of single-strength APW. APW enrichments were incubated overnight at 35 ±2°C (18 +/- 2 hrs). A 3-mm loopful from the top 1 cm of each APW tube was struck for isolation onto TCBS, mCPC, and *Vibrio* Chromagar plates. Concurrently with plating, a BAX ® PCR assay was performed from each MPN tube. TCBS and Chromagar plates were incubated at 35 ±2°C and mCPC at 39-40 °C overnight.

Additionally, five 25g samples from the same master sample were directly stomached (2 min at 100 rpm) with APW. For enrichment and plating, the 25g enrichments were treated as described above for MPN analysis.

V. parahaemolyticus appear as round, opaque, green or bluish colonies (usually), 2 to 3 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies are, large, opaque, and yellow (usually). Isolates were struck for purity on T_1N_3 agar plates and subjected to initial screening by oxidase and string tests. Isolates giving expected reactions were subjected to further screening using the API 20E test kit as modified in the FDA-BAM by using 2% NaCl as the diluent.

Raw Scallops (*V. vulnificus*)

Raw scallops were spiked with *V. vulnificus* strain TD3149 at a level likely to be informative of method performance (in which at least one dilution of the MPN analysis was fractionally positive) when compared to the reference MPN method. For the FDA BAM method, from the spiked master samples, five replicates of 50g of scallops were weighed into blender jars and homogenized at high speed for 90 sec and used for analysis. Scallops were held at 4°C for 48-72 hrs to acclimate the introduced *Vibrio*. PBS (450 ml) was added and blended for 1 min at 8,000 RPM. This constituted the 1:10 dilution. One further serial dilution was prepared in PBS for a final 1:100 dilution (in testing of artificially contaminated product, since very low spike levels were used, no further dilutions were performed). 3 x 10 ml portions of the 1:10 dilution were transferred into 3 tubes containing 10 ml of 2X APW. This represented the 1 g portion. Similarly, 3 x 1 ml portions of the 1:10 and 1:100 dilutions were inoculated into 10 ml of single-strength APW. APW enrichments were incubated overnight at 35 ± 2°C (18 +/- 2 hrs). A 3-mm loopful from the top 1 cm of each APW tube was struck for isolation onto TCBS, mCPC, and *Vibrio* Chromagar plates. Concurrently with plating, a BAX ® PCR assay was performed from each MPN tube. TCBS and Chromagar plates were incubated at 35 ±2°C and mCPC at 39-40 °C overnight (18 +/- 2 hrs).

Additionally, five 25g samples from the same master sample were directly stomached (2 min at 100 rpm) with APW. For enrichment and plating, the 25g enrichments were treated as described above for MPN analysis.

V. vulnificus appear as purple colonies on mCPC agar. Isolates were struck for purity on T_1N_3 agar plates and subjected to initial screening by oxidase and string tests. Isolates giving expected reactions were subjected to further screening using the API 20E test kit as modified in the FDA-BAM by using 2% NaCl as the diluent.

Oysters (*V. parahaemolyticus* and *V. vulnificus*)

BAX ® lysates were prepared as described above for scallops (with the exception that dilutions were carried out to 10^{-6}) from samples tested using the MPN procedures of the FDA-BAM in collaboration with the FDA Dauphin Island Seafood Laboratory. The FDA-BAM protocol with *tlh* (thermo-labile hemolysin) pcr based isolate confirmation for *V. parahaemolyticus* and with *vvh*-a (cytolysin) pcr based isolate confirmation for *V. vulnificus* was used for these studies. BAX ® results were compared to the results from the appropriate species specific FDA-BAM PCR for the presence of *V. parahaemolyticus* and *V. vulnificus* in the MPN tubes. To demonstrate the utility of the protocol across a wide level of contamination density, three sets of oysters were examined. One set was stored overnight after harvest

at 3°C, another set at 25°C overnight, and a third set at 35°C. For molluscan shellfish, ~12 animals were pooled and blended 90 sec with an equal vol of PBS (1:2 diln). A 1:10 dilution was prepared by weighing (weighing is recommended because air bubbles in the 1:2 dilution prevent accurate volumetric transfer) of the 1:2 homogenate to 4 X ml of PBS. Additional 10-fold dilutions were prepared volumetrically (i.e. 1ml of 1:10 to 9.0ml of PBS for a 1:100 dilution).

Three 100 ml portions (the 10g samples) were added to 100 ml 2X APW. Three 10 ml portions of the 1:10 dilution were inoculated into 3 tubes containing 10 ml of 2X APW. This represented the 1 g portions. Similarly, 3 x 1 ml portions of the 1:10, 1:100, 1: 1000, and 1:10,000 dilutions were inoculated into 10 ml of single-strength APW. APW was incubated overnight (18 +/- 2 hrs) at 35 ±2°C. A 3-mm loopful was struck from the top 1 cm of all APW tubes onto TCBS, mCPC, and CC agars.

1.1 TCBS plates were incubated at 35 ±2°C overnight (18 +/- 2 hrs) while mCPC and CC plates were incubated at 39-40°C. *V. parahaemolyticus* appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies are, large, opaque, and yellow. Most strains of *V. parahaemolyticus* will not grow on mCPC or CC agar. On mCPC and CC agars, *V. vulnificus* colonies are round, flat, opaque, yellow, and 1 to 2 mm in diameter. Presumptive isolates (three typical isolates per species per MPN tube where available) were purified as described previously and inoculated onto T_1N_3 plates and into 96 well plates for freezing and subsequent FDA-BAM colony confirmation pcr testing.

1.1.1 Isolates with typical morphology from each MPN tube were identified as *V. parahaemolyticus* or *V. vulnificus* by pcr as described in the FDA-BAM and the following sections.

Confirmation of *V. vulnificus* by polymerase chain reaction

1. Isolates obtained by the MPN procedure plating were confirmed by PCR as described in the FDA-BAM.
2. Primers for PCR vvhA (519 base amplicon) are from base 785 to 1303 of the cytolysin gene. The following primers should be used:

   Vvh-785F 5' ccg cgg tac agg ttg gcg ca 3'
   Vvh-1303R 5'cgc cac cca ctt tcg ggc c 3'

3. The follow reaction was used:

Reagent	Reaction vol.
dH2O	28.2 µl
10X Buffer.MgCl2	5.0 µl
dNTPs	8.0 µl
primer mix (6 primers)	7.5 µl
template	1.0 µl
Taq polymerase	0.3 µl
Total vol	50.0 µl

4. The following PCR conditions were used:

   PCR conditions:

denature	94°C 10 min	
denature	94° C 1 min	
anneal	62°C 1 min	25 cycles
extend	72°C 1 min	
final extend	72°C 10 min	
hold	8°C indefinite	

5. Agarose gel analysis of PCR products. For each isolate, 10 µl PCR product was combined with 2 µl 6X loading gel and loaded into wells of a 1.5% agarose gel containing 1 µg/ml ethidium bromide submerged in 1X TBE. A constant voltage of 5 to 10 V/cm was applied. Gels were illuminated with a UV transluminator (Gel Doc 1000 System, BioRad, Hercules, CA) and bands were visualized relative to molecular weight marker migration. Positive and negative culture

controls and reagent controls were included with each PCR run. Isolates were confirmed with the presence of a 519 bp for the species specific pcr product.

Confirmation of *V. parahaemolyticus* by polymerase chain reaction

1. Isolates obtained by the MPN procedure plating were confirmed by PCR as described in the FDA-BAM.
2. The following primer sets were used (final concentration in each reaction for each primer 0.2µM):
   tlh gene species specific (450 bp)
   L-TL 5' aaa gcg gat tat gca gaa gca ctg 3'
   R-TL 5' gct act ttc tag cat ttt ctc tgc 3'
3. The following PCR reagents were used:

Reagent	Reaction vol.
dH2O	28.2 µl
10X Buffer.MgCl2	5.0 µl
dNTPs	8.0 µl
primer mix (6 primers)	7.5 µl
template	1.0 µl
Taq polymerase	0.3 µl
Total vol	50.0 µl

4. The following PCR conditions were used:
   PCR conditions:

denature	94°C 3 min	
denature	94° C 1 min	
anneal	60°C 1 min	25 cycles
extend	72°C 2 min	
final extend	72°C 3 min	
hold	8°C indefinite	

5. Agarose gel analysis of PCR products. For each isolate, 10 µl PCR product was combined with 2 µl 6X loading gel and loaded into wells of a 1.5% agarose gel containing 1 µg/ml ethidium bromide submerged in 1X TBE. A constant voltage of 5 to 10 V/cm was applied. Gels were illuminated with a UV transluminator (Gel Doc 1000 System, BioRad, Hercules, CA) and bands were visualized relative to molecular weight marker migration. Positive and negative culture controls and reagent controls were included with each PCR run. Isolates were confirmed with the presence of the 450 bp band for the species specific pcr product.

Table 1. BAX vs. Reference Results for Presence/Absence Testing

Sample type	MPN or Spike Level	Samples	BAX pos	BAX Confirmed	Reference pos	Sensitivity[1]	Specificity[2]	Chi Square[3]
Tuna	0.5 MPN/25g (*V. cholerae*)	20	3	3	3	100%	100%	-
	1.9 MPN/25g (*V. cholerae*)	20	13	13	13	100%	100%	-
	3.75 MPN/25g (*V. cholerae*)	20	19	19	19	100%	100%	-
	0 cfu/25g	5	0	0	0		100%	
Tuna (Independent Laboratory)	6 MPN/25g (*V. cholerae*)	20	9	9	9	100%	100%	-
	0 cfu/25g	5	0	0	0		100%	
Frozen raw shrimp	Naturally contaminated (*V. cholerae*)	20	5	5	5	100%	100%	-

[1] Sensitivity - Total number of confirmed positive test portions by the method divided by total number of confirmed positive test portions by both the alternative and reference methods.

[2] Specificity - Total number of analyzed negative test portions by the method divided by total number of confirmed negative test portions by both the alternative and reference methods.

[3] McNemar Chi-Square test statistic used for calculating significance

Table 2. BAX System Results for Samples with Presence/Absence and MPN Testing								
	Presence/Absence in 25g sample			MPN (3 tube, 3 dilution – 1g, 0.1g, 0.01g)				
Sample type	Inoculation level	BAX positive / confirmed	Reference positive / confirmed	Sample	BAX positive (1g, 0.1g, 0.01g)	Reference positive (1g, 0.1g, 0.01g)	BAX MPN[1]	Reference MPN[1]
Cooked shrimp (*V. parahaemolyticus*)	1.8 cfu/g	5/5	5/5	1	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				2	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				3	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				4	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				5	1, 0, 0	1, 0, 0	0.36/g	0.36/g
Cooked shrimp (*V. parahaemolyticus*)	18 cfu/g	5/5	5/5	1	2, 0, 0	2, 0, 0	0.92/g	0.92/g
				2	2, 2, 0	2, 2, 0	2.1/g	2.1/g
				3	2, 0, 0	2, 0, 0	0.92/g	0.92/g
				4	3, 0, 0	3, 0, 0	2.3/g	2.3/g
				5	2, 1, 0	2, 1, 0	1.5/g	1.5/g
Scallops (*V. vulnificus*)	1.4×10^4 cfu/g	5/5	5/5	1	1, 0, 0	1, 0, 0	0.36/g	0.36/g
				2	0, 0, 0	0, 0, 0	<0.3/g	<0.3/g
				3	2, 0, 0	2, 0, 0	0.92/g	0.92/g
				4	0, 0, 0	0, 0, 0	<0.3/g	<0.3/g
				5	0, 0, 0	0, 0, 0	<0.3/g	<0.3/g

[1] MPN values determined using the FDA-BAM MPN tables.

Table 3. BAX System Results for Oysters with MPN Testing *V. parahaemolyticus* (3 tube, 8 dilution)				
Sample Set	BAX positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	Reference positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	BAX MPN[1]	Reference MPN[1]
3°C	3, 3, 3, 1, 0, 0, 0, 0	3, 3, 3, 1, 0, 0, 0, 0	42 MPN/g	42 MPN/g
25°C	3, 3, 3, 3, 3, 3, 3, 2	3, 3, 3, 3, 3, 3, 3, 2	1.1×10^6 MPN/g	1.1×10^6 MPN/g
35°C	3, 3, 3, 3, 3, 3, 3, 3	3, 3, 2, 3, 3, 3, 3, 3	$>1.1 \times 10^6$ MPN/g	$>1.1 \times 10^6$ MPN/g *

[1] MPN values determined using the FDA-BAM MPN tables.

***An MPN of 3,3,3 for the Reference MPN was used for the 10^{-4}, 10^{-5} and 10^{-6} replicates. This MPN calculation assumes that the one 10^{-1} g MPN tube from which no confirmed *V. parahaemolyticus* strain was recovered was a failure to pick a true typical isolate present in the background of non-*V. parahaemolyticus* which exhibited typical morphology for the target. Since all three replicates for the MPN tubes up to 5 orders of magnitude more dilute than the 10-1 tube were culture confirmed, it is unlikely that the culture result from this one discordant tube was correct.**

Table 4. BAX System Results for Oysters with MPN Testing *V. vulnificus* (3 tube, 8 dilution)				
Sample Set	BAX positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	Reference positive (10g, 1g, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})	BAX MPN[1]	Reference MPN[1]
3°C	3, 3, 1, 0, 0, 0, 0, 0	3, 3, 1, 0, 0, 0, 0, 0	4.6 MPN/g	4.6 MPN/g
25°C	3, 3, 3, 3, 3, 1, 0, 0	3, 3, 3, 3, 3, 1, 0, 0	4,200 MPN/g	4,200 MPN/g
35°C	3, 3, 3, 3, 3, 2, 0, 1	3, 2, 3, 3, 3, 2, 0, 1	14,000 MPN/g	14,000 MPN/g *

[1] MPN values determined using the FDA-BAM MPN tables

*** An MPN of 2,0,1 for the Reference MPN was used for the 10^{-4}, 10^{-5} and 10^{-6} replicates. This MPN calculation assumes that the one 1 g MPN tube from which no confirmed *V. vulnificus* strain was recovered was a failure to pick a true typical isolate present in the background of non-*V. vulnificus* which exhibited typical morphology for the target. Since all three replicates for the MPN tubes up to 3 orders of magnitude more dilute than the 10-1 tube were culture confirmed, it is unlikely that the culture result from this one discordant tube was correct..**

Table 5. BAX vs. Reference Results Aggregate									
Sample type	Target Level by MPN or cfu per 25 gram	Samples or Number of MPN Tubes	BAX pos	Reference pos	Sensitivity %[1]	Specificity %[2]	False Pos %[3]	False Neg %[4]	Chi Square[5]
Tuna	0.5 MPN/25g	20	3	3	100	100	0	0	-
	1.9 MPN /25g	20	13	13	100	100	0	0	-
	3.75 MPN /25g	20	19	19	100	100	0	0	-
	0 cfu/25g	5	0	0		100	0	0	-
Tuna (Independent Laboratory Study)	MPN/25g	20	9	9	100	100	0	0	-
	0 cfu/25g	5	0	0		100	0	0	-
Frozen raw shrimp	Naturally contaminated	20	5	5	100	100	0	0	-
Cooked shrimp (MPN)	1.8 cfu/g	45	5	5	100	100	0	0	-
Cooked shrimp (25g)	1.8 cfu/g	5	5	5	100		0	0	-
Cooked shrimp (MPN)	18 cfu/g	45	14	14	100	100	0	0	-
Cooked shrimp	18 cfu/g	5	5	5	100		0	0	-

(25g)									
Frozen Scallops (MPN)	1.4 x 10^4 cfu/g	45	3	3	100	100	0	0	-
Frozen Scallops (25g)	1.4 x 10^4 cfu/g	5	5	5	100		0	0	-
Oysters 3°C	Naturally contaminated – *V. parahaemolyticus*	24	10	10	100	100	0	0	-
Oysters 25°C Abuse		24	23	23	100	100	0	0	-
Oysters 35°C Abuse		24	24	23	100	96	4	0	0
Oysters 3°C	Naturally contaminated – *V. vulnificus*	24	7	7	100	100	0	0	-
Oysters 25°C Abuse		24	16	16	100	100	0	0	-
Oysters 35°C Abuse		24	18	17	100	94	6	0	0

[1] Sensitivity - Total number of confirmed positive test portions by the method divided by total number of confirmed positive test portions by both the alternative and reference methods.
[2] Specificity - Total number of analyzed negative test portions by the method divided by total number of confirmed negative test portions by both the alternative and reference methods.
[3] False negative rate is calculated as BAX (-) Ref (+) BAX enrichment samples / Tot Ref (+) samples
[4] False positive rate is calculated as BAX (+) Ref (-) / Tot Ref (-) samples
[5] McNemar Chi-Square test statistic used for calculating significance of results

Results and Discussion of Food Studies

Data from these studies exhibits near complete equivalence between test and reference method results. In all studies except the oyster trials, complete equivalence was found. From two enrichments in the oyster studies, there was a discordant result, one for *V. parahaemolyticus* and one for *V. vulnificus*. In both of these cases the result occurred in an MPN tube that was well under the highest dilution that tested positive and was thus likely indicative of a failure to be able to isolate the target when it was truly present in the enrichment. Since selective and differential media for Vibrio do not give complete inhibition against many other genre there was likely a relatively high number of non-target similar appearing bacterial colonies on the plate, and none of the selected colonies were found to be the target species by phenotypic characterization from these two enrichment tubes.

Since the BAX ® test kit returns a result in about 24 hours versus the 3-5 days needed for culture based methods; the test kit can lead to a significantly faster increase in release of product.

Inclusivity / Exclusivity Study

Choice of Strains

V. cholera (n=46), *V. parahaemolyticus* (n=47), and *V. vulnificus* (n=33) strains were tested by the BAX ® assay for inclusivity. Most isolates were originally obtained from naturally contaminated food and environmental samples (many from the laboratory of Dr. Judy Johnson, collected when she was on faculty at the University of Maryland) and an effort is being made to more accurately determine source for non-ATTC isolates shown below. Additionally, 36 strains were obtained through an ongoing retail shrimp study at Qualicon. Identifications were confirmed biochemically using either the API 20E test kit as modified in the FDA-BAM or using the biochemical characterization scheme described in Table 1 of the FDA-BAM *Vibrio* chapter (9), some *V. cholera* isolates (see table 6) were also characterized by serology.

Culture Enrichment

For each inclusivity strain, one colony from an overnight T_1N_3 agar plate was inoculated into a tube containing alkaline peptone water (APW) and incubated at 37°C overnight, giving a cell density of approximately 10^8 cfu/ml. Isolates were diluted 1:1000 in APW to reach the target enrichment level of 10^5 cfu/mL before processing in the BAX® system.

Each non-*Vibrio* exclusivity strain was incubated at 37°C overnight in Brain Heart Infusion (BHI) broth. Isolates were diluted 1:10 in BHI before processing in the BAX® system. *Vibrio* strains in the exclusivity panel were grown at 35°C overnight in APW, then diluted 1:10 in APW before processing in the BAX® system.

Results

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*

Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
VcJVY212		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcJVB52		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc5439/62		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc569B		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcS171		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcNAG12		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcATCC25874		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc8		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcB1307 Dacca		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcA5		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
VcI10		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc646 Ogawa01		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
Vc395 Classical Ogawa01		Unknown	UF	*V. cholerae*	Pos	Neg	Neg
TD3192		Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
TD7000	ATCC 9459	Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
DD9892		Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
DD13084	ATCC 14035	Unknown	Qualicon	*V. cholerae*	Pos	Neg	Neg
TD3161		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3162		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3163		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3164		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3165		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3167		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3170		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3171		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3173		Unknown	Qualicon	*V. cholerae (non-O1, O139)*	Pos	Neg	Neg
TD3180		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3183		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3185		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3186		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*							
Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
TD3187		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3858		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3859		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3860		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3861		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3862		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3863		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3864		Unknown	Qualicon	*V. cholerae O1*	Pos	Neg	Neg
TD3203		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
TD3211		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
TD3213		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
TD3214		Unknown	Qualicon	*V. cholerae O139*	Pos	Neg	Neg
VpTx2103		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
VpTx3547		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
VpDAL1094		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
Vp17802		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
Vp43996		Unknown	UF	*V. parahaemolyticus*	Neg	Pos	Neg
DD2633	ATCC 17802	Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3129		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3130		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3131		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3132		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3133		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3134		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3135		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3153		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3154		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3155		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3156		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3157		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3159		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
TD3160		Unknown	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*							
Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
Vv FLA141		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
Vv FLA126		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA134		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
Vv Fla 129		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA127		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA135		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA115		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA149		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvB3-313/98		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA121		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA137		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvB3-302/99		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA119		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA116		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA102		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvB2-2		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
VvFLA108		Unknown	UF	*V. vulnificus*	Neg	Neg	Pos
TD3121		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3148		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3149		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3204		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3207		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3208		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3210		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3212		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3217		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD3219		Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
TD4527	ATCC 27562	Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13082	ATCC BAA-86	Unknown	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13231		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg
DD13232		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg
DD13208		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg

Table 6. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*							
Strain ID	Other strain designation	Source	Location of testing	Species (serotype)	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
DD13209		Shrimp	Qualicon	*V. cholera*	Pos	Neg	Neg
DD13212		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13216		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13217		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13218		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13211		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13222		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13223		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13224		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13225		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13226		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13228		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13229		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13230		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13233		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13234		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13235		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13236		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13204		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13207		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13200		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13202		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13201		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13203		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13211		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13214		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13215		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13210		Shrimp	Qualicon	*V. parahaemolyticus*	Neg	Pos	Neg
DD13205		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13206		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13227		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos
DD13213		Shrimp	Qualicon	*V. vulnificus*	Neg	Neg	Pos

Table 7. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*

Strain ID	Other strain designation	Source	Species	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
DD2558		Unknown	*Citrobacter freundii*	Neg	Neg	Neg
DD383		Unknown	*Citrobacter freundii*	Neg	Neg	Neg
DD2560		Unknown	*Citrobacter kosei*	Neg	Neg	Neg
DD2561		Unknown	*Citrobacter kosei*	Neg	Neg	Neg
DD12835		Unknown	E. coli O157:H7	Neg	Neg	Neg
DD1450		Unknown	E. coli O157:H7	Neg	Neg	Neg
DD1979		Unknown	E. coli O157:H7	Neg	Neg	Neg
TD8136		Unknown	E. coli O157:H7	Neg	Neg	Neg
DD2554		Unknown	*Enterococcus faecalis*	Neg	Neg	Neg
DD6523		Unknown	*Klebsiella oxytoca*	Neg	Neg	Neg
DD2546		Unknown	*Klebsiella pneumoniae*	Neg	Neg	Neg
DD1144		Unknown	*Listeria monocytogenes*	Neg	Neg	Neg
DD1283		Unknown	*Listeria monocytogenes*	Neg	Neg	Neg
DD1309		Unknown	*Listeria monocytogenes*	Neg	Neg	Neg
DD3572	ATCC 9459	Unknown	*Listeria innocua*	Neg	Neg	Neg
DD3376		Unknown	*Listeria ivanovii*	Neg	Neg	Neg
DD2874	ATCC 14035	Unknown	*Listeria seeligeri*	Neg	Neg	Neg
DD3354		Unknown	*Listeria welshimeri*	Neg	Neg	Neg
DD3411		Unknown	*Listeria welshimeri*	Neg	Neg	Neg
DD2357		Unknown	*Proteus mirabilis*	Neg	Neg	Neg
DD374		Unknown	*Proteus mirabilis*	Neg	Neg	Neg
DD13148		Unknown	*Pseudomonas aeruginosa*	Neg	Neg	Neg
DD3982		Unknown	*Pseudomonas aeruginosa*	Neg	Neg	Neg
DD3019		Unknown	*Salmonella ser.* Dublin	Neg	Neg	Neg
DD706		Unknown	*Salmonella ser.* Enteritidis	Neg	Neg	Neg
DD1261		Unknown	*Salmonella ser.* Newport	Neg	Neg	Neg
DD13060		Unknown	*Salmonella ser.* Senftenburg	Neg	Neg	Neg
DD586		Unknown	*Salmonella ser.* Typhimurium	Neg	Neg	Neg
DD1083		Unknown	*Shigella flexneri*	Neg	Neg	Neg
DD699		Unknown	*Shigella soneii*	Neg	Neg	Neg
DD10156		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg
DD7426		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg

Table 7. Inclusivity Results for *Vibrio cholerae/parahaemolyticus/vulnificus*

Strain ID	Other strain designation	Source	Species	Result *V. cholera*	Result *V. parahaemolyticus*	Result *V. vulnificus*
DD9775		Unknown	*Staphylococcus aureus*	Neg	Neg	Neg
DD11233		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3146		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3195		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3200		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD3658		Unknown	*Vibrio alginolyticus*	Neg	Neg	Neg
TD4501		Unknown	*Vibrio anguillarum*	Neg	Neg	Neg
TD4498		Unknown	*Vibrio carchariae*	Neg	Neg	Neg
TD3194		Unknown	*Vibrio damsela*	Neg	Neg	Neg
TD4524		Unknown	*Vibrio damsela*	Neg	Neg	Neg
DD2631		Unknown	*Vibrio fluvialis*	Neg	Neg	Neg
TD4526		Unknown	*Vibrio fluvialis*	Neg	Neg	Neg
TD4497		Unknown	*Vibrio harveyi*	Neg	Neg	Neg
DD11232		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
DD13083		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD3137	ATCC 17802	Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD3147		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD3216		Unknown	*Vibrio mimicus*	Neg	Neg	Neg
TD4500		Unknown	*Vibrio natriegens*	Neg	Neg	Neg
TD4528		Unknown	*Vibrio pelagia*	Neg	Neg	Neg
TD4523		Unknown	*Vibrio tubiashii*	Neg	Neg	Neg
DD2399		Unknown	*Yersinia aldovae*	Neg	Neg	Neg
DD592		Unknown	*Yersinia enterocolitica*	Neg	Neg	Neg

Results – ALL TARGET *VIBRIO* ISOLATES GAVE EXPECTED POSITIVE RESULTS AND ALL NON-*VIBRIO* AND NON-TARGET *VIBRIO* SPECIES GAVE EXPECTED NEGATIVE RESULTS.

Stability Study

Methodology – BAX ® system test kits were evaluated in experiments to determine a reasonable shelf-life using both accelerated and non-accelerated storage conditions (see table below). *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, *V. parahaemolyticus* TD 4496, and *V. vulnificus* DD 13082 were assayed using purified DNA at a level equivalent to one order of magnitude over the product's claimed sensitivity level (i.e. 10^5 cfu/mL) by the BAX ® assay. Additionally, two non-target *Vibrio* and non-*Vibrio* strains, *Pseudomonas aeruginosa* DD 962 and *Vibrio mimicus (non-target Vibrio species)* DD 13083 were tested using purified DNA at levels corresponding to 10^8 cfu/ml in an enriched sample. Three replicates of each strain at each temperature condition at each time point were assayed. Also, for each condition, for each time point, three non-spiked lysis buffer controls were tested.

Results – All results except for one *V. vulnificus* test at the 23 day 25°C treatment gave the expected result (tests spiked with positive target tested BAX ® positive while non-target and non-spiked tests tested BAX ® negative). It is not known why this one result was atypical, though it is possible this was due to a procedural error such as a pipette tip not properly affixed during the 5 µl lysate preparation step or an accidental loading of a non-target replicate into what was supposed to be a target reaction. The results of the accelerated stability study showed no difference in the performance of this test kit after being stored for 122 days at 25°C and 37ºC as compared to the 4ºC control. Based on these results and applying the Q_{10} rule of the Arrhenius equation, a 32 month shelf life has been assigned to these test kits.

Predicted Stability = Accelerated Stability X $2^{\Delta t/10}$
For example: Stability of a product at 50°C is 32 days.
Recommended storage temperature is 25°C and n = (50 - 25)/10 = 2.5
Qn = (2)2.5 = 5.66 The predicted shelf life is 32 days X 5.66 = 181 days

Accelerated stability studies are continuing and it is anticipated that the next lot of test kits will be assigned a 36 month shelf life. Real-time testing at 4ºC has shown stability for 122 days and is continuing.

Table 8. Summary of stability study

Time Point (days)	Storage Temp (°C)	BAX ® Positive *Vibrio cholera/parahaemolyticus/ vulnificus*	BAX ® Positive non-target organisms and non-spiked controls
23	4	15/15	0/9
	25	14/15	0/9
	37	15/15	0/9
60	4	15/15	0/9
	25	15/15	0/9
	37	15/15	0/9
122	4	15/15	0/9
	25	15/15	0/9
	37	15/15	0/9

Lot-to-lot study

Methodology –BAX® system test kits from three lots with different expiration dates were tested in an experiment to determine any evidence of lot-to-lot performance differences. *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, and *V. vulnificus* DD 13082 were assayed using dilutions of overnight cultures at levels equivalent to approximately one order of magnitude over the product's claimed sensitivity level (i.e. ~10^5 cfu/mL) by the BAX ® assay. Additionally, two non-target *Vibrio* and non-*Vibrio* strains, *Pseudomonas aeruginosa* DD 962 and *Vibrio mimicus (non-target Vibrio species)* DD 13083 were tested using cells at levels of approximately 10^8 cfu/ml. Two replicates of each strain at each temperature condition at each time point were assayed. Also, for each condition, for each time point, two non-spiked lysis buffer controls were tested.

Table 9. Lot to Lot Test Kit Comparison

Lot #	Expiration Date	*Vibrio* spiked positives	Non-*Vibrio* spiked positives
030508	12/05/2010	8/8	0/6
061008	02/09/2011	8/8	0/6
8263	08/23/2011	8/8	0/6

Results – This lot to lot comparison study found no evidence of performance differences.

Ruggedness Study

Methodology –The BAX® system was evaluated to determine whether it performs as expected despite variations in operational parameters. Since the entire amplification and detection phases are fully automated, independent variables were selected from the enrichment and sample preparation phases. Eight variables believed to have the largest potential for impact on performance were selected, as shown in Table 10 with associated low and high levels:

Table 10. Variables in ruggedness study

Variable	Normal level	Low level	High level
1) Sample volume	5 µL	4	6
2) Incubation temperature (lysis)	37ºC	34	40
3) Incubation time (lysis)	20 minutes	15	30
4) Inactivation temperature (lysis)	95ºC	91	99
5) Inactivation time (lysis)	10 minutes	8	12
6) Total hydration volume	30 µL	27	33
7) Enrichment temperature	35ºC	32	38

For assay factors (1-6) each factor was varied, both high and low level as well as a normal level, for three replicates of 6 strains (4 different *Vibrio target strains* and 2 different non-*target strains*). Additionally, two non-inoculated samples were assayed for each variable/level studied.

For inoculated samples, *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, and *V. vulnificus* TD 3121 were serially diluted to just above the product's claimed sensitivity level (i.e. 10^5 cfu/ml of enriched culture) and prepared for the BAX® assay. Additionally, two non-target *Vibrio* and non-*Vibrio* strains, *Vibrio mimicus (non-target Vibrio species)* TD 3147 and *Salmonella* Newport DD 1261 were grown and diluted to attain inoculation levels of ~10^7-10^8 cfu/ml as described in the lot to lot study. Uninoculated samples were freshly prepared with APW and treated in an analogous manner to the inoculated samples.

For the enrichment factor (7) low levels of ~10 cfu of *V. cholera* TD 3858, *V. cholera* TD 3192, *V. parahaemolyticus* TD 3129, and *V. vulnificus* TD 3121 and high levels (~10^5 cfu) of non-target strains *Salmonella* Newport DD 1261 and *Vibrio mimicus (non-target Vibrio species)* TD 3147 were added to 225 ml aliquots of APW with replicates for each variable for each strain and assayed for high (n=2), low (n=2), and normal (n=2) conditions.

Table 11. Results of ruggedness study

Variable	Normal level	Positive *Vibrio*	Positive Non- *Vibrio*	Positive Uninoc.	Low level	Positive *Vibrio*	Positive Non- *Vibrio*	Positive Uninoc.	High level	Positive *Vibrio*	Positive Non- *Vibrio*	Positive Uninoc.
1) Sample volume	5 µL	12/12	0/6	0/2	4	12/12	0/6	0/2	6	12/12	0/6	0/2
2) Incubation temperature	37ºC	12/12	0/6	0/2	34	12/12	0/6	0/2	40	12/12	0/6	0/2
3) Incubation time	20 min	12/12	0/6	0/2	15	12/12	0/6	0/2	30	12/12	0/6	0/2
4) Inactivation temperature	95ºC	12/12	0/6	0/2	91	12/12	0/6	0/2	99	12/12	0/6	0/2
5) Inactivation time	10 min	12/12	0/6	0/2	8	12/12	0/6	0/2	12	12/12	0/6	0/2
6) Total hydration volume	30 µL	12/12	0/6	0/2	27	12/12	0/6	0/2	33	12/12	0/6	0/2
7) Enrichment temperature	35ºC	8/8	0/4	0/2	32	8/8	0/4	0/2	38	8/8	0/4	0/2

Results – The results of the ruggedness study are shown in Table 11. All *Vibrio*-inoculated samples returned positive results. All non-*Vibrio* inoculated and un-inoculated samples were negative. These results indicate that the variables studied did not affect the performance of the BAX® system assay within the ranges tested.

Discussion

In initial development studies, some enriched samples were found to test positive by the BAX® pcr assay but negative by the reference culture method. Often, this is the case when non-target competitive flora, either non-*Vibrio*, or non-target *Vibrio* species are present in an enrichment with cell densities at a much higher level than the target organism. In such cases, an additional plating media, CHROMagar *Vibrio*, has been found to be useful. For each sample tested for most studies (with the exception of the oyster studies performed at Dauphin Island), a CHROMagar *Vibrio* plate was also struck from each enriched sample to reflect this fact. In one study (the naturally contaminated frozen raw shrimp work) two samples were found to be pcr positive/culture negative. For these samples that tested pcr positive, but from which no confirmed colonies of a positive species were found from the FDA-BAM media, more colonies than required by the FDA BAM procedure were picked from the TCBS, mCPC and CHROMagar *Vibrio* plates into cluster tubes containing 500 µl APW (up to 24 per sample per media where available). Individual isolates were allowed to grow in the cluster tubes overnight at room temperature and tested by BAX® assay. Presumptive positive cluster tubes were struck onto TCBS or T_1N_3 agar and confirmed using the FDA-BAM methods. Both of these samples were then found to be positive using this enhanced protocol, yielding at least one confirmed *V. cholera* isolate. Qualicon has also demonstrated the presence of atypical *V. parahaemolyticus* strains (confirmed by DNA sequence-based characterization) that do not present with typical characteristics on *Vibrio* selective and differential agars. All enrichments which tested positive by PCR, with the exception of two MPN tubes from the oyster study, were also positive for typical confirmed colonies on one or more of the three agars above. In the oyster studies, only three typical colonies per MPN tube were selected as per the FDA-BAM protocols, and a greater number of colonies selected per tube would have made the experiment unmanageable. This highlights a potential issue with the reference method in that typical colony morphology on plates is a critical step in the reference method and the complex microbial ecology of an oyster can potentially lead to less than optimal results when non-target isolates with a typical phenotype on *Vibrio* selective agars are present in significant numbers relative to the levels of target *Vibrio*. In other non-AOAC studies conducted at Qualicon some instances of PCR positive enrichments have yielded phenotypically atypical isolates that test positive by PCR. These isolates have been characterized by sequence-based identification (microSeq ®, Applied Biosystems, Foster City, CA) as target *Vibrio* species and are being shared with the community of *Vibrio* experts for further characterization (data not shown). The above described work supports continued work on the natural phenotypic and genetic variation of pathogenic species of *Vibrio* occurring in foods.

Conclusion

The data in these studies, within their statistical uncertainty, support the product claims of the BAX® System PCR Assay for Detecting *Vibrio cholera, parahaemolyticus, and vulnificus* with the tested foods, including raw frozen shrimp, cooked shrimp, raw oysters, raw ahi tuna, and raw scallops.

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