

Detection and Quantification of Total and Potentially Virulent *Vibrio parahaemolyticus* Using a 4-Channel Multiplex Real-Time PCR Targeting the *tl*, *tdh*, and *trh* Genes and a Novel PCR Internal Control

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ABSTRACT

Vibrio parahaemolyticus (*Vp*) is an estuarine bacterium that is the leading cause of shellfish-associated cases of bacterial gastroenteritis in the US. The thermolabile hemolysin gene (*tl*) is a species-specific marker for *Vp*; the thermostable direct hemolysin gene (*tdh*) and the thermostable-related hemolysin gene (*trh*) are both currently used as pathogenicity markers for this organism since most clinical isolates of *Vp* possess one or both of these genes. Our laboratory developed a real-time 4-channel multiplex PCR assay for the simultaneous detection and quantification of these three target genes plus a novel internal control in a single 40-minute reaction using the Cepheid Smart Cycler® (Sunnyvale, CA). The assay was tested for specificity against a panel of more than 100 strains representing seventeen different bacterial species. Only *Vp* strains possessing the appropriate target genes produced amplified target and generated fluorescent signal. The robustness of the assay was confirmed using a pure culture *Vp* template suspended in the following matrices: PBS, direct and concentrated oyster mantle fluid, oyster tissue enrichment, shrimp tissue enrichment, and crab tissue enrichment. The assay was shown to have a dynamic range of detection of 1–10^6 cfu (colony forming unit) per reaction (with simultaneous detection of each target gene), and was demonstrated to be quantitative with a precision of detection within two-fold when tested using pure cultures of various *Vp* strains possessing *tdh*, *trh*, or both *tdh* and *trh*. Combining this real-time PCR assay with rapid and repeatable methods for sample and template preparation and concentration will allow same day direct enumeration of total and potentially virulent *Vp* from environmental sources and seafood.

INTRODUCTION

Vibrio parahaemolyticus (*Vp*) is found worldwide in estuarine waters and is the leading cause of gastroenteritis from seafood in the US, with most infections occurring from the consumption of raw or mishandled seafood. Most previous methods for the detection of *Vp* have involved labor and resource intensive testing of individual isolates by various phenotypic assays.

The *tl* (thermolabile hemolysin) gene is a species-specific marker for *Vp*, while the *tdh* (thermostable direct hemolysin) and *trh* (thermostable-related hemolysin) genes are two current pathogenicity markers for *Vp*. Several DNA-based assays targeting these

genes have recently been developed and utilized for the detection and enumeration of *Vp*; however, none of these methods are capable of same day simultaneous enumeration of both total and pathogenic strains from seafood or environmental samples. There is a need within the public health sector and the seafood industry for methods that are capable of rapid, sensitive, and accurate identification and quantification of *Vp*—especially those strains that may be pathogenic. Such a method could potentially be utilized for the monitoring of seafood products to increase food safety, and would be a valuable research tool to assist in understanding the occurrence and distribution of this organism in the marine environment.

With the advent of real-time (kinetic) PCR analysis, using appropriate standards and sample preparation techniques it is now possible to directly quantify the number of bacteria (or other organisms) in a sample without the need for post-PCR analysis methods such as gel electrophoresis. However, one of the risks associated with testing samples by PCR is the occurrence of false negatives. While a positive and negative control are normally run for every PCR master mix to ensure the integrity of the reagents, inhibition of the PCR by the sample matrix may cause an individual test sample to report a negative result, even if there is target template present in the reaction. In quantitative real-time PCR this even more of a concern, as partial PCR inhibition may lead to inaccurate quantification results. Therefore, it is necessary to include an internal positive control in each individual reaction to prevent the reporting of false negatives and to allow accurate adjustments to quantitative data. In the present report we describe a 4-channel real-time multiplex PCR assay developed on the Smart Cycler system from Cepheid. This assay includes a novel exogenous internal control and is designed for the robust and simultaneous detection and quantification of total (*tl*+) and potentially pathogenic (*tdh*+ or *trh*+) strains of *Vp*.

OBJECTIVES

1. Development of a real-time multiplex PCR assay for the simultaneous detection of total (*tl*+) and pathogenic (*tdh*+ and/or *trh*+) *Vp* in microbiological enrichments.
2. Optimization of the assay for the simultaneous enumeration of total and pathogenic *Vp* in natural (unenriched) samples.

- Determine assay applicability in testing of various seafood and environmental samples—including oysters & oyster mantle fluid, shrimp, crab, seawater, sediment, and others.

METHODS

Assay design considerations

- Multiple variants of *tdh*, all potentially capable of sufficient TDH production for illness (promoter efficiency-related).
- tdh1* and *tdh2* gene variants have been associated with K+ strains, *tdh2* causes hemolytic activity in K+ strains.
- tdh* also found in *V. hollisae*, *V. fluvialis*, *V. mimicus*, *V. cholerae*, others.
- Plasmid-borne *tdh* (rare) in some *Vp* and other *Vibrios*.
- trh* associated with virulence, often present in association with *tdh* in pathogenic strains.
- Several *trh* variants—*trh1*, *trh2*, others, > 84% nt identity.
- trh* has ~70% nt identity with *tdh*.
- tdh* and *trh* are in close proximity on the chromosome in *tdh+*, *trh+* strains—possible pathogenicity island.
- While *tl* is a *Vp*-specific marker, it has significant similarity to *tdh* and *trh* in some regions of the gene.
- PCR amplicons of sizes that could be easily distinguished using agarose gel electrophoresis.
- Primer and probe sets that were compatible in multiplex without primer dimer formation and that produced PCR reactions of similar reaction efficiencies.

Bacterial cultures and genomic DNA preparation

Selected *V. parahaemolyticus* strains [(FIHES98, TX2103 (*tdh+*), AQ4037 (*trh+*), F11-3A (*tdh+*,*trh+*), and 91A-4950 (*tdh+*,*trh+*)] used for real-time PCR bacterial enumeration experiments were grown for 6 hours at 35 °C in 5 mL alkaline peptone water (APW) (1.0% peptone, 1.0% NaCl, pH 8.5 ± 0.2). Crude cell lysates were prepared from 1 mL aliquots of each of these cultures by boiling for 15 minutes in 1.5 mL microcentrifuge tubes. The genomic DNA contained in these lysates was later used as template in the real-time PCR assay. Overnight plate counts from T1N3 plates (1.0% tryptone, 3.0% NaCl, 2.0% agar) containing spread dilutions of each six-hour culture were used to determine the original cfu/mL for each preparation of boiled template.

All additional *Vibrio* strains utilized in this study were grown overnight at 35 °C in APW, while all other bacterial strains were grown overnight at 35 °C in tryptic soy broth (1.7% pancreatic digest of casein, 0.3% enzymatic soy digest, 0.25% dextrose, 5.0 g/L NaCl, 2.5 g/L K₂HPO₄, pH 7.3 ± 0.2). Purified genomic DNA was prepared from many strains using the MagNA Pure LC robotic DNA extraction instrument from Roche (Indianapolis, IN). A Bacterial

DNA Isolation Kit (III) was used for the extraction process, which produced 100 µL of eluted DNA sample from 100 µL of enrichment culture. The purity and concentration of each DNA sample was determined by UV spectrophotometry and fluorometry with PicoGreen (Molecular Probes, Inc., Eugene, OR), respectively.

Design of primers and fluorogenic probes for real-time PCR

The complete nucleotide sequences (open reading frame regions only) for all reported variants of the *tl*, *tdh* and *trh* genes of *Vp* were aligned and compared using Lasergene MegAlign™ software (clustal alignment, PAM250 distance tables) from DNASTAR (Madison, WI). An additional newly-identified variant of the *trh* gene (manuscript in preparation) was also included in the alignments. Primer Express Software from Applied Biosystems (Foster City, CA) was used to design oligonucleotide primers and TaqMan® or TaqMan MGB fluorogenic probes targeting regions identified by the alignments as unique to each of these genes. Multiple primer sets were designed and tested for specificity and compatibility in multiplex. Information on the primers and probes utilized in the assay can be seen in the following table:

Target	Primers ^{2/} probe	Amplicon size (bp)	Probe type	Probe fluorophore	Probe quencher
<i>tl</i> gene	tl-F, tl-R, tl-P	207	5'-nuclease ³	TX-Red or Rox ⁵	Black Hole Quencher™-2
<i>tdh</i> gene	tdh-F, tdh-R, tdh-P	233	TaqMan MGB ⁴	FAM	Non-fluorescent Quencher
<i>trh</i> gene	trh-F, trh-R, trh-P	273	TaqMan MGB ⁴	TET	Non-fluorescent Quencher
Internal control	NR	NR	NR	Cy-5	NR

NR, Not for public release at this time—manuscript and patent application in progress.
²All primers were synthesized by either Integrated DNA Technologies (Coralville, IA) or Invitrogen (Carlsbad, CA).
³Synthesized by Integrated DNA Technologies.
⁴Synthesized by Applied Biosystems (Foster City, CA); MGB—minor groove binder.
⁵Both fluorophores have been utilized for this assay, using the ROX channel of the original Smart Cycler and the TX-Red channel of the Smart Cycler II.

Table 1. Primers and probes.

Exogenous internal control

An exogenous internal control of novel design was incorporated into this assay. A manuscript describing this internal control is in preparation, and a patent application is in process. For the present study, the control was set to report amplification at ~20 PCR cycles when testing either living or boiled cells of pure *Vp* cultures in APW or phosphate-buffered saline (PBS), which were both shown not to inhibit the reaction.

Real-time PCR amplification

The real-time PCR cycling protocol, fluorescent detection parameters, and reaction component concentrations were carefully optimized for the simultaneous detection and quantification of the *tl*, *tdh*, and *trh* genes of *Vp*. PCR was conducted in a 25 μ L volume using the following reaction components (final concentrations shown): 1X PCR Amplification Buffer [10X buffer consisted 200 mM Tris-HCl (pH 8.4) and 500 mM KCl] (Invitrogen), 5 mM of MgCl₂, 400 nM of each of the dNTPs (Roche, Indianapolis, IN), 200 nM of each primer (described above), 150 nM of each of the four fluorogenic probes (*tl*, *tdh*, *trh*, and internal control), and 2.25 μ L Platinum™ Taq polymerase (Invitrogen). The remainder of the reaction volume consisted of PCR-grade H₂O, target DNA template (either 2 μ L of boiled cells or 5 ng of purified DNA), and the internal control reagents. Real-time PCR thermal cycling was conducted using the Smart Cycler II system from Cepheid.

Cycling Parameters

The two-step touchdown cycling parameters utilized are shown below:

Time/temperature	Number of cycles
(95 °C for 60 seconds)	1 cycle—Denaturation/taq activation
(95 °C for 5 seconds, 64 °C for 45 seconds)	1 cycle—touchdown
(95 °C for 5 seconds, 63 °C for 45 seconds)	1 cycle—touchdown
(95 °C for 5 seconds, 62 °C for 45 seconds)	1 cycle—touchdown
(95 °C for 5 seconds, 61 °C for 45 seconds)	1 cycle—touchdown
(95 °C for 5 seconds, 60 °C for 45 seconds)	1 cycle—touchdown
(95 °C for 5 seconds, 59 °C for 45 seconds)	40 cycles—amplification

Table 2. Cycling parameters.

The Smart Cycler II instrument was programmed to measure the accumulated fluorescence in each reaction tube at the end of each amplification cycle using the FTTC-25 dye calibration set and the default software parameters, except that the Manual Threshold Fluorescent Units setting was changed to 8.0 units above background. A sample was therefore considered to be positive when a signal of at least eight fluorescence units above baseline (after background correction) was observed in the FAM, TET, TX-Red, or CY-5 channels of the instrument within 45 cycles. Positive controls of *Vp* strains possessing all three target genes plus the internal control and a negative control (sterile dH₂O added as template in the reaction) were prepared for each PCR master mix. For each reaction, a plot of the cycle threshold (Ct) vs. fluorescence (and log fluorescence) was examined for the FAM, TET, TX-Red, and CY-5 channels of the Smart Cycler II instrument. Reactions lacking the internal control probe were also performed on a Smart Cycler I (the first version of the instrument) using the FAM, TET, and ROX channels (*tdh*, *trh*, and *tl* detection, respectively).

Generation of Standard Curves

For determination of the assay sensitivity, dynamic range, and quantitative capabilities, *Vp* strain 91A-4950 was used to generate standard curves for each target during multiplex real-time PCR amplification (Figures 8–10). Dilutions (10⁻¹–10⁻⁷) in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3 \pm 0.2) were prepared from boiled cells from six-hour cultures with known cfu/mL values (see above). The assay was run in duplicate for each dilution, using 2 μ L of template per reaction.

Specificity and Enrichment Testing

Using boiled cells and/or purified DNA, the assay was tested for specificity against a panel of > 100 bacterial isolates including: *Escherichia coli*, *Vibrio alginolyticus*, *V. cholerae*, *V. fluvialis*, *V. holislae*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus*, *V. vulnificus*, *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *Salmonella sp.* (Figure 5). The robustness of the assay was evaluated using enrichments of *Vp*-spiked oyster homogenate (Figure 12), oyster mantle fluid (Figure 13), and shrimp homogenate (Figure 14). Typically, *Vp*-spiked samples were blended for 90 seconds with 9X their weight of APW and incubated overnight at 35 °C before testing.

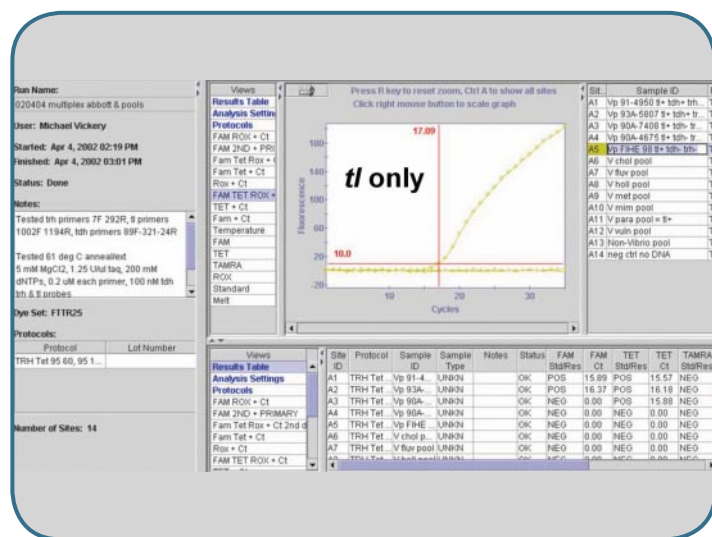


Figure 1. *tdh*-, *trh*- *Vp* strain. A screen shot of Ct vs. Fluorescence from the Smart Cycler system showing multiplex amplification of *tl*, *tdh*, and *trh* (ROX, FAM, and TET channels, respectively).

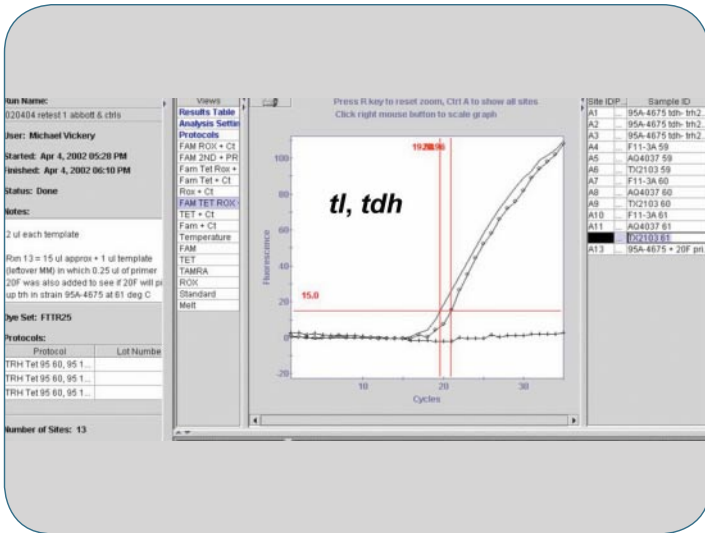


Figure 2. *tdh+*, *trh-* Vp strain. Ct vs. fluorescence is shown for the multiplex amplification of *t1*, *tdh*, and *trh* from a Vp strain (*t1+*) possessing the *tdh* gene but lacking the *trh* gene (*tdh+*, *trh-*).

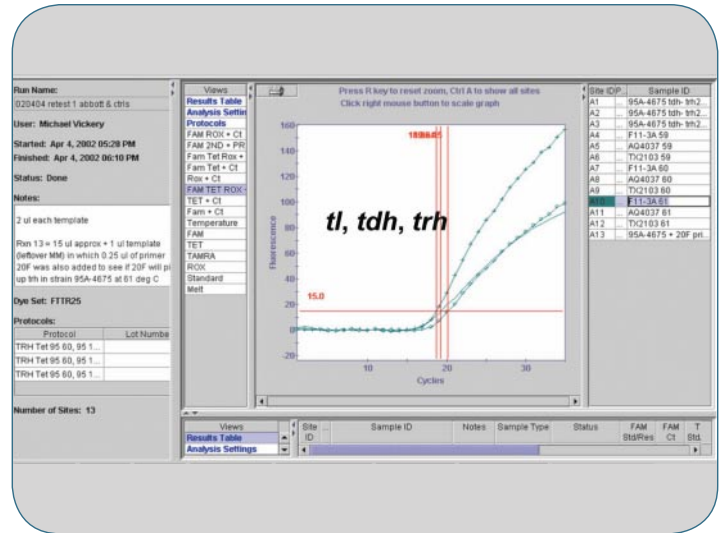


Figure 4. *tdh+*, *trh+* Vp strain. Ct vs. fluorescence is shown for the multiplex amplification of *t1*, *tdh*, and *trh* from a Vp strain (*t1+*) possessing both the *tdh* gene and the *trh* gene (*tdh+*, *trh+*).

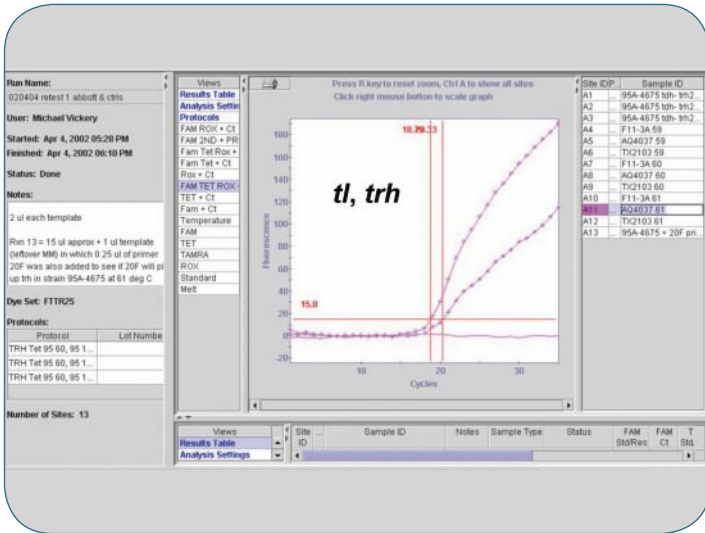


Figure 3. *trh+*, *tdh-* Vp strain. Ct vs. fluorescence is shown for the multiplex amplification of *t1*, *tdh*, and *trh* from a Vp strain (*t1+*) possessing the *trh* gene but lacking the *tdh* gene (*tdh-*, *trh+*).

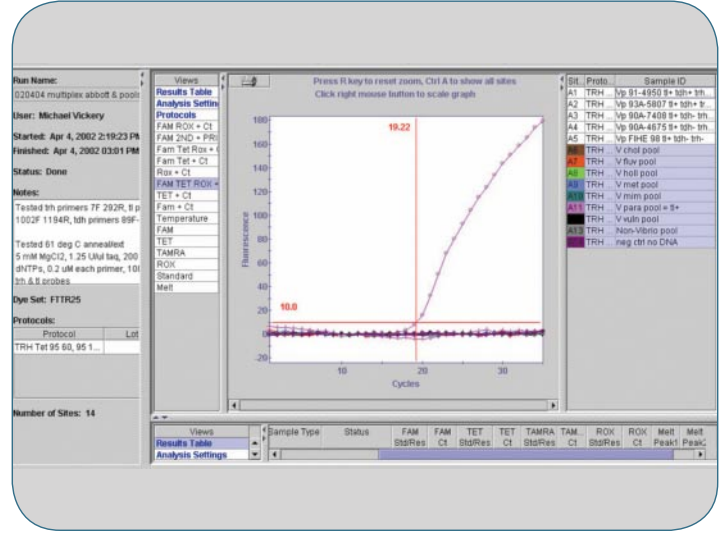


Figure 5. Specificity. Ct vs. fluorescence is shown for multiplex amplification of *t1*, *tdh*, and *trh* against a panel of diverse bacterial isolates (boiled cells). None of the non-Vp isolates amplified or reported. The Vp pool tested in this run contained only isolates lacking the *tdh* and *trh* genes.

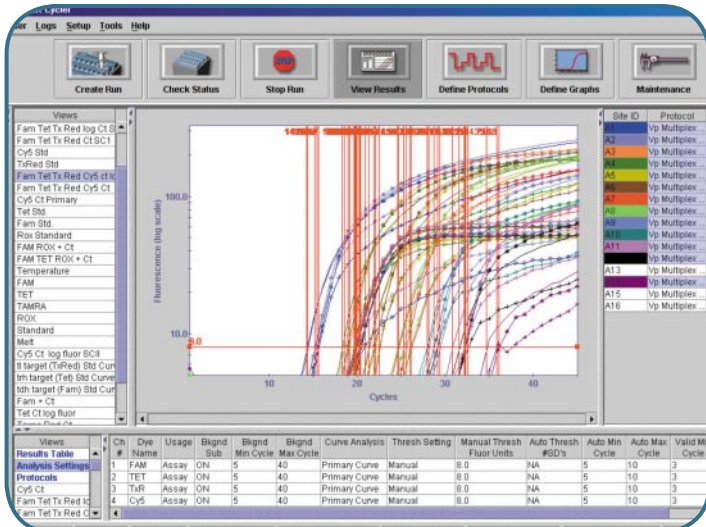


Figure 6. Four-channel multiplex amplification. A screen shot from the Smart Cycler II system software showing a 6-log template dilution range (two replicates each) for multiplex amplification of the *tl*, *tdh*, and *trh* gene targets plus the internal control (TX-Red, FAM, TET, and CY-5 channels, respectively).

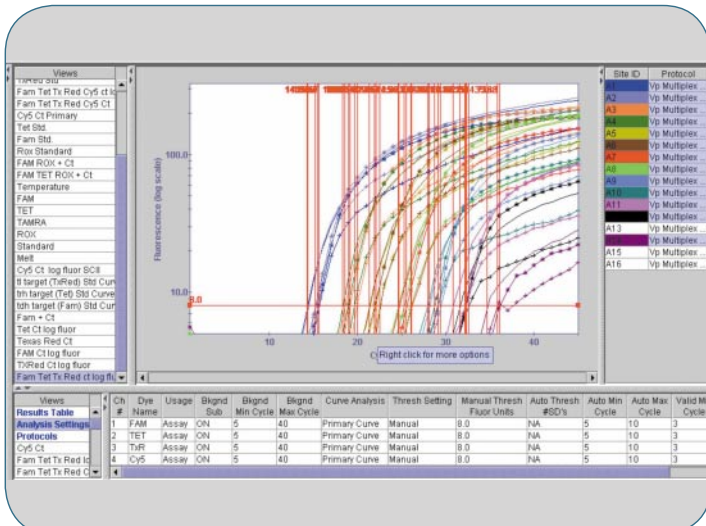


Figure 7. Six-log dynamic range. Ct vs. log fluorescence is shown for multiplex amplification of the *tl*, *tdh*, and *trh* gene targets. This is the same data presented in Figure 6 except the internal control plot has been removed for clarity. Note the dynamic range of the assay ($> 10^6$ cfu equivalents to 1 cfu equivalent) and the Ct value similarity for each replicate at each dilution—such that the replicates are hard to distinguish).

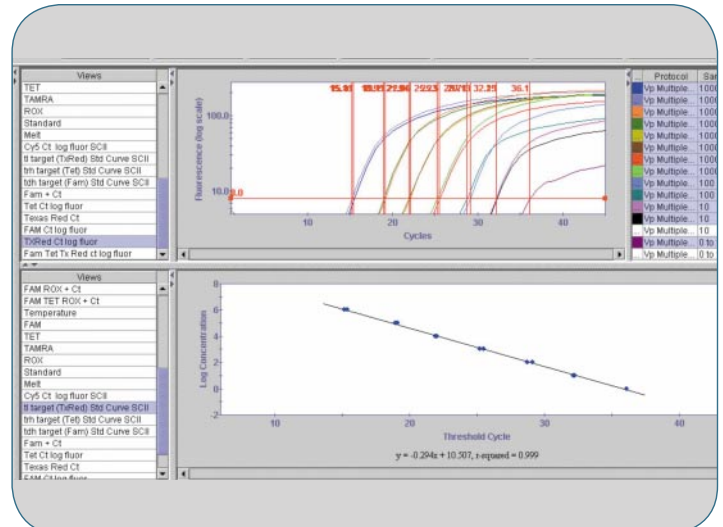


Figure 8. *tl* standard curve. Ct vs. log fluorescence is shown for the Vp *tl* gene target shown in Figure 6, along with a plot of the log cell # vs. threshold cycle (the standard curve). Note the correlation coefficient of 0.999 for the standard curve (indicative of how well the data points fit the line).

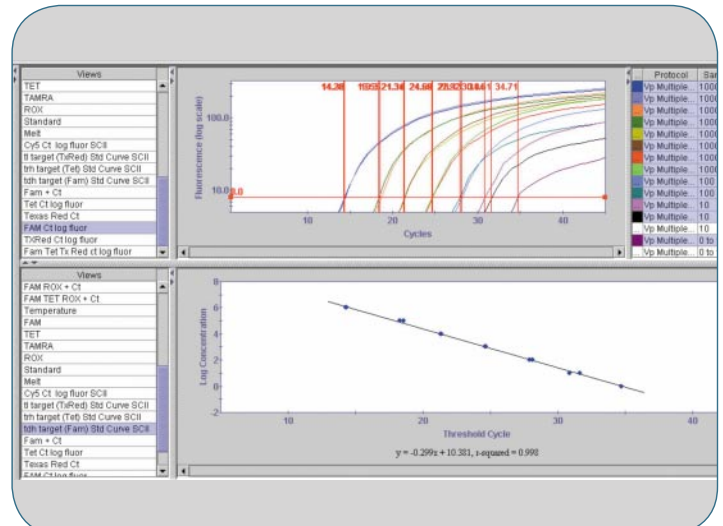


Figure 9. *tdh* standard curve. Ct vs. log fluorescence is shown for the Vp *tdh* gene target shown in Figure 6 along with a plot of the standard curve. Note the correlation coefficient of 0.998.

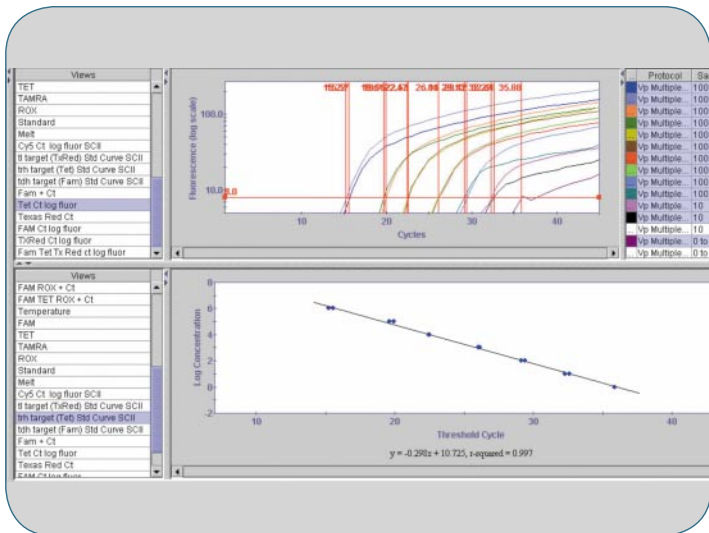


Figure 10. *trh* standard curve. Ct vs. log fluorescence is shown for the *Vp trh* gene target shown in Figure 6, along with a plot of the standard curve. Note the correlation coefficient of 0.997.

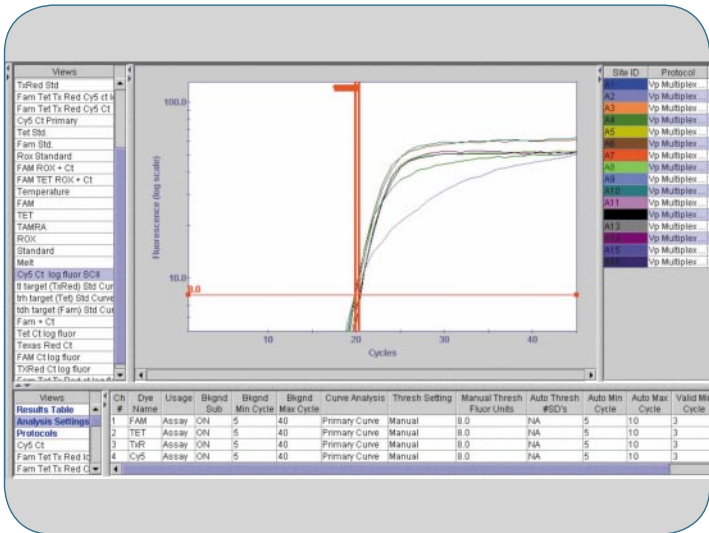


Figure 11. Internal control. Ct vs. log fluorescence is shown for the internal control presented in Figure 6. Note that the cycle threshold value for the internal control remains constant at ~20 cycles over the full dynamic range of the multiplex assay—during simultaneous quantification of three target genes.

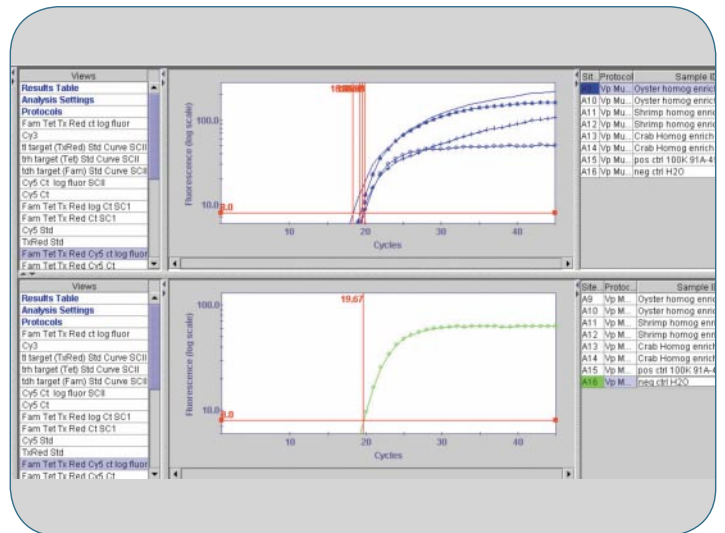


Figure 12. Oyster homogenate enrichment. The upper graph is a plot of Ct vs. log fluorescence for an oyster homogenate enrichment spiked with cells from a *tdh+*, *trh+* *Vp* strain. The lower graph is the same plot for the negative control (no *Vp* template). By comparison of the graphs it is evident that the oyster enrichment (1 μ L) did not significantly inhibit the PCR reaction, as the internal control Ct values were approximately the same for the enrichment and the negative control.

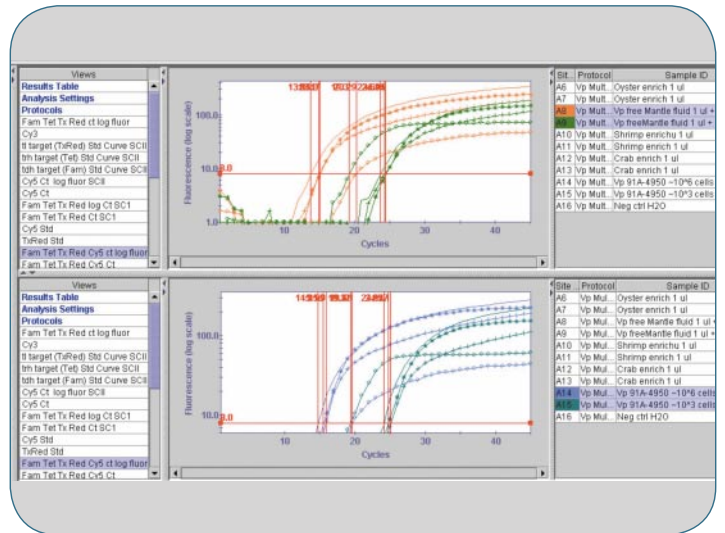


Figure 13. Mantle fluid. The upper graph is a plot of Ct vs. Log Fluorescence for *Vp*-free oyster mantle fluid (1 μ L) spiked with 10^6 and 10^3 cells from a *tdh+*, *trh+* *Vp* strain. The lower graph is the same plot for a control reaction of *Vp* cells only. It is evident that the mantle fluid did not inhibit the reaction, as both the target and internal control Ct values were approximately the same for the spiked mantle fluid and the control cells.

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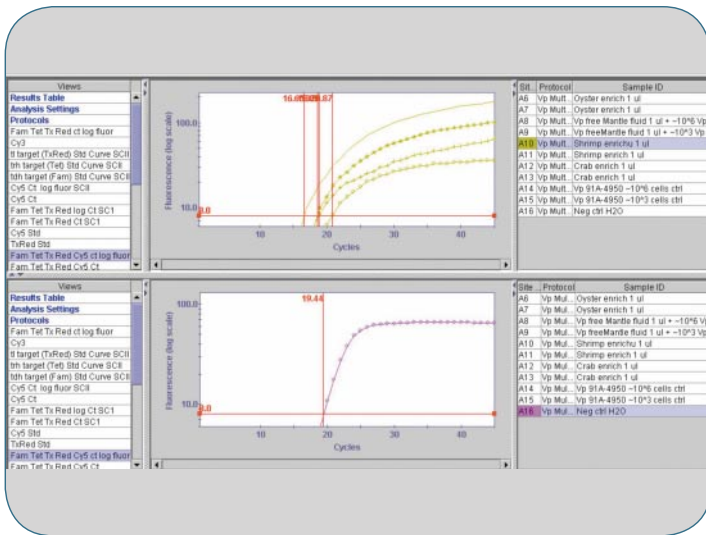


Figure 14. Shrimp homogenate enrichment. The upper graph is a plot of Ct vs. log fluorescence for a shrimp homogenate enrichment spiked with cells from a *tdh+*, *trh+* *Vp* strain. The lower graph is the same plot for the negative control (no *Vp* template). It is evident that the shrimp enrichment (1 μ L) did not significantly inhibit the PCR reaction, as the internal control Ct values were about the same for the enrichment and the negative control.

CONCLUSIONS

1. The assay is highly specific for detection of the *tl*, *tdh*, and *trh* genes of *Vibrio parahaemolyticus*.
2. Using pure cultures, the assay can simultaneously detect and quantify all three target genes with a high degree of accuracy, and has a 6-log dynamic range from $>10^6$ cells to a single cfu while quantifying all three targets simultaneously.
3. The assay is capable of detection of *Vp* in oyster mantle fluid and seawater, and in enrichments of oyster homogenate, shrimp homogenate, crab homogenate, and other samples.
4. The quantitative accuracy of the *Vp* multiplex when using unenriched seafood and environmental samples is presently under evaluation.
5. The internal control utilized in this assay successfully prevents the reporting of false negatives, and potentially also can be used as a quantitative internal control to estimate PCR inhibition.

ASM 2003 Annual Meeting, Poster #Q-082

0080-01

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