UCDAVIS
CENTER FOR
PRODUCE SAFETY

2008 RFP FINAL PROJECT REPORT, DUE APRIL 1, 2010

Project Title
Enhancing the effectiveness of human pathogen testing systems for the advancement of practical produce safety research and commercial management

Project Period
January 1, 2009 through April 1, 2010

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Objectives
Objective 1: Conduct a comprehensive analysis of available protocols and emerging technologies to stabilize and preserve enrichment samples and to develop a standard protocol for sample preparation and extraction.

Objective 2: Design a probe based version of a specifically designed primer set that will detect the presence of pathogenic STEC and EHEC and validate its use in produce plant tissue as well as other environmental samples.

Objective 3: Use the tools developed to evaluate the appropriateness of current metrics by tracking the survival, dispersal, natural transfer coefficients, and intervention tolerances of enteric pathogens more broadly.

Layman's Summary: This project accomplished its three primary objectives; 1) Refine and validate a novel, rapid method of screening diverse environmental and product samples for diverse pathogenic *E. coli*; 2) Verify the function of a simple sample "banking" method to allow delayed pathogen testing; and 3) Application of the developed tools within a standardized scheme for investigation of natural contamination events of leafy greens. To accomplish the first objective, diverse samples were inoculated with very low concentrations of human pathogenic *E. coli*. The highly specific DNA probe was verified to detect all *E. coli* O157:H7 and over 40 non-O157 types most associated with human illness. Sample DNA "banking" using a commercial system not requiring expensive freezing equipment, called FTA Cards, was effective with leafy greens for over two months with limited loss of detection efficiency. This technology will be used to more broadly secure grower permissions for farm access to efficiency. This technology will be used to more broadly secure grower permissions for farm access to conduct detailed grid-analysis of a crop, separated in time from marketing. Putting the pieces together, a large number of naturally contaminated samples, including irrigation water, animal fecal matter, manure-laden soil, compost, and leafy greens field contamination events were tested during the project period. The probe for pathogenic *E. coli* proved useful for rapid molecular screening, confirmation tests, and mass screening of colonies for inclusion or exclusion to identify the source of the initial pathogen test reactions that suggested a commercially contaminated crop.

Objective 1 and 2: These substantially co-dependent objectives were addressed together and iteratively rather than strictly sequentially. Therefore they will be presented together. A previously developed pathogen assay for pathogenic *E. coli* (Suslow Lab), utilizing the widely applied SYBR-Green based PCR detection reporter, targeted two genetic sequences known to be associated with the virulence of pathogenic *E. coli*. During the early stages of implementing Objective 2, converting the SYBR-assay to a more specific probe-assay, this existing real-time PCR assay was used to screen multiple *E. coli* O157:H7, non-O157 pathogenic *E. coli* as well as multiple produce related and environmental bacteria from our culture collection. Pathogen detection or absence of detection requires operator analysis of the dissociation curve generated to determine successful amplification of the genetic targets (TPEC; for Total Pathogenic *E. coli*, and *eae*A; a widely used marker for pathogenic *E. coli* including *E. coli* O157:H7). An enrichment culture or a pure isolate having both targets would produce a very specific dissociation profile with peaks at the precise melt temperatures for each amplicon (amplified PCR product) (Figure 1A). All *E. coli* O157:H7 strains tested were positive for this diagnostic profile. A group of 30 pathogenic and non pathogenic, gram negative produce-related or environmental strains as well as a few strains of gram positive bacteria were also tested and found to be negative (Table 1). We assembled a library of 40 non-O157 pathogenic *E. coli* strains that included the six priority O-serotypes the FDA and CDC have identified as being most associated with human clinical illness and outbreaks (O111, O26, O145, O103, O121 and O45). These strains were provided by Dr. Robert Mandrell, USDA/ARS, Albany, California and Dr. Mick Bosilevac, USDA/ARS, Nebraska. Of the 40 non-O157 pathogenic *E. coli* strains tested

(Table 2A), eight were clearly positive for the diagnostic profile and eight were clearly negative. The remaining 24 strains could be divided into two groups based on their dissociation curve profiles. Fifteen of the strains were labeled as "G" and the other nine were labeled as "X" for the purpose of grouping in this study (Figure 1B and 1C). The PCR products associated with these profiles were sequenced and it was determined that the desired target was amplified. In the case of the SYBR Green based PCR assay, the distinction between a positive and negative reading is based entirely on the dissociation curve profile. In the case of 75% of the non-O157 pathogenic *E. coli* cultures tested, these profiles deviated from the reference profile. We had determined that this version of the PCR detection tool in a complex product or environmental sample with high levels of non-target microbes will result in aberrant dissociation curves making the distinction between a negative and positive very challenging.

During this interval, steps towards converting this SYBR based system to a highly specific TaqMan MGB probe based system were completed. All previously tested non-O157 pathogenic *E. coli* cultures were retested with these newly designed primers and probes (Table 2B). 32/40 of the cultures were TPEC positive however, only 18 of them were both TPEC and *eae*A positive. Due to this discrepancy our next step involved redesigning the *eae*A gene primers and probe. This was done by sequencing the *eae*A gene amplicons from each of the strains and aligning them to fit a more conserved region specific to numerous serogroups of non-O157 pathogenic *E. coli*. These new *eae*A probe and primers were capable of amplifying the correct targets in all 32 of the TPEC positive cultures (Table 2C), when tested as a single target PCR assay. Eight of the 40 cultures gave a negative result with the new *eae*A probe. These cultures also gave negative results with the TPEC probe, the SYBR green PCR assay, as well as several conventional diagnostic tests for other virulence genes such as the shigatoxins. Many of these 8 strains were derived from ATCC reference collections with original deposit dating back to the 1950's. We believe that rather than suggesting the TPEC/*eae*A PCR system is not fully inclusive for key pathogenic *E. coli* this outcome suggests these isolates may have been incorrectly sub-cultured over the years in passing from lab to lab or have lost their virulence regions during transfer or storage and therefore should not be considered as legitimate pathogenic *E. coli* cultures.

Although the original intent was to design and apply a multiplex PCR assay (more than one confirming target in the same reaction), it became evident that pathogenic *E. coli* strains that were TPEC positive were also uniformly *eae*A positive. One outcome of the final detection design was to reveal that all future PCR assays could focus specifically on the conserved TPEC target.

The option to conduct such PCR detection assays with TPEC and other pathogen test kits or related methods, in a planned-delay analysis, was identified as an enabling and facilitating technique for on-farm or processed product surveys and investigative research efforts (Objective 1). The ability to offer an immediate sample processing but deferred detection analysis, by economically stabilizing the DNA, has been an invaluable asset when securing permission from

growers and handlers for on-farm studies in comprehensive field investigations of potential pathogen contamination. Prior to application of this PCR assay to naturally contaminated production lots or samples, it was necessary to determine its overall efficiency in a variety of lab inoculated produce and produce-related samples. For this, ten grams Red and Green leaf lettuce were placed into 90 ml of enrichment buffer (mEHEC). This was pulsified (a specialized, microbial dislodging system) for 30 seconds and then inoculated with either 10 CFU/sample of a marked strain of *E. coli* O157:H7 (kanamycin resistant and UV fluorescent) or a strain of *E. coli* O111. After 8 and 18h, aliquots were applied to Whatman FTA® Elute cards (Whatman, Clifton, NJ). The purpose of applying the enrichments to FTA cards (used in blood sample DNA preservation) was to investigate their potential as a stabilizing storage vehicle to preserve samples at room temperature in comparison to standard boiled samples. Enrichments inoculated with *E. coli* O157:H7 were also plated to enumerate the final population of inoculated bacteria.

The TPEC PCR assay from FTA card punches detected the presence of DNA from the inoculated pathogenic *E. coli* after an 8h incubation period in both Red and Green leaf lettuce samples (Table 3). In addition to presence or absence, the TaqMan TPEC system allows for absolute quantitation using a standard target DNA curve assay. A strong correlation was observed between the predicted values obtained using TaqMan TPEC and the enumerated marked *E. coli* O157:H7 (Figure 2). When comparing the use of boiled samples versus extracts from FTA cards as PCR templates, we did observe a consistently lower quantity of target DNA among the latter, though not always limiting in a presence/absence test (Figure 3).

In further experiments, two replicates of red and green leaf lettuce were prepared by inoculating with 5,000, 50,000, 500,000 and 5,000,000 CFU/leaf for each pair of *E. coli* O157:H7. One set was directly ground with 2ml of buffer and applied to the FTA cards while the other set was pulsified with 2ml of buffer and applied to the cards. One major factor differentiating this set of experiments from the others is that these samples were not enriched. Our goal was to determine the approximate concentration of bacteria on a single leaf, without enrichment, necessary for PCR detection. DNA was extracted from each card as a template for PCR analysis. During this phase of Objective 1, a TaqMan® probe and primers for *uid*A targeting the +93 single nucleotide polymorphism specific for *E. coli* O157:H7, as described and utilized in a FDA reference method for detection of *E. coli* O157:H7 was optimized for use in our lab. FTA card extraction and evaluation by PCR was done on a monthly basis over six months to determine their capacity to store DNA at room temperature without degradation. A slight indication of interference by pigmentation of red leaves to detection of the target pathogen using the PCR assay was observed. In addition, application of the pulsified leaf samples may hold an advantage over direct application of the ground leaf samples to the FTA cards (Table 4). However, overall data indicates that at least Log 5 CFU/leaf of target bacteria is necessary for successful detection. As this is not a realistic occurrence in pre-harvest contamination, as far as we are aware of have experienced, this system could not likely be applied successfully to single leaf samples without prior enrichment.

In addition to leafy vegetables, we also tested our TaqMan primers and probes on a diverse set of samples collected from the Salinas Valley region. These samples included 5 different soil textures (Silt loam, Sandy loam, clay loam, sandy and loamy clay), 4 different types of water (irrigation water direct from emitter, irrigation runoff, groundwater reservoir, and run-off detention pond), 3 different types of organic amendments (aged dairy manure, manure compost and thermal-treated chicken manure pellets) and 2 types of commercial liquid fertilizers (hydrolyzed fish emulsion and green manure extract emulsion). These samples were used to evaluate the efficiency of the TaqMan MGB probes in very complex materials. Three replicates of selected samples were prepared by inoculating with 100 CFU/sample of *E. coli* O157:H7. Following enrichment, one of these replicates was filtered; the second was spun down (1000RPM for 15mins) and third was not further treated. These were applied to FTA® Elute cards and allowed to dry. The water samples were also inoculated and then filtered and enriched using laboratory standard operating procedures and then applied to FTA cards. These samples were analyzed using TaqMan *uid*A PCR assay. The extraction and PCR analysis was done on a monthly basis over a period of six months. Post-enrichment processing by filtration or centrifugation did not improve detection efficiency when compared to the untreated samples (Table 5). As anticipated, PCR detection of *E. coli* O157:H7 in complex samples such as fertilizers was significantly compromised. Results indicated that this method may not be appropriate for samples such as hydrolyzed fish emulsion and other general organic fertilizers, however one possible explanation for the lack of detection was the poor growth of the inoculated isolates in these matrices.

In some of the samples detection of the target from newly extracted FTA card DNA was lost over time indicating possible degradation of DNA. However, when considering factors such as space, expense, and ease of shipping, FTA cards do appear to have a clear advantage over tubes of frozen samples. To initially differentiate the contribution of non-homogeneity of the sample on a card from DNA degradation, to explain the lower than anticipated consistency from a single sample extracted from different locations on an FTA card, we conducted the following experiment. A typical Whatman FTA Elute card is 7 cm long and 3 cm wide with four 1.5cm diameter circles, each upon which 50µl of enrichment are added. These cards are dried thoroughly before a sample of DNA can be extracted from them. An 18h lettuce enrichment sample with approximately Log 9 CFU/ml of *E. coli* O157:H7 was applied to each circle on four FTA cards. After the cards had dried, DNA was extracted from twelve 3mm punches taken from each card in specified locations seen in Figure 4. TaqMan *uid*A PCR assay was conducted on these DNA extracts. Results indicated that there was no significant difference between the quantities of target DNA obtained from each punch regardless of where the punch was taken. Assessment of water samples indicated efficient detection capacity with irrigation water direct from the emitter. However within applications to highly turbid water the detection was not as efficient (Table 6). Detection was also consistent among all five of the soil samples. Uninoculated loamy clay, manure and compost were enriched and tested as negative controls. Both loamy clay and compost were negative for *E. coli* O157:H7 but manure was positive. Since manure was obtained from a dairy source, it is possible that it was naturally contaminated with *E.*

coli O157:H7. Table 7 provides two examples (Irrigation water and Chicken compost; thermally treated pellets) to indicate how the use of absolute quanititation using the TaqMan PCR assay enabled approximate enumeration of target DNA in each FTA card extract over a period of four months. When evaluating absolute quantitation data using the TaqMan *uid*A PCR assay, there were some major inconsistencies indicating the need for an alternate PCR assay. We have currently begun use of a TaqMan *rfb*E PCR assay to differentiate *E. coli* O157:H7 specifically from a positive TPEC detection which thus far appears to be much more consistent. We applied this PCR assay on an extraction of DNA from the 10 month old FTA cards described above and observed positive detection of *E. coli* O157:H7 in many of the samples (Table 8). Since we do not have data from earlier time points it is difficult to make comparisons between samples however, distinguishing between positive and negative samples using this assay was unambiguous and therefore this system is highly preferred.

Objective 3: This objective involved application of the developed tools for investigation of natural contamination events of leafy greens. After developing the probe and applying it successfully on a variety of lab inoculated samples the technology was ready for challenge studies with diverse environmental and leafy vegetable samples from Central Coast production regions. Each trial is described below in no particular order of analysis. The eventual goal of this objective was to apply the tools that have been designed and validated in objectives 1 and 2 to evaluate the appropriateness of selected current metrics used by the Leafy Green Handler Marketing Agreement (LGMA). Two risk-based evaluations were conducted with fields that had evidence of animal intrusion and another set of contaminated finished spinach product was also provided for evaluation. In addition, the detection system was applied to over one hundred blinded samples of Feedlot Surface Material as well as a few samples of bovine fecal material highly likely to be contaminated with *E. coli* O157:H7 and other pathogenic *E. coli*.

Risk based field evaluation 1: Leaf material, water, soil and animal feces were collected from a field where there had been evidence of animal intrusions. These samples were enriched and tested using a commercially available rapid testing tool as a comparison. One of the leaf samples and one of the water samples were positive for *E. coli* O157:H7. Culture confirmations were successful with these samples. These samples were also tested before and after enrichment using the TPEC screen. In this trial 3/33 of the enriched leaf samples were TPEC positive. Results indicated that although the TaqMan TPEC PCR assay is highly sensitive, there is a definite need for enrichment of samples prior to detection. On CHROMagar O157, colonies of *E. coli* O157:H7 appear mauve in color. We were able to take advantage of this characteristic in avoiding these colonies in order to increase our chances of identifying non-O157 pathogenic *E. coli*. We isolated 320 suspect blue colonies for the TPEC screen however were unable to identify any colony confirmations with this sample set.

Risk based field evaluation 2: Spinach leaf material and soil samples were collected from a field that had encountered incidences of human pathogens from commercial laboratory testing. All samples were enriched and tested using the commercial rapid test for *E. coli* O157:H7 and the

TaqMan TPEC screen. None of the samples were positive for *E. coli* O157:H7 and 18 of the 60 plant samples were TPEC positive. From each of these positive samples, we isolated eight dark blue colonies and screened them again using the TPEC PCR assay. We were able to isolate single TPEC positive colonies from four of these samples. These colonies have been purified and stored at -80ºC, and will be sent for O serogrouping. These enrichments were also screened separately for *Salmonella* targeting the *inv*A gene. Two of the eight soil samples were positive for *Salmonella* however colony confirmations were not successful. We have currently designed and preliminarily applied TaqMan probes and primers for the *inv*A gene which we plan to multiplex with the TPEC screen or use as a singleplex assay for pathogenic screening of future samples.

Finished product contamination: In this trial *E. coli* O157:H7 contaminated spinach, as a washed and packed product, was provided for further analysis. Samples were enriched and tested using a commercially available rapid testing system which detected 12 of the 26 samples to be presumptively positive for *E. coli* O157:H7. The TPEC screen was capable of detecting the presumptive target in 11 of the samples. Our goal was to try to isolate non-O157 pathogenic *E. coli*, if present, from these samples, which is extremely challenging. This challenge is due to the fact that these bacteria rarely possess any significant distinguishing characteristics from non pathogenic *E. coli* as well as other coliforms. We tested approximately 320 colonies that appeared as different shades of blue on CHROMagar O157 and were able to isolate one suspect colony that was sent for O serogrouping to the E. coli Reference Center at Pennsylvania State University. The isolate was typed as O105 verotoxin producing *E. coli* that has been isolated from cows and human clinical patients.

Feedlot Surface Material: At an early stage in the redesign and improvement of the TPEC and *eae*A probes and primers, we sought to verify the applicability of the TPEC screen on a more reliably contaminated and complex natural matrix. We obtained a number of blind-coded feedlot surface material (FSM) samples from Dr. Berry, USDA MARC Clay Center. These samples were prescreened by Dr. Berry and a high percentage was known to be positive for *E. coli* O157:H7 and likely to contain non O157 pathogenic *E. coli*. Initial tests in our lab involved enriching the samples for analysis with a commercially available rapid detection tool. Using this system, none of the samples were positive. We concluded that due to the high content of PCR inhibitors in the FSM, this technology, which is usually very sensitive and reliable, was not able to detect the presence of *E. coli* O157:H7. This commercial tool makes use of immunomagnetic separation (IMS) beads and although these beads gave negative readings using their PCR based system, we were able to isolate *E. coli* O157:H7 from some of those same beads. These results demonstrate the essential need for alternate rapid methods for detection of pathogens in complex samples such as FSM. Our TaqMan TPEC tool detected the presence of TPEC in all 60 of the first set of samples. We also received a second set of blinded FSM samples provided at a later date which we enriched (for a shorter period of time; 8h) in two different broths (mEHEC and TSB). These samples were then tested using the commercial rapid detection tool and were found to be negative for *E. coli* O157:H7. In addition to boiling the enrichments prior to PCR analysis,

MoBIO® soil extraction kit was used to extract and purify DNA in a separate set. Results indicated that mEHEC had no advantage over TSB in the enrichment process. We also observed that the MOBIO soil extraction kit was not beneficial to sample preparation before PCR analysis in fact; it may have slightly reduced the detection efficiency using the TPEC screen. 19 of the 20 samples were TPEC positive however due to the high content of *E. coli* O157:H7 it was very challenging to identify non-O157 pathogenic *E. coli* from the 100 colonies that we tested.

Bovine fecal material: Fresh bovine fecal samples were provided by Dr. Hoar of UC Davis School of Veterinary Medicine. These samples were enriched and tested using the TPEC screen. Prior to PCR analysis, enrichments were treated in three ways to reduce the possibility of PCR inhibitors. One set was boiled, the other was diluted and the third was treated with a Triton extraction method. Results indicated that boiling the samples prior to analysis was the most efficient method for PCR template preparation. Three of the 5 samples were TPEC positive and were therefore diluted and plated out to identify TPEC positive colonies. Approximately 100 colonies with different shades of blue, white and pink were tested using the TPEC screen. Some of the single colonies that were dark blue with a purplish halo were TPEC +ve. These colonies were purified and frozen. These five isolates were also sent for O serotyping to the E. coli Reference Center. All colonies were typed as O15 uropathogenic *E. coli* associated with cystitis and bacteremia in humans.

After several rounds of improved inclusivity optimization, we are very confident in the practical value of the TPEC screen for diverse applications related to produce food safety. Coupled with a comparable screen for *Salmonella,* it would be an extremely useful tool for research and potentially for commercial preharvest and product screening. Currently we are using this rapid screen and FTA Card storage for leafy green grower requested environmental investigations as preliminary indicator for hot spots in field contamination.

What unexpected outcomes (positive and negative) resulted from the project, and how did you manage them?

Three significant unexpected outcomes were encountered during this project period that substantially affected the rate of progress and, in turn, the projected timeline for budget expenditures and management.

1. In progressing from the general SYBR Green PCR assay for TPEC and *eae*A it quickly became apparent that the non-O157 EHEC reference culture collection presented inconsistent results for inclusion of known pathogenic *E. coli* in the planned detection scheme. These exceptions undermined the confidence and future utility of the test method for use in on-farm or product testing for this project and transferability to other researchers and commercial testing. This was resolved by consecutive rounds of optimization, as explained in the body of the report, and repeated, multi-platform comparisons with recognized PCR methods of recognition and differentiation of *E. coli* O157:H7 and related non-O157 EHEC.
2. At the outset of the project, we relied on the functionality and specificity of the *uid*A PCR method to initiate long-term evaluations of the FTA Card DNA-storage method with diverse plant, preharvest crop inputs, and environmental samples in combination with the TPEC+*eae*A screen available at the time. Through our research analysis we determined that the *uid*A detection method, while highly useful for pure cultures and low microbial background samples, it was not sufficiently robust, in our hands, with many important sample matrices. In combination with the hurdles explained in Point #1, these unexpected outcomes undermined the interpretation of sample stability testing and application to on-farm investigations during the project period. Most of this ambiguous data is not presented in this report. After months of effort to understand and resolve the detection problems, our solution was to accelerate the development and validation of an alternative *E. coli* O157:H7 specific TaqMan probe and primers based on the widely used *rfb*E gene, as described in the body of the report.
3. Throughout this project period and as improvements in the TPEC system were eventually achieved, we were limited by the lack of opportunities to test the planned, full detection protocol within industry identified locations under Objective 3. Many factors likely contributed to this which was beyond our control. Lack of such opportunities was one of the rationales for investing time and effort in arranging for blinded samples of Feedlot Surface Material rather than continue to wait for access to suspected pathogen contaminated fields. Eventually, though late in the project period, persistent interactions with industry contacts and the ability to commit to delayed analysis of product, we were able to conduct a few investigations using the developed tools.

Collaborative efforts involved in planning and/or implementing this project.

Non-O157 pathogenic *E. coli* strains were provided by Dr Robert Mandrell and Dr Mick Bosilevac. Naturally contaminated environmental samples were provided by Dr Elaine Berry and Dr Bruce Hoar. Naturally contaminated produce and produce related samples were provided from risk based field evaluations with collaborative efforts of Dr Trevor Suslow and his research staff and graduate students.

Did you have the necessary funds to fully implement this project?
Yes, however we had some delays in accomplishing some of our objectives as explained above and therefore need to use some of the funding beyond the deadline of the allocated time period in order to more fully address each objective.

Describe any changes that occurred to the original budget.
Due to the unexpected results and delays encountered in the execution of this project and a number of administrative impediments that result in cross-project invoicing during periods when a dedicated project account is not available, the original budget was grossly underutilized and a significant percent of the allocated funds will be returned to CPS. We request that CPS consider allowing a period to document and justify a cost recovery for a portion of the unspent allocation that was invoiced to other project accounts in the Suslow Lab.

Give a brief narrative breakdown of how the grant funds were spent.
Grant funds were spent on salary and benefits for PI who was responsible for project coordination and reporting. Grant also supported salary and benefits for one junior specialist who assisted in PCR trials and development involved in the project as well as the salary of one hourly student assistant who was primarily responsible for media preparation and sample processing. Funds were also spent on materials and supplies required for the project such as Whatman FTA supplies, microbiological media, chemical media, molecular analysis and PCR reagents and kits as well as other basic laboratory disposables.

List publications and presentations resulting from this grant. Provide a digital copy with the report. State if there have been no publications or publications.
No publications at this time however, one manuscript will be prepared and research will be presented in the form of a poster at ASM in May 2010. Poster and manuscript will be provided once they have been prepared.

Presentations - Information from results in progress were shared by Co-PI Suslow with diverse audiences at the following meetings:

- National Restaurant Association Quality Assurance Executive Study Group. *Decoding the Microbiology of Fresh Produce.* April 1, 2009. Sacramento,. CA
- Third Governor's Conference on Ensuring Food Safety. *On-Farm Issues and Methods to Minimize the Risk of STECs and other Enteric Pathogens*. May 5th, 2009. Lincoln, NE
- Central Coast Leafy Greens Grower Food Safety Research Update. May 19th, 2009.Salinas , CA
- Overview of Produce and Pathogens. Presented at AOAC International 125th Annual Meeting. June 26th, 2009. Washington D.C.
- Central Coast Leafy Greens Grower Food Safety Research Update. Aug 3, 2009. Santa Maria.
- *E. coli* survival and epidemiology. California Leafy Greens Research Board. Mid-term Research Reports. Seaside, CA. October 13, 2009.
- *E. coli* survival and epidemiology. California Leafy Greens Research Board. Annual research reports. Coalinga. March 16, 2010.

Appendix:

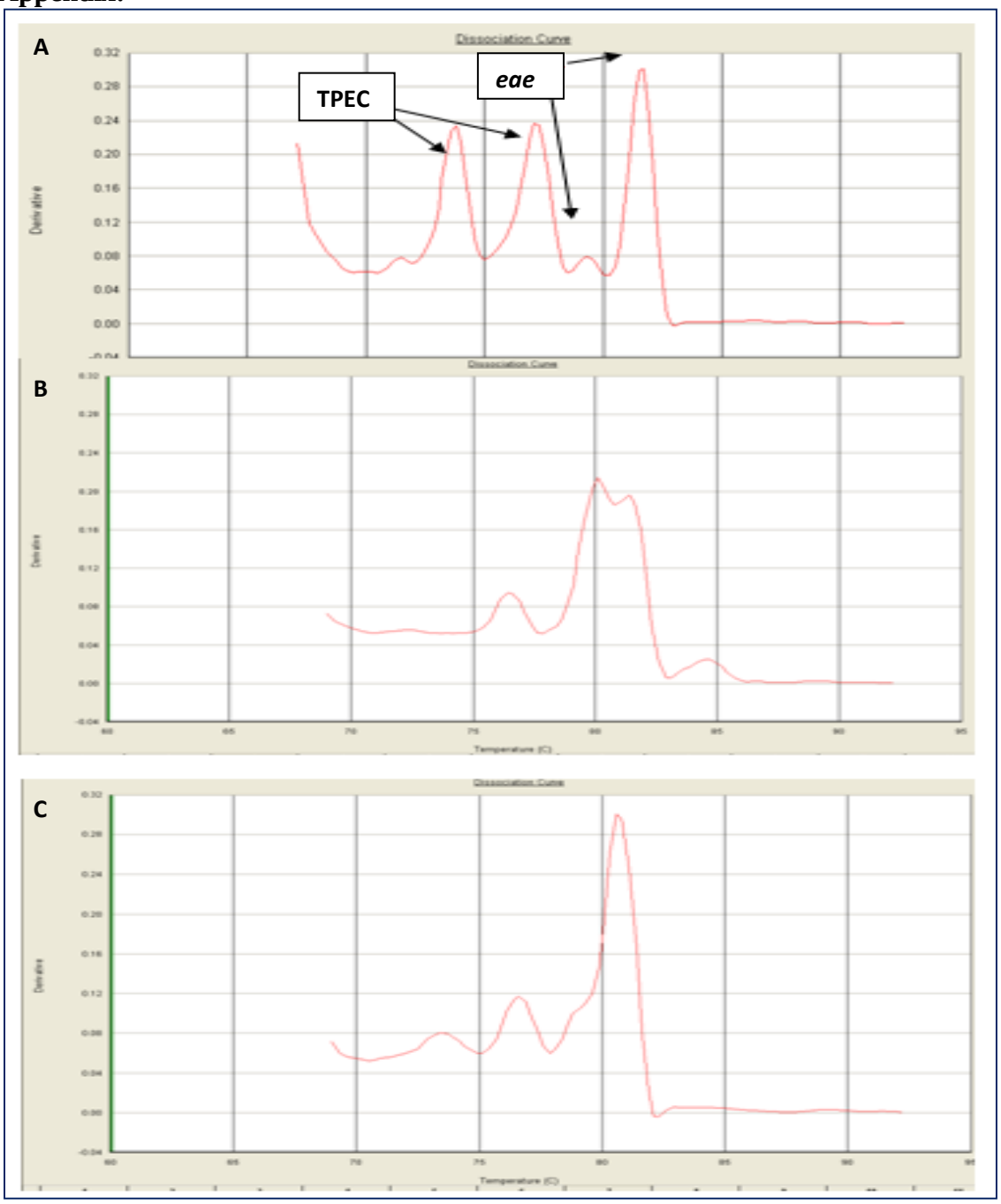

Figure 1: Dissociation curve profiles of multiplex SYBR green PCR analysis of pure colonies from culture collection; (A) *E. coli* O157:H7 reference culture positive (B) Group G (C) Group X. Rate of change of relative fluorescence units are plotted on the X-axis and melting temperature is plotted on the Y-axis.

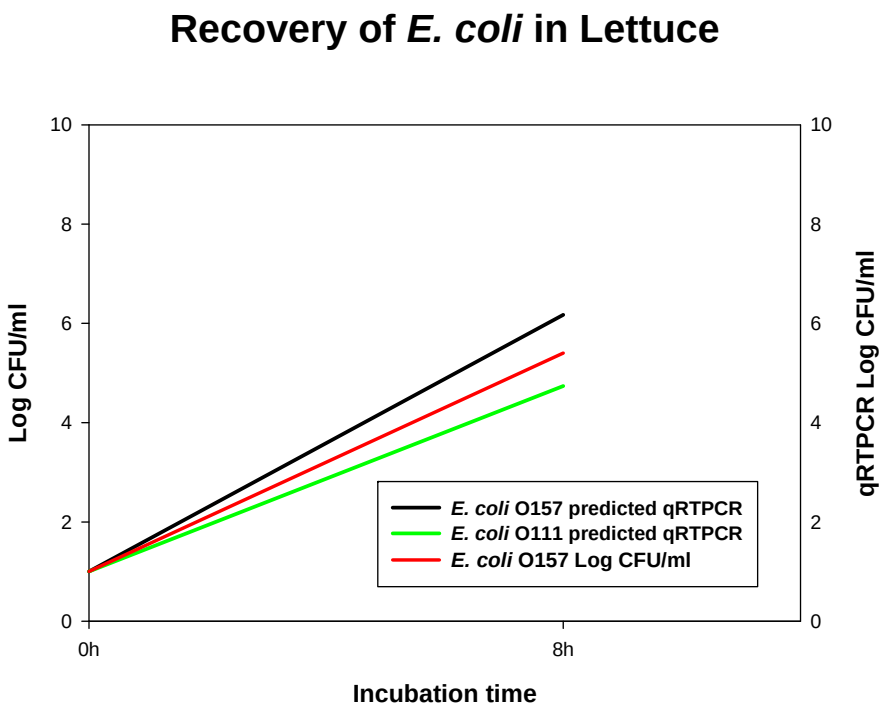

Figure 2: Growth of *E. coli* O157:H7 and *E. coli* O111 following inoculation of lettuce at 10 CFU/sample during enrichment over an 8h incubation period as predicted by TaqMan TPEC absolute quantitation analysis as well as approximate enumeration of *E. coli* O157:H7 using agar plate counts.

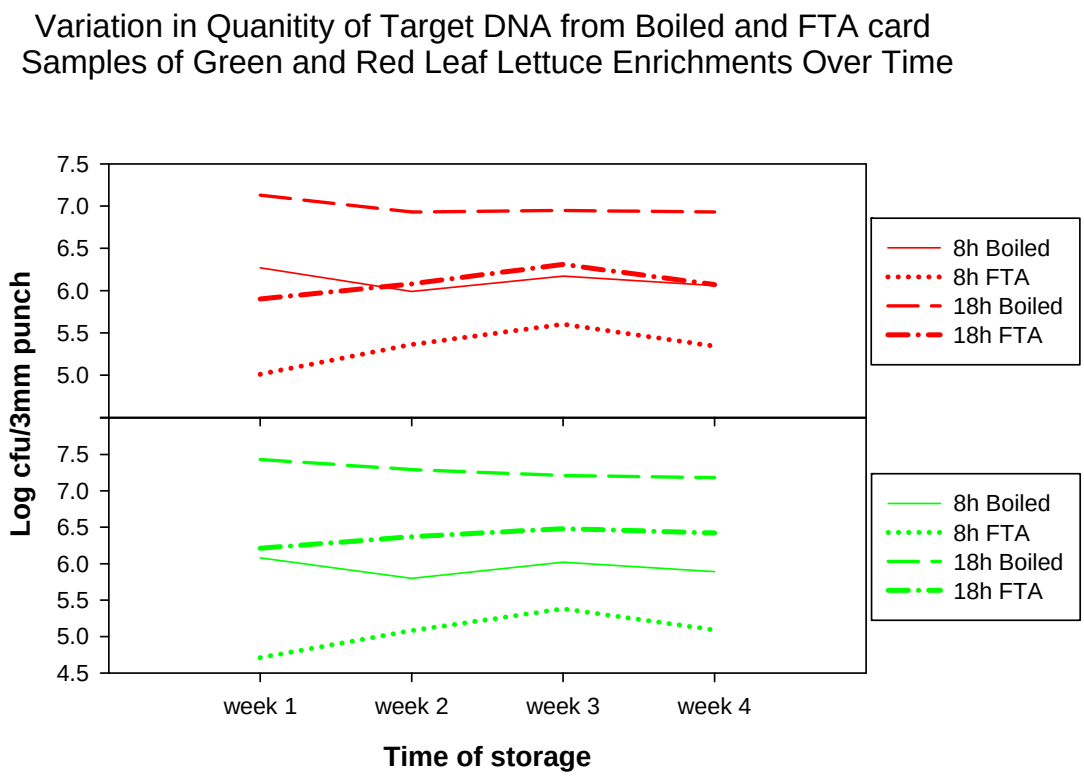

Figure 3: Growth of 10 CFU/sample of pathogenic *E. coli* in lettuce enrichments over an 18h incubation period as predicted by TaqMan TPEC PCR assay. PCR template from boiled samples as well as FTA card extracts were tested over a period of 4 weeks.

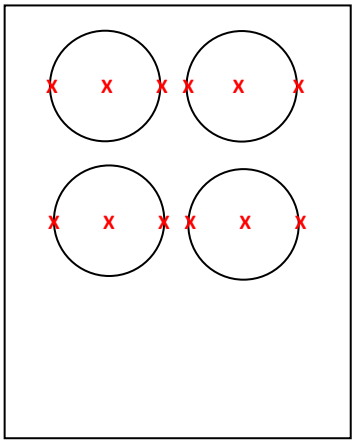

Figure 4: A typical Whatman FTA® Elute card marked at the regions where punches were taken to determine consistency of DNA at different locations within the same card

S/No.	Strain Information	TPEC
1	*Escherichia coli* - water	-
2	*Pseudomonas fluorescens* biotype G - lettuce	-
3	*Citrobacter youngae* - Cantaloupe rind	-
4	*Citrobacter freundii* ATCC 8090	-
5	*Escherichia coli* - soil	-
6	*Proteus vulgaris* - ATCC 13315	-
7	*Pectobacterium chrysanthemi* - run off onion sprouts	-
8	*Serratia liquefaciens/grimesii*	-
9	*Pantoea agglomerans* - Asparagus hydrocooling	-
10	*Enterobacter cloacae*	-
11	*Bacillus cereus*	-
12	*Bacillus megaterium*	-
13	*Buttiauxella izardii*	-
14	*Citrobacter braakkii*	-
15	*Enterobacter aerogenes*	-
16	*Erwinia carotovora pv. carotovora*	-
17	*Escherichia coli* K-12	-
18	*Escherichia hermanii*	-
19	*Hafnia alvei*	-
20	*Klebsiella planticola*	-
21	*Klebsiella terrigena*	-
22	*Leclercia adecarboxylate*	-
23	*Pseudomonas aeruginosa*	-
24	*Rahnella aquatilis*	-
25	*Raoultella terrigena*	-
26	*Staphylococcus aureus*	-
27	*Staphylococcus epidermidis*	-
28	*Salmonella enterica* serovar Montevideo	-
29	*Salmonella enterica* serovar Poona	-
30	*Salmonella enterica* serovar Typhimurium	-

Table 1: Summary of non-target environmental and pathogenic isolates of bacteria screened with TPEC PCR assay

S/No.	Strain Information		(A) SYBR TPEC, *eae*A	(B) TaqMan TPEC, *eae*A	(C) Red. *eae*A
1	RM1712	O137:H41	-ve	-	-
2	RM1715	O103:H2	-ve	-	-
3	RM2032	O26: H11	G	T+	+
4	RM2048	O45:H2	G	T+	+
5	RM2050	O128:H7	G	-	-
6	RM2060	O111:H11	G	T+	+
7	RM2064	O111:H2	X	T+, E+	+
8	RM2068	O55:H6	X	T+, E+	+
9	RM2164	O55:H6	X	T+, E+	+
10	RM3653	O111:NM	+ve	T+, E+	+
11	RM2079	O111:HN	G	T+	+
12	RM2162	O55:NM	X	T+, E+	+
13	RM2163	O 55:H7	+ve	T+, E+	+
14	RM2166	O 111:H2	X	T+, E+	+
15	RM2169	O 119:NM	X	T+, E+	+
16	RM2170	O 142:H6	X	T+, E+	+
17	RM2171	O 21:NM	-ve	-	-
18	RM2172	O 29:NM	X	T+, E+	+
19	RM2245	O111:H8	-ve	-	+
20	RM2246	O111:?	+ve	T+, E+	+
21	RM3648	O145:NM	G	T+	+
22	RM2014	O157:H43	-ve	-	-
23	RM2016	O26:H11	G	T+	+
24	RM2020	O128:H7	-ve	-	-
25	RM2022	O111:H21	-ve	-	-
26	RM2024	O55:HN	X	T+, E+	+
27	RM2034	O111:H2	G	T+	+
28	RM2031	O26:HN	+ve	T+, E+	+
29	MB -1	O26H11	G	T+	+
30	MB -2	O26 H11	G	T+	+
31	MB -3	O45 human	G	T+	+
32	MB -4	O45 beef	-ve	T+E+	+
33	MB -5	O111 H8 human	+ve	T+E+	+
34	MB -6	O111 NM human	+ve	T+E+	+
35	MB -7	O103 H2 human	G	T+	+
36	MB -8	O103 beef	G	T+	+
37	MB -9	O121 human	G	T+	+
38	MB -10	O121 beef	G	T+	+
39	MB -11	O145 NM human	+ve	T+E+	+
40	MB -12	O145 beef	+ve	T+E+	+

Table 2: Summary of presumptive reference pathogenic *E. coli* strains screened using the PCR assays described in this study. (A) SYBR green based multiplex PCR assay targeting TPEC and *eae*A; (B) TaqMan based multiplex PCR assay targeting TPEC and *eae*A; (C) Redesigned TaqMan based PCR singleplex assay targeting *eae*A. Templates for all PCR assays included 1µl of a single colony resuspended in 200µl of sterile water which is heated at 95ºC for 10 minutes

		Week1		Week 2		Week 3		Week 4	
	Sample	**Boiled**	**FTA**	**Boiled**	**FTA**	**Boiled**	**FTA**	**Boiled**	**FTA**
8h	**Green 1**	6.06	4.70	5.79	5.06	6.00	5.48	5.88	5.11
	Green 2	6.07	4.58	5.77	5.06	5.99	5.32	5.86	5.08
	Green 3	6.12	4.84	5.84	5.14	6.07	5.35	5.94	5.08
	Red 1	6.27	4.96	5.95	5.35	6.16	5.54	6.04	5.31
	Red 2	6.17	4.88	5.89	5.23	6.09	5.52	5.98	5.23
	Red 3	6.37	5.18	6.13	5.51	6.27	5.73	6.17	5.49
18h	**Green 1**	7.40	6.13	7.28	6.43	7.20	6.49	7.17	6.48
	Green 2	7.51	6.18	7.35	6.60	7.26	6.52	7.25	6.45
	Green 3	7.38	6.31	7.25	6.08	7.16	6.42	7.14	6.32
	Red 1	6.96	5.89	6.66	5.89	6.76	6.19	6.69	5.92
	Red 2	7.20	5.80	7.00	6.13	7.00	6.31	7.00	6.13
	Red 3	7.23	6.01	7.13	6.23	7.09	6.42	7.09	6.15
Control	**Green-8**	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3
	Red-8	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3
	Green-18	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3
	Red-18	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3

Table 3: Summary of TaqMan TPEC PCR analysis data from individual replications of Red and Green leaf lettuce inoculation trials. Template used for PCR assays were either from boiled enrichment samples or from Whatman FTA elute card extracts. These Log values indicate the quantity of fluorescence signal which is an approximate indication of the quantity of initial target DNA in the sample. Results are reported over a four week period

	Detection of *E. coli* O157:H7 using TaqMan *uid*A PCR analysis					
Lettuce type + Inoculum	**Month 1**	**Month 2**	**Month 3**	**Month 4**	**Month 5**	**Month 6**
Red 5 x 10^3	-	-	-	-	-	-
Red 5 x 10^4	-	+	-	+	+	-
Red 5 x 10^5	+	+	+	+	+	+
Red 5 x 10^6	+	+	+	+	+	+
Green 5 x 10^3	-	-	-	+	-	-
Green 5 x 10^4	+	+	+	-	+	-
Green 5 x 10^5	+	+	+	+	+	+
Green 5 x 10^6	+	+	+	+	+	+
Green 5 x 10^3 direct	-	-	-	-	-	-
Green 5 x 10^4 direct	+	-	-	-	-	-
Green 5 x 10^5direct	+	+	-	+	+	+
Green 5 x 10^6 direct	+	+	+	+	+	-
Red 5 x 10^3 direct	-	-	-	-	-	-
Red 5 x 10^4 direct	+	-	-	-	-	-
Red 5 x 10^5 direct	+	+	-	-	+	-
Red 5 x 10^6 direct	+	+	+	+	+	+
Control green	-	-	-	-	-	-
Control green direct	-	-		-	-	-
Control red	-	-	-	-	-	-
Control red direct	-	-	-	-	-	-

Table 4: Summary of TaqMan *uid*A PCR analysis data from Red and Green leaf lettuce. These samples were inoculated with different quantities of *E. coli* O157:H7 and then either applied directly to FTA cards (direct) or 2ml of buffer was added with a 30 second pulsification prior to addition onto FTA cards. Cards were allowed to dry before extraction of DNA for PCR analysis which was done over a 6 month period

	Detection of *E. coli* O157:H7 using TaqMan *uid*A PCR analysis					
Source + Preparation	**Month 1**	**Month 2**	**Month 3**	**Month 4**	**Month 5**	**Month 6**
Compost filter	+	+	-	-	-	-
Compost spin	+	-	+	+	-	-
Compost direct	+	-	+	+	-	-
Loamy clay filter	+	+	+	+	+	+
Loamy clay spin	+	+	+	+	+	+
Loamy clay direct	+	+	+	+	+	+
Manure filter	+	+	+	+	+	+
Manure spin	+	+	+	-	+	+
Manure direct	+	-	-	+	-	+
HF filter	-	-	-	-	-	-
HF spin	-	-	-	-	-	-
HF direct	-	-	-	-	-	-
BGP filter	-	-	-	-	-	-
BGP spin	-	-	-	-	-	-
BGP direct	-	-	-	-	-	-
Sandy loam filter	+	+	+	+	+	+
Sandy loam spin	+	+	+	+	+	+
Sandy loam direct	+	+	+	+	+	+
-ve Loamy clay	-	-	-	-	-	-
-ve Manure	+	+	+	-	-	-
-ve Compost	-	-	-	+	-	-

Table 5: Summary of TaqMan *uid*A PCR analysis data from a diverse set of samples collected from the Salinas Valley region. These samples were inoculated with 100 CFU/sample of *E. coli* O157:H7, broth-culture enriched and either filtered, centrifuged or directly applied to FTA® Elute cards. The cards were allowed to dry before extraction of DNA for PCR analysis over a period of 6 months

	Detection of *E. coli* O157:H7 using TaqMan *uid*A PCR analysis					
Source	**Month 1**	**Month 2**	**Month 3**	**Month 4**	**Month 5**	**Month 6**
Coyote feces	+	-	+	-	-	-
Irrig water 1	+	+	+	+	+	+
Irrig water 2	+	+	+	+	+	+
Runoff 1	-	-	-	-	-	-
Runoff 2	+	-	+	-	-	-
Reservoir 1	+	+	+	+	-	-
Reservoir 2	+	+	+	+	-	-
Reservoir in compost 1	+	-	+	+	-	-
Reservoir in compost 2	+	-	+	+	+	-
Chicken compost 1	+	+	+	+	+	+
Chicken compost 2	+	+	+	+	+	+
Silt loam 1	+	+	+	+	+	+
Silt loam 2	+	+	+	+	+	+
Sandy loam 1	+	+	+	+	+	+
Sandy loam 2	+	+	+	+	+	+
Clay loam 1	+	+	+	+	+	+
Clay loam 2	+	+	+	+	+	-
Sandy 1	+	+	+	+	+	+
Sandy 2	+	+	+	+	+	+
Loamy clay 1	+	+	+	+	+	+
Loamy clay 2	+	+	+	+	+	-
-ve Loamy clay	-	-	-	-	-	-
-ve Manure	+	+	+	-	-	-
-ve Compost	-	-	-	+	-	-

Table 6: Summary of TaqMan *uid*A PCR analysis of 4 different types of water, 5 different soil textures, coyote feces, and chicken compost enrichments. These replicate samples were inoculated with 100 CFU/sample of *E. coli* O157:H7, broth-culture enriched, applied to FTA® Elute cards and then dried before extraction of DNA for PCR analysis over a period of 6 months.

	Absolute quantitation of *E. coli* O157:H7 using TaqMan *uid*A PCR analysis			
	Month 1	**Month 2**	**Month 3**	**Month 4**
Source	**Qty**	**Qty**	**Qty**	**Qty**
Irrig water 1	5.90	5.70	5.72	5.68
Irrig water 2	5.24	5.36	5.16	4.46
Chicken compost 1	3.23	3.31	3.48	3.58
Chicken compost 2	4.98	5.07	5.25	5.12
-ve Loamy clay	< 1	< 1	< 1	< 1

Table 7: Summary of TaqMan *uid*A PCR absolute quantitation analysis from irrigation water and chicken compost from Salinas Valley region. Samples were inoculated with 100 cfu/sample of *E. coli* O157:H7, allowed to enrich, applied to FTA® Elute cards and then dried before extraction of DNA for PCR analysis over a period of 4 months.

Detection of *E. coli* O157:H7 using TaqMan *rfb*E PCR analysis								
Source	**Month 10**		**Source**	**Month 10**		**Source**	**Month 10**	
	P/A	**Qty**		**P/A**	**Qty**		**P/A**	**Qty**
Red 5 x 10^3	-	< 3	**Compost F**	-	< 3	**Irrig water 1**	+	6.51
Red 5 x 10^4	-	< 3	**Compost S**	-	< 3	**Irrig water 2**	+	6.25
Red 5 x 10^5	+	4.34	**Compost D**	-	< 3	**Runoff 1**	-	< 3
Red 5 x 10^6	+	4.89	**Loamy clay F**	+	4.33	**Runoff 2**	-	< 3
Green 5 x 10^3	-	< 3	**Loamy clay S**	+	4.93	**Reservoir 1**	+	4.72
Green 5 x 10^4	+	4.00	**Loamy clay D**	+	5.24	**Reservoir 2**	-	< 3
Green 5 x 10^5	+	4.33	**Manure F**	+	4.95	**Reservoir in compost 1**	-	< 3
Green 5 x 10^6	+	4.89	**Manure S**	+	4.41	**Reservoir in compost 2**	-	< 3
Green 5 x 10^3 D	-	< 3	**Manure D**	-	< 3	**Chicken compost 1**	+	5.20
Green 5 x 10^4 D	-	< 3	**HF F**	-	< 3	**Chicken compost 2**	+	6.03
Green 5 x 10^5D	+	4.18	**HF S**	-	< 3	**Silt loam 1**	+	5.40
Green 5 x 10^6 D	+	4.63	**HF D**	-	< 3	**Silt loam 2**	+	5.60
Red 5 x 10^3 D	-	< 3	**BGP F**	-	< 3	**Sandy loam 1**	+	5.02
Red 5 x 10^4 D	+	3.91	**BGP S**	-	< 3	**Sandy loam 2**	+	5.50
Red 5 x 10^5 D	+	4.17	**BGP D**	-	< 3	**Clay loam 1**	+	5.82
Red 5 x 10^6 D	+	4.78	**Sandy loam F**	+	4.89	**Clay loam 2**	+	4.98
Control green	-	< 3	**Sandy loam S**	+	4.70	**Sandy 1**	+	4.90
Control green D	-	< 3	**Sandy loam D**	+	4.67	**Sandy 2**	+	5.32
Control red	-	< 3	**-ve Compost**	-	< 3	**Loamy clay 1**	+	4.35
Control red D	-	< 3	**-ve Loamy clay**	-	< 3	**Loamy clay 2**	+	4.82
			-ve Manure	-	< 3	**Animal feces**	-	< 3

Table 8: Summary of TaqMan *rfb*E PCR absolute quantitation analysis a diverse set of samples from the Salinas Valley region. Samples were inoculated with 100 cfu/sample of *E. coli* O157:H7, allowed to enrich, applied to FTA® Elute cards. Presence/absence data from extracts of 10 month-old FTA cards is included.