

ABSTRACT

Background: Idaho Technology has developed the *E. coli* O157:H7 LT Food Security System (FSS) for the rapid detection of *E. coli* O157:H7 in 25g and 375g samples of ground beef and 25g spinach samples. This system uses a newly developed assay that is specific for *E. coli* O157:H7 and which contains an internal amplification control. It utilizes Idaho Technology's R.A.P.I.D.® LT real time PCR instrument with analytic software. Protocols developed for this system were designed to minimize the complexity and length of enrichment, use non proprietary media, allow for 5x1 post-enrichment sample pooling for 25g samples and for the use of 375g composited ground beef samples. Sample preparation for testing uses a simple mechanical lysis and dilution step. *E. coli* O157:H7 is a food-borne pathogen that causes hemorrhagic colitis and hemolytic-uremic syndrome. Improved means of detection of *E. coli* O157:H7 is a high priority for food producers, suppliers and policy makers.

Methods: Ground beef in both 25g and 375g formats and 375g loose leaf spinach samples were inoculated with *E. coli* O157:H7 combined with media, enriched and tested. Data will be presented that several variables of this system have been evaluated. These variables include: different *E. coli* O157:H7 strains, ground beef fat contents, growth media, enrichment times and sample preparation methods.

Results: Buffered peptone water was chosen as the optimal media for the enrichment of *E. coli* O157:H7 in all protocols developed for the *E. coli* O157:H7 LT FSS. Enrichment times are as low as 8 hours for spinach and ground beef samples. Rapid downstream processing and PCR analysis mean final results can be obtained within 9.5 hours from sample to result. The system specifically detects *E. coli* O157:H7 strains.

Conclusion: Current efforts include evaluating this system for method comparison, sensitivity and more extensive specificity. This PCR-based system is expected to provide an *E. coli* O157:H7 detection system that is fast, specific, reliable and sensitive.

E. COLI O157:H7 LT FSS OVERVIEW



Current methods can take 3-4 days to identify *E. coli* O157:H7 in food. The goal of Idaho Technology's *E. coli* O157:H7 FSS is to provide a sensitive and robust system that is faster than currently available detection systems.

TEST METHODOLOGY

The *E. coli* O157:H7 LT FSS identifies *E. coli* O157:H7 bacteria through a series of sequential steps that include sample collection, enrichment, sample preparation, cell lysis to release DNA, DNA amplification in the Idaho Technology R.A.P.I.D. LT instrument, and automatic result interpretation by the R.A.P.I.D. LT software.

The *E. coli* O157:H7 LT FSS protocols include a post enrichment pooling option. Pooling provides a number of significant benefits to the user. These include: increased sample throughput (i.e. up to 5 times), substantial reduction in material and labor costs, and the integrity of the original sample is maintained.

This pooling method relies on combining up to 5 sub-samples to create a single composite sample. If a positive pooled sample is obtained, then the individual post-enrichment samples can be re-analyzed to achieve rapid confirmation.

Salmonella and *Listeria* species tests for the R.A.P.I.D. LT FSS have been evaluated and certified as Performance Tested Methods by the AOAC Research Institute.

INTRODUCTION

The assay used in the *E. coli* O157:H7 LT FSS is designed for real-time PCR using the R.A.P.I.D. LT instrument and probes for DNA sequence specific detection of *E. coli* O157:H7. A unique DNA sequence of the *E. coli* O157 genome is amplified using specific primers. This amplified product is then detected by fluorescence using a specific set of probes. This technique allows for two initial levels of specificity in terms of amplification and real time detection. The probes consist of two oligonucleotides that hybridize to a specific internal sequence of the amplification product during the annealing phase of the PCR cycle. One probe is labeled at the 5' end with LC-Red 640 and has a phosphorylated 3' end (to prevent extension of the probe sequence). The second probe is labeled at the 3' end with fluorescein. Upon hybridization to specific amplified DNA sequences, the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores and the emission of a signal at 640 nm (Figure 1). The intensity of this signal is proportional to the amount of target amplification product generated during PCR (Figure 2a). The effectiveness of PCR is ensured within each reaction by an internal amplification control which is detected by probes that emit a 705 nm signal (Figure 2b).

Discrimination of the amplified *E. coli* O157 product between H7 and non H7 DNA sequences is provided by a post-PCR melt program. In this program, the temperature of the reaction is slowly raised and the decrease in fluorescence is measured as the annealed product and probe denature, i.e. melt off. The probes of the *E. coli* O157:H7 LT FSS are designed to be completely complementary to a segment of H7 sequence, if the reaction is H7 positive then a more thermodynamically stable product is created. This stable product has a higher observed melting temperature (Tm) peak than that of the probe with mismatched non H7 product (Figure 2c). These peaks are analyzed by the software and a Positive or Negative call is reported to the user. This then provides a third level of specificity for identifying the target as *E. coli* O157:H7.

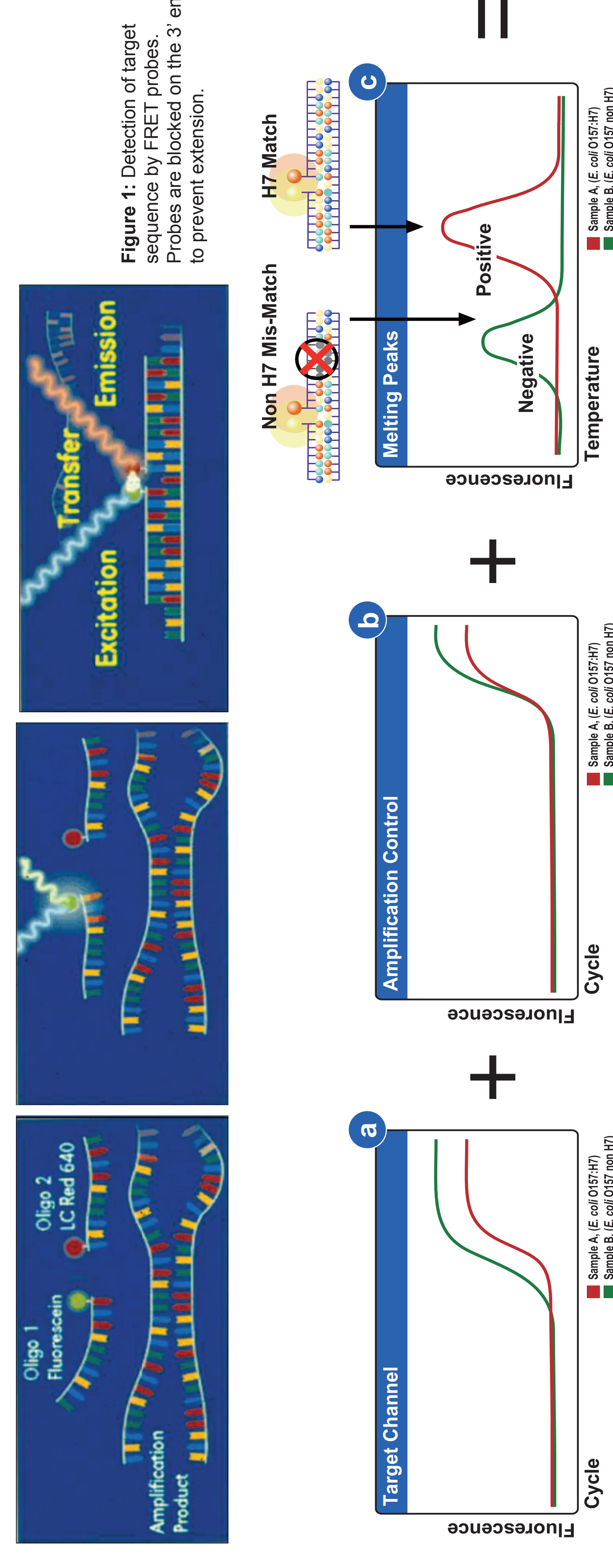
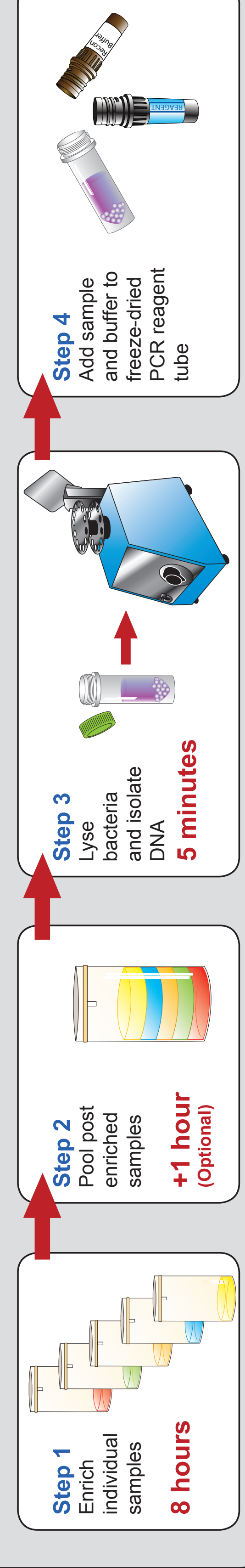


Figure 2. (a) Real time amplification curves of *E. coli* O157 DNA reported in target channel, (b) Valid amplification control curves reported in another channel, (c) Discrimination of amplified product by melt peak (Tm) analysis, (d) Automatic interpretation and analysis by software.

Figure 3: *E. coli* O157:H7 LT FSS Protocol



PROTOCOL DEVELOPMENT

Materials and methods evaluated include:

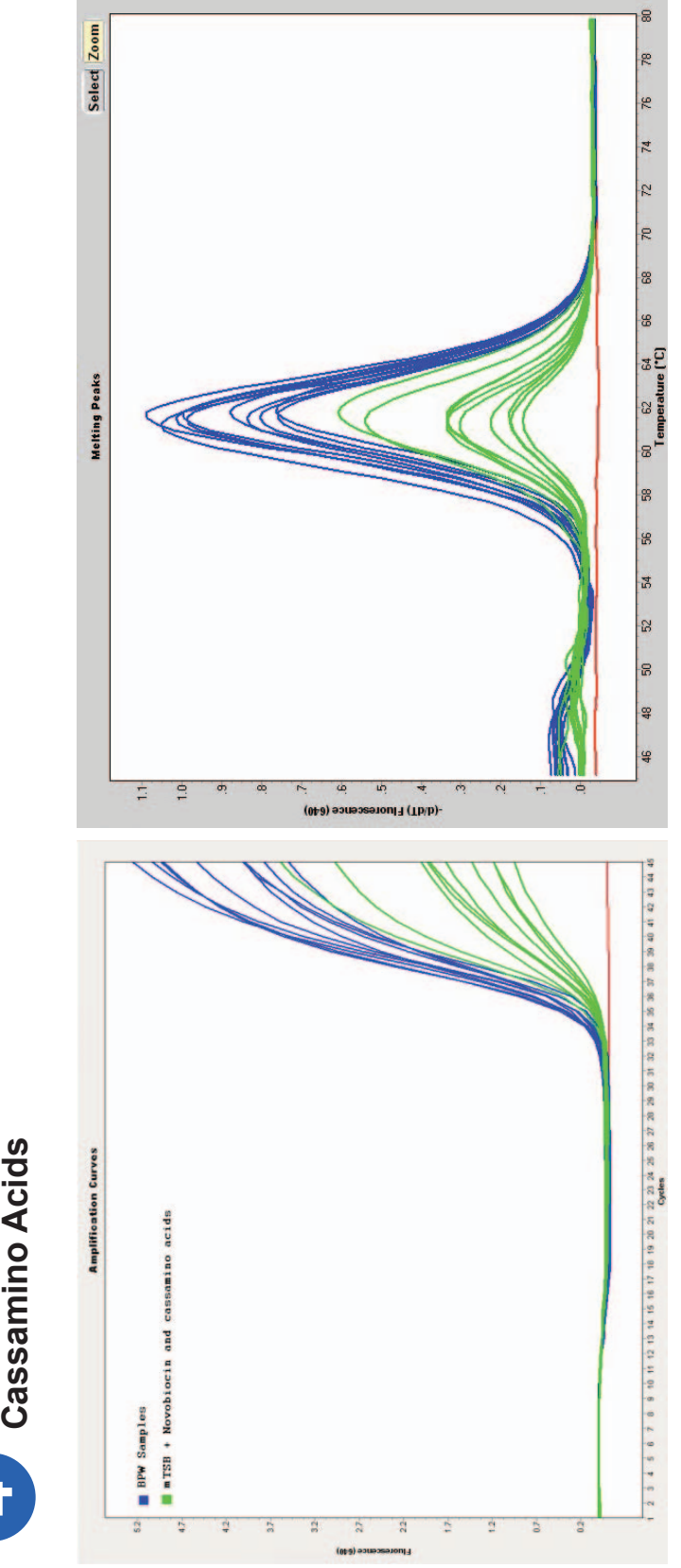
GROWTH MEDIA AND TIME

A wide variety of liquid enrichment media were examined for the *E. coli* O157:H7 LT FSS (including TSB, mTSB, mTSB, mTSB with Novobioicin, and Buffered Peptone Water (BPW). BPW was chosen for its attributes in PCR compatibility, its speed and reliability of enrichment and its low relative cost (Figures 4, 5, and 6).

Minimum length of enrichment was investigated for *E. coli* O157:H7 in 25g and 375g raw ground beef as well as 25g uncooked spinach. For the 25g protocols 8 hours was determined to be a reliable length of enrichment for detection of low levels of *E. coli* O157:H7. For 375g ground beef samples, 16 hours has been initially investigated for detection (Figures 4, 5, and 6).

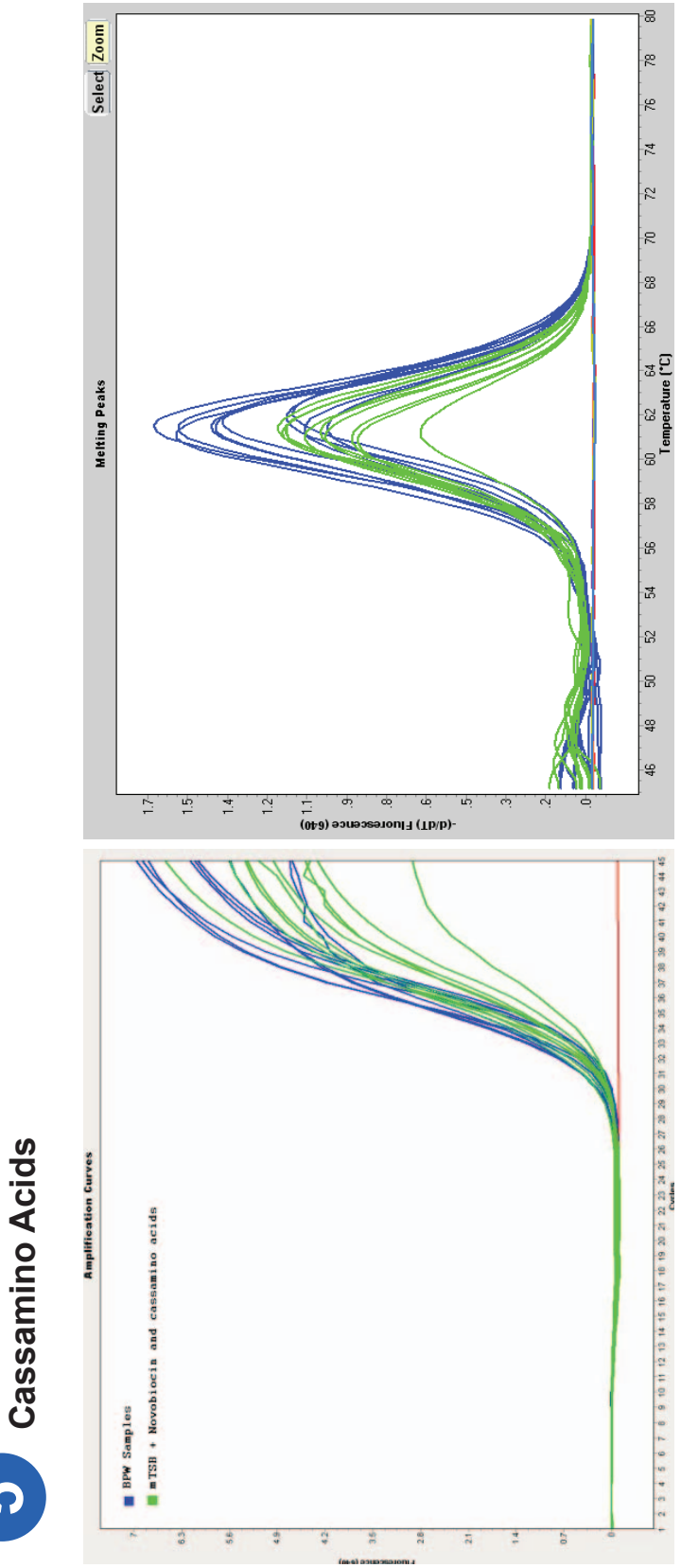
4 Ground Beef: 7 hour Enrichment in BPW compared to mTSB with Novobioicin and Cassamino Acids

Figure 4. Ground beef samples (25g) inoculated with ~ 10 CFU *E. coli* O157:H7 and enriched for 7 hours in TSB with Novobioicin and mTSB with Novobioicin and cassamino acids (green). PCR amplification curves are shown on the left, melt peaks are on the right. The mTSB with Novobioicin water show higher growth levels earlier in enrichment.



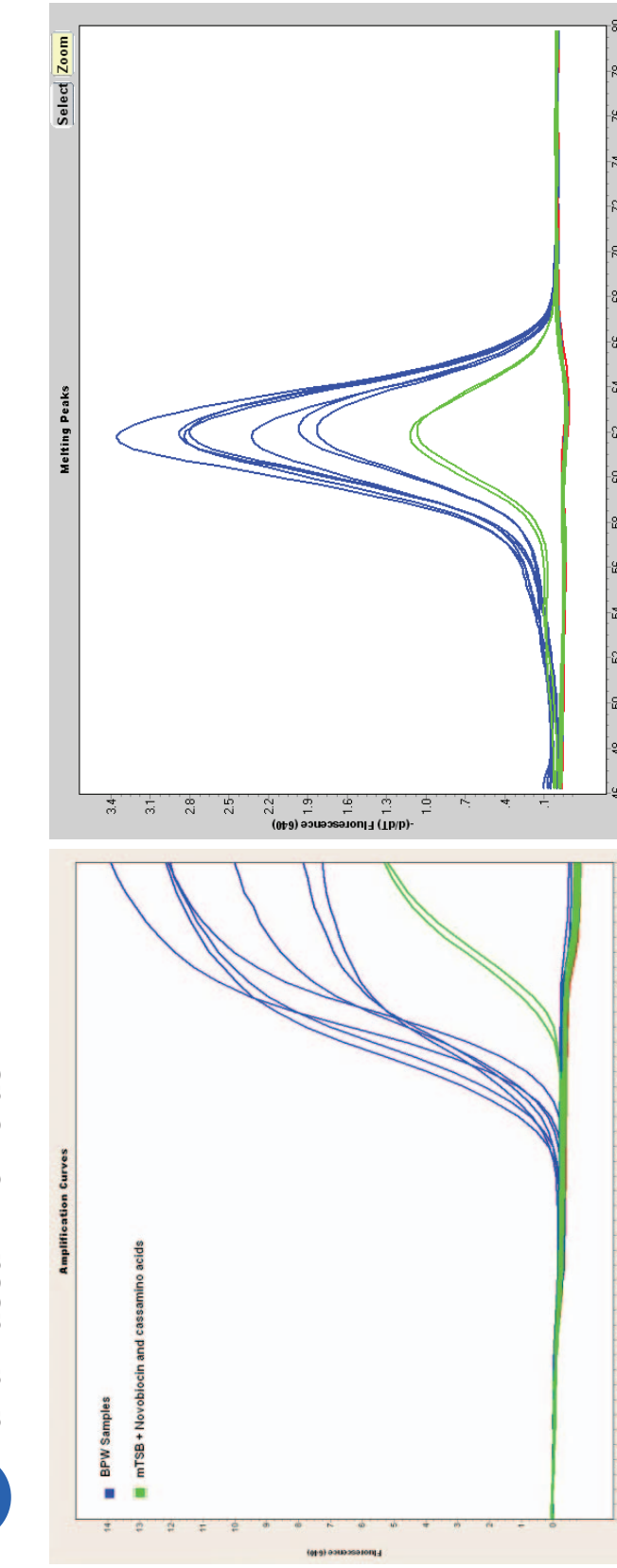
5 Ground Beef: 8 hour Enrichment in BPW compared to mTSB with Novobioicin and Cassamino Acids

Figure 5. Samples from figure 4. Ground beef samples (25g) inoculated with ~ 10 CFU *E. coli* O157:H7 and enriched for 8 hours in buffered peptone water (BPW) with Novobioicin and cassamino acids (green).



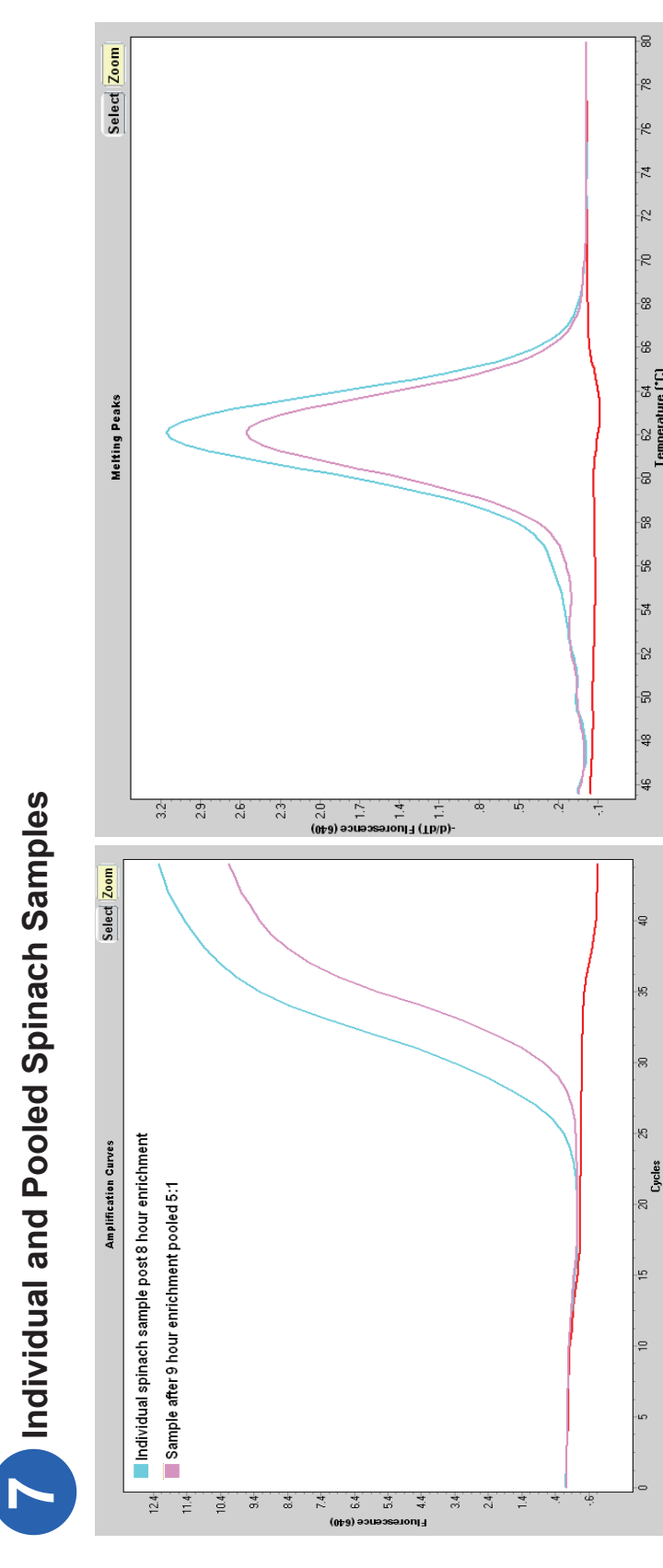
6 Spinach Samples: 8 hour Enrichment in BPW compared to mTSB with Novobioicin and Cassamino Acids

Figure 6. Spinach samples (25g) inoculated with ~ 10 CFU *E. coli* O157:H7 and enriched for 8 hours in buffered peptone water (BPW) with Novobioicin and cassamino acids (green, 2/10 positive).



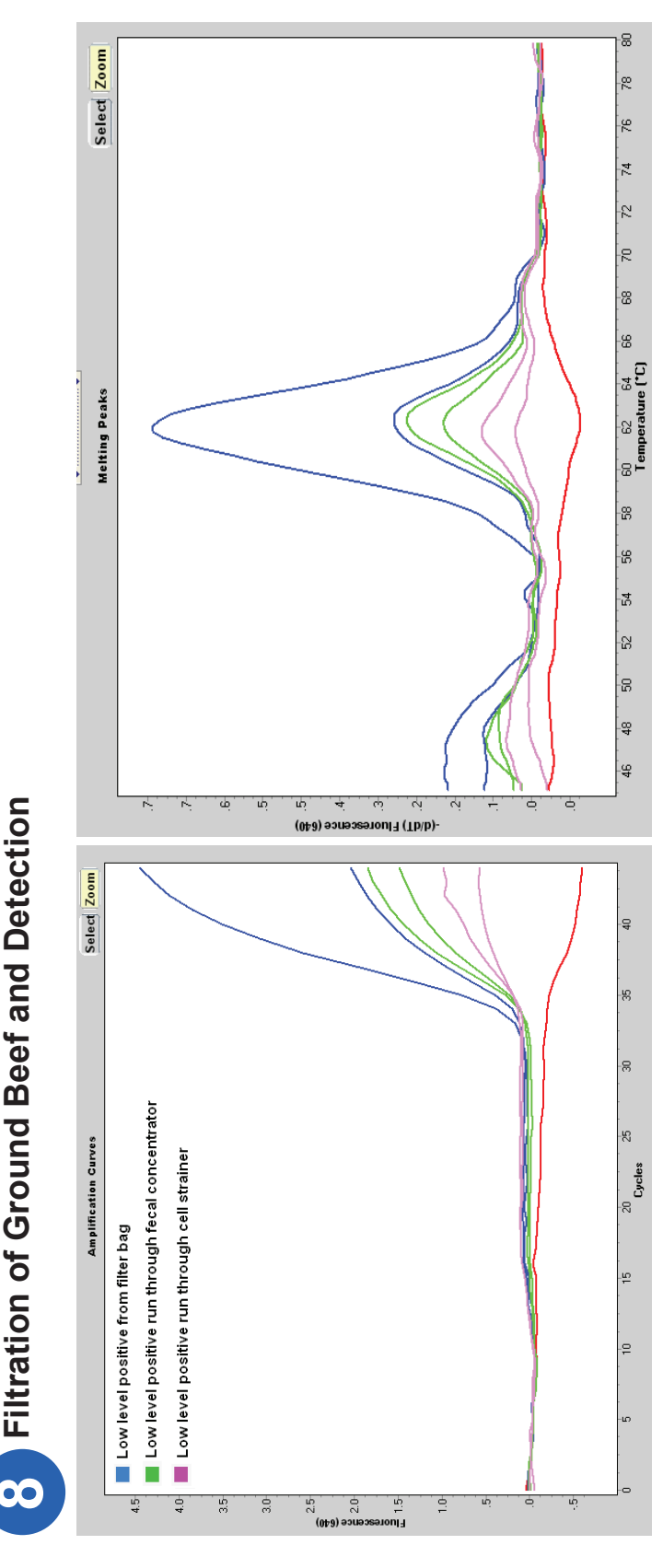
7 Individual and Pooled Spinach Samples

Figure 7. Single spinach sample (25g) from figure 6 inoculated with ~ 1 CFU *E. coli* O157:H7 and enriched for 8 hours in buffered peptone water (turquoise). After a total of 8 hours of enrichment, the positive sample was pooled with 4 negative samples and tested (pink).



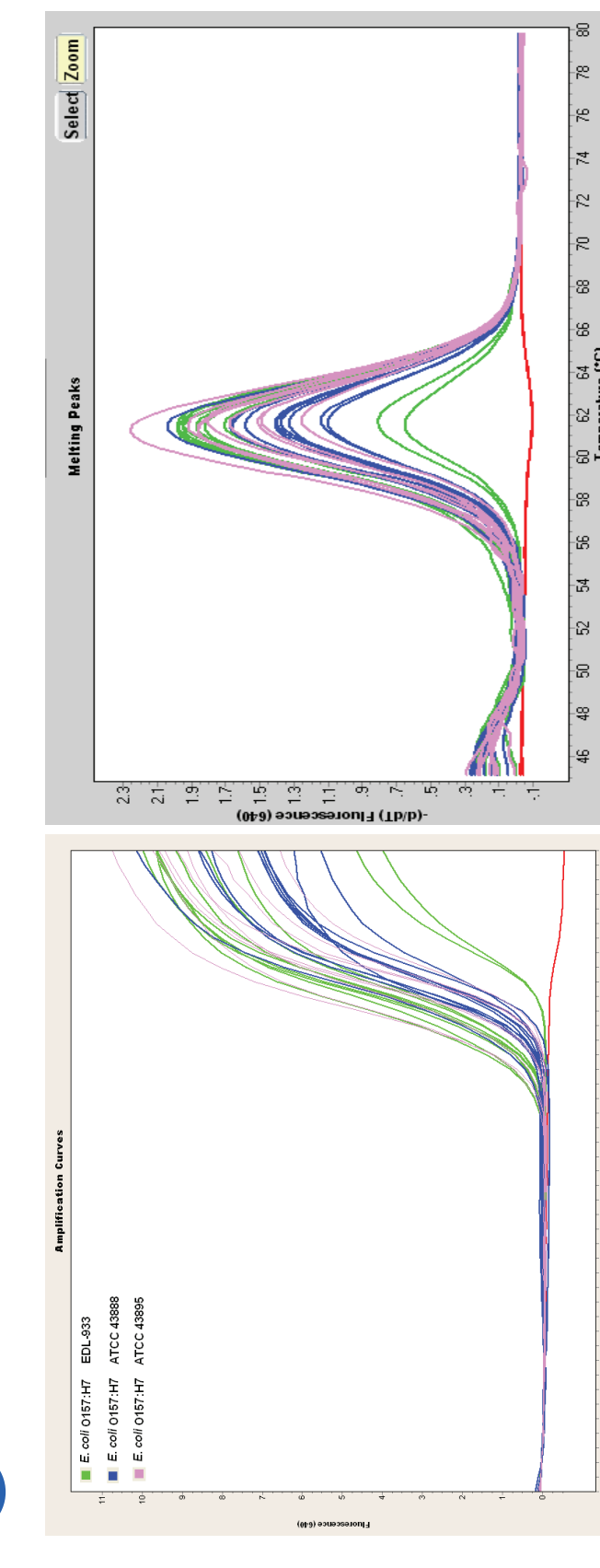
8 Filtration of Ground Beef and Detection

Figure 8. Low level ground beef positive (375g) enriched for 16h, and then aliquoted directly from the BPW into 100 µm cell strainer (green), or run through 100 µm cell strainer (pink). Filtration beyond that provided by filler bag appears to hinder detection.



9 Multiple *E. coli* O157:H7 Strains Evaluated

Figure 9. Ground beef samples (25g) inoculated with ~ 10 CFU of three *E. coli* O157:H7 strains and enriched in BPW for 8 hours.



POOLING

Post enrichment sample pooling of 25g samples was also investigated. A pooled positive sample was made by combining the positive sample with 4 negative samples A somewhat longer enrichment (9 hours) was determined to be optimal for detection in pooled samples (Figure 7).

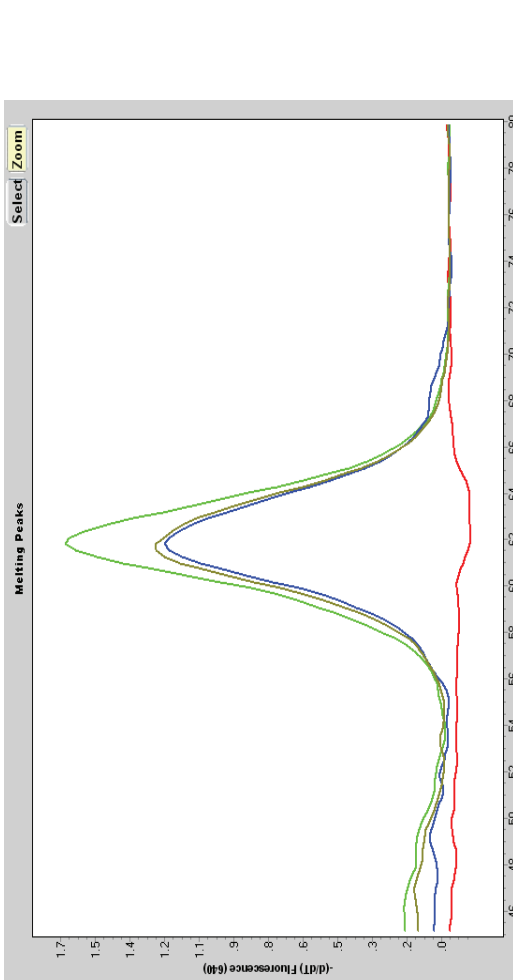
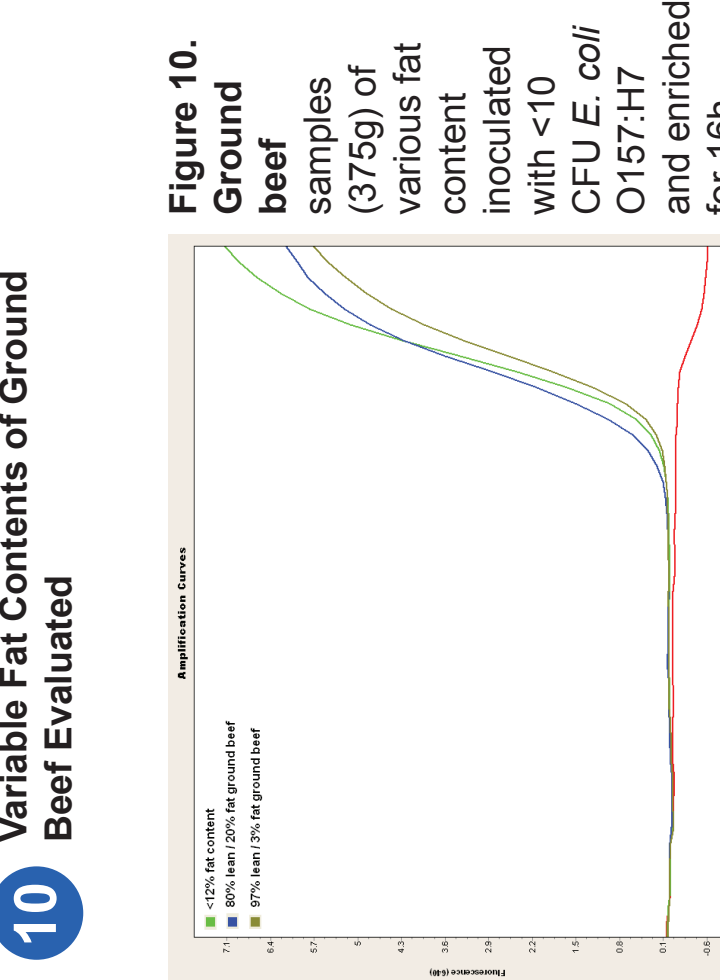
FILTRATION, CONCENTRATION AND SEPARATION TECHNIQUES

Some techniques led to declines in the ability to detect *E. coli* O157:H7 (see Figure 8) while others had promising results. However, gains in speed of detection were minimal with these technologies and led to increased chances of cross contamination and user error from sample handling.

MATRIX AND STRAIN RELATED VARIABLES

Once initial protocols for raw ground beef and uncooked spinach were determined, they were further tested with additional variables such as: long (48-72 hour) sample equilibration prior to enrichment, the use of multiple *E. coli* O157:H7 strains (Figure 9), variable fat content in ground beef (Figure 10) and food samples from a variety of producers.

10 Variable Fat Contents of Ground Beef Evaluated



REFERENCES

1. The reference method for spinach is the Eighth edition, revision A, Chapter 4a of the FDA BAM (<http://www.cfsan.fda.gov/~abam/bam-4a.html>).
2. The reference method for ground beef is the Third Edition, revision A, Chapter 4a of the FDA BAM (http://www.fda.usda.gov/OPD/MLG_5_04.pdf).

CONCLUSION

The developmental experiments conducted by Idaho Technology have resulted in protocols for use with the *E. coli* O157:H7 LT FSS that are:

- Rapid
 - 8 hours for 25g raw ground beef or uncooked spinach
 - 16 hours (this protocol is still being optimized) for 375g raw ground beef
 - Accommodates 5:1 post-enrichment pooling (at 9 hours of incubation)
 - There is no lengthy sample purification step
- Inexpensive
 - Uses a single enrichment of buffered peptone water without supplements
 - Uses simple filtration (strained filter bag only)
 - Does not require additional consumables (filters, immunomagnetic beads etc.)
- Robust
 - The protocol performs well with multiple strains of *E. coli* O157:H7
 - The protocol performs well despite matrix related variables (long equilibrations, variable fat content, multiple suppliers)

- Simple
 - Minimizes sample handling steps and thus risk of cross contamination (single enrichment, no separation or filtration step, no sample purification step)
 - The 25g protocols for raw ground beef and uncooked spinach are ready for inclusion in the *E. coli* O157:H7 LT FSS. The system is ready to be evaluated for its overall performance in the detection of *E. coli* O157:H7.

Current Evaluation

- The *E. coli* O157:H7 LT FSS system and protocols are currently undergoing AOAC Performance Tested Methods evaluation. This evaluation includes:
- Method comparison between the *E. coli* O157:H7 LT FSS and uncooked spinach.
 - Specificity of the *E. coli* O157:H7 LT FSS:
 - Inclusivity: 61 strains of *E. coli* O157:H7 will be tested.
 - Exclusivity: 44 strains of non-*E. coli* O157:H7 bacteria, including closely related taxa will be tested.
 - Ruggedness of system parameters to minor user error and protocol deviations.
 - Lot-to-lot consistency and shelf stability.