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Validation of the *E. coli* O157 GDS method for analysis of UCFM and cooked meat samples

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Scientific Interpretive Summary

This SIS is prepared by MPI risk assessors to provide context to the following report for MPI risk managers and external readers

Validation of the *E. coli* O157 GDS method for analysis of Uncooked Comminuted Fermented Meat (UCFM) and cooked meat samples ESR Report FW 10095

Escherichia coli O157:H7 is a food borne pathogen of high priority worldwide and is subject to food monitoring programmes in many countries. It has been classified as a food adulterant in the US since 1994.

A wide range of molecular methods for screening food products for the presence of pathogens, such as *E. coli* O157:H7, are now available, providing a rapid and cheap analysis for food processors. These methods must be validated for both sensitivity (detection of low numbers of bacteria) and specificity (detection of a given pathogen) in specific foods or matrices.

Uncooked Comminuted Fermented Meat (UCFM) and pre-cooked meat products have properties that as well as being inhibitory to microbial growth in the product itself, could also interfere with the assay. For example, both product types can have a high fat content and can contain a range of additive ingredients such as garlic, pepper, salts and sugars, phenolic compounds and emulsifiers; all of which potentially can inhibit microbial growth. In addition, UCFM can contain high levels of microflora used for fermentation of the product (for example lactic acid bacteria and other mesophilic aerobic bacteria) which may compete or inhibit *E. coli* O157:H7 during the enrichment phase.

The Biocontrol Assurance GDS® Assay for O157 uses a combination of selective enrichment, immuno-magnetic separation (IMS) and real time PCR (RT-PCR) to detect the presence of *E. coli* O157:H7. In this study, the assay was compared to standard IMS-culture methods. 8 different products (2 pre-cooked patties, 2 pre-cooked sausages and 4 UCFM products) and 4 strains of *E. coli* O157:H7 (inoculated at 10-50 cells per gram of sample) were used. Several modifications to the manufacturers' instructions were made.

For both pre-cooked products, all *E. coli* O157:H7 spiked samples were identified as positive by Assurance GDS®. The generic *E. coli* control was not detected. For UCFM with high pH and low water activity or added ingredients (garlic or pepper), all *E. coli* O157:H7 spiked samples were identified as positive. Initially high background microflora found in UCFM did appear to impact detection of one of the strains of O157 used in the study, however it was also noted that fewer colonies were detected when plated, suggesting impaired growth during enrichment or IMS capture rather than the PCR assay itself.

The impact of anti-bacterial properties of spices that had been noted in a number of previous studies was not evident in this study; however this may be due to the concentration being too low in the product to impact on O157 enrichment. If the spice was inhibitory for growth but not bactericidal, this would lead to a false negative result where detection by PCR would not have occurred. Validation for all screening methods for different food types is therefore important.

Based on this study, the authors concluded that Assurance GDS was able to detect low levels of *E. coli* O157:H7 in a range of UCFM and pre-cooked meat products and is therefore useful as a screen for detection for *E. coli* O157:H7. However, care is needed in interpreting the results.

Client Report
FW10095

Final Report

Validation of the *E. coli* O157 GDS method for analysis of UCFM and cooked meat samples

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Final Report

Validation of the *E. coli* O157 GDS method for analysis of UCFM and cooked meat samples

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SUMMARY

Food adulterants are increasingly a concern for the New Zealand meat exporter, and a condition on which export products are accepted or rejected. *Escherichia coli* O157:H7 has been designated a food adulterant by the United States Department of Agriculture, Food Safety Inspection Service (USDA /FSIS) since 1994. Raw beef products are currently tested for the presence of *E. coli* O157:H7 before export to the USA under the MAF US Overseas Market Access Requirement (US OMAR) (NZFSA, 2009a). This requirement was recently under consideration for extension by the US to incorporate Ready-To-Eat (RTE) products, including dry or semi-dry fermented sausage (also known as uncooked comminuted fermented meats (UCFM)) and fully cooked meat patties intended for export to the United States (USDA 2009). While a decision was made not to implement this requirement, the decision was made during the course of the work described here.

The New Zealand Food Safety Authority commissioned a preliminary validation study to investigate the validity of a PCR screen method to detect a range of *E. coli* O157:H7 isolates from a variety of products. Products were chosen based on possession of physico-chemical characteristics that may be inhibitory to the enrichment, isolation or detection of *E. coli* O157:H7. The chosen assay was the Assurance GDS Assay for *E. coli* O157:H7 as this assay has been approved for raw ground beef and beef trim because of the high degree of specificity and selectivity afforded by this assay (Mills *et al.*, 2009). The Assurance GDS system is a combination of selective enrichment followed by immuno-magnetic separation (IMS), and Real Time PCR amplification using specific proprietary primers for *E. coli* O157:H7. Sample which are positive by this assay need to be confirmed by approved conventional assays using IMS, selective plating and latex bead agglutination (NZFSA, 2009a).

The aim of this work was to determine the suitability of the Assurance GDS system for reliably detecting low numbers of *E. coli* O157:H7 on a variety of pre-cooked or UCFM products. This study shows that the Assurance GDS *E. coli* O157:H7 system is suitable for testing partially cooked and pre-cooked meat patties and sausages and UCFM products.

1 INTRODUCTION

The United States Department of Agriculture, Food Safety Inspection Service (USDA FSIS) has recently instigated a sampling protocol requiring a variety of Ready-To-Eat (RTE) products, including dry or semi-dry fermented sausages (also known as uncooked comminuted fermented meats (UCFM)) and fully cooked meat patties be tested for *Escherichia coli* O157:H7 (USDA FSIS Directive 10,240.4 RTE001). Should this requirement need to be incorporated into the MAF US Overseas Market Access Requirement (US OMAR) a robust, validated, method of analysis will also be required.

UCFM products have intrinsic physico-chemical properties such as low water activity (a_w and low pH that are inhibitory to the survival and growth of pathogens (i.e. pH < 5.2 and a_w < 0.95 (NZFSA, 2009a)). The temperatures involved in producing cooked meat patties should render these products pathogen-free. However, there is the opportunity for post-processing contamination as, although the recommendation is to reheat both the pre-cooked patties and sausages, UCFM products do not require further heating before consumption. It is therefore argued that testing is necessary to ensure that these products are pathogen-free.

Tests stipulated in the US OMAR are required to be conducted using approved analytical methods that have been appropriately validated for the matrix tested. The Assurance GDS *E. coli* O157:H7 (BioControl, Seattle, USA) PCR screen method (GDS) has recently been validated and approved for raw ground beef, beef trim, orange juice, apple juice, fresh vegetables, and sprout process water (AOAC, 2005). This rapid test method was recently approved by MPI as the method of choice for meat processors to test for *E. coli* O157:H7 in New Zealand because of its high degree of sensitivity and specificity compared to other methods (NZFSA, 2009a).

Presently, the regulatory limits for pathogens in UCFM and cooked meat products do not include *E. coli* O157:H7 (NZFSA, 2010), so therefore there are no approved methods, rapid or conventional, for its detection in these products. A literature search and discussions with BioControl did not identify that the GDS PCR screen method has been previously validated for use in the detection of *E. coli* O157:H7 in UCFM and cooked meat products. The GDS PCR screen assay performed best in validation studies of a range of rapid test methods (Mills

et al., 2009) and is currently the sole approved test method for ground beef. New Zealand meat processors therefore have access to laboratories using this method.

Validation is required as these products may have properties that inhibit aspects of the GDS PCR screen assay as well as potentially changing conditions during the enrichment process that may affect the growth of *E. coli* O157:H7. Properties inherent in fermented products include low a_w and pH, as previously mentioned and the high background micro-flora which may inhibit the growth of *E. coli* O157:H7 during enrichment. Two levels of fat content in UCFM products were also examined. Additionally, the presence of potentially inhibitory ingredients such as garlic, pepper and cheese (Rossen et al 1992; Wilson, 1997) were considered. A high background microflora may introduce a physical inhibitory step in the IMS step. Finally, the PCR efficacy may be inhibited by phenolic compounds and ingredients such as salts, sugars and emulsifiers (Rossen et al 1992; Wilson, 1997). Therefore, this work sought to validate the GDS *E. coli* O157:H7 PCR screen assay on samples of UCFM and cooked meat patties and cooked sausages. The results were to be compared to those obtained by IMS-culture (NZFSA, 2009a) and efficacy comparisons performed.

2 METHODS

2.1 Bacterial Isolates

Four isolates of *E. coli* O157:H7 were chosen by source, shiga toxin subunit variant, acid tolerance, and detectability by both PCR and lateral flow type screen kits. A non-O157 *E. coli* isolate was chosen as a negative control (Table 1).

Table 1. *E. coli* O157:H7 strain information and basis for selection

					Genotype information						
No	**Strain**	**Serotype**	**Source**	**Xba Type**	**stx1**	**stx2**	**stx2c**	**stx2d**	**eaeA**	**ehxA**	**Reason for choosing**
1	CPH0510332-7	O157:H7	Bobby veal Shank	92	1	1	0	1	1	1	stx2d, stx1 and stx2
2	ERL06-2497 (N427)	O157:H7	Bobby calf	95	1	1	0	0	1	1	Difficult to detect in Lateral Flow Immunoassays; stx1 and stx2
3	NZRM4159	O157:H7	Bovine	40**	0	1	0	0	1	1	Acid resistant, pH 3.5; Water activity sensitive; stx2 only
4	96/2998*	O157:[H7]†	Human	18	1	1	1	0	1	1	Water activity sensitive; we know this strain survives in UCFM (information up to 1 hour); stx1 and stx2, stx2c
5	NZRM 916	Non-O157									Negative control

* Australian strain used in UCFM PCR validation study (unpublished)

** most prevalent type of *E. coli* O157:H7 isolated from meat/bobby calves in New Zealand (NMD database)

† *E. coli* O157:[H7] strain 96/2998 is a non-motile isolate positive for H7-specific PCR amplification, but negative by H-serotyping.

Frozen bacterial isolates, kept at -80 °C, were streaked for recovery onto Tryptic Soy Agar (TSA) which is Trypticase Soy Broth (TSB: Oxoid, Basingstoke, UK) containing 1.5 % (w/v) agar, and MacConkey Agar plus Sorbitol, Cefixime, and Tellurite plates (CT-SMAC: Fort Richard Media, Auckland, NZ) incubated for 18-24 hours at 37°C. Plates were checked for purity and typical colony morphology before use. TSA plates were stored at 4 °C for a maximum of 14 days for use as inoculum seed plates.

2.2 Food Matrices

Three food types were selected for this study: UCFM products, cooked meat patties and cooked sausages (Table 2). A range of UCFM products were analysed to cover a series of characteristics that would represent either inhibitory ingredients for growth of *E. coli* O157:H7 on the matrix, or cause inhibition of the immunomagnetic separation (IMS) and / or PCR components of the GDS assay. Two types of cooked meat patties and cooked sausages were

chosen for ingredients that might inhibit either bacterial growth or components of the GDS assay also. A total of eight matrices was tested after agreement with MPI.

Table 2. Product Characteristics

Sample	Product	Distinguishing Characteristics/Ingredients
1	Pre-Cooked Beef Patties	Cheese
2	Pre-Cooked Beef Patties	Plain
3	Pre-Cooked Sausages	Cheese
4	Pre-Cooked Sausages	Savoury (including dehydrated onion, parsley, dehydrated garlic and spice extracts)
5	UCFM	High pH, low a_w
6	UCFM	Pepper
7	UCFM	Garlic
8	UCFM	High background flora

All products were purchased locally. Frozen precooked meat patties were stored frozen at -20°C and UCFM and pre-cooked sausage products were kept at 4 °C before testing. Frozen pre-cooked meat patties were defrosted at refrigeration temperatures for 24 h before testing.

One set of UCFM samples was required to contain a high background bacterial flora to observe any possible effects from competition. This was achieved by selecting a UCFM product identified from a previous project (UCFM National survey) as containing a high microflora and enriching overnight in modified TSB supplemented with casamino acids and Novobiocin (mTSB+n; Fort Richard Media, Auckland, NZ) at 42°C to ensure that a typical microflora which would grow in the selective enrichment broth was obtained. One ml of this overnight enrichment (~10^9 CFU ml^{-1}) in stationary phase was then added to sample enrichment broths of UCFM. This was to ensure that a balanced microflora in large numbers in stationary phase would be present, as would be expected in the UCFM at retail.

2.3 Inoculation Procedure

Each food matrix was inoculated with one of four *E. coli* O157:H7 isolates in triplicate and with one generic *E. coli* (NZRM 916). A single colony was taken from a prepared TSA plate (section 2.1), inoculated into 10 ml of TSB and incubated for 18 hours at 37 °C. This initial broth culture was then diluted 1:10,000 into Phosphate Buffered Saline (PBS; Fort Richard Media, Auckland, NZ) and enumerated by plating onto TSA with incubation at 37 °C overnight.

Food samples were cut aseptically into small pieces and 25 g weighed into sterile petri dishes in preparation for inoculation. The surfaces of food samples were then inoculated with 10 µl of each of the diluted isolates and incubated at room temperature for 10 minutes to allow stabilisation of the inoculum on the matrix. At this point, the enriched background microflora inoculum was also added to the appropriate samples. The samples were then aseptically transferred into filter stomacher bags and 225 ml of mTSB+n added, HBF added if required, the sample stomached and incubated at 42°C for 24 ± 1h. All strains were inoculated onto each matrix at low concentrations of approximately 10 – 50 CFU g^{-1} as calculated in retrospect from the TSA enumeration plates.

After enriching for 24 h at 42°C, two subsamples were taken, one for validation of the presence of *E. coli* O157:H7 using the BioControl Assurance GDS system. The second volume was screened for the presence of *E. coli* O157:H7 using the previous beef and veal monitoring programme IMS-culture method (NZFSA, 2009a). Samples were retained frozen at -20 °C after sampling in case of the need for further testing.

2.4 Assurance GDS for *E. coli* O157:H7 Assay

The Assurance GDS for *E. coli* O157:H7 method is a gene detection-based assay that uses specific primers and proprietary probes directed against a highly conserved DNA sequence in the target organism. A modification of the AOAC official Method 2005.04 was followed. Briefly, 25 g of sample is incubated in 225 ml of pre-warmed mTSB+n instead of mEHEC media. This modification follows the protocol as outlined in MAF Part 12 United States of America: Technical procedures for Monitoring *Escherichia coli* O157:H7 Programme in Bulk Manufacturing Beef. A longer incubation period of 24 ± 1 h (as compared to the 15 – 22 h outlined in the above protocol) was elected to allow maximal time for bacterial stress recovery and growth from the low bacterial concentrations inoculated. Previous experience had shown

an increased lag phase of some strains when enriched in mTSB+n, and that the extended incubation time did not adversely affect the viability of cells (Pers. comm. Angela Cornelius, ESR). After enrichment, populations of target microorganisms are concentrated by using a proprietary concentration device (pick pen) based IMS. After washing and resuspension in buffer, the concentrate was transferred to a reaction vessel containing amplification reagents. The vessel was then sealed and placed in an instrument which allows for simultaneous amplification and detection. All tests, positive and negative, are indicated at the end of analysis, as well as the results of a procedural internal control which is contained in every reaction vessel.

The validation of the BioAssurance GDS system was performed over four runs consisting of 32 samples per run. For each run, samples were prepared in four blocks of eight. Two matrices, inoculated with the five isolates were tested in each run, and included overnight cultures of all isolates in broth, serving as internal positive controls to demonstrate that the GDS system was capable of detecting the isolates used, as well as a PBS+Tween blank for a negative internal control. Each combination of matrix and bacterial isolate was performed in triplicate with the exception of the generic *E. coli* NZRM 916 which was inoculated in a single assay.

2.5 Confirmation of Presence of *E. coli* O157:H7 by Immunomagnetic Separation

The method followed for confirmation of the presence of *E. coli* O157:H7 was as outlined in MAF Part 12 United States of America: Technical procedures for Monitoring *Escherichia coli* O157:H7 Programme in Bulk Manufacturing Beef. Briefly, 500 µl of enriched sample was processed in duplicate using Dynabeads Anti-*E.coli* O157 and the Dynal BeadRetriever™ (Dynal Biotech, Carlton South, Australia) according to the manufacturer's instructions. At the end of the program run, the bead-bacteria complex was spread over one half of a CT-SMAC agar plate, then streaked for isolation over the second half of the plate. The plates were then incubated at 37 °C for 18-24 h. Isolates were then tested for agglutination with the *E. coli* O157 Latex test. (Oxoid, Basingstoke, UK).

Each sample set was carried out with positive isolate controls in broth and one PBS buffer (blank) negative control.

2.6 Further Investigations with High Fat and High background Flora Samples

Initial results noted that there may be some problems with the ability of the system to detect *E. coli* O157 in high fat/high background flora samples. This was examined further by testing

high (~30%) and standard (~20%) fat concentration salamis in the presence of the naturally occurring and added microflora. All four categories of sample were inoculated separately in duplicate with the five test strains, enriched and then tested as described above.

It was necessary to show that paprika did not inhibit the growth of *E. coli* O157:H7, and so some experiments were conducted to examine this. A "mild" salami was purchased and 25 g portions weighed into stomacher bags. All samples, except a control were individually inoculated with a low concentration of the five test strains. Paprika was added to five bags to achieve a concentration of 8% in the enrichment (10 times that possible if all of the spice in a chorizo sausage was paprika) and five were not amended. 225 ml of mTSB+n was added to each sample, a portion removed to obtain a viable count on triplicate plates of CT-SMAC (or MacConkey Agar for the non-STEC *E. coli*) and the rest of the sample incubated at 42°C for 24h. Following enrichment dilutions were performed in BPW and viable counts on CT-SMAC or CBA performed.

To reduce the effects on space constraints, as all work was conducted in a biohazard cabinet, the work was divided into two parts which were completed on separate days.

3 RESULTS AND DISCUSSION

3.1 Pre-Cooked Meat Patties and Pre-Cooked Sausages

The two pre-cooked meat patties tested (with and without cheese) as well as the two pre-cooked sausages tested (cheese and savoury) were able to support the growth of the four *E. coli* O157:H7 strains, and did not inhibit the GDS assay (Table 3). A representative graph set and results table for these products is shown in Appendix 1. There were no false negative or positive results by the GDS system for these foods. The generic *E. coli* control (NZRM 916) was not detected. These results indicate that with these types of products, the GDS assay is a suitable rapid method for the detection of *E. coli* O157:H7.

Table 3. GDS and IMS/CT-SMAC confirmation results for detection of *E. coli* O157 in ready-to-eat cooked meat samples.

Matrix	***E. coli* strain**	**Initial inoculum level CFU g^{-1}**	**GDS Results**	**Confirmation by IMS / Latex**
Cooked meat patties + Cheese	CPH0510332-7	47.2	+	+
	ERL06-2497	52.8	+	+
	NZRM4159	64.4	+	+
	96/2998	48.4	+	+

	NZRM 916	59.6	-	-
Cooked meat patties – Plain	CPH0510332-7	47.2	+	+
	ERL06-2497	52.8	+	+
	NZRM4159	64.4	+	+
	96/2998	48.4	+	+
	NZRM 916	59.6	-	-
Pre-cooked Sausages + Cheese	CPH0510332-7	36.0	+	+
	ERL06-2497	37.6	+	+
	NZRM4159	36.0	+	+
	96/2998	44.4	+	+
	NZRM 916	50.8	-	-
Pre-cooked Sausages - Savoury	CPH0510332-7	36.0	+	+
	ERL06-2497	37.6	+	+
	NZRM4159	36.0	+	+
	96/2998	44.4	+	+
	NZRM 916	50.8	-	-

3.2 UCFM Products

The UCFM products tested included a product with high pH and low a_w values (as compared to a mean determined from a national survey of UCFM products); two containing either pepper or garlic; and one with additional high background microflora. These products were agreed with MPI beforehand to encompass parameters that might affect either the growth of *E. coli* O157:H7 during enrichment, the IMS separation, or the PCR steps.

3.2.1 UCFM – High pH, Low a_w

The UCFM product chosen for high pH and low a_w properties, as detailed in the project specification, was able to support the growth of the four *E. coli* O157:H7 strains and did not inhibit the GDS assay (Table 4). The generic *E. coli* control (NZRM 916) was not detected.

This product was chosen after examining the results from the first tranche of a National UCFM survey. Results for pH and a_w were collated for all UCFM products assayed and the upper and lower 10 percentile figures determined (respectively), resulting in establishing a lower limit of pH 5.6 or higher, and an upper figure of 0.84 a_w or lower.

Table 4. GDS and IMS/CT-SMAC confirmation results for detection of *E. coli* O157 in high pH, low a_w UCFM samples.

Matrix	***E. coli* strain**	**Initial inoculum level CFU g^{-1}**	**GDS Results**	**Confirmation by IMS / Latex**
UCFM : High pH (5.6), Low a_w (0.75)	CPH0510332-7	61.2	+	+

	ERL06-2497	52.8	+	+
	NZRM4159	62.4	+	+
	96/2998	55.2	+	+
	NZRM 916	57.2	-	-

A representative graph set and results table for these products is shown in Appendix 1.

3.2.2 UCFM – With added Garlic or Pepper

An initial analysis of UCFM samples containing pepper and garlic both had one of three replicates produce negative GDS results when spiked with *E. coli* O157:H7 NZRM4159 and CPH0510332-7 respectively (Table 5). When the GDS Results Report Graphs were examined, it was noted that the traces indicated that there was amplification product for these samples, but that they had not amplified to the pre-set cut-off value taken to represent a positive result by the end of 32 cycles of amplification (Appendix 2). When these samples and isolate combinations were retested (using frozen portion of the original samples), all repeats were positive (Figure 1).

These results indicate that the low inoculum concentration is the reason for the false negative result. The same inoculum was used for each matrix tested in that run, as well as the two other replicates, which were all positive. Additionally, when the frozen samples were retested, the results were positive. When the Internal Control Results are examined (Appendix 2), a lag in amplification for the internal control is also observed for each sample in that particular block of eight samples. On examining the PCR tubes after the run, this block of eight samples appeared to contain far fewer of the IMS beads in the reaction tubes than the other three blocks. Therefore, it is believed there was a technical error in reagent or sample processing.

Table 5. GDS and IMS/CT-SMAC confirmation results for detection of *E. coli* O157 in UCFM samples containing garlic and pepper.

Matrix	***E. coli* strain**	**Initial inoculum level CFU g^{-1}**	**GDS Results**	**Confirmation by IMS / Latex**
UCFM + Pepper	CPH0510332-7	42.4	+	+
	ERL06-2497	47.6	+	+
	NZRM4159	44.0	+[1]	+

	96/2998	38.8	+	+
	NZRM 916	44.4	-	-
UCFM + Garlic	CPH0510332-7	42.4	+[1]	+
	ERL06-2497	47.6	+	+
	NZRM4159	44.0	+	+
	96/2998	38.8	+	+
	NZRM 916	44.4	-	-

[1] One replicate tested negative, repeat test in triplicate, all replicates positive; beef and bobby veal *E. coli* O157:H7 isolates

The graphs obtained for this run indicated that there was an anomaly in these results and therefore we retested. Commercial laboratories would also be able to diagnose sample processing issues if trained and encouraged to do so, and therefore consequently retesting to obtain accurate results. There did not appear to be any inhibition of the enrichment, IMS process or the PCR amplification steps by the ingredients in then UCFM products.

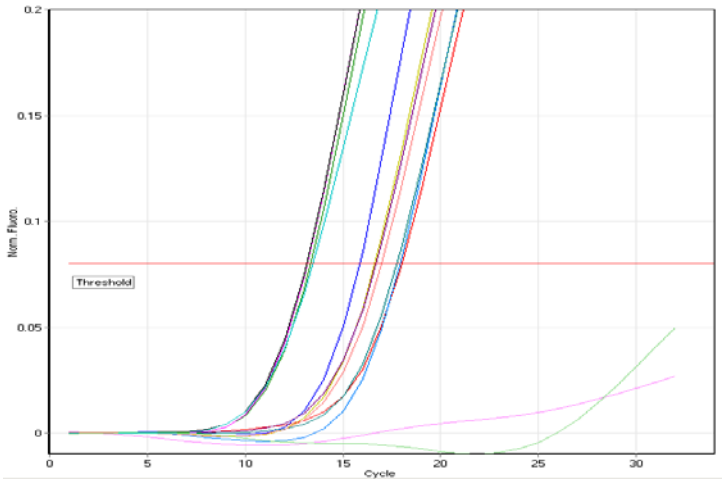

Figure 1. Assurance GDS Test Results for repeated UCFM containing garlic and pepper.

Previous samples that had been negative, gave positive results in this second run.

3.2.3 UCFM – High Background Flora

A UCFM product containing a high background flora (HBF) was tested to determine whether competing microflora would result in lower specificity or sensitivity of the GDS assay. A random UCFM product was chosen from a contemporaneous national survey for which physico-chemical and microbiological information were available. The product was purchased, sampled and incubated in mTSB+n overnight. One ml of this overnight enrichment was added to 225ml pre-warmed enrichment broth, prior to incubation for the trial proper. This was estimated to contain 1.0×10^9 CFU ml^{-1} and would equate to an additional 4.0×10^7 CFU g^{-1}

when the 25g sample size was taken into consideration. As these products are fermented, it was assumed that there was already a high number of both aerobic mesophilic micro-organisms and lactic acid bacteria present. Literature to support this assumption include Drosinos (2005) and Fontán *et al* (2007a,b) where the microflora of various fermented meat products was found to be between 10^8 and 10^9 CFU g^{-1}. The addition of an overnight culture to imitate a high background flora would therefore ensure a healthy and competitive background flora, rather than increase concentration necessarily.

Three of the four *E. coli* O157:H7 isolates were detectable at the limits inoculated on this matrix (Table 6). However, *Escherichia coli* O157:[H7] 96/2998, a human isolate, was included in this study because of its a_w sensitivity, ability to survive in UCFM and toxin profile, was not detected. When the GDS Result Report Charts are examined, the time for amplification is delayed for all combinations of matrices and this *E. coli* O157:H7 strain (Figure 2; complete report is in Appendix 3).

Table 6. GDS and IMS/CT-SMAC confirmation results for detection of *E. coli* O157 in UCFM with added microflora.

Matrix	***E. coli* strain**	**Initial inoculum level CFU g^{-1}**	**GDS Results**	**Confirmation by IMS / Latex**
UCFM: "High background" flora	CPH0510332-7	61.2	+	+
	ERL06-2497	52.8	+	+
	NZRM4159	62.4	+	+
	96/2998	55.2	-[1]	+
	NZRM 916	57.2	-	-

[1] All 3 replicates were negative; bobby veal *E. coli* O157:H7 isolate

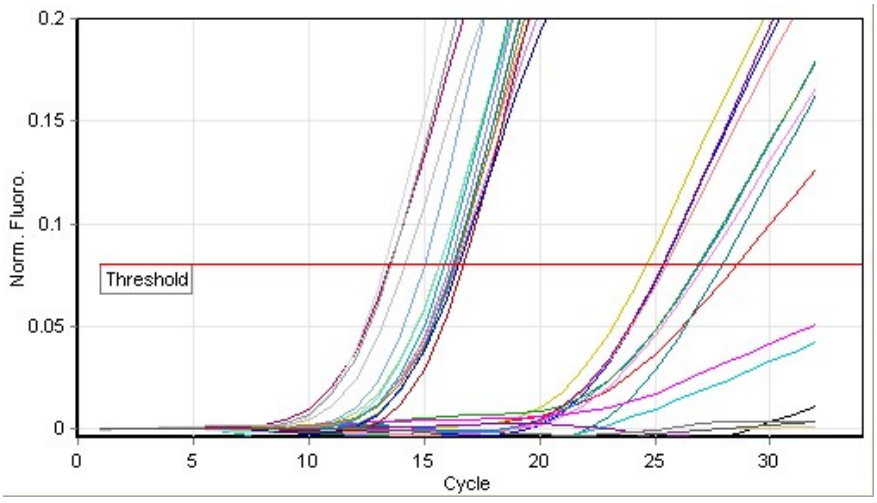

Figure 2. Assurance GDS Test Sample Results for UCFM with high pH, low Aw, and high background micro-flora products. The products are divided into two obviously different groups,

with the high background microflora spiked UCFM the 2nd group that has delayed amplification (approximately 20 – 25 cycles), including negative results when spiked with *E. coli* O157:[H7] 96/2998. The complete Assurance GDS Result Report with colour key is included in Appendix 3.

The strain positive controls (in broth) and the Internal Control Results for all samples in this run were all positive, indicating that the assay was run according to specification, and that the negative results obtained were not due to errors made during preparation (Appendix 3). Additionally, the conventional IMS-culture method was able to detect *E. coli* O157:H7 in this matrix (Table 6). However, it was noted that fewer presumptive colonies were detected after plating onto CT-SMAC post IMS than from the IMS samples from UCFM High pH, low a_w spiked matrices which were analysed in the same run. This might indicate inhibition of these *E. coli* O157:H7 cells during the enrichment or sub-optimal capture during IMS.

A factor that was not included in the scope of this validation study was the fat content of the meat products. The presence of fat has not previously been shown to be an inhibitory factor in PCR analyses of meat and meat products to the author's knowledge. A study looking at PCR inhibition in a series of diluted milk samples found the calcium content, not the fat content, to be inhibitory (Bickley *et al.*, 1996). However, a study by Rossen *et al* (1992) found that PCR was sensitive to cheese samples containing a high fat content. Investigation in the UCFM product that was spiked with high background flora, revealed that this product contained approximately double the amount of fat than other UCFM products tested (35.5 %, compared to 19.8%). While there is no evidence that fat content of this particular UCFM played an inhibitory role in the GDS analysis, further investigation was carried out.

3.3 Further Examination of High Fat and HBF UCFM Samples

Problems were encountered with the supply of sufficient UCFM with a constant and known fat content. The original samples (section 3.2) were purchased from a small manufacturer and repeated fat content testing showed considerable variability between batches and, notably, with respect to the sample used in the previous work (section 3.2). To remedy this, a salami was sought from a major manufacturer which contained a high fat content, and one was located with fat approximating 30% (although there was again moderate batch to batch variation). However, this sample was a chorizo containing paprika, a spice which may inhibit the growth of the target isolates.

When assessing the effects of paprika on enrichment, the uninoculated control yielded counts of <100 CFU/g *E. coli* O157:H7 in samples tested before and after enrichment. All of the *E. coli* isolates were able to grow in the presence or absence of 8% paprika, increasing from 10^4 CFU ml^{-1} prior to incubation to 10^9 CFU ml^{-1} after incubation. The Chorizo product was therefore used in further experiments on high fat salamis as the paprika did not inhibit growth.

The counts of *E. coli* O157:H7 inocula recovered (2 ml spread over six plates) from the samples (normal fat +HBF, normal fat –HBF, high fat +HBF, high fat –HBF) had a mean of 609 CFU $sample^{-1}$ (SD +/- 494 CFU $sample^{-1}$), equating to 24.4 CFU g^{-1} (SD +/-19.8 CFU g^{-1}). The generic *E. coli* strain was added at a comparable concentration. These data corresponded well with the calculated inoculum which was 653 CFU $sample^{-1}$ (SD +/- 109 CFU $sample^{-1}$), and were at similar/slightly lower concentrations than in the previous work (Tables 3-6).

For the first batch, samples supplemented with HBF received an additional mean 1.5 x 10^9 CFU $sample^{-1}$ as measured by LAB count, or mean 4.9 x 10^9 CFU $sample^{-1}$ as measured by APC. The salami samples themselves contained (normal fat: 21%) 3.3 x 10^9 CFU g^{-1} as measured by both APC and LAB methods and (high fat: 28%) 3.9 x 10^8 CFU g^{-1} by APC and 3.4 x 10^8 CFU g^{-1} by LAB count methods.

The first set of samples included those inoculated with *E. coli* O157 isolates 1-3, two uninoculated controls and pure cultures of isolates 1-3. The results for the duplicate samples of inoculated salami were that, when *E. coli* O157 was added to the sample at the concentrations cited above, then a GDS positive result was recorded and successful IMS recovery of typical *E. coli* O157 colonies was made on CT-SMAC. These presumptive isolates were confirmed on a per sample basis by latex agglutination. Uninoculated controls (two) were negative by GDS and did not yield suspect colonies by IMS and plating. The three pure cultures of *E. coli* O157:H7 were all scored as "detected" by GDS.

For the second batch, samples supplemented with HBF received an additional mean 4.7 x 10^8 CFU $sample^{-1}$ as measured by LAB count, or mean 2.9 x 10^9 CFU $sample^{-1}$ as measured by APC. The salami samples themselves contained (normal fat: 21%) 2.3 x 10^9 CFU g^{-1} as measured by APC and 2.5 x 10^9 CFU g^{-1} by the LAB count and (high fat: 28%) 4.4 x 10^8 CFU g^{-1} by APC and 4.2 x 10^8 CFU g^{-1} by LAB count methods. These values are very similar to those obtained in the first run. This is because the same batches of salami were used for both.

The second set of samples included salamis inoculated with the fourth *E. coli* O157 isolate, two uninoculated controls, salamis inoculated with generic *E. coli* and pure cultures of the last *E. coli* O157 isolate and the generic *E. coli*. The results for the duplicate samples of inoculated salami were that, when *E. coli* O157 was added to the sample at the concentrations cited above, then a GDS positive result was recorded and successful IMS recovery of typical *E. coli* O157 colonies was made on CT-SMAC. These presumptive isolates were confirmed on a per sample basis by latex agglutination. Controls (two) were negative by GDS and did not yield suspect colonies by GDS. One of two controls inoculated with generic *E. coli* was negative by GDS and no *E. coli* O157 isolates were obtained. The other was positive by GDS but no typical colonies were obtained on CT-SMAC following IMS. This result can therefore be regarded as a GDS false positive result. The *E. coli* O157 culture was scored as "detected" by GDS, and the generic *E. coli* (NZRM 916) was "not detected".

3.4 *E. coli* O157:H7 Isolates

Four *E. coli* O157:H7 isolates were included in this validation study which varied in their source (beef and veal), shiga-toxin subunit variations, acid tolerance and ability to be detected by both PCR and lateral flow type screen kits (Table 1). A non-O157 *E. coli* isolate was chosen as a negative control. With the exception of the human isolate *E. coli* O157:[H7] 96/2998 on the high background microflora containing UCFM product, all strains were detected after enrichment when inoculated at low levels ($3.6 – 6.4 \times 10^1$ CFU g^{-1}). While the 96/2998 strain is non-motile and negative by H-serotyping, it was detected by conventional IMS and plating techniques on all matrices. This strain was also detectable at similar inoculation levels on the remaining matrices by the GDS PCR assay. In repeated experiments (section 3.3) there were no difficulties in detecting this, or any other, of the four isolates used.

Data are lacking on the effects of additives on the recovery of *E. coli* O157:H7 from processed foods. However with respect to the isolation of *Salmonella* spp. isolation Andrews *et al.* (2001) discuss the need for adding neutralising agents such as potassium sulphite for analysis of garlic and onion, and dilution of herbs and spices containing toxic substances for which no appropriate neutralising agent has been identified. In the current study, ingredients of concern (garlic, pepper, dehydrated onion and garlic, parsley and spice extracts) were considered to be sufficiently diluted in the food matrices to negate potential toxicity concerns. Additionally, the IMS-culture method resulted in 100% recovery, and so it can be confidently concluded that

these additives had no effect on the growth and recovery of *E. coli* O157:H7 in the studied matrices. This was shown in specific studies on the addition of paprika to enrichment broth.

3.5 Specificity, Selectivity and Efficiency of the GDS System

The performance parameters for screen tests accepted for use by the USDA-FSIS, as published in the O157-MP technical procedures are described in Parts 12 and 13 section 6.3 of the US OMAR (MAF, 2009).

To ascertain the level of specificity that the GDS method affords, the following calculation is used:

$$\text{Specificity} = \text{true negative} / (\text{true negative} + \text{false positive}) \times 100\%$$

The GDS system gave no false positives for any of the negative controls in the sampling regime. For each run, the negative controls included a non-O157:H7 *E. coli* strain inoculated onto each matrices being tested, a culture of the same strain in broth, as well as a uninoculated PBS+Tween control. Therefore, for each run, three negative controls were incorporated. Specificity can then be determined to be 100% in the scope of this limited validation study.

Selectivity, is determined by the calculation:

$$\text{Selectivity} = \text{true positive} / (\text{true positive} + \text{false negative}) \times 100\%$$

A value can be determined separately for each matrix. Results from GDS analysis of precooked sausages and meat patties, UCFM containing either garlic or pepper, and UCFM of high pH, low a_w, all gave 100% true positive readings, and no false negative results. Selectivity, therefore, can be determined to be 100%. However, when the UCFM matrices containing high background microflora / high fat content was analysed, 3/12 results were false negatives, giving a Selectivity result of 75%, which is less than the 98% requirement.

In the initial series of experiments (Tables 3-6) a total of 112 spiked samples was tested, alongside 16 negative controls (after negative results due to presumed technical error are discounted). The Efficiency of the GDS system is determined by the percentage of test results overall that are true-positives and true-negatives giving a result of 96.9%, higher than the

minimum requirement of 94%. If the negative results attributed to technical error are included, then an Efficiency of 95.3% was achieved.

Data from the additional work with salamis of different fat and HBF compositions (section 3.3) there were only eight negative samples tested, one yielding a false positive result. This corresponds to a sensitivity of 88.9%, while the selectivity for inoculated samples was 100% (i.e. there were no false negative results). The level of specificity quoted is based on a small number of samples and so does not warrant a comparison with the OMAR requirements.

4 CONCLUSIONS

The Assurance GDS system for *E. coli* O157:H7 assay worked sufficiently well for both partially cooked and pre-cooked meat patties, as well as pre-cooked sausages. These products contained what would be considered a typical range of ingredients, including cheese and savoury ingredients that might be considered inhibitory at one of the steps (enrichment, IMS, PCR amplification) of this process.

Potentially inhibitory ingredients such as pepper, garlic and paprika did not interfere with the assay to produce false negative results. There were some indications from the data in Table 6 that for one isolate the HBF may have resulted in false negative results. However, when repeated in greater depth no evidence of the sample composition interfering with the results was obtained; the system seemed equally effective with high/normal fat, HBF/normal flora products. The method was therefore satisfactory for UCFM products as well.

The need for technical competency and quality control in interpreting results is particularly important to capture results which are false positive. The GDS system relies on the detection of specific genes which may be possessed by organisms which are not STEC, and so the isolation of organisms from the sample is required before a sample can be scored as positive. However, the date presented here suggest that the GDS system is a useful screen for the detection of *E. coli* O157 in various meat samples given that a certain proportion of false positive results is inevitable.

False negative results present a more significant problem as they will allow contaminated product to be released. Such false negative results for the GDS system have been reported for

minced beef and lettuce when the inoculum concentration is low[1], although that study is now quite old. Remembering that false negative results are recognised when the cultural method detects the pathogen while the PCR does not, false negative results can be caused by the presence of chemical which interfere with the PCR. However, this should be reflected in failure of the internal control.

It is interesting to note that the presence of a variety of spices (pepper, garlic and paprika) did not affect the results, whereas there are many reports in the literature on the anti-bacterial properties of spices (e.g. Gupta and Ravishankar, 2005). The potential for these ingredients present at concentrations plausibly present in foods to interfere with enrichment cultures would be of interest. It is also possible that active ingredients may be more of a concern in different types of food if they are not, for example, water soluble.

[1] www.aoac.org/omarev1/2005_04.pdf

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APPENDIX 1. ASSURANCE GDS RESULTS REPORT FOR PRE-COOKED MEAT PATTIES, BUT ALSO REPRESENTATIVE FOR PRE-COOKED SAUSAGES AND UCFM PRODUCTS WITH HIGH PH, LOW AW VALUES.

BIOCONTROL

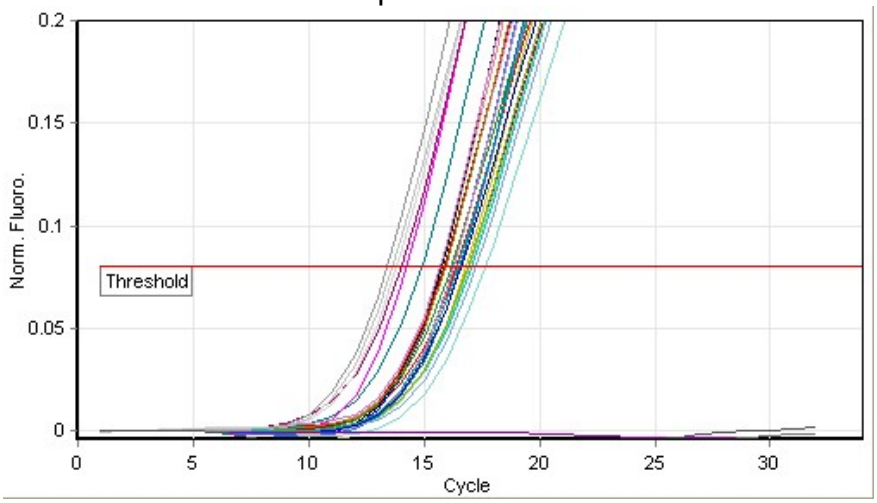
Assurance GDS Results Report

Run Information

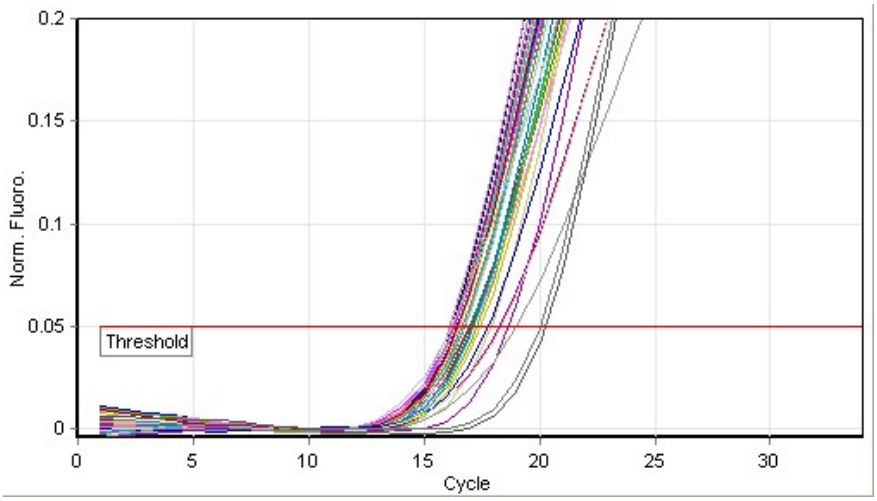
Run Name	BioControl Assay 2010-09-23
Run Start	9/23/2010 9:47:03 AM
Run Finish	9/23/2010 11:06:58 AM
Operator	Assurance GDS User
Notes	
Run On Software Version	Rotor-Gene 6.1.96
Run Signature	The Run Signature is valid.

Assay: E coli O157:H7

Assurance GDS Test Sample Results



Assurance GDS Internal Control Results



Results

No.	Colour	Name	Result	Assay	Kit Lot Number	Description
1		5A-1	Positive	E coli O157:H7	052410-24	Patties + Cheese
2		5A-2	Positive	E coli O157:H7	052410-24	Patties + Cheese
3		5A-3	Positive	E coli O157:H7	052410-24	Patties + Cheese
4		5B-1	Positive	E coli O157:H7	052410-24	Patties + Cheese
5		5B-2	Positive	E coli O157:H7	052410-24	Patties + Cheese
6		5B-3	Positive	E coli O157:H7	052410-24	Patties + Cheese
7		5C-1	Positive	E coli O157:H7	052410-24	Patties + Cheese
8		5C-2	Positive	E coli O157:H7	052410-24	Patties + Cheese
9		5C-3	Positive	E coli O157:H7	052410-24	Patties + Cheese
10		5D-1	Positive	E coli O157:H7	052410-24	Patties + Cheese
11		5D-2	Positive	E coli O157:H7	052410-24	Patties + Cheese
12		5D-3	Positive	E coli O157:H7	052410-24	Patties + Cheese
13		5E	Negative	E coli O157:H7	052410-24	Patties + Cheese
14		6A-1	Positive	E coli O157:H7	052410-24	Plain Patties
15		6A-2	Positive	E coli O157:H7	052410-24	Plain Patties
16		6A-3	Positive	E coli O157:H7	052410-24	Plain Patties
17		6B-1	Positive	E coli O157:H7	052410-24	Plain Patties
18		6B-3	Positive	E coli O157:H7	052410-24	Plain Patties
19		6B-3	Positive	E coli O157:H7	052410-24	Plain Patties
20		6C-1	Positive	E coli O157:H7	052410-24	Plain Patties
21		6C-2	Positive	E coli O157:H7	052410-24	Plain Patties
22		6C-3	Positive	E coli O157:H7	052410-24	Plain Patties
23		6D-1	Positive	E coli O157:H7	052410-24	Plain Patties
24		6D-2	Positive	E coli O157:H7	052410-24	Plain Patties
25		6DF-3	Positive	E coli O157:H7	052410-24	Plain Patties
26		6E	Negative	E coli O157:H7	052410-24	Plain Patties
27		A	Positive	E coli O157:H7	052410-24	controls
28		B	Positive	E coli O157:H7	052410-24	controls
29		C	Positive	E coli O157:H7	052410-24	controls
30		D	Positive	E coli O157:H7	052410-24	controls
31		E	Negative	E coli O157:H7	052410-24	controls
32		Blank	Negative	E coli O157:H7	052410-24	PBS+Tween

5 = Patties + Cheese, 6 = Plain Patties, a = *E. coli* O157:H7 CPH0510332-7, b = *E. coli* O157:H7 ERL06-2497 (N427), c = *E. coli* O157:H7 NZRM4159, d = *E. coli* O157:[H7] 96/2998, e = *E. coli* NZRM 916

APPENDIX 2. ASSURANCE GDS RESULT REPORT FOR PEPPER AND GARLIC UCFM Products

BIOCONTROL

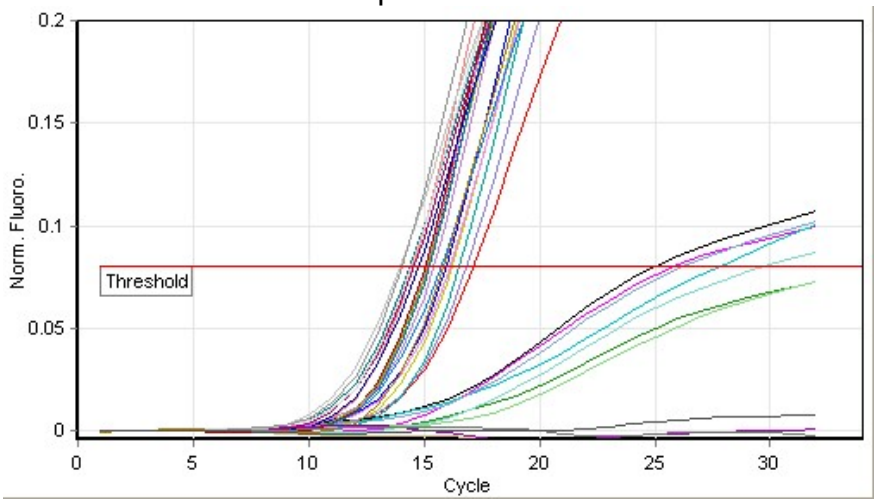
Assurance GDS Results Report

Run Information

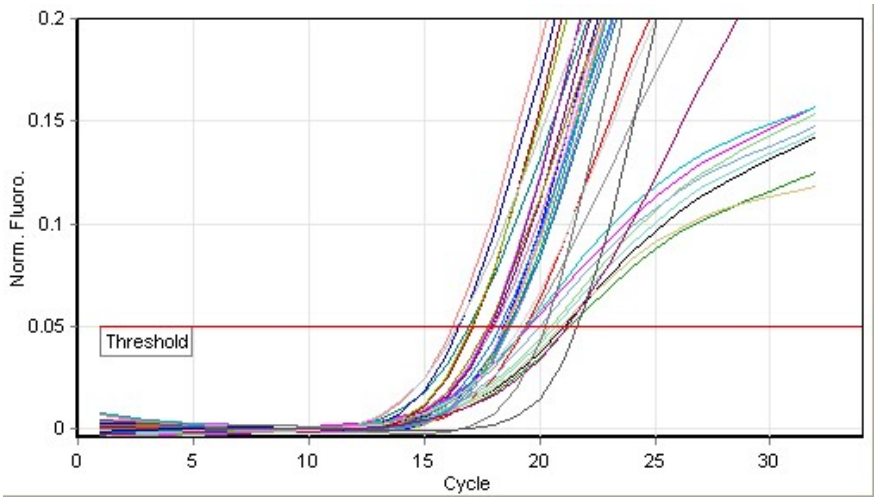
Run Name	BioControl Assay 2010-09-30
Run Start	9/30/2010 11:12:24 AM
Run Finish	9/30/2010 12:32:14 PM
Operator	Assurance GDS User
Notes	
Run On Software Version	Rotor-Gene 6.1.96
Run Signature	The Run Signature is valid.

Assay: E coli O157:H7

Assurance GDS Test Sample Results



Assurance GDS Internal Control Results



Results

No.	Colour	Name	Result	Assay	Kit Lot Number	Description
1		3A-1	Positive	E coli O157:H7	052410-24	Pepper UCFM
2		3A-2	Positive	E coli O157:H7	052410-24	Pepper UCFM
3		3A-3	Positive	E coli O157:H7	052410-24	Pepper UCFM
4		3B-1	Positive	E coli O157:H7	052410-24	Pepper UCFM
5		3B-2	Positive	E coli O157:H7	052410-24	Pepper UCFM
6		3B-3	Positive	E coli O157:H7	052410-24	Pepper UCFM
7		3C-1	Positive	E coli O157:H7	052410-24	Pepper UCFM
8		3C-2	Positive	E coli O157:H7	052410-24	Pepper UCFM
9		3C-3	Negative	E coli O157:H7	052410-24	Pepper UCFM
10		3D-1	Positive	E coli O157:H7	052410-24	Pepper UCFM
11		3D-2	Positive	E coli O157:H7	052410-24	Pepper UCFM
12		3D-3	Positive	E coli O157:H7	052410-24	Pepper UCFM
13		3E	Negative	E coli O157:H7	052410-24	Pepper UCFM
14		4A-1	Negative	E coli O157:H7	052410-24	Garlic UCFM
15		4A-2	Positive	E coli O157:H7	052410-24	Garlic UCFM
16		4A-3	Positive	E coli O157:H7	052410-24	Garlic UCFM
17		4B-1	Positive	E coli O157:H7	052410-24	Garlic UCFM
18		4B-2	Positive	E coli O157:H7	052410-24	Garlic UCFM
19		4B-3	Positive	E coli O157:H7	052410-24	Garlic UCFM
20		4C-1	Positive	E coli O157:H7	052410-24	Garlic UCFM
21		4C-2	Positive	E coli O157:H7	052410-24	Garlic UCFM
22		4C-3	Positive	E coli O157:H7	052410-24	Garlic UCFM
23		4D-1	Positive	E coli O157:H7	052410-24	Garlic UCFM
24		4D-2	Positive	E coli O157:H7	052410-24	Garlic UCFM
25		4D-3	Positive	E coli O157:H7	052410-24	Garlic UCFM
26		4E	Negative	E coli O157:H7	052410-24	Garlic UCFM
27		A	Positive	E coli O157:H7	052410-24	controls
28		B	Positive	E coli O157:H7	052410-24	controls
29		C	Positive	E coli O157:H7	052410-24	controls
30		D	Positive	E coli O157:H7	052410-24	controls
31		E	Negative	E coli O157:H7	052410-24	controls
32		Blank	Negative	E coli O157:H7	052410-24	PBS+Tween

3 = Pepper UCFM, 4 = Garlic UCFM, a = *E. coli* O157:H7 CPH0510332-7, b = *E. coli* O157:H7 ERL06-2497 (N427), c = *E. coli* O157:H7 NZRM4159, d = *E. coli* O157:[H7] 96/2998, e = *E. coli* NZRM 916

APPENDIX 3. GDS Report For UCFM Products With A High Background Micro-Flora, And High pH / Low Aw Values.

BIOCONTROL

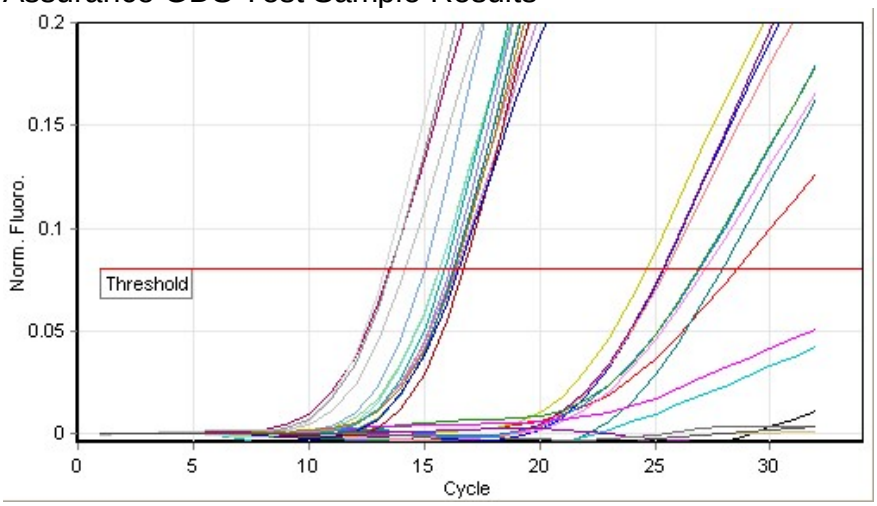
Assurance GDS Results Report

Run Information

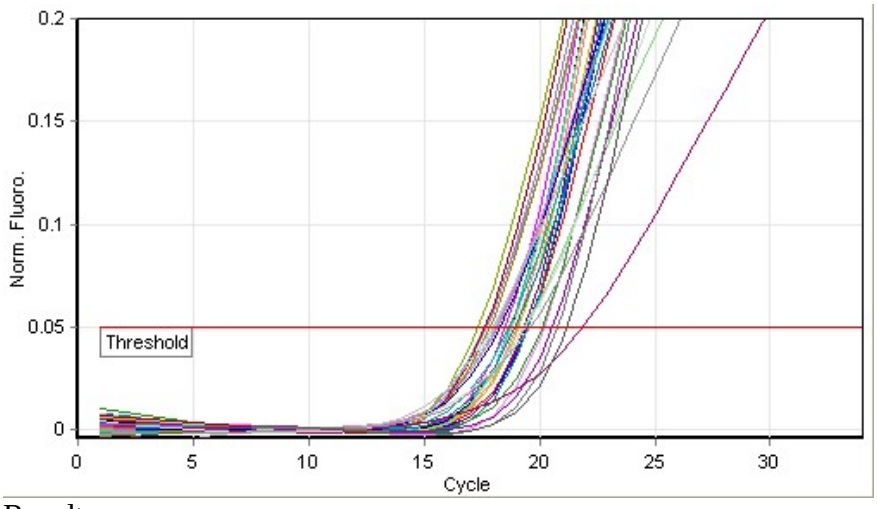
Run Name	BioControl Assay 2010-10-05 (1)
Run Start	10/5/2010 10:06:36 AM
Run Finish	10/5/2010 11:25:54 AM
Operator	Assurance GDS User
Notes	
Run On Software Version	Rotor-Gene 6.1.96
Run Signature	The Run Signature is valid.

Assay: E coli O157:H7

Assurance GDS Test Sample Results



Assurance GDS Internal Control Results



Results

No.	Colour	Name	Result	Assay	Kit Lot Number	Description
1		1a-1	Positive	E coli O157:H7	052410-24	UCFM + High background flora
2		1a-2	Positive	E coli O157:H7	052410-24	UCFM + High background flora
3		1a-3	Positive	E coli O157:H7	052410-24	UCFM + High background flora
4		1b-1	Positive	E coli O157:H7	052410-24	UCFM + High background flora
5		1b-2	Positive	E coli O157:H7	052410-24	UCFM + High background flora
6		1b-3	Positive	E coli O157:H7	052410-24	UCFM + High background flora
7		1c-1	Positive	E coli O157:H7	052410-24	UCFM + High background flora
8		1c-2	Positive	E coli O157:H7	052410-24	UCFM + High background flora
9		1c-3	Positive	E coli O157:H7	052410-24	UCFM + High background flora
10		1d-1	Negative	E coli O157:H7	052410-24	UCFM + High background flora
11		1d-2	Negative	E coli O157:H7	052410-24	UCFM + High background flora
12		1d-3	Negative	E coli O157:H7	052410-24	UCFM + High background flora
13		1e	Negative	E coli O157:H7	052410-24	UCFM + High background flora
14		2a-1	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
15		2a-2	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
16		2a-3	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
17		2b-1	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
18		2b-2	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
19		2b-3	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
20		2c-1	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
21		2c-2	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
22		2c-3	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
23		2d-1	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
24		2d-2	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
25		2d-3	Positive	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
26		2e	Negative	E coli O157:H7	052410-24	UCFM + High pH, Low Aw
27		a	Positive	E coli O157:H7	052410-24	controls
28		b	Positive	E coli O157:H7	052410-24	controls
29		c	Positive	E coli O157:H7	052410-24	controls
30		d	Positive	E coli O157:H7	052410-24	controls
31		e	Negative	E coli O157:H7	052410-24	controls
32		blank	Negative	E coli O157:H7	052410-24	PBS+Tween

1 = High background flora UCFM, 2 = High pH, low Aw UCFM, a = *E. coli* O157:H7 CPH0510332-7, b = *E. coli* O157:H7 ERL06-2497 (N427), c = *E. coli* O157:H7 NZRM4159, d = *E. coli* O157:[H7] 96/2998, e = *E. coli* NZRM 916