VALIDATION REPORT

Validation Title: Revalidation of screen test kits for *Escherichia coli* O157:H7.

Validation Report Number: MIC 0808V January 2009

Report prepared by: Warwick Aspin and Justine Inverarity

AsureQuality Limited
Auckland Laboratory
PO Box 41
Auckland
New Zealand

EXECUTIVE SUMMARY

An independent single laboratory study was performed by AsureQuality Auckland Laboratory to revalidate *Escherichia coli* O157:H7 screen kits following method amendments to the sample size and enrichment procedures in the New Zealand Food Safety Authority (NZFSA) *E. coli* O157:H7 monitoring programme for red meat. Revalidation included all screen kits currently approved for use, i.e. Reveal 20 hour (Neogen Corp, USA), VIP (Biocontrol, USA), BAX MP (Qualicon, USA) and Tecra VIA (3M Microbiology, USA). The amended procedure using mTSB with casaminoacids and novobiocin at an enrichment rate of 1000mL broth to 375g beef tissue and incubated at 42°C for 15-22 hours, was compared to the old procedure using modified EC Broth with novobiocin, 1125mL to 125g beef tissue incubated at either 37°C or 42°C (depending on the screen method of choice) for 20 hours. In total, thirty three enrichments were prepared using fresh beef (from a lot previously determined to be *E. coli* O157:H7 free), and each enrichment was seeded with a low dose (<10cfu) of *E. coli*. Ten different strains of *E. coli* O157:H7 were used, including five variants from New Zealand sources, and one non-O157:H7 *E. coli* was used as a negative control. All eleven strains were tested using each enrichment protocol and screen kit test combination. All screen methods were directly compared against each other, i.e. aliquots from the same enrichment broth, were tested in parallel by each of the screen methods.

All four screen methods successfully detected *E. coli* O157:H7 in all samples seeded with target organism using the new enrichment procedures. However, the Reveal and VIP test kits expressed weak signal for strains N635 and N427 after 15hours incubation using the new enrichment procedures. As a result of this observation, an increase in the minimum enrichment incubation time is recommended when using these test kits. Further research is required to identify the minimum incubation time required to achieve a definitive expression using the new enrichment procedures.

Reveal, Tecra and BAX MP screen methods successfully detected *E. coli* O157:H7 in all samples seeded with target organism using the old enrichment procedures, however VIP, did not detect *E. coli* O157:H7 in one sample seeded with *E. coli* O157:H7 (strain N635). Further work is also recommended to investigate the performance of VIP with respect to *E. coli* O157:H7 strain N635 using the old enrichment procedures.

INTRODUCTION

The official culture method published in FDA Bacteriological Analytical Manual (1) for detecting *Escherichia coli* O157:H7 in foods is laborious relative to rapid screening methods commercially available today, requiring 2-3 days to obtain a negative result. The traditional method is hindered by the reliance on selective culture plating, and an over night process, required to visually assess the presence or absence of the viable *E. coli* O157 within the cultured enrichment broth. Enzyme Linked Immunosorbant Assay (ELISA), Lateral Flow and more recently PCR assays have been used successfully for rapid screening of foods for *E. coli* O157. (2,3)

The New Zealand Food Safety Authority (NZFSA) has approved on an interim basis the use of four such assays, Reveal 20hour (NeogenCorp, USA) VIP (Biocontrol, USA), BAX MP (Qualicon, USA) and Tecra VIA (3M Microbiology, USA) for use in the *E. coli* O157:H7 monitoring programme for red meat. Each rapid screening assay has been designed using highly specific targets for *E. coli* O157:H7, in the form of either cell wall antigens (Reveal, VIP and Tecra VIA) or genetic markers (BAX MP).

In response to recent initiatives by the United States to further control *E. coli* O157:H7 in the US beef supply, NZFSA and industry have agreed with FSIS to implement changes to it's microbiological monitoring programme to facilitate continued market access to the US.

Guided by findings made by Guerini *et al* (USDA Agricultural Research Service, Clay Center, Nebraska, US)(4), NZFSA and industry opted to implement a change to the standardised enrichment protocol for *E. coli* O157:H7 testing procedures and align with market access requirements stated by US. The enrichment media, modified EC broth was replaced by modified TSB with casamino acid, and novobiocin (20mg/L) was retained as a selective agent. The analytical sample size was increased from 125g to 375g of beef tissue and the volume of enrichment broth was reduced from 1125ml to 1000ml per enrichment. The ratio of sample to suspension fluid volume has therefore decreased. The incubation temperature was standardised for all screen methods to 42±1°C and the incubation time was changed from 18-24 hours to 15-22 hours.

Each of the approved screen assays had existing validation data supporting various parameters of the new enrichment protocol; however none of the kits had been evaluated using the specific protocol implemented by NZFSA.

In addition, FSIS required replacement of the standard BAX *E. coli* O157:H7 test with the BAX *E. coli* O157:H7 MP test. The MP version of the assay uses several additional markers on the *E. coli* O157:H7 genome for greater assurance that new, unusual or modified varieties of this complex organism are detected. (5)

The study objective was to revalidate, except BAX, the commercial *E. coli* O157:H7 screen kits in conjunction with the amended enrichment protocol described above.

METHOD

Bacterial Strains. Ten strains of *E. coli* O157:H7 were used in these studies. Two were reference cultures (one toxigenic NZRM 2988 and one non-toxigenic NZRM 3614), five were variant wild types from the New Zealand meat industry (designated as N373, N427, N635, N754 and N776), and three were freezed dried vials containing non-toxigenic *E. coli* O157:H7 (NZRM 3614), provided by AsureQuality Proficiency Programmes. A generic non-O157:H7 *E. coli* strain (NZRM 916) was also used as a negative control.

The wild type strains were provided by AgResearch and were authenticated using phenotypical expression.

Overnight cultures were prepared for all strains by streaking onto Tryptone Soy Agar (TSA) and incubating at 37±1°C for 18-22 hours. Purity was determined and a 10^8 suspension was prepared using 10ml of maximum recovery diluent (MRD). The suspension was serially diluted to prepare a cell concentration of approximately 100 cfu/ml. To assist achievement of the target inoculation for the trial (<10cfu), an enumeration of suspension was made using TSA and each culture suspension was refrigerated (2-5°C) overnight.

The freeze dried vials were hydrated using 3ml of MRD and vortexed for 2 minutes. Vials were allowed to stand at room temperature for 10minutes and vortexed immediately prior to the inoculation of the enrichment samples.

Study Protocol One - New sample size and enrichment protocol; 375g beef tissue and 1000ml of modified TSB with casamino acids + novobiocin. Incubation at 42±1°C and tested at 15 hours and 22 hours.

A set of eleven enrichments were prepared by adding 375g of beef tissue to 1000ml of mTSB with casamino acids + novobiocin, pre-warmed to 35°C. Each of the eleven standardized cultures were inoculated into one of the enrichments. The enrichments were mixed for 2 minutes, and then transferred to a 42°C incubator. At 15 hours of incubation an aliquot of each enrichment was removed and tested using all four screen test kits Reveal, VIP, Tecra VIA and BAX MP. At 22 hours of incubation a further aliquot for the enrichment was removed and again tested using all four test kits.

Study Protocol Two - Old sample size and enrichment protocol; 125g beef tissue and 1125ml of modified EC broth + novobiocin. Incubation at 37±1°C or 42±1°C and tested at 20 hours.

Two sets of eleven enrichments were prepared by adding 125g of beef tissue to 1125ml of mEC + novobiocin, pre-warmed to 35°C. Each of the eleven standardized cultures were inoculated into a pair of enrichments. The enrichments were mixed for 2 minutes. One set of enrichments were incubated at 37±1°C for 20 hours and then tested using Reveal and VIP screen kits. The other set of enrichments were incubated at 42±1°C for 20 hours and then tested using Tecra VIA and BAX MP.

Verification of Inoculation dose - At the time of enrichment seeding, each of the culture suspension doses was enumerated to quantify the level of inoculation. Spread plate technique with TSA and incubation at 37±1°C for 22±2 hours was used. Five replicates of each suspension dose were plated. Colony counts were made and the results were averaged to provide an estimate of the dose level achieved for each organism.

Post enrichment *E. coli* O157:H7 enumeration - The *E. coli* concentration was determined for each enrichment after incubation using a direct plating technique with CT SMAC. Enrichments were effectively mixed and a 1ml aliquot was removed. Serial dilution was performed using maximum recovery diluent and 0.1ml of each dilution was plated onto the surface of duplicate CT-SMAC agar plates. The inoculum was evenly distributed across the plate surface using a sterile spreader and the plates were left at room temperature for 30 minutes to allow sample to absorb into the agar. Plates were then inverted and incubated at 37±1°C for 22±2 hours. Typical sorbitol negative colonies were counted as *E. coli* O157:H7.

RESULTS

Table 1: *E. coli* O157:H7 counts for inoculation doses

Organsim	**cfu/dose**	**Average cfu/dose**
N373	9/5/6/4/6	6
N427	8/4/5/5/7	5.8
N635	6/12/8/6/11	8.6
N754	7/7/5/5/7	6.3
N776	7/9/5/6/10	7.4
stx+ (2988)	10/8/9/7/8	8.6
stx - (3614)	4/7/6/4/8	5.8
ILCP 1 (PO7.13)	77/72/89/67/73	75.6
ILCP 2 (PO7.14)	59/57/64/68/68	63.2
ILCP 3 (PO7.15)	50/61/55/29/48	48.6
E. coli 916	13/7/6/8/7	8.2

Table 2: Study One – New sample size and enrichment protocol, Modified TSB incubated at 42±1˚C for 15 hours.

Sample	**TECRA**	**BAX MP**	**VIP**	**Reveal**	**Post enrichment count cfu/ml**
N373	Positive	Positive	Positive	Positive	>$2.5x10^8$
N427	Positive	Positive	Positive*	Positive*	$2.0x10^5$
N635	Positive	Positive	Positive*	Positive*	$4.3x10^5$
N754	Positive	Positive	Positive	Positive	$9.7x10^5$
N776	Positive	Positive	Positive	Positive	$8.5x10^6$
stx+ (2988)	Positive	Positive	Positive	Positive	$1.1x10^7$
stx - (3614)	Positive	Positive	Positive	Positive	$3.0x10^7$
ILCP 1 (PO7.13)	Positive	Positive	Positive	Positive	$1.0x10^8$
ILCP 2 (PO7.14)	Positive	Positive	Positive	Positive	$6.6x10^6$
ILCP 3 (PO7.15)	Positive	Positive	Positive	Positive	$2.0 x10^7$
E. coli 916	Negative	Negative	Negative	Negative	N/A

* Weak expression

Table 3: Study One – New sample size and enrichment protocol, Modified TSB incubated at 42±1˚C for 22 hours.

Sample	**TECRA**	**BAX MP**	**VIP**	**Reveal**	**Post enrichment count cfu/ml**
N373	Positive	Positive	Positive	Positive	>2.5x10^8
N427	Positive	Positive	Positive	Positive	1.3x10^6
N635	Positive	Positive	Positive*	Positive	1.9x10^6
N754	Positive	Positive	Positive	Positive	5.9x10^6
N776	Positive	Positive	Positive	Positive	1.5x10^7
stx+ (2988)	Positive	Positive	Positive	Positive	3.9x10^7
stx - (3614)	Positive	Positive	Positive	Positive	1.8x10^8
ILCP 1 (PO7.13)	Positive	Positive	Positive	Positive	>2.5x10^8
ILCP 2 (PO7.14)	Positive	Positive	Positive	Positive	1.7x10^7
ILCP 3 (PO7.15)	Positive	Positive	Positive	Positive	2.4 x10^7
E. coli 916	Negative	Negative	Negative	Negative	N/A

* Weak expression

Table 4: Study Two: Old sample size and enrichment protocol, Modified EC broth incubated at 37±1˚C or 42±1˚C for 20 hours.

Sample	**TECRA** 42±1˚C	**BAX MP** 42±1˚C	**VIP** 37±1˚C	**Reveal** 37±1˚C	**Post enrichment O157 count cfu/ml**	
					37˚C	**42˚C**
N373	Positive	Positive	Positive*	Positive*	3.1x10^5	1.9x10^7
N427	Positive	Positive	Positive	Positive	2.6x10^7	7.2x10^5
N635	Positive	Positive	**Negative**	**Negative**	2.7x10^6	1.0x10^8
N754	Positive	Positive	Positive	Positive	1.7x10^7	1.0x10^8
N776	Positive	Positive	Positive	Positive	1.6x10^7	6.4x10^7
stx+ (2988)	Positive	Positive	Positive	Positive	7.3x10^6	1.0x10^8
stx - (3614)	Positive	Positive	Positive	Positive	2.1x10^6	2.0x10^8
ILCP 1 (PO7.13)	Positive	Positive	Positive	Positive	2.9x10^7	1.0x10^8
ILCP 2 (PO7.14)	Positive	Positive	Positive	Positive	1.8x10^6	8.1x10^6
ILCP 3 (PO7.15)	Positive	Positive	Positive	Positive	2.4x10^6	1.3x10^7
E.coli 916	Negative	Negative	Negative	Negative	N/A	N/A

* Weak expression

DISCUSSION

The performance of two enrichment protocols for the detection of *E. coli* O157:H7 were compared in conjunction with four different commercial screen test kits.

In the first study, modified TSB with casamino acids and novobiocin was incubated at 42±1°C then tested after 15 hours and 22 hours using all screen test kits. All four screen test kits, VIP, Reveal, Tecra VIA and BAX MP, expressed positive detection of *E. coli* O157:H7 in all samples seeded with *E. coli* O157:H7 strains, at both 15 hours and 22 hours of incubation.

The screen test kits showed some variation in the strength of positive expression, with VIP and Reveal showing weak signal for two samples spiked with *E. coli* O157:H7 (N635 and N427) after 15hours incubation. The "test line" expression was in fact very weak for the enrichment seeded with strain N635 in both kits. It was felt the expression could possibly be misinterpreted as negative because it was only after observing the test devices at the end of the recommended development time period and at different light angles that the interpretation of positive was concluded. The weak positive observations were supported by a retest screen result conducted 20-30minutes after the original result. The post enrichment concentration levels for enrichments seeded with N635 and N427 after 15hours incubation were 4.3×10^5 cfu/ml and 2.0×10^5 cfu/ml respectively, suggesting the threshold of detection for these *E. coli* O157:H7 strains is between 10^5 and 10^6 using VIP and Reveal screening kits.

The laboratory used electronic interpretation for BAX MP and Tecra VIA screen methods and both tests expressed positive results for all samples containing *E. coli* O157:H7. Both methods showed lower expression for samples containing strains N635 and N427 after 15hours incubation; however the signal was above the cut-off threshold for a positive determination. The cell concentration required to initiate a positive signal may be the same for all methods evaluated, however the BAX MP and Tecra VIA methods gain an increase in objectivity for this study by virtue of the optical readers. Optical readers are possible for lateral flow devices however were not available to the laboratory for this study.

All screening kits showed moderate or strong positive expression for samples containing *E. coli* O157:H7 at 22 hours incubation, with exception of VIP for strain N635 (also supported by a retest) which again showed weak expression. There was no noticeable difference in the strength of the signal at 22 hours compared to the test conducted at 15 hours incubation. The post enrichment *E. coli* O157 level was 1.9×10^6 cfu/ml.

It is recommended that further evaluation be conducted to verify the performance of VIP and Reveal at 15hours of enrichment incubation. This represents the minimum time allowed for incubation and the study outcomes need to be further reviewed with respect to this study data. The NZFSA *E. coli* O157:H7 method should to be amended to state a minimum incubation time of 22hours when using VIP or Reveal kits.

In the second study, modified EC (mEC) with novobiocin was incubated at 37±1°C and 42±1°C then tested after 20 hours using all screen test kits. All strains except N635 were detected by all four kits, albeit a weak reaction of N373 with the VIP and Reveal kits. In contrast, N635 was not detected by the VIP and Reveal kits (false negative) despite post enrichment counts at 37°C and 42°C of 10^6 and 10^8 respectively. Two different analysts were unable to observe a positive "line" in the test window. A retest for both kits was immediately conducted and the outcome supported the original result. A false negative rate of 15% was recently reported by Feldsine et. al. (6) relating to raw ground beef testing using VIP *E. coli* 0157:H7. Incubation conditions and target organism level were both similar to the parameters used in this study, however the enrichment used was different, using modified EHEC broth.

In both studies, all screen kits expressed negative results for enrichments seeded with the non-O157 *E. coli* strain and also in all cases a control line was present, for lateral flow devices, validating the kit quality control requirements.

Post enrichment *E. coli* O157:H7 concentration levels showed an increase in *E. coli* O157:H7 numbers is achieved by incubating for 20 hours at the higher temperature of 42±1°C compared to enrichment at 37±1°C using mEC broth. The post enrichment *E. coli* O157:H7 levels between mTSB and mEC were comparable, when incubated at 42±1°C, suggesting mTSB has at the very least an equivalent performance to mEC broth as an enrichment media. Note: mTSB levels were on average approximately 1 log higher, however these enrichments had 2 hours longer incubation.

All freeze-dried samples routinely used in the New Zealand *E. coli* O157:H7 Proficiency Programme returned positive results for all screen kits using both the old and new enrichment protocols.

CONCLUSION

The data reported here within demonstrates all *E. coli* O157:H7 screen kits approved for use in New Zealand Food Safety Authority (NZFSA) *E. coli* O157:H7 monitoring programme are compatible with the new enrichment and sampling protocol adopted by NZFSA.

However, due to the weak positive reaction expressed by Reveal and VIP when testing strains N635 and N427 after 15hours enrichment, it is strongly recommended the minimum enrichment time be increased. Further research should be conducted to determine the required incubation time to achieve a definitive expression for these *E. coli* O157:H7 strains.

ACKNOWLEDGEMENTS

The evaluation was conducted at AsureQuality Auckland Laboratory by Warwick
Aspin, Technical Manager and Justine Inverarity, Scientific Analyst.

The protocol was designed with the assistance of Dr Roger Cook, NZFSA and John Mills, AgResearch. Wild E. coli O157:H7 strains were obtained from AgResearch culture collection.

The ILCP samples were provided by Sandra Mott, AsureQuality Proficiency.

The study was funded by NZFSA.

REFERENCES

(1) U.S. Food and Drug Administration (1998 revision A) Bacteriological Analytical Manual, 8th Ed., AOAC INTERNATIONAL, Arlington, VA

(2) Feldstine, P.T., et al. (1997) J.AOAC 80, 517-528

(3) Bird, C.B., et al. (2001) J. AOAC 84, 719-735

(4) Guerini, M.N., et al. (2006) J.Food Protection 69, 1007-1011

(5) BAX System User Guide, 2005, DuPont Qualicon, USA

(6) Feldstine, P.T., et al. (2008) J.AOAC 91, No. 2, 360-364