final report

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Australian Implementation of NeoSeek for molecular detection and confirmation of Shiga toxin producing Escherichia coli

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Abstract

The project is for the implementation and verification of NeoSeek at Neogen Australasia, in Gatton Australia. NeoSeek is a multiplexed polymerase chain reaction (PCR) of 89 targets followed by analysis of Matrix assisted laser desorption ionisation – time of flight (MALDI-TOF) mass spectrometry of the PCR products to determine the presence of harmful Shiga toxin-producing *Escherichia coli*. This molecular approach requires an Agena Mass Spectrometer, which has already been purchased and is in Gatton. The project aims to achieve site acceptance testing of the testing protocol, verification of laboratory quality systems and production of data on Australian samples that will further enable the Australian industry to make informed choice of testing method.
This final report particularly describes applications to AOAC and DAWE for acceptance of the method as a screening test in USA and Australia.

Executive summary

Australian meat samples for export to the United States of America are required to undergo microbiological testing for certain *Escherichia coli* strains that can cause disease in humans. *E. coli* that produce Shiga toxins (stx) are termed Shiga toxigenic *E. coli* (STEC). *E. coli* O157:H7 was the first STEC required by the US for export testing in 2007, however an additional six serogroups, including O26, O45, O103, O111, O121 and O145 (commonly referred to as non-O157) were added to testing requirements in 2012.

There are currently 21 laboratories in Australia approved by Department of Agriculture, Water and the Environment (DAWE) to run the screening tests, and 4 labs approved to run the confirmation tests.

The current methods for STEC testing in Australia consist of an initial screening test which identifies potential positives, and then a separate confirmation step if a potential positive (PP) is detected. The most common methods of this initial screen in Australia are the BAX system real-time PCR STEC Suite (Hygiena) for the screening, and the Assurance GDS MPX STEC assays (BioControl) for confirmation. There is a relatively low rate of confirmation of these potential positives (19% in 2019), which has raised questions about the accuracy of the current screening test methods.

For a sample to be called an STEC, it must include the O-group, *stx* and *eae* in the one cell. Current methods presently detect combinations of target organisms within an individual sample, which can lead to samples being called as PP's when in fact they do not contain STECs. PP Samples that lead to an STEC-clear confirmation, have led to questions being raised about the initial screening test over-calling positives, as well the confirmation test potentially missing positives. Both of these scenarios are problematic for the Australian meat export industry, as sending samples for confirmation testing adds time and cost to the process, potentially leading to downgraded product, and missed STECs in confirmation testing could lead to contaminated product leaving Australia and resulting in point of entry detections in the USA by the US Food Safety and Inspection Service (FSIS).

In addition to this, the current testing regime requires both a screening test and a confirmation test, should a potential positive be detected. The additional confirmation test adds a minimum of 24 hours to the process which increases storage fees as well as the delay in shipment.

Of most value to the Australian meat export industry is a test that is able to minimize the amount of PP samples, whilst maintaining a high power of discrimination in detecting STEC samples. NeoSeek STEC can be run as a single-step confirmatory method, with a proven high rate of accuracy and low rate of false potential positives.

The NeoSeek technology uses PCR coupled with mass spectrometry-based multiplexing to develop a genetic profile for bacteria in a meat sample, and then compares those results with the known genetic makeup of the reference *E. coli* strains to identify and differentiate the target strains. NeoSeek assays more than 86 specific genetic markers to provide faster results than conventional culture methods. NeoSeek STEC test has received AOAC approval (#081901)[1] and is an accepted method in the US with FSIS. NeoSeek is currently being run in the US at the Neogen Geneseek Lincoln, Nebraska laboratory, and the overarching aim of this project was to get the NeoSeek assay up and running as a DAWE and NATA approved method at the Neogen Australasia Laboratory based in Gatton, Queensland.

NeoSEEK will be offered in Australia as both a screening test (NeoSEEK Screen) and a confirmation test (NeoSEEK Confirm). The screening test will have a 24 hour turnaround, as will the confirmation.

If the screening test has been performed with NeoSEEK, then the confirmation results can be processed within 2 hours. If the test is screened elsewhere and run on NeoSeek Confirm, the results can be processed in 24 hours.

DAWE approval Neogen Australasia as a confirmatory laboratory to run NeoSeek for export meat testing is currently pending. Further, the addition of the STEC test to the current NATA scope of accreditation(Accreditation number 20553) is currently pending.

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1 Background

1.1 Current STEC testing in Australia and introduction of NeoSeek STEC testing into Australia

Current STEC testing regimes in Australia consist primarily of BAX or GDS methods, with a confirmatory step if STECs are detected from screening. The corfirmation test adds cost, days to the process and possible downgrading of goods. Examination of current testing methods has showed that there is a low confirmation rate (around 20%) for those samples that produce potential positive results from the screening test, which has also raised some questions over the accuracy and efficacy of current methods. In addition, culture method confirmation of non-O157 STEC is a time consuming, expensive and laborious process as the small group of STEC it is trying to identify are very similar to harmless *E. coli.*

Most STEC testing protocols look for *stx*, *eae* and O serogroup genes. A positive screening test therefore only indicates that these genetic targets are present in the sample, it can't tell us if they are in the same cell or if that cell is an *E. coli*.

A survey of STEC in Australian cattle faeces conducted in 2013 had a low conversion rate of PP to confirmed positives. Of the 1,500 samples tested, 44.5% were PP for non-O157 STEC but only 1.3% were culture confirmed as non-O157 STEC[1].

2 Project objectives

1. NeoSeek service is available through Neogen's Gatton, Australia facility.
2. NeoSeek in Gatton meets the precision, specificity, and accuracy previously attained in the Lincoln, NE laboratory.
3. NeoSeek in Gatton meets proficiency requirements.
4. NeoSeek in Gatton is added to the ISO 17025 accreditation scope.
5. Application is made to AOAC for acceptance of the method as a screening test
6. Application is made to DAWR for acceptance of the method for export purpose
7. Comparison is made between NeoSeek performance and the performance of common testing methods used in Australia

2.1 Project Milestones

2.1.1 NeoSeek service is available through Neogen's Gatton, Australia facility

All standard operating procedures have been transferred over to the staff at the Gatton Facility. Three staff members have also travelled to the Lincoln, Nebraska laboratory during 2019 in order to train on the procedures, and at current there are four laboratory staff fully trained to run the NeoSeek STEC panel. As NeoSeek runs on the Agena platform, we had a team of staff already highly trained in the running of the Agena platform.

[1] https://www.mla.com.au/research-and-development/search-rd-reports/final-report-details/Product-Integrity/Understanding-confirmation-test-failures-for-detecting-pathogenic-E-coli/1167

2.1.2 NeoSeek in Gatton meets the precision, specificity, and accuracy previously attained in the Lincoln, NE laboratory.

Staff at the Gatton laboratory collaborated with CSIRO Food safety laboratory at Coopers Plains in Brisbane, in order to run additional proficiency samples, and determine accuracy and effectiveness of the assay being run in the Gatton lab.

Fifty samples were provided by CSIRO. These samples consisted of multiple mixed strains as well as single strains. These were constructed using isolates from CSIRO Food Safety labs culture collection and most had been previously sequenced. Isolates were recovered from protect beads and subsequently tested by real-time PCR for the presence of stx 1, 2 and eae using the primers and probes outlined in the FSIS Laboratory Guidebook (MLG 5C Appendix 4). Big6 serotypes were confirmed using real-time PCR (again MLG 5C) and subsequently confirmed by latex agglutination. O157 isolates were only confirmed using latex agglutination.

We received the samples in duplicate aliquots both as enrichment aliquots and cell lysates. The samples were processed in the Gatton laboratory according to the relevant SOPs, and the results analysed and first-round scored. The Agena Typer files were then sent to Neogen Geneseek Operations in Lincoln, Nebraska, where they were second-scored and the STEC results were translated. The results were then sent to the CSIRO Food Safety & Stability group within CSIRO Agriculture & Food for comparison analysis. Abbreviated results are listed in Table 1.

The results of the comparison showed that all of the strains were correctly characterized by the NeoSeek test, with one exception. It was noted that the O121 strain had been called as ‘non’ for each of the samples in the group. There were 8 results affected by this, and all samples were called by the NeoSeek test consistently as ‘non’. The CSIRO laboratory had expected this sample to be called ‘STEC’. It was noted that previous testing in Lincoln laboratory had identified a rare O121 strain originating from New Zealand where the SNPs had called the sample ‘non’, but all virulence factors were present, which suggests regional differences in strains. Through all subsequent NeoSeek testing, this strain has not been identified again. In light of this, we propose to further review O121 calls for New Zealand and Australia samples before making the final call.

Sample ID	O103	O111	O121	O145	O157	O26	O45	Eae	Stx	NLE	Alpha	Gamma2	Beta	Epsilon	Gamma1
Ne-01	STEC				STEC		STEC	Eae+	Stx+	NLE+				epsilon	gamma1
Ne-02				STEC	STEC			Eae+	Stx+	NLE+					gamma1
Ne-03			non	STEC		STEC		Eae+	Stx+	NLE+			beta	epsilon	gamma1
Ne-04						STEC		Eae+	Stx+	NLE+			beta	epsilon	
Ne-05	STEC				STEC	STEC	STEC	Eae+	Stx+	NLE+			beta	epsilon	gamma1
Ne-06						STEC		Eae+	Stx+	NLE+			beta	epsilon	
Ne-07	STEC	STEC				STEC		Eae+	Stx+	NLE+			beta	epsilon	
Ne-08				STEC				Eae+	Stx+	NLE+			beta	epsilon	gamma1
Ne-09			non	STEC		STEC		Eae+	Stx+	NLE+			beta	epsilon	gamma1
Ne-10			non					Eae+	Stx+	NLE+				epsilon	
Ne-11		STEC						Eae+	Stx+	NLE+	alpha	gamma2		epsilon	
Ne-12		STEC						Eae+	Stx+	NLE+	alpha		beta	epsilon	
Ne-13				STEC				Eae+	Stx+	NLE+	alpha			epsilon	gamma1
Ne-14			non					Eae+	Stx+	NLE+	alpha			epsilon	
Ne-15		STEC						Eae+	Stx+	NLE+		gamma2			
Ne-16							STEC	Eae+	Stx+	NLE+	alpha			epsilon	
Ne-17															
Ne-18							STEC	Eae+	Stx+	NLE+				epsilon	
Ne-19						STEC		Eae+	Stx+	NLE+			beta	epsilon	
Ne-20		STEC						Eae+	Stx+	NLE+			beta		
Ne-21	STEC							Eae+	Stx+	NLE+				epsilon	
Ne-22	STEC				STEC		STEC	Eae+	Stx+	NLE+				epsilon	gamma1
Ne-23	STEC	STEC	non				STEC	Eae+	Stx+	NLE+		gamma2		epsilon	
Ne-24		STEC				STEC		Eae+	Stx+	NLE+			beta		
Ne-25							STEC	Eae+	Stx+	NLE+			beta	epsilon	
Ne-26		STEC	non		STEC			Eae+	Stx+	NLE+		gamma2		epsilon	gamma1
Ne-27								Eae+	Stx+	NLE+				epsilon	
Ne-28						STEC		Eae+	Stx+	NLE+	alpha		beta		
Ne-29	STEC							Eae+	Stx+	NLE+			beta	epsilon	
Ne-30					STEC			Eae+	Stx+	NLE+					gamma1
Ne-31		STEC		STEC	STEC	STEC		Eae+	Stx+	NLE+		gamma2	beta		gamma1
Ne-32								Eae+	Stx+		alpha				
Ne-33						non		Eae+		NLE+			beta		
Ne-34			non					Eae+	Stx+	NLE+			beta	epsilon	
Ne-35			non			STEC		Eae+	Stx+	NLE+			beta	epsilon	
Ne-36								Eae+	Stx+	NLE+			beta	epsilon	
Ne-37		STEC						Eae+	Stx+	NLE+		gamma2	beta	epsilon	
Ne-38						STEC		Eae+	Stx+	NLE+			beta		
Ne-39						STEC		Eae+	Stx+	NLE+	alpha		beta	epsilon	
Ne-40						STEC		Eae+	Stx+	NLE+			beta		
Ne-41						STEC		Eae+	Stx+	NLE+			beta		
Ne-42	STEC	STEC				STEC		Eae+	Stx+	NLE+			beta	epsilon	
Ne-43								Eae+	Stx+	NLE+			beta	epsilon	
Ne-44		STEC					STEC	Eae+	Stx+	NLE+		gamma2		epsilon	
Ne-45								Eae+	Stx+	NLE+	alpha			epsilon	
Ne-46								Eae+	Stx+	NLE+				epsilon	
Ne-47					STEC			Eae+	Stx+	NLE+			beta	epsilon	gamma1
Ne-48	STEC							Eae+	Stx+	NLE+	alpha			epsilon	
Ne-49				STEC				Eae+	Stx+	NLE+					gamma1
Ne-50						STEC		Eae+	Stx+	NLE+			beta	epsilon	

Table 1. NeoSeek results obtained from CSIRO samples.

2.1.3 NeoSeek in Gatton meets proficiency requirements

The Gatton laboratory participates in proficiency testing with the Lincoln Laboratory, who send six enrichment broth samples to Gatton twice yearly to ensure the results obtained are concordant. Results are analysed and compiled by the Veterinary Diagnostics Director based in Lincoln, NE. In addition, Neogen are enrolled in twice yearly external, independent proficiency tests with SGS Vanguard Sciences, and the Gatton laboratory recieves aliquots of these samples for processing. This test involves five samples sent at a time, for a total of ten, completely blind samples. The spikes include any of the 7 serotypes included in the NeoSeek test over the course of the year.

2.1.4 NeoSeek in Gatton is added to the ISO 17025 accreditation scope.

Neogen Australasia currently holds NATA accreditation for the majority of the tests run in the Gatton Laboratory, including tests run on the Agena platform. (Accreditaion number 20553). Application has been made to NATA for addition of NeoSEEK to the scope of accreditation.

2.1.5 Application is made to AOAC for acceptance of the method for export purpose

NeoSeek received AOAC validation in September 2019. AOAC's validation of NeoSeek for STEC (#081901) was the first AOAC PTM validation under the new serviced-based method guidelines, and was done in collaboration with MLA.

2.1.6 Application is made to DAWE for acceptance of the method for export purpose

Neogen Australasia has applied to DAWE for acceptance of the method. DAWE are currently awaiting confirmation from FSIS (June 2020)

2.1.7 Comparison is made between NeoSeek performance and the performance of common testing methods used in Australia

It has been estimated that the average annual cost of STECs in Australia (2013-2017) aside from the mandatory testing is $3.2 million. Only 20% of this is directly attributed to testing costs, the remaining majority of the cost is attributed to downgrading of lots due to detection of STECs.

In the study undertaken by Neogen for the FSIS Letter of No Objection, blind ground beef trim samples as single and double spikes were produced by an independent lab and sent to Neogen for NeoSeek STEC analysis as well as being analysed by an independent laboratory via current methods. The analysis was done in accordance with MLG 5B.01. Five were reported as negative by the MLG 5B.01 method but reported as positive by NeoSeek STEC. These five samples initially screened positive by the MLG but were unable to be found on the plates after IMS. The combined evidence between the third party lab performing the spike and enrichment, the MLG screen being presumptive positive and Neoseek identifying the organisms indicates these as false negatives. The results of this study are listed in Table 2. Whilst NeoSeek has, in some studies confirmed more than the MLG, this does indicate that the MLG method leaves the producers at higher risk of sending contaminated meat to commerce resulting in the potential for much higher cost associated with recall. In 2019, there were 30 food product recalls in Australia by FSANZ due to microbial contamination, with *E. coli* being in the top three microbial contaminants.

	True Positive (NeoSEEK vs. MLG 5B.01)		True Negative (NeoSEEK vs. MLG 5B.01)	
Reported Positive	48	43	0	0
Reported Negative	0	5	8	8
Total	48	48	8	8
Relative Sensitivity	100%	88.6%		
Relative Specificity			100%	100%

Low Inoculum (1-2 cfu/test portion)	Correct Identification MLG 5B.01	Correct Identification NeoSEEK
True Positive STEC (non-O157:H7)	15	17
False Positive STEC	0	0
True Negative (non-STECs)	4	4
False Negative	2	0
Total Inoculations	21	21
High Inoculum (5-10 cfu/test portion)	**Correct Identification MLG 5B.01**	**Correct Identification NeoSEEK**
True Positive STEC (non-O157:H7)	28	31
False Positive STEC	0	0
True Negative (non-STECs)	4	4
False Negative	3	0
Total Inoculations	35	35

Table 2. Results of the FSIS No-Objection Letter Study for NeoSeek

3 Discussion

At 30 June, 2020, there are still two milestones for which responses are outstanding. Once approval processing delays are over, an approved system will be operable.

1. NeoSeek service is available through Neogen's Gatton, Australia facility.
   This milestone has been met. The test is current up and working and the laboratory is ready to proceed with processing samples.

2. NeoSeek in Gatton meets the precision, specificity, and accuracy previously attained in the Lincoln, NE laboratory.

   This milestone has been met. Proficiency has been determined by running samples in parallel with Lincoln as well as running external proficiency samples. We have shown (#) that the processing of samples in the Gatton laboratory is 100% concordant with the samples run in the Lincoln, NE Laboratory.

3. NeoSeek in Gatton meets proficiency requirements.

   This milestone has been met. The Gatton laboratory will be participating with the same external proficiency test as GeneSeek. This test is completely independent and external, and run by Vanguard Sciences.

4. NeoSeek in Gatton is added to the ISO 17025 accreditation scope.

   This milestone is in progress. We have completed the validation report for NATA and have been in communication with NATA regarding adding it to our current scope of accreditation. We have met all of the ISO17025 requirements for running this test. NATA have advised us that they require NeoSeek to be added to the DAWE list of approved tests before formally assessing this addition to the scope.

5. Application is made to AOAC for acceptance of the method as a screening test

   This milestone has been met. AOAC acceptance has been granted for NeoSEEK (#081901).

6. Application is made to DAWE for acceptance of the method for export purpose

   This milestone is in progress. Application has been made to DAWE and they are currently awaiting a response indicating no objection for this test to be used by FSIS. There have been delays on this response due to the COVID-19 situation, and a positive response is expected in the very near future.

7. Comparison is made between NeoSeek performance and the performance of common testing methods used in Australia
   This milestone has been met. Results have been lised above in 2.1.7 and show that NeoSEEK is consistently accurate compared to current methods.

4 Conclusions/recommendations

If the current screening tests were run through NeoSEEK as compared to current test methods, there would be a cost saving to industry of approximately $1,627,000 per year. In addition, if both screening and confirmations were run on NeoSEEK then there would be a considerable time saving of a minimum 22 hours where the costs of storage would be saved, and product could be shipped out promptly. The time for testing and confirming on NeoSEEK is by far ahead of any current tests on the market.

In addition, due to the fundamental difference in methodology of NeoSEEK, specifically the limitations with IMS being able to determine which individual sample has the correct combination of O-group, stx and eae in the one cell – it can be postulated that NeoSEEK will result in a higher accuracy of both screening and confirmation tests. The exact extent of this will have to be determined going forward after sufficient rates of PP's and confirmations have been determined. If this proves to be accurate then NeoSEEK will have significant consequences in terms of reductions of PP's as well as capturing confirmed samples that could have otherwise slipped through.

	NeoSeek used instead of screening test	
Scenario	**Current situation**	**NeoSeek Australia**
Screening test method	BAX or GDS	NeoSeek method
# of screening tests per annum	30,000	30,000
Screening testing cost - $/test	120	100
Confirmation test method	culture based	N/A
# of confirmation tests per annum	750	750
Confirmation testing cost - $/test	1500	350
Total testing cost	$4,725,000	$3,262,500
change in cost		-$1,462,500
Transport time for sample to laboratory - days	0.5	0
Time taken for test on recepit of sample by laboratory - days	7	0
Storage cost - $/carton/day	0.072	0.072
# cartons/lot	406.519	406.519
Total storage cost	$164,640	$0
change in cost		-$164,640
# of downgraded lots	77.05	77.05
Weight of carton - kg	27.4	27.4
Weight of lot - kg	11,139	11,139
Price of non-downgraded product - $/kg	5.81	5.81
Price of downgraded lot - $/kg	2.905	2.905
Total downgraded lot cost	$2,493,291	$2,493,291
change in cost		$0
Total cost $/annum	$7,382,931	$5,755,791
change in costs		-$1,627,140
Total cost $/ test	$246.10	$191.86
change in costs		-$54

5 Key messages

Changing from current methods (BAX/GDS) to NeoSEEK will result in a significant cost saving from producers, in addition to a higher accuracy in results. The higher accuracy will result in a greatly lessened risk of any missed confirmations being detected by FSIS at the port of entry, which in turn is a benefit for the meat producer as well as the greater public. Additionally, NeoSeek provides additional data for future work and improvements in addressing more prevalent or problematic virulence markers, particular sero groups, *eae* and *stx*.

It is recommended that producers switch to NeoSeek for screening and confirmation testing, which is result in both cost savings in terms of total money spent on testing and storage fees.

6 Bibliography

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