Study Report

Comparative Evaluation of the RapidFinder STEC Detection Workflow and USDA-FSIS MLG Method for the Analysis of Sponge Samples from Large Beef Production Facilities Potentially Containing Shiga toxin-Producing *Escherichia coli*

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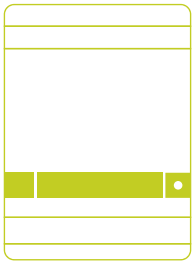

Applied Biosystems™ 7500 Fast Real-Time PCR Instrument

Summary

This technical research report describes an evaluation of the Applied Biosystems™ RapidFinder™ STEC Detection Workflow (Thermo Fisher Scientific) (alternative method) for determining the presence of Shiga toxin-producing *Escherichia coli* (STEC) in carcass sponge samples. Results from this Real-Time PCR workflow were compared to the USDA-FSIS MLG 5B.05 method (reference method) for the detection of STECs. These comparisons were performed across 61 production facility samples provided by a large North American beef producer for whom this workflow evaluation study was performed.

Sixty-one sponge samples were received by Thermo Fisher Scientific Austin laboratory from two production facilities of the same producer in different states (21 from facility "D" and 40 from facility "F"). Each sample bag contained a previously wetted sponge in Buffered Peptone Water (BPW). 100ml of pre-warmed Tryptone Soya Broth (TSB) was then added to those sample bags and incubated for 10 hours at 42+/-1ºC in a forced air incubator. Following enrichment, 200µl of sample were removed from the bag and added to the sample plate. After a semi-automated preparation on the Applied Biosystems™ MagMAX™-96 Magnetic Particle Processor (Thermo Fisher Scientific), 30µl of the elution was added to the Applied Biosystems™ RapidFinder™ STEC Screening Real-Time PCR Assay (Thermo Fisher Scientific). If samples returned positive results for *stx* and *eae* or O157:H7 then 30µl of the elution were also added to the Applied Biosystems™ RapidFinder™ STEC Real-Time PCR Confirmation Assay (Thermo Fisher Scientific). The assays were run on an Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) using the respective Applied Biosystems™ RapidFinder™ Express Software v1.2 (Thermo Fisher Scientific) cycling conditions for each STEC assay. The RapidFinder Express v1.2 software was also used to determine the positive/negative calls for each sample well. 1ml of the original enrichment was retained for the USDA-FSIS MLG 5B.05 reference method, performed by an external expert lab.

Of the 61 samples that were sent to Thermo Fisher Scientific for evaluation, nine were found to be presumptive positive for STEC by the RapidFinder Real-Time PCR assays and were sent to the expert laboratory for analysis. All nine of these samples were also positive by the MLG screening reference assay, as performed by the expert lab. Upon running the MLG confirmation assay, six of the nine were able to be confirmed. Additionally, a further 10 samples were sent to the expert laboratory which did NOT present a presumptive positive result by the RapidFinder Real-Time PCR method. In all of these cases, a virulent STEC was not able to be isolated thereby confirming the RapidFinder results. The three samples which did not produce a STEC isolate by the MLG Confirmation method were found to be positive by the RapidFinder STEC method and the MLG Screening method, and either virulence genes or a relevant

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O-type was detected with the MLG Confirmation. It is the belief of both the author of this report and the expert laboratory that the failure of the reference method to produce an isolate does not preclude the probability that a STEC was, in fact, present in these samples.

Introduction

Escherichia coli O157:H7 was first recognized as a human pathogen in 1982 and until relatively recently was the only *E. coli* serotype mandated for testing by the USDA. In June 2012, the USDA declared six additional Shiga-toxin producing *E. coli* serogroups (O26, O45, O103, O111, O121, and O145 – so called the "Big 6") as adulterants in ground beef and beef trim, if they also contain virulence genes for Shiga toxin 1 and/or 2 (*stx1*, *stx2*) and intimin (*eae*).

Thermo Fisher Scientific has developed the RapidFinder STEC Detection Workflow, which includes a two-stage real-time PCR method that meets USDA regulations to detect *E. coli* O157:H7 and the Big 6 serogroups of non-O157 Shiga toxin-producing *E. coli*. Applied Biosystems™ TaqMan™ Real-time PCR Assays were designed against each of the six non-O157 STEC O-antigen genes (O-group) and the virulence factors *stx1*, *stx2*, and *eae*. Each assay was tested against 132 STEC inclusion isolates and 284 exclusion isolates to determine assay sensitivity and specificity. Assays demonstrating 100% specificity and sensitivity were multiplexed with the Applied Biosystems™ MicroSEQ™ *E. coli* O157:H7 Assay and optimized across two PCR reactions.

1.1 Assay Evaluation

The final optimized lyophilized assays were tested in collaboration with the USDA-ARS Eastern Regional Research Center against 375g ground beef samples spiked with as low as 2 CFU of three separate representative *E. coli* isolates for each O-group and enriched in TSB for 10 and 15 hours. DNA was extracted from each sample using Applied Biosystems™ PrepSEQ™ sample preparation (Thermo Fisher Scientific) and automated using the Applied Biosystems™ MagMAX™ Express-96 instrument (Thermo Fisher Scientific). Real-time PCR was performed on the Applied Biosystems™ 7500 Fast Real-Time PCR system using RapidFinder Express software v1.2 or higher (Thermo Fisher Scientific). Positive samples were confirmed positive by the USDA FSIS Microbiology Laboratory Guidebook (MLG) method (5B.05), which includes isolation by immunomagnetic separation (IMS) using a mixture of Applied Biosystems™ Dynabeads™ (Thermo Fisher Scientific) coated with anti-*E.coli* serogroup specific antibodies and identification based on additional Real-Time PCR, latex agglutination, and biochemical testing performed on isolated colonies from modified Rainbow™ Agar O157 (mRBA) (Biolog Inc., Hayward, CA).

Both assays detected all inclusion strains and showed no cross-reactivity to any of the exclusion strains tested. The *stx* assays detected all known variants of *stx1* and *stx2*, including *stx2f* and *stx2g*. The optimized workflow showed equivalent detection to the MLG reference method.

1.2 Field Testing

Although the assay under evaluation has performed well against curated isolates of outbreak origin, the author of this report acknowledges that its ability to detect STECs in field samples must also be evaluated. To this end, an agreement with a major beef producer in the United States was reached, whereby the producer would provide Thermo Fisher Scientific with carcass sponge samples from active beef production facilities in two different states. This report details the testing and analysis of these samples, of which 61 were provided (21 from facility 'D' and 40 from facility 'F').

Method Evaluation

2.1 Purpose of Evaluation

The beef producer's interest was to determine the correlation between positive detections by the alternative and reference methods. Because of the diverse microbial composition of beef samples, molecular detection methods will tend to not be able to distinguish between the following scenarios:

- A virulent Big 6 O-type carrying the *stx* and *eae* genes is present
- A non-virulent Big 6 O-type is present concurrently with some other bacteria that carries the *stx* and/or *eae* genes.

This challenge tends to result in false positive virulent Big 6 O-type calls, leading to higher costs for beef producers. The purpose of this study was to determine when a positive Big 6 detection is made by the alternative method; does that same sample also produce a virulent O-type isolate with the MLG reference method?

2.2 Sample Preparation and Enrichment

Samples were delivered to the Thermo Fisher Scientific Austin site by overnight mail on cold packs. The samples were received in individually wrapped, unlabeled homogenizer bags containing a sponge and approximately 20ml of Buffered Peptone Water (BPW) (Thermo Fisher Scientific). The bags were then labeled according to their source; D or F, representing the cities from which the samples came. They were then numbered sequentially D1-21 and F1-40, though in no particular order, as they were not shipped as such.

Thermo Scientific™ Oxoid™ Tryptone Soya Broth (TSB) (Thermo Fisher Scientific), was pre-warmed at 42°C for 24 hours prior to enrichment. 100ml of TSB was added to each homogenizer bag, the sponge was squeezed in the medium, and the bag was re-sealed. The bags were then placed in a forced air incubator for 10 hours at 42°C. Following enrichment, 1ml was removed for reference laboratory testing. 200µl was added to the sample plate. No aliquot was set aside for direct plating.

2.3 Sample Processing on the MagMAX Express 96

The sample plate was pre-filled prior to sample addition with 200µl of Proteinase-K Lysis Buffer and 10µl of Proteinase-K. Three reagent control wells were also designated. Following the addition of 200µl of sample, the sample plate was placed on the MagMAX Express instrument along with the appropriate wash plates, elution plate, and tip comb. The "STEC" script was selected on the instrument (4400799_PrepSEQ_STEC) and the run commenced. After 10 minutes of incubation on the instrument, 200µl of Lysis Buffer were added to each well of the sample plate. The sample plate was placed back on the instrument and the run continued. After 2 minutes, 350µl of Binding Buffer and magnetic bead mixture were added to each well of the sample plate. The sample plate was placed back on the instrument and the run continued until completion. The total time of the MagMAX instrument run is 38 minutes.

2.4 Sample Processing by the Reference Method

One millilitre samples were submitted to an expert laboratory by Thermo Fisher Scientific to confirm the presence of one of the Big 6 (O26, O45, O103, O111, O121, O145) Shiga-like toxin producing *Escherichia coli*. A 30µl aliquot of the primary enrichment from each sample was screened using a USDA/FSIS approved commercially available Real-Time PCR assay (DuPont™ Nutrition and Health, BAX™ System). Each sample was screened for the presence of STEC virulence factors (*stx1/2* and *eae*) and then further screened for specific serogroups. Regardless of the screening result, all samples were subjected to isolation by immunomagnetic separation (IMS) in a ferromagnetic column with paramagnetic beads. Due to the amount of

sample submitted, a modification to the reference method was required. For each sample, 500µl aliquots, rather than the recommended 1ml aliquots, were transferred to microcentrifuge tubes containing a 50µl suspension of one of the Big 6 STEC immunomagnetic beads (which was determined during the RT-PCR screening process). The solution was placed onto a Thermo Scientific™ Labquake™ agitator (Thermo Fisher Scientific) and rotated for 15 minutes. After rotation, the bead and sample solution was transferred to a MACS™ large cell separation column (Miltenyi Biotec) and washed four times with E buffer before the final elute was collected with 1ml of E Buffer into a sterile tube. Following the IMS procedure, a 1:10 dilution and a 1:100 dilution of each IMS suspension in E Buffer were spread plated onto mRBA. A 450µl aliquot of each remaining sample was transferred into a microcentrifuge and mixed with 25µl of a 1N HCl solution. The microcentrifuge tubes were briefly mixed by vortexing, placed onto the Labquake agitator and rotated for 1 hour. After rotating, a 475µl aliquot of E Buffer was added to each sample tube.

The acid washed IMS suspension and a 1:10 dilution of the acid washed IMS suspension in E Buffer were plated onto mRBA. All mRBA plates were incubated for 20-24 hours at 35±1°C. After incubation, plates were observed for typical colonies and any mRBA plates containing typical colonies were tested for serogroup-specific latex agglutination. Up to five isolated colonies were streaked to Sheep Blood Agar (SBA) and incubated for 18-24 hours at 35±1°C. After incubation, SBA plates were observed for purity. Isolated colonies from SBA were confirmed positive for the presence of virulent non-O157 STEC by Real-Time PCR. The serogroup was also confirmed by latex agglutination.

2.5 Target Detection by Applied Biosystems Real-Time PCR

Thirty microlitres of the resulting elution from the PrepSEQ Nucleic Acid on the MagMAX Express 96 sample purification step were added directly to the wells of a RapidFinder STEC Screening Assay, which contains lyophilized bead mixtures of all of the components needed for PCR. The filled PCR strip tube was then capped and mixed by agitating on a benchtop vortexer for 10 seconds on high speed, then centrifugated at 2000rpm (400xg) for 30 seconds to place all of the material at the bottom of the tube. The Real-Time PCR analysis was performed on the 7500 Fast instrument.

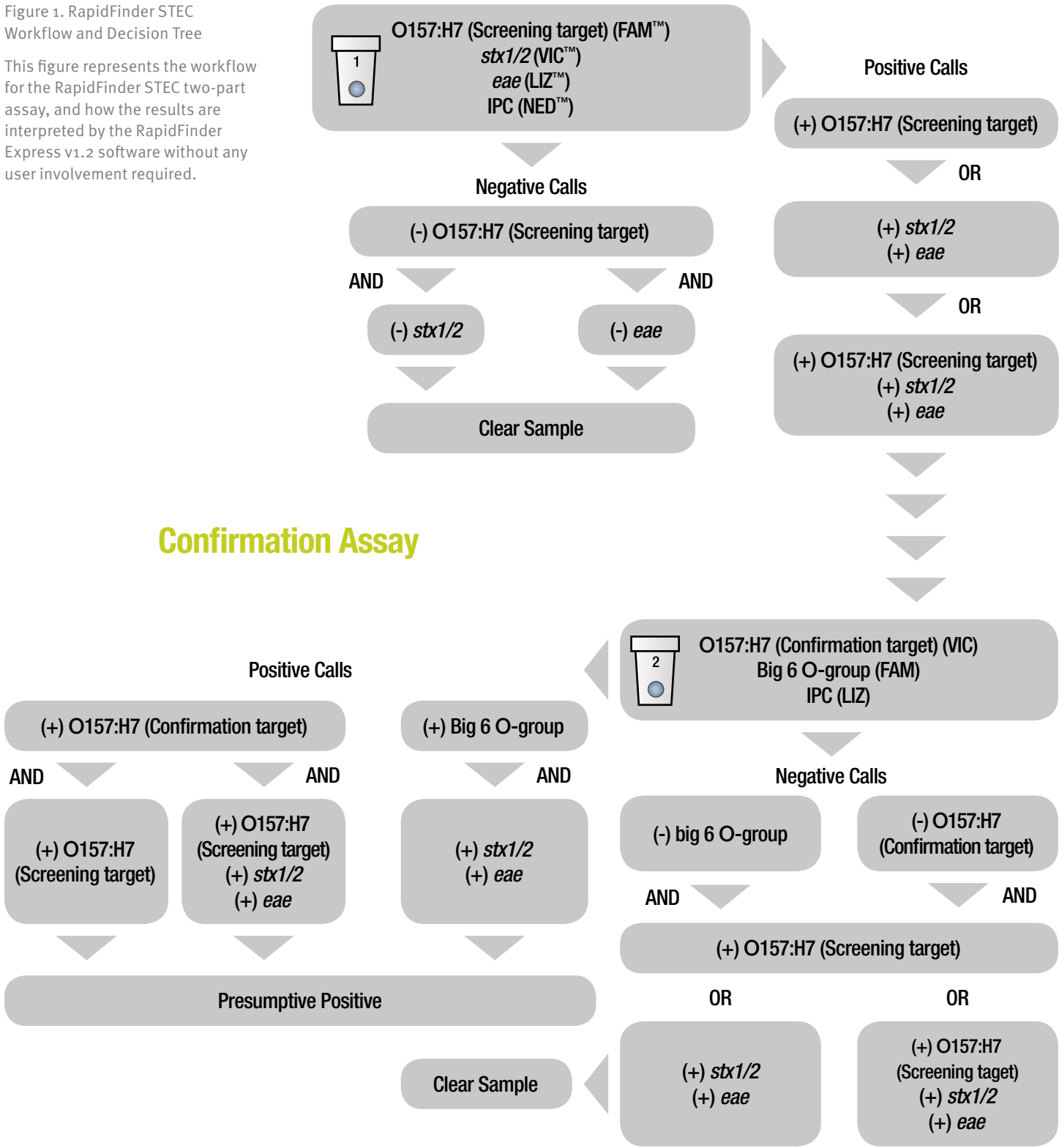
Although the protocol for this two-part assay states that the RapidFinder STEC Confirmation Assay should only be run in cases where the RapidFinder STEC Screening assay is positive, all 61 samples were also subjected to the Confirmation Assay. The significance of this will be discussed later in this report. 30µl of the PrepSeq Nucleic Acid elution were added to the lyophilized assay beads for the RapidFinder STEC Confirmation Assay. Per the protocol, the filled PCR strip tube was capped and mixed by vortexing then centrifuged as it is for the RapidFinder STEC Screening Assay. However, the vortexing and centrifugation steps are repeated for the RapidFinder STEC Confirmation Assay.

The default RapidFinder Express v1.2 software settings for each respective assay were used for the PCR runs. The PCR run takes approximately 40-50 minutes. Only the samples that were positive for either or both of the RapidFinder Screening and Confirmation Assays were sent to the expert laboratory for further evaluation and confirmation.

Screening Assay

Figure 1. RapidFinder STEC Workflow and Decision Tree

This figure represents the workflow for the RapidFinder STEC two-part assay, and how the results are interpreted by the RapidFinder Express v1.2 software without any user involvement required.



Evaluation Results

Sample	Test Type	Result	Sample	Test Type	Result
D1	RapidFinder STEC	+	D15	RapidFinder STEC	- *
	MLG Screening	+		MLG Screening	- (NV O103)
	MLG Confirmation	+ (O103)		MLG Confirmation	- (NV O103)
D2	RapidFinder STEC	+	D16	RapidFinder STEC	- †
	MLG Screening	+		MLG Screening	- †
	MLG Confirmation	+ (O26/O45)		MLG Confirmation	-
D3	RapidFinder STEC	-	D17	Rapid Finder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
D4	RapidFinder STEC	+	D18	RapidFinder STEC	-
	MLG Screening	+		MLG Screening	
	MLG Confirmation	+ (O121/O103)		MLG Confirmation	
D5	RapidFinder STEC	-	D19	RapidFinder STEC	- *
	MLG Screening			MLG Screening	- (NV O103)
	MLG Confirmation			MLG Confirmation	- (NV O103)
D6	RapidFinder STEC	- *	D20	RapidFinder STEC	-
	MLG Screening	- (NV O103)		MLG Screening	
	MLG Confirmation	- (NV O103)		MLG Confirmation	
D7	RapidFinder STEC	+	D21	RapidFinder STEC	+
	MLG Screening	+ (O45)		MLG Screening	+
	MLG Confirmation	-		MLG Confirmation	+ (O103)
D8	RapidFinder STEC	+	F1	RapidFinder STEC	-
	MLG Screening	+		MLG Screening	
	MLG Confirmation	+ (O103)		MLG Confirmation	
D9	RapidFinder STEC	- *	F2	RapidFinder STEC	-
	MLG Screening	- (NV O103)		MLG Screening	
	MLG Confirmation	- (NV O103)		MLG Confirmation	
D10	RapidFinder STEC	-	F3	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
D11	RapidFinder STEC	-	F4	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
D12	RapidFinder STEC	+	F5	RapidFinder STEC	-
	MLG Screening	+		MLG Screening	
	MLG Confirmation	+ (O121/O103)		MLG Confirmation	
D13	RapidFinder STEC	-	F6	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	

Sample	Test Type	Result	Sample	Test Type	Result
D14	RapidFinder STEC	+	F7	RapidFinder STEC	-
	MLG Screening	+ (O103)		MLG Screening	
	MLG Confirmation	- (NV O103)		MLG Confirmation	
F8	RapidFinder STEC	-	F22	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F9	RapidFinder STEC	-	F23	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F10	RapidFinder STEC	-	F24	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F11	RapidFinder STEC	-	F25	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F12	RapidFinder STEC	-	F26	RapidFinder STEC	- *
	MLG Screening			MLG Screening	- (NV O103)
	MLG Confirmation			MLG Confirmation	- (NV O103)
F13	RapidFinder STEC	-	F27	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F14	RapidFinder STEC	-	F28	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F15	RapidFinder STEC	-	F29	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F16	RapidFinder STEC	-	F30	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F17	RapidFinder STEC	-	F31	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F18	RapidFinder STEC	-	F32	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F19	RapidFinder STEC	-	F33	RapidFinder STEC	+
	MLG Screening			MLG Screening	+ (O26)
	MLG Confirmation			MLG Confirmation	- (NV O26)

Sample	Test Type	Result	Sample	Test Type	Result
F20	RapidFinder STEC	-	F34	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F21	RapidFinder Stec	- *	F35	RapidFinder STEC	- *
	MLG Screening	- (NV O103)		MLG Screening	- (NV O103)
	MLG Confirmation	-		MLG Confirmation	-
F36	RapidFinder STEC	-	F40	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	
F37	RapidFinder STEC	- *	Water	RapidFinder STEC	-
	MLG Screening	- (NV O103/O45)		MLG Screening	
	MLG Confirmation	- (NV O103/O45)		MLG Confirmation	
F38	RapidFinder STEC	- *	Water	RapidFinder STEC	-
	MLG Screening	- (NV O103/O45)		MLG Screening	
	MLG Confirmation	- (NV O103/O45)		MLG Confirmation	
F39	RapidFinder STEC	-	Water	RapidFinder STEC	-
	MLG Screening			MLG Screening	
	MLG Confirmation			MLG Confirmation	

Table 1. Results Summary

This data represents the tests applied to each sample: the two-part RapidFinder STEC method, the MLG screening assay, and the MLG confirmation assay. Results that were negative for both the RapidFinder STEC Screening and Confirmation Assays were not sent to the expert laboratory for evaluation; thus, their respective results for those assays are greyed out. Please refer to the following to interpret the notation within the table:

- : A negative detection call, though there is occasionally more detail on these samples.

+: A positive detection call. In the cases of the MLG Confirmation, the specific O-types identified are listed.

- *: An overall negative detection call was made, but the RapidFinder STEC Confirmation Assay detected a relevant O-type.

- †: An overall negative detection call was made, but the RapidFinder STEC Screening Assay detected both virulence genes.

NV: Non-virulent O-type

Three discrepancies were observed in the data between the RapidFinder STEC method result call and the MLG confirmation (highlighted in orange). Each of these samples show agreement between the alternative method and the MLG screening assay, and show some level of detection in the MLG confirmation. The samples are as follows:

D7: Positive detections by both parts of the RapidFinder STEC method and MLG Screening assay, showing O45 detection. Confirmation assay showed positive detection of virulence genes, but did not show an *E.coli* O-type.

D14: Positive detections by both parts of the RapidFinder STEC method and MLG Screening Assay, showing virulence gene and *E.coli* O103 detection. Confirmation shows O103 detection and positive *eae* detection, but not *stx*.

F33: Positive detections by both parts of the RapidFinder STEC method and MLG Screening Assay, showing virulence gene and O26 detection. Confirmation shows *E.coli* O26 detection and positive *eae* detection, but not *stx*.

3.1 Real-Time PCR Results

How to Interpret RapidFinder Express v1.2 and SDS 1.4.2.1 Results

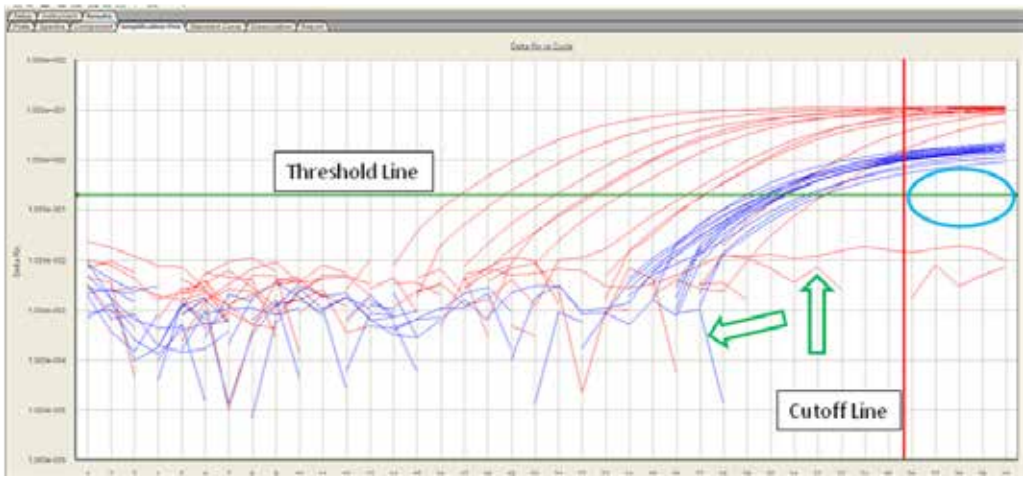


Figure 2.
This represents a detailed readout of the raw PCR data that is optionally displayed in SDS 1.4.2.1 through RapidFinder Express software, in multi-componenting view. By default, a "Positive/Negative" graphical readout would inform the user whether a sample was positive for STEC or not. However, sometimes it is necessary to display the raw PCR data in the case of a "Warning" result. This usually occurs as the result of inhibition in the PCR reaction. Alternatively, if one wished to observe the raw data prior to acting on a presumptive positive result, this functionality allows one to do so.

The green horizontal line represents the threshold for this assay (RapidFinder Express Software and SDS 1.4.2.1 will set this by default and should not be changed). The red vertical line represents the Ct cut-off value for this assay (also set by default by the software and should not be changed). In order for a sample to be called "positive" by the software, a given PCR amplification curve (shown here as the curved blue and red lines) must cross the threshold line before it reaches the cut-off line. In this case, we see that both the Internal Positive Control (IPC) signal in blue, and the target detector signal in red, cross the threshold before the cut-off. If they did not, we would see curves crossing the green line in the area of the light blue circle in the figure; these samples would be called "negative" by the software. The green arrows indicate inhibited samples. The general guidance is to remove the inhibitor through dilution and repeat the PCR.

3.2 Further Analysis

3.2.1 Further Investigating RapidFinder STEC Results

We observed many results during this study that gave us some reason for further consideration. Specifically, we found that nine of the samples were RapidFinder STEC Screening Assay negative, however we proceeded to test with the RapidFinder STEC Confirmation Assay against the recommended protocol and discussed positive results.

This has not been seen historically in our hands. This indicates that the assay was not able to detect the virulence genes, but was able to detect a relevant O-type of the Big 6 serogroups. It should be said that this is not a result that we, or customers, would typically see because it is not representative of the prescribed workflow. If a user were to obtain a negative result from the RapidFinder STEC Screening Assay, our workflow dictates that the sample can be cleared and running the RapidFinder STEC Confirmation Assay is not necessary.

However, given the unique opportunity here to use real-world samples, we decided to run both assays. Because we obtained so many RapidFinder STEC Screening Assay negative/ RapidFinder STEC Confirmation Assay positive results, we decided to send these samples to the expert laboratory as well to ensure that our method was functioning properly. These samples can be identified in Table 1 by the designation, "-*" under the RapidFinder STEC Assay results.

In all cases of obtaining this result, the expert laboratory also reported a negative result. The expert laboratory further described these samples as containing a non-virulent relevant O-type. In other words, the expert laboratory confirmed our data by obtaining the same results, so we are confident that the assay is performing as designed. Additionally, we obtained one (1) result that was RapidFinder STEC Screening Assay positive and RapidFinder STEC Confirmation Assay negative. This sample is identified in Table 1 as "- †", indicating that the RapidFinder Detection Workflow was able to detect virulence genes, but no O-type of relevance. Again, the expert laboratory was able to confirm our data by generating the same results, indicating that the candidate assay is performing as designed.

3.2.2 RapidFinder Results Not Confirmed

Aside from the 10 samples discussed in the previous section, there were nine samples that were RapidFinder STEC Screening Assay and RapidFinder STEC Confirmation Assay positive by the alternative method, which were then sent to the expert laboratory to be confirmed. Of these, six were able to be confirmed with the MLG Confirmation method. The three exceptions have been described in the legend for Table 1: D7, D15, F33. Upon review of these samples after the results were delivered from the expert laboratory, there were no errors found in the RapidFinder STEC Assay result interpretation (i.e., no software or human error), and each target that was designated as "positive" was clearly positive. In other words, these were not borderline Ct values when the raw data was observed. The MLG screening assay results were also clearly positive. Some parts of the MLG confirmation assay were also positive, indicating that at least some of the findings of the previous two assays can be confirmed and are valid.

Conclusions and Discussion

The objective of this study was to evaluate the RapidFinder STEC Detection Workflow on real world samples provided by a major US beef producer, and have the results of that evaluation confirmed by an expert laboratory against the reference method. The question of interest was, if a positive detection was obtained with the alternative method, could that result be confirmed by the MLG reference method by way of at least one Big 6 isolate? 61 samples were assayed for the presence of *E. coli* O157:H7 and the Big 6 STEC O-types. Of these, nine were designated by the RapidFinder STEC method as positive, which indicates that both Assays of the two-part Workflow showed a positive detection. These samples were sent to the expert laboratory to be confirmed by the reference method. Of these, six were confirmed by the MLG method.

The MLG confirmation data showed at least some concordance with the RapidFinder STEC method and the MLG screening assay data. It was concerning to see two PCR assays detect the *stx* gene clearly, yet have a third fail to detect it, especially as both MLG PCR assays are

identical. In speaking with the expert laboratory, we were assured that the failure of the MLG confirmation assay to detect a virulent O-type does not preclude the possibility that one was present. Because of the method of selecting colonies from an agar plate for testing, there exists the very real possibility that sampling error played a part in the failure to detect the virulent O-type because of the following factors:

- The high amount of background growth on the agar plates reported by the expert laboratory
- The fact that non-virulent O-types tend to be indistinguishable from virulent strains of the same O-type on an agar plate
- Only five colonies are picked for analysis

Additionally, ten negative samples were sent to the expert laboratory due to a suspicion that the RapidFinder STEC method was not functioning as intended. This was indicated by the observation that these samples had disparate results between the RapidFinder STEC Screening and RapidFinder STEC Confirmation Assays that have not historically been seen during development. As is discussed above, we found that, for all of the samples that aroused suspicion, the RapidFinder STEC Detection Workflow is working precisely as designed.

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