

Design and Evaluation of Two-Stage Multiplex Real-Time PCR Method for Detecting O157:H7 and non-O157 STEC Strains from Beef Samples

Michelle S Swimley¹, Lori Bagi², Sharon A. Matheny¹, Aisha Abdul-Wakeel², Craig Cummings¹, Robert S. Tebbs¹, and Pina Fratamico², ¹Thermo Fisher Scientific, Austin, TX, USA, ²USDA-ARS-ERRC, Wyndmoor, PA, USA

INTRODUCTION

E. coli O157:H7 was first recognized as a human pathogen in 1982 and until recently was the only *E. coli* strain 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) 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*).

PURPOSE

To develop a complete Applied Biosystems[™] RapidFinder[™] STEC Detection Workflow, including 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*.

METHODS

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 strains and 284 exclusion strains 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. The final optimized lyophilized assays were tested against 375-g ground beef samples spiked with as low as 2 CFU of three separate representative *E. coli* strains for each O-group and enriched with TSB for 10 and 15 hours. DNA was extracted from each sample using Applied Biosystems[™] PrepSEQ[™] sample preparation and an automated Applied Biosystems[™] MagMAX[™] Express 96 instrument. Real-time PCR was performed on the Applied Biosystems[™] 7500 Fast real-time PCR system using RapidFinder Express software. Presumptive 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 Invitrogen[™] Dynabeads[™] 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).

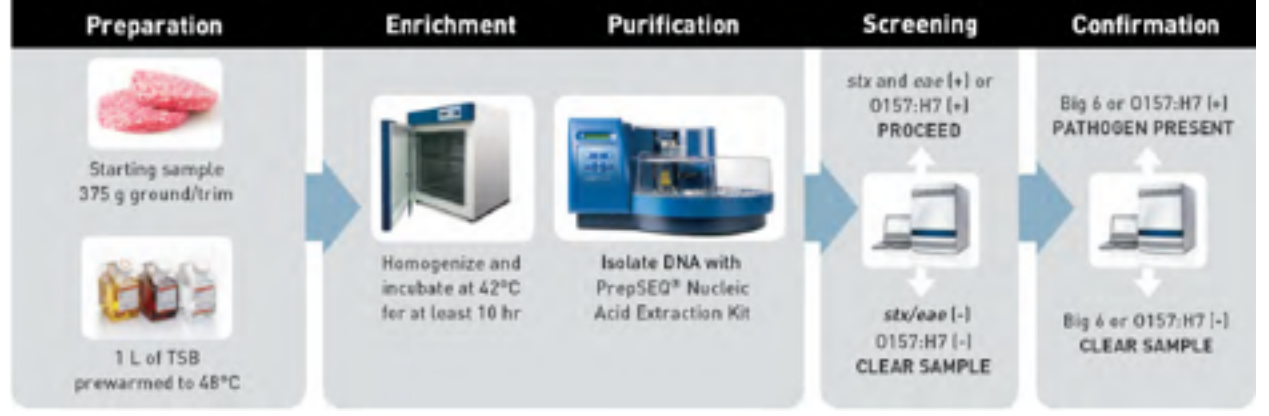
RESULTS

All 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.

SIGNIFICANCE

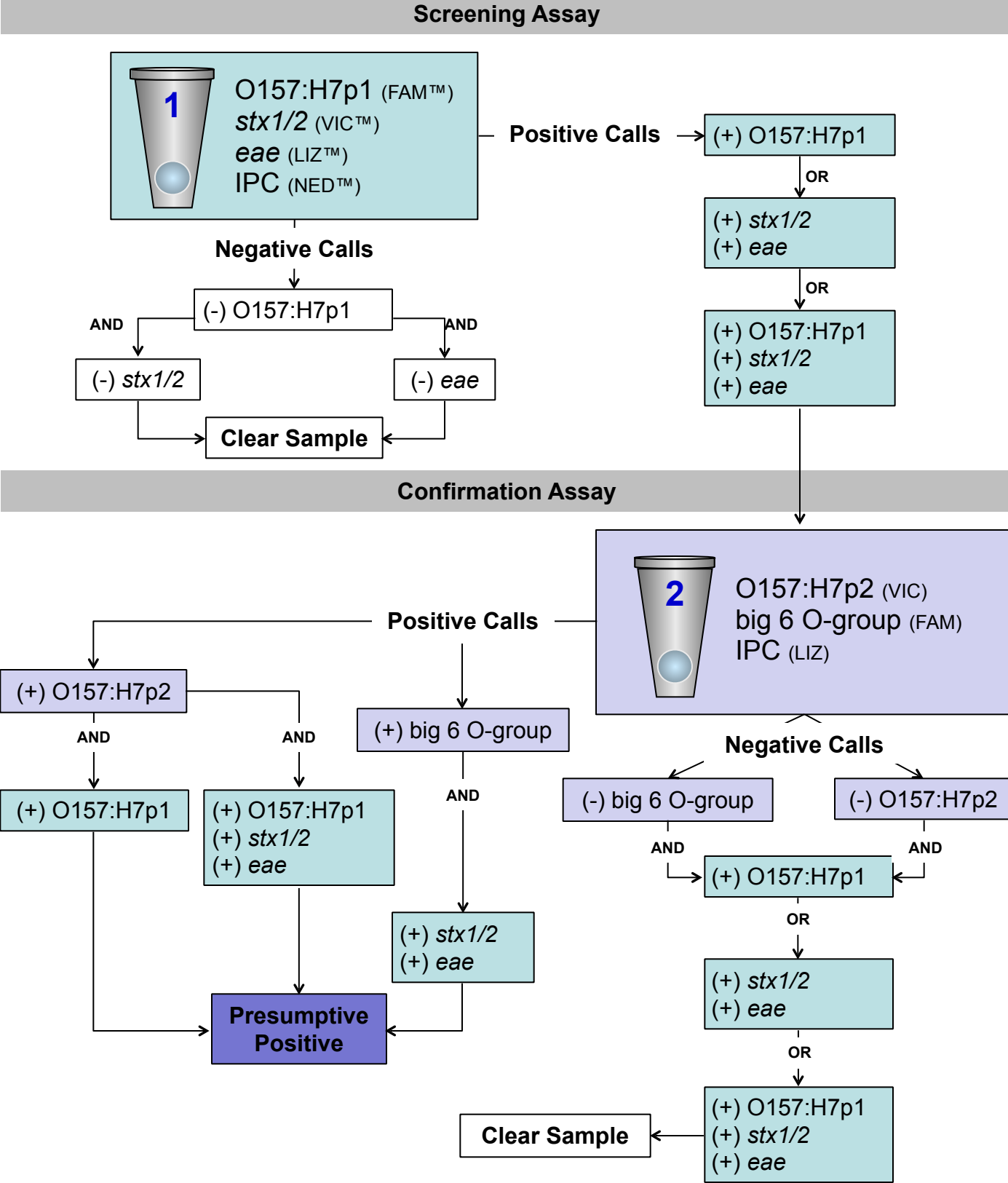
The multiplex real-time PCR assays developed are part of a complete food testing solution for routine and rapid detection of *E. coli* O157:H7 and the "big 6" non-O157 STEC strains in beef samples.

Figure 1. RapidFinder[™] STEC Complete Workflow



The RapidFinder STEC Detection Workflow provides confident results through a simplified, same-day workflow for detecting *E. coli* O157:H7 and Big 6 serogroups of non-O157 *E. coli* found in ground beef and beef trim samples.

Figure 2. RapidFinder[™] STEC Product Configuration



If sample is positive for O157:H7 part 1 and/or *stx1/2* and *eae*, proceed to confirmation assay, otherwise sample is cleared as negative. If sample is positive for both O157:H7 part 1 and O157:H7 part 2, sample is considered presumptive positive for O157:H7. If sample is positive for *stx1/2*, *eae* and big 6 O-group, sample is considered presumptive positive for "big 6" STEC. Samples that are considered presumptive positives should be confirmed by MLG reference method.

Table 1. RapidFinder STEC Screening Assay detects all known *stx* variants

Strain	Serotype	Stx subtype(s)	<i>stx1</i> assay	<i>stx2</i> (14254)	<i>stx2</i> (29134)	<i>stx2</i> (29133)	Multiplex Screening Assay		
							<i>stx1/2</i>	<i>eae</i>	O157:H7
AA1	O174:H8	1c, 2b	25.56	25.95	-	24.17	32.44	-	
BB2	O55:H7	1a	24.79	-	-	25.84	24.75	38.92	
CC3	O128ac[H2]	2f	-	-	25.41	24.41	23.29	-	
DD4	O177:[H25]	2c, 2d	-	24.57	-	25.52	25.21	-	
EE5	O111:[H8]	1a, 2a	25.44	26.7	-	24.70	24.17	-	
FF6	O113:H4	1c, 2b	25.53	24.27	-	22.65	30.29	-	
GG7	O103:H2	1a	25.09	-	-	25.36	24.11	-	
HH8	O26:H11	1a	26.61	-	-	24.79	23.28	-	
II9	O41:H26	1d	25.26	-	-	25.91	-	-	
JJ10	O157:H7	2c	-	25.52	-	26.92	24.46	24.47	
OS622	O138	2e	-	25.99	-	26.90	-	-	
B2F1	O91:H21	2d	-	23.78	-	25.73	-	-	
D3509	O2:H25	2g	-	-	24.60	27.86	-	-	

Table 2. RapidFinder STEC assays show high sensitivity and specificity for *E. coli* O157:H7 and Big 6 non-O157 STEC strains

Organism	Serotype/ species	# strains		% Detected
		Thermo Fisher	USDA	
Inclusion				
<i>E. coli</i>	O157:H7	8	5	100
<i>E. coli</i>	O26	13	9	100
<i>E. coli</i>	O45	11	16	100
<i>E. coli</i>	O103	13	7	100
<i>E. coli</i>	O111	13	7	100
<i>E. coli</i>	O121	10	7	100
<i>E. coli</i>	O145	7	6	100
Exclusion				
<i>E. coli</i>	non-Big-6 STEC, non-O157:H7	65	180	0
<i>Salmonella</i>	typhi, typhimurium, enteritidis, others	8	12	0
<i>Bacillus</i>	cereus, brevis, subtilis, spp.	0	4	0
<i>Citrobacter</i>	freundii	0	1	0
<i>Pseudomonas</i>	aeruginosa	0	1	0
<i>Pseudomonas</i>	fluorescens	0	1	0
<i>Shigella</i>	dysenteriae	3	1	0
<i>Shigella</i>	flexneri	0	1	0
<i>Shigella</i>	sonnei	0	1	0
<i>Legionella</i>	pneumoniae	1	0	0
<i>Listeria</i>	monocytogenes	1	1	0
<i>Listeria</i>	welshmeri	0	1	0
<i>Serratia</i>	spp.	0	1	0
<i>Vibrio</i>	cholerae	1	0	0

Figure 3. Culture Confirmation of presumptive positive samples

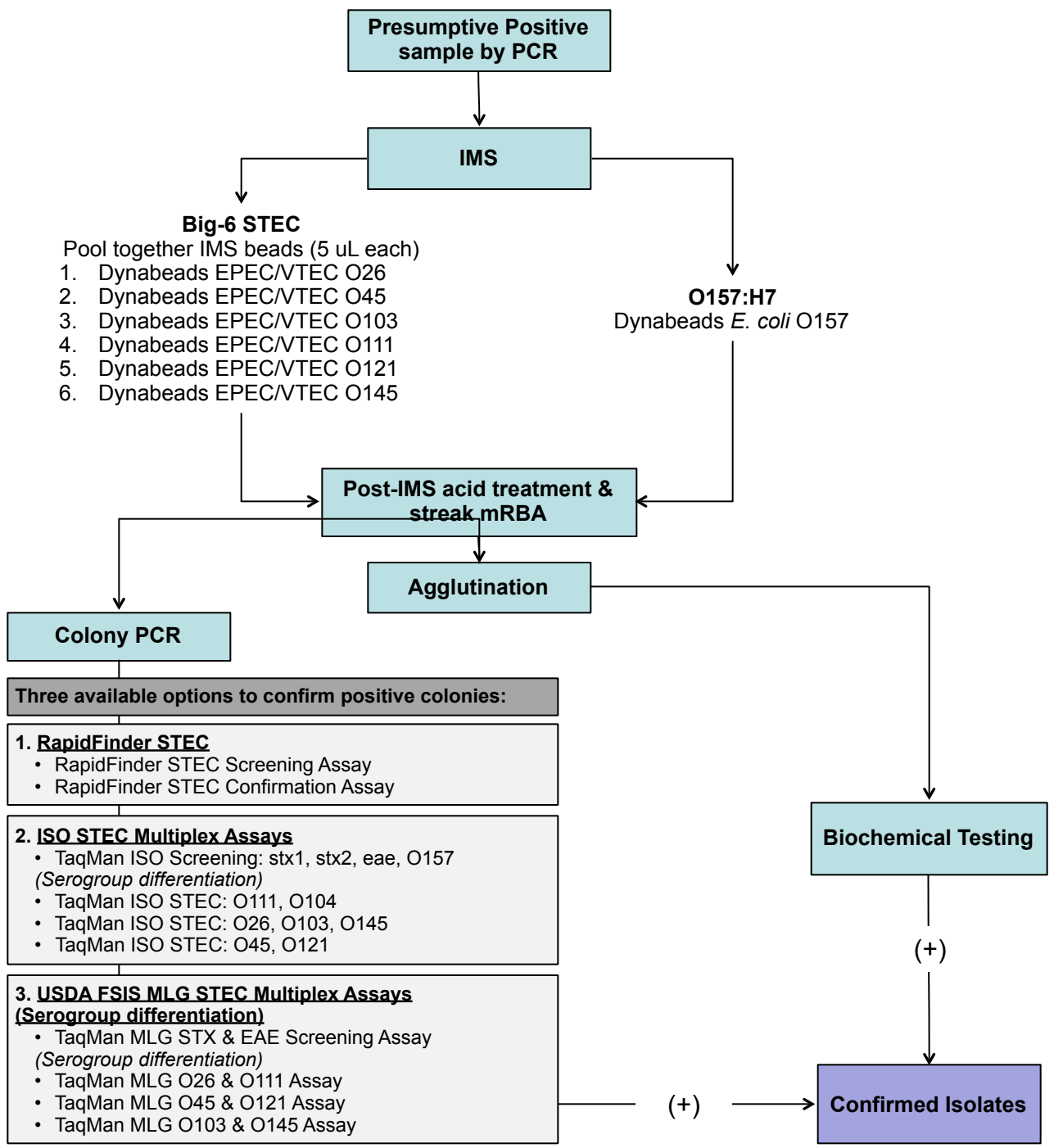


Table 3. USDA Evaluation of RapidFinder STEC Workflow

O-Type	Strain	Strain ID	Analysis using RapidFinder Express Software				Final Call	RapidFinder STEC Workflow	MLG Reference Method
			RapidFinder STEC Screening Assay		RapidFinder STEC Confirmation				
			eae	stx1/2	O157p1	O157p2			
O26	O26:H11	00971	+	+	-	-	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O26:H11	00971	+	+	-	-	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O26:H11	05-6544	+	+	-	-	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O26:H11	05-6544	+	+	-	-	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O26:NM	TB352	+	+	-	-	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O26:NM	TB352	+	+	-	-	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
O45	O45:H2	10-2360	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O45:H2	10-2360	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O45:H2	05-6545	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O45:H2	05-6545	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O45:H2	96-3285	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O45:H2	96-3285	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
O103	O103:H8	04162	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O103:H8	04162	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O103:H25	03-2444	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O103:H25	03-2444	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O103:H2	04-2446	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O103:H2	04-2446	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
O111	O111:H8	01387	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O111:H8	01387	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O111:NM	00-4748	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O111:NM	00-4748	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O111:NM	98-8338	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O111:NM	98-8338	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
O121	O121:H19	08023	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O121:H19	08023	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O121:H19	03-2832	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O121:H19	03-2832	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O121:NM	03-4064	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O121:NM	03-4064	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
O145	O145:H28	07865	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O145:H28	07865	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O145:NM	03-4699	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O145:NM	03-4699	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O145:NM	83-75	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
	O145:NM	83-75	+	+	-	+	Positive (eae, stx, big-6)	Confirmed Big-6 STEC	Confirmed Big-6 STEC
O157	O157:H7	06E20128	+	+	-	+	Positive (O157:H7)	Confirmed O157:H7	Confirmed O157:H7
	O157:H7	06E20128	+	+	-	+	Positive (O157:H7)	Confirmed O157:H7	Confirmed O157:H7
	O157:H7	380-94	+	+	-	+	Positive (O157:H7)	Confirmed O157:H7	Confirmed O157:H7
	O157:H7	380-94	+	+	-	+	Positive (O157:H7)	Confirmed O157:H7	Confirmed O157:H7
	O157:H7	EDL933	+	+	-	+	Positive (O157:H7)	Confirmed O157:H7	Confirmed O157:H7
	O157:H7	EDL933	+	+	-	+	Positive (O157:H7)	Confirmed O157:H7	Confirmed O157:H7
O55	O55:H7	05-0376	+	+	-	-	Negative	Confirmed Negative	Confirmed Negative
	O55:H7	05-0376	+	+	-	-	Negative	Confirmed Negative	Confirmed Negative
O121 (stx-)	O121	3056-85	+	-	-	NT	Negative	Confirmed Negative	Confirmed Negative
	O121	3056-85	+	-	-	NT	Negative	Confirmed Negative	Confirmed Negative
uninoculated ctrl	-	-	-	-	-	NT	Negative	Confirmed Negative	Confirmed Negative
	-	-	-	-	-	NT	Negative	Confirmed Negative	Confirmed Negative

*NT = not tested; based on results of screening assay these samples were cleared as negative and not tested with confirmation assay.
 **O157:H7p2 assay shows cross-reactivity with some Big 6 O-groups but not with any non-Big 6 O-groups.
 ***Parenthesis under Final Call column indicate interpretation of the positive call by RFE software.

CONCLUSIONS

- The RapidFinder STEC Screening and Confirmation Real-Time PCR assays are part of a complete food testing solution designed to make food pathogen detection as rapid and easy as possible.
- Ease-of-use with a streamlined, lyophilized format.
- Minimal hands-on-time with automated MagMAX Express 96 protocol.
- Total time to results is <12 hours for raw ground beef or beef trim.
- Results of validation studies demonstrate 100% detection of inclusion strains and no detection of exclusion strains.
- Pooling together IMS beads allows for streamlined culture confirmation of big 6 presumptive positive samples.
- Internal positive control significantly reduces the risk of false-negatives.

REFERENCES

1. Detection and Isolation of non-O157 Shiga-Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges (MLG 5B.05). United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science. Effective: 6/29/14

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