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Multiplex Real-Time PCR Method To Identify Shiga Toxin Genes *stx1* and *stx2* and *Escherichia coli* O157:H7/H$^-$ Serotype

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A multiplex real-time PCR method to simultaneously detect the *stx1* and *stx2* genes of Shiga toxin-producing *Escherichia coli* and a unique conserved single-nucleotide polymorphism in the *E. coli* O157:H7/H$^-$ *uidA* gene has been developed. There is more than 98.6% sensitivity and 100% specificity for all three gene targets based on a panel of 138 isolates. The PCR efficiencies were ≥1.89, and as few as 6 CFU/reaction could be detected.

Shiga toxin-producing *Escherichia coli* (STEC) strains are recognized as an important group of enteric pathogens capable of causing serious illnesses and death (15, 18, 20, 26). More than 100 *E. coli* serotypes may produce Shiga toxins (24). Several sporadic cases, large outbreaks, and illnesses worldwide have been associated with STEC organisms, primarily the *E. coli* O157:H7 serotype, making this an important class of food-borne pathogens (1, 7, 8, 9, 21, 22, 30, 33, 34, 35).

The Shiga toxins produced by STEC are generally considered the principal virulence characteristic responsible for serious illnesses associated with this organism. Therefore, the presence of these cytotoxins or their genes (*stx1* and *stx2*) is the focus of many assays for STEC organisms. Due to the predominance of the O157:H7 STEC serotype associated with human illness, many methods also focus on the detection and identification of this serogroup. A highly conserved point mutation at position 93 of the *uidA* (β-glucuronidase) gene occurs in O157:H7 and nonmotile O157 strains, including atypical O157:H$^-$ clones implicated in German hemolytic-uremic syndrome outbreaks (11, 12, 13, 23). The detection of the *stx1* and *stx2* genes and the single-nucleotide polymorphism (SNP) at position 93 are the basis of a multiplex mismatch amplification mutation assay (6) and oligonucleotide probe hybridization tests (10).

Real-time PCR applications offer the advantages of being more sensitive and rapid by not requiring post-PCR procedures to detect amplification products used in conventional PCR-based procedures. Recent advances using minor-groove binder (MGB) modifications to significantly increase duplex stability have improved SNP detection in real-time PCR applications (19). The goal of this project was to develop an STEC multiplex real-time PCR method that can specifically detect *stx1* and *stx2* genes with a multiplex 5′ nuclease approach and simultaneously detect the presence of *E. coli* O157:H7/H$^-$ strains based on a unique and conserved SNP in the *uidA* gene by using a 3′ MGB probe. The real-time PCR MGB probe approach used here combines the advantages of a rapid PCR process similar to the mismatch amplification mutation assay without reducing PCR efficiency and the ability to optimize stringent hybridization requirements for SNP detection in one assay.

Primer pairs and internal fluorescent probes were selected and designed with Primer Express (Applied Biosystems, Foster City, Calif.) (Table 1) based on sequences submitted to GenBank. Sequence comparison and lineups were generated with the GCG program (Wisconsin Package, version 10.3; Accelrys Inc., San Diego, Calif.). Primers were synthesized by standard methods (IDT, Coralville, Iowa, and Sigma-Genosys, The Woodlands, Tex.). The *stx1* probe (stx1P990) was 5′ end labeled with 6-carboxy-X-rhodamine (ROX) and 3′ end labeled with Black Hole Quencher (BHQ2) (Biosearch Technologies, Novato, Calif.); the *stx2* probe (stx2P1249) was 5′ labeled with 6-carboxyfluorescein (FAM) and 3′ end labeled with BHQ1 (IDT). The *uidA* O157:H7/H$^-$ genotype MGB probe (uidAP266) was 5′ end labeled with 6-carboxy-4,7,2′,7′-tetrachloro-fluorescein (TET) and MGB nonfluorescence quencher moieties at the 3′ end (Applied Biosystems).

The four sets of optimal reaction conditions identified previously with the *uidA* O157:H7/H$^-$ genotype real-time PCR assay (36) were tested with a selected group of isolates in a multiplex format (Tables 2 and 3). The most stringent magnesium concentration (2 mM) and temperature (63°C for 25 s of annealing and extension) combination resulted in a false-negative result for *stx2* with strain EDL 933. Among the other three sets of conditions tested, there were no false-negative or false-positive results for any of the test isolates. The set of conditions including 3 mM MgCl, 63°C for 25 s annealing and extension, and 0.025 μM *uidA* probe resulted in the lowest average end fluorescence, 2.684, for the non-O157:H7/H$^-$ isolates (C600, CFSAN$400, and ATCC 13337), and therefore these conditions were selected for testing additional strains.

However, under these conditions, STEC strains that were non-O157:H7/H$^-$ and *stx2* positive had considerable spectral overlap between the adjacent FAM (*stx2*) and TET (*uidA* O157:H7/H$^-$ genotype) channels. Reducing the FAM (*stx2*) probe concentration to 0.025 μM resulted in no false-positive results in the TET (*uidA* O157:H7/H$^-$ serotype) or FAM (*stx2*) channels (Table 4). The end fluorescence values in the FAM (*stx2*) channel for *stx2*-positive isolates decreased, but the *stx2*

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TABLE 1. Primer and probe sequences used in a multiplex real-time PCR assay to detect *stx1*, *stx2*, and the position 93 *uidA* SNP of *E. coli* O157:H7/H$^-$

Primer or probe[a]	GenBank accession no.	Sequence (5′→3′)	T_m (°C)
stx1F934	M19473	GTGGCATTAATACTGAATTGTCATCA	58
stx1R1042	M19473	GCGTAATCCCACGGACTCTTC	60
stx2F1218	X07865	GATGTTTATGGCGGTTTTATTTGC	60
stx2R1300	X07865	TGGAAAACTCAATTTTACCTTTAGCA	58
uidAF241	AF305917	CAGTCTGGATCGCGAAAACTG	59
uidAR383	AF305917	ACCAGACGTTGCCCACATAATT	59
stx1P990	M19473	Rox-TGATGAGTTTCCTTCTATGTGTCCGGCAGAT-BHQ2	69
stx2P1249	X07865	6FAM-TCTGTTAATGCAATGGCGGCGGATT-BHQ1	69
uidAP266	AF305917	TET-ATTGAGCAGCGTTGG-MGB/NFQ	66

[a] Primer and probe names are composed of the name of the target gene, a letter indicating forward primer (F), reverse primer (R), or probe (P), and the 5′ base position of the oligonucleotide.

(FAM)-positive isolates remained positive even at the reduced probe concentration, with an average cycle threshold (Ct) value of 19.82, compared to 19.61 ($n = 14$). Real-time fluorogenic multiplex assays can be complicated by several variables related to the fluorescence emission spectra generated by each probe. While the introduction of dark quencher molecules, where fluorescence resonance energy transfer (FRET) is to the infrared rather than the UV spectrum, allows less nonspecific background fluorescence in multiplex assays, the emissions of the reporter dyes in adjacent channels must still be carefully optimized. In this multiplex format, the optimal probe concentration was reduced to 0.025 μM for the two probes (*stx2* and *uidA*) located in adjacent channels (FAM and TET).

The optimized method utilized 10 mM Tris HCl (pH 8.3), 50 mM KCl (PCR Gold Buffer II; Applied Biosystems), 200 μM (each) dGTP, dCTP, dTTP, and dATP, 3.0 mM $MgCl_2$, a 0.25 μM concentration of each primer (stx1F934, stx1R1042, stx2F1218, stx2R1300, uidAF241, and uidAR383) (Table 1), a 0.1 μM concentration of the *stx1* probe (stx1P990ROX), a 0.025 μM concentration of the *stx2* probe (stx2P1249FAM), a 0.025 μM concentration of the *uidA* O157:H7/H$^-$ serotype probe (uidAP266TET-MGB), 1.25 U of AmpliTaq Gold (Applied Biosystems), and 0.5 μl of sample template in a total volume of 25 μl. The amplification program included an initial polymerase activation step, 10 min at 94°C, and 40 cycles of 20 s at 94°C and 25 s at 63°C, performed on a Smart Cycler thermal cycler (Cepheid, Sunnyvale, Calif.). Fluorescence values were recorded in each round during the 25-s, 63°C annealing-extension step in the FAM, TET, and ROX channels. Ct values were based on primary curve analysis using manual threshold settings set at 15.0 fluorescence units, with default background subtraction.

TABLE 2. Amplification conditions used for multiplex PCR of *stx1*, *stx2*, and the position 93 *uidA* SNP of *E. coli* O157:H7/H$^-$

Parameter set[a]	MgCl concn (mM)	Annealing and extension temp (°C)	Probe concn (μM)		
			stx1	*stx2*	*uidA*
A	3	63	0.1	0.1	0.025
B	4	63	0.1	0.1	0.025
C	2	61	0.1	0.1	0.025
D	2	63	0.1	0.1	0.05

[a] For the Ct and end fluorescence values obtained with each set of conditions, see Table 3.

The optimized method was tested with 138 isolates, which had various *stx1*, *stx2*, and *uidA E. coli* O157:H7/H$^-$ genotypes that had been previously determined. The assay specificity was 100% of this multiplex real-time PCR for all three targets with 138 isolates, and the assay sensitivity was 98.6, 100, and 100% for *stx1*, *stx2*, and *uidA* O157:H7/H$^-$ targets, respectively. All of the isolates except one produced the correct genotypic pattern with this real-time multiplex PCR method (Table 5). The one isolate that did not was CFSAN$407 (*E. coli* O15:H27), which gave a false-negative result for the *stx1* (ROX) gene. Although this isolate did not cross the threshold, there appeared to be some amplification, which resulted in an end fluorescence value of 27.727 in the ROX channel. The appropriate *stx1* genotype with a Ct value of 22.60 was achieved when the annealing-extension temperature was reduced to 60 from 63°C. In addition, sequencing of the *stx1* gene from this strain revealed two mismatches with the probe and one mismatch with the reverse primer, perhaps contributing to the reduced reaction efficiency. The *stx1* gene sequence of this strain had the greatest similarity with the *stx1* variant GenBank sequences AY135685, AJ314839, and AJ314838 (2).

The test panel included 52 *E. coli* O157:H7 and two *E. coli* O157:H$^-$ isolates, all of which were detected by the unique *E. coli* O157:H7/H$^-$ *uidA* position 93 component of this multiplex assay. A real-time PCR assay that targets the *rfbE* gene (lipopolysaccharide O side chain of *E. coli* O157) would not distinguish between *E. coli* O157:H7 and *E. coli* O157 with other H-flagellin antigens (14). The assay reported here did not detect *E. coli* O157 with other flagellin serotypes, including three *E. coli* O157:H16 isolates and an *E. coli* O157:H45 isolate. Other real-time PCR assays target the eae_{O157} (γ-intimin) gene and detect *E. coli* O55:H7 and *E. coli* O55:NM strains in addition to *E. coli* O157:H7 and *E. coli* O157:H$^-$ strains (25, 32). This assay did not detect two strains of the closely related *E. coli* O55:H7/H$^-$ serotype.

The sensitivity of the multiplex format was reliable, with as few as 6 CFU/reaction within 40 cycles for *E. coli* O157:H7 strain EDL 933. Serial dilutions of the EDL 933 template also demonstrated the potential quantitative ability of this multiplex real-time PCR application, with an average shift in Ct values of 3.66 for each 10-fold dilution (Fig. 1). The real-time

TABLE 3. Ct and end fluorescence (dR) values for *stx1*, *stx2*, and the position 93 *uidA* SNP of *E. coli* O157:H7/H$^-$ with four amplification parameters sets[a]

Gene and isolate	Genotype	Result obtained with amplification parameter set:							
		A		B		C		D	
		Ct	dR	Ct	dR	Ct	dR	Ct	dR
stx1									
EDL 933	+	20.14	131.115	19.59	182.974	20.89	112.544	22.36	76.967
CFSAN$400	+	18.98	143.727	18.76	209.545	19.79	129.558	20.52	88.951
ATCC 43889	−	−	0.112	−	−1.916	−	−0.572	−	0.216
C600	−	−	0.938	−	−2.059	−	1.171	−	−2.317
ATCC 13337	−	−	1.094	−	2.505	−	1.288	−	−1.102
Neg. control	−	−	1.202	−	−1.314	−	4.606	−	−0.855
stx2									
EDL 933	+	18.8	235.545	18.46	336.727	19.76	149.934	−[b]	4.127[b]
CFSAN$400	−	−	−1.075	−	0.806	−	−0.580	−	−2.229
ATCC 43889	+	18.1	315.091	17.59	361.455	18.91	246.909	21.36	89.976
C600	−	−	0.402	−	−1.644	−	0.351	−	0.4498
ATCC 13337	−	−	−0.074	−	−0.389	−	−0.383	−	0.785
Neg. control	−	−	−0.276	−	1.405	−	0.545	−	−1.384
uidA									
EDL 933	+	22.14	91.467	21.12	110.560	23.76	75.75	23.82	86.992
CFSAN$400	−	−	2.185	−	2.313	−	1.606	−	1.633
ATCC 43889	+	20.77	117.005	19.97	123.825	21.56	101.536	21.37	128.908
C600	−	−	5.138	−	5.084	−	6.649	−	4.009
ATCC 13337	−	−	0.728	−	2.201	−	0.450	−	−0.760
Neg. control	−	−	0.660	−	−1.343	−	−0.143	−	−0.210

[a] Multiplex PCR conditions were as described in the text; for the conditions that correspond to parameter sets A, B, C, and D, see Table 2. +, positive; − and Neg., negative.
[b] False-negative result.

PCR efficiency was calculated for each gene in the multiplex based on the slope of the lines using the formula $10^{-1/\text{slope}}$ (5, 27, 28). The calculated efficiencies for each of the components of this multiplex were similar, with values of 1.89, 1.91, and 2.01 for the *stx1*, *stx2*, and *uidA* O157 genotypes, respectively. Optimal PCR efficiency would be equal to 2.00 and generate a slope of −3.32. The sensitivity for each component of the multiplex reaction can be compared based on the *y* intercept (5, 27, 28). In this case, the *stx1* and *stx2* genes had *y* intercepts of 39.395 and 39.427, respectively, while the *uidA* O157 genotype was slightly less sensitive, with a *y* intercept of 42.369.

The average Ct values for each amplification product (*stx1*, *stx2*, and *uidA* O157 genes) were similar regardless of whether the amplifications were run individually or in the multiplex format (Fig. 2). The average Ct values for the individually run reactions were 19.62, 19.08, and 21.67 for the *stx1*, *stx2*, and

TABLE 4. Effect of reduced *stx2* (FAM) probe concentration on background end fluorescence (dR) in the FAM and TET channels[a]

Isolate	Result in channel with indicated *stx2* probe concn (μM)									
	stx2 (FAM)					*uidA* SNP (TET)				
	Genotype	0.1		0.025		Genotype	0.1		0.025	
		Ct	dR	Ct	dR		Ct	dR	Ct	dR
13A71	Pos.	19.33	315.7	19.99	104.456	Neg.	−	33.897	−	28.3
13A74	Pos.	19.93	389.5	20.36	111.5	Neg.	24.79[b]	44.9	−	24.042
13A75	Pos.	19.72	409	19.68	106.828	Neg.	−	31	−	18
13A76	Pos.	19.7	404.422	20.09	103.389	Neg.	−	35.238	−	22.571
13B47	Pos.	21.43	275.952	20.85	96.564	Pos.	22.91	82.714	22.31	77.714
CDC 3493-88	Pos.	20.24	268.344	19.86	102	Neg.	−	42	−	12.993
13C06	Pos.	19.61	306.429	19.69	106.65	Neg.	−	34.571	−	33.9
13C10	Pos.	19.34	414.762	19.83	116.75	Neg.	−	35.022	−	23.25
CFSAN$406	Pos.	16.81	366	17.63	104	Neg.	21.76[b]	51.3	−	27
CFSAN$407	Pos.	21.08	277.571	21.12	105.5	Neg.	−	34.182	−	26.3
3377–85	Pos.	19.14	344.25	19.18	114.381	Neg.	−	14.1	−	24.1
3493–88	Pos.	19.93	302.25	20.32	94.714	Neg.	−	23.75	−	7.560
3024–94	Pos.	19.76	445.133	19.93	119.286	Neg.	−	26	−	12.052
EDL 933	Pos.	18.54	413	18.92	102.5	Pos.	22.91	68.091	23.24	70.286

[a] Pos., positive; − and Neg., negative.
[b] False-positive result due to spectral overlap from the *stx2* (FAM) probe at 0.1 μM.

TABLE 5. Ct values for 138 isolates representing different genotypes for *stx1*, *stx2*, and the *uidA E. coli* O157:H7/H$^-$ position 93 SNP

Organism	Serotype (no. of isolates tested)	Genotype[k]			Avg Ct (SD)		
		stx1	*stx2*	*uidA* O157:H7/H$^-$	*stx1*	*stx2*	*uidA* O157:H7/H$^-$
EHEC	O157:H7 (39)[a–g]	+	+	+	19.68 (0.548)	18.98 (0.560)	23.77 (1.248)
	O157:H7 (1)	+	−	+	19.41	−	22.21
	O157:H7 (11)[a,b,c,e,f]	−	+	+	−	19.71 (1.469)	23.29 (1.017)
	O157:H7 (1)	−	−	+	−	−	23.30
	O157:H$^-$ (1)[b]	+	+	+	18.63	20.85	22.31
	O157:H$^-$ (1)[f]	−	+	+	−	18.83	22.25
STEC	O68:H$^-$ (1)[e]	+	+	−	19.43	19.11	−
	O48: (1)				18.62	19.99	−
	O45:H2 (1)[g]				19.32	19.89	−
	O137:H41 (3)[g,h]				18.12 (0.125)	19.96 (0.326)	−
	O111:H$^-$ (3)[e,f]				19.47 (0.567)	19.21 (0.480)	−
	O22:H8 (1)[f]				17.87	17.63	−
	O15:H27 (1)[f]				−[i]	21.12	−
	O4:H$^-$ (1)[g]				18.92	19.18	−
	O26:H11 (7)[e,f,h]	+	−	−	19.43 (0.757)	−	−
	O26:H$^-$ (2)[e]				20.45 (0.488)	−	−
	O45:H2 (1)[g]				20.18	−	−
	O85:H$^-$ (1)[e]				19.86	−	−
	O103:H2 (1)[e]				19.82	−	−
	O103:H6 (1)[e]				19.46	−	−
	O111:H11 (1)[f]				18.89	−	−
	O125:H$^-$ (2)[f,g]				19.68 (0.092)	−	−
	O126:H27 (1)[g]				18.66	−	−
	O146:H21 (2)[g]				19.63 (0.269)	−	−
	Strain with *stx1* insert (1)[a]				18.99	−	−
	O14:H19 (1)[g]	−	+	−	−	18.61	−
	O28:H35 (1)[h]				−	19.83	−
	O48:H21 (1)[h]				−	18.40	−
	O55:H7 (1)[f]				−	21.97	−
	O104:H21 (4)[d,g]				−	20.02 (0.247)	−
	O121:H19 (1)[g]				−	18.99	−
	O165:H25 (3)[e]				−	20.00 (0.442)	−
	Strain with *stx2* insert (1)[g]				−	19.10	−
Nontoxigenic *E. coli*	Non-O157:H7 (2)[g]	−	−	−	−	−	−
	O55:H7 (1)				−	−	−
	O157:H16 (3)[a]				−	−	−
	O157:H45 (1)				−	−	−
Shigella dysenteriae	(1)	+	−	−	21.39	−	−
Hafnia alvei	(2)	−	−	−	−	−	−
Morganella morganii	(1)	−	−	−	−	−	−
Citrobacter freundii	(2)	−	−	−	−	−	−
Leclercia adecarboxylata	(1)	−	−	−	−	−	−
Shigella sonnei	(1)[a]	−	−	−	−	−	−
Shigella boydii	(1)[a]	−	−	−	−	−	−
Shigella flexneri	(1)[a]	−	−	−	−	−	−
Salmonella group 30	(1)	−	−	−	−	−	−
Salmonella enterica serovar Lansing, group P	(1)	−	−	−	−	−	−
Klebsiella pneumoniae	(1)	−	−	−	−	−	−
Listeria monocytogenes	(1)	−	−	−	−	−	−
Listeria innocua	(1)	−	−	−	−	−	−
Listeria ivanovii	(1)	−	−	−	−	−	−
Listeria seeligeri	(1)	−	−	−	−	−	−
Listeria welshimeri	(1)	−	−	−	−	−	−
Vibrio cholerae	O1 Inaba (1)	−	−	−	−	−	−
Vibrio parahaemolyticus	O4 (1)	−	−	−	−	−	−
Vibrio vulnificus	(1)	−	−	−	−	−	−
Staphylococcus aureus	(1)	−	−	−	−	−	−
Rhodococcus equi	(1)	−	−	−	−	−	−
Lactobacillus sp.	(2)	−	−	−	−	−	−

Continued on facing page

TABLE 5—*Continued*

Organism	Serotype (no. of isolates tested)	Genotype			Avg Ct (SD)		
		stx1	*stx2*	*uidA* O157:H7/H$^-$	*stx1*	*stx2*	*uidA* O157:H7/H$^-$
Salmonella enterica serovar Typhimurium	(1)[a]	–	–	–	–	–	–
Streptococcus pyogenes	(1)	–	–	–	–	–	–
Alcaligenes faecalis	(1)	–	–	–	–	–	–
Salmonella enterica serovar Choleraesuis	(1)	–	–	–	–	–	–
Yersinia enterocolitica	(2)	–	–	–	–	–	–
Enterobacter cloacae	(1)	–	–	–	–	–	–
Avg[j]					19.51 (0.713)	19.32 (0.906)	23.56 (1.225)

[a] Isolate received from the Washington State Department of Health.
[b] Isolate received from the Oregon State Department of Health.
[c] Isolate received from the King County Public Health Department.
[d] Isolate received from the Montana Public Health Laboratory.
[e] Isolate received from Phil Tarr, Children's Hospital Seattle, Wash.
[f] Isolate received from Peter Feng, Center for Food Safety and Nutrition, Food and Drug Administration.
[g] Isolate received from Joy Wells, Centers for Disease Control and Prevention.
[h] Isolate received from Sharon Abbott, California Health Department.
[i] False-negative result obtained under these reaction conditions.
[j] Overall average Ct for positive isolates (n = 74, 76, and 54 for *stx1*, *stx2*, and *uidA*, respectively).
[k] +, positive; −, negative.

uidA O157 genotypes, respectively, based on 14 replicates. When the real-time PCR was run in a multiplex format, the average Ct value shifted only to 19.23 for *stx1*, 18.81 for *stx2*, and 22.69 for *uidA* O157 genotype, also based on 14 replicates.

The specificity of the *stx1* and *stx2* assay components of this real-time multiplex PCR is attributed to the specificity of the primers and probes for sequences present only in STEC strains. In contrast, the primers in the *uidA* O157:H7/H$^-$ assay are designed to amplify a 143-bp fragment of the *uidA* gene that occurs in nearly all *E. coli* strains. The O157:H7/H$^-$ specificity is conferred by the specificity of an internal MGB probe for the conserved SNP at position 93. For use in 5′ nuclease assays such as this one, the MGB is attached to the 3′ end of the probe. Because the specificity of the *uidA* MGB probe needs to be more tightly controlled than that of the *stx1* or *stx2* probe, the optimization of the entire multiplex assay is more contingent on the *uidA* assay requirements than the *stx1* or *stx2* components. The similar slopes observed for all three multiplex assay components (*stx1*, *stx2*, and *uidA*) indicate that the PCR efficiencies for all three are generally equivalent even in a multiplex format. The Ct value lag of 3 and the increased y intercept of the *uidA* component relative to those of *stx1* and *stx2* could be attributed more to differences in the annealing efficiency of the internal probes than to the PCR efficiency.

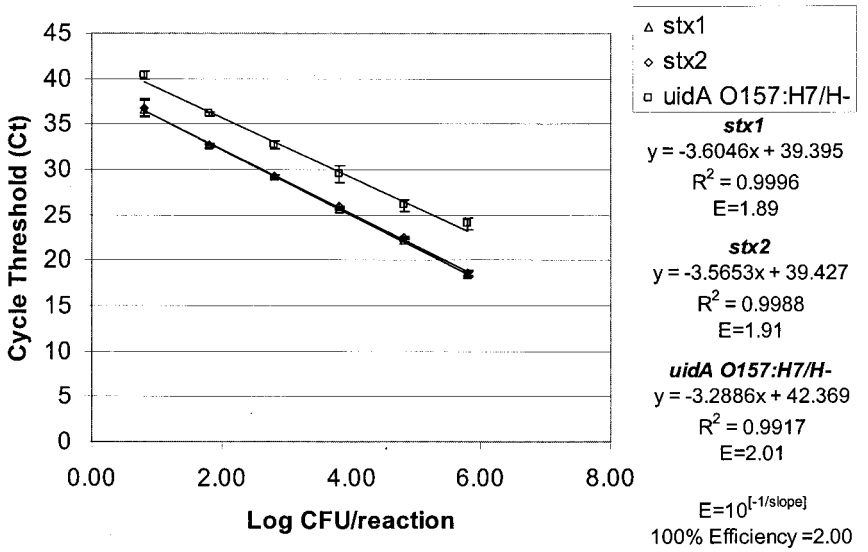

FIG. 1. Standard curve for the multiplex real-time PCR analysis for the *stx1* and *stx2* genes and the *E. coli* O157:H7/H$^-$ *uidA* SNP at position 93 tested with *E. coli* O157:H7 isolate EDL 933. The standard deviations are based on three PCR amplifications.

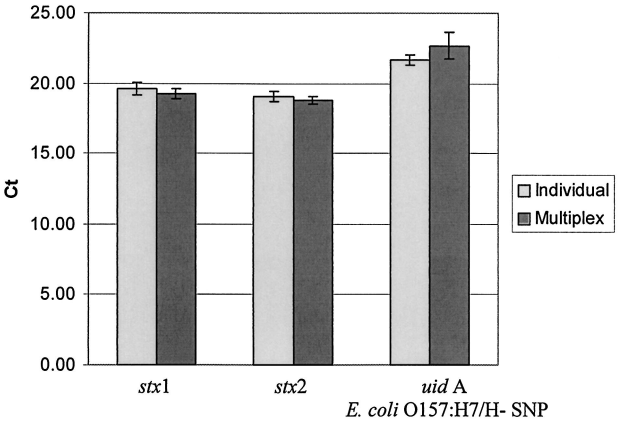

FIG. 2. Average Ct values for amplifications of *stx1*, *stx2*, and the *E. coli* O157:H7/H$^-$ *uidA* SNP at position 93 for reactions run individually and in multiplex format for *E. coli* O157:H7 strain EDL 933, based on 14 replicates.

Other real-time PCR STEC methods have focused primarily only on detection of the *stx1* and *stx2* genes (3, 4, 17, 29, 31, 32). Some real-time PCR assays may include additional components to identify EHEC by targeting the intimin (*eaeA*), enterohemolysin (E-*hly*), and O-antigen (*rfbE*) genes (14, 16, 25, 31, 32). This is the first multiplex real-time PCR method to specifically target the highly conserved SNP at position 93 of *E. coli* O157:H7/H$^-$ *uidA* and the *stx1* and *stx2* genes. Real-time PCR methods for detection of STEC and EHEC use a variety of fluorogenic detection approaches, including the use of SYBR green (Molecular Probes, Inc., Eugene, Oreg.) and melt curve analyses (17), 5′ nuclease assay probes (16, 25, 31, 32), FRET hybridization with melt curves to distinguish *stx2* and *stx2e* (4, 29), and molecular beacons (3, 14). Several instrument platforms, including Light Cycler (4, 17, 29), Smart Cycler (3), ABI Prism (14, 25, 32), and the I-Cycler (16), have been used to support these assays. This multiplex assay uses fluorogenic probes in a 5′ nuclease assay format and was optimized on a Smart Cycler instrument. Overall, this multiplex real-time PCR method can be used for the rapid detection of all STEC strains and other Shiga toxin-producing bacteria by targeting the *stx1*, *stx2*, and variant genes as well as providing specific identification of the O157:H7/H$^-$ serotype, the predominant STEC serotype associated with human illness.

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