



Laboratory Guidebook Notice of Change

Chapter **new**, revised, or archived: MLG 5C Appendix 4.00

Title: Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-Time PCR Assay

Effective Date: 02/04/2019

Description and purpose of change(s):

This Appendix lists the primer and probe sequences and reagent concentrations for the Real-Time PCR Assay in MLG 5C Appendix 3 PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay

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Revision: Original	Replaces: NA	Effective: 02/04/2019

Procedure Outline

A4 5C.1 Sequences for non-O157 STEC Primers and Probes for Use on ABI® 7500 FAST

A4 5C.1.1 Primer Sequences

A4 5C.1.2 Probe Sequences

A4 5C.2 Real-time PCR Assay Reagent Concentrations

A4 5C.2.1 PCR Assay for *stx* and *eae* Genes

A4 5C.2.2 PCR Assay for Serogroup-specific Genes from O antigen Gene Cluster

A4 5C.2.2.1 Serogroup-specific PCR Assay for O26 and O111

A4 5C.2.2.2 Serogroup-specific PCR Assay for O45 and O121

A4 5C.2.2.3 Serogroup-specific PCR Assay for O103 and O145

A4 5C.3 Reference Tables for Component Volumes of Real-time PCR Primers/Probes Master Mixes for Use in FSIS Field Service Laboratories

A4 5C.1 Sequences for non-O157 STEC Primers and Probes for Use on ABI® 7500 FAST

A4 5C.1.1 Primer Sequences

Primers and probes are obtained from Integrated DNA Technologies (Coraville, Iowa).

Note: Stx and Eae primers contain degenerate nucleotides at several positions (key is listed below the sequences)

Stx F (Forward) 5' TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3'

Stx R (Reverse) 5' CCC CAG TTC ARW GTR AGR TCM ACD TC 3'

Eae F (Forward) 5' CAT TGA TCA GGA TTT TTC TGG TGA TA 3'

EaeR (Reverse) 5' CTC ATG CGG AAA TAG CCG TTM 3'

Mixed nucleotide key: Y (C,T), W (A,T), R (A,G), M (A,C), D (A,G,T)

16SRna-F (Forward) – 5' CCT CTT GCC ATC GGA TGT G 3'

16SRna-R (Reverse) – 5' GGC TGG TCA TCC TCT CAG ACC 3'

Wzx O26-F (Forward) – 5' GTA TCG CTG AAA TTA GAA GCG C 3'

Wzx O26-R (Reverse) – 5' AGT TGA AAC ACC CGT AAT GGC 3'

Wzx O45-F (Forward) – 5' CGT TGT GCA TGG TGG CAT 3'

Wzx O45-R (Reverse) – 5' TGG CCA AAC CAA CTA TGA ACT G 3'

Wzx O103-F (Forward) – 5' TTG GAG CGT TAA CTG GAC CT 3'

Wzx O103-R (Reverse) – 5' ATA TTC GCT ATA TCT TCT TGC GGC 3'

WbdI O111-F (Forward) – 5' TGT TCC AGG TGG TAG GAT TCG 3'

WbdI O111-R (Reverse) – 5' TCA CGA TGT TGA TCA TCT GGG 3'

Wzx O121-F (Forward) – 5' AGG CGC TGT TTG GTC TCT TAG A 3'

Wzx O121-R (Reverse) – 5' GAA CCG AAA TGA TGG GTG CT 3'

Wzx O145-F (Forward) – 5' AAA CTG GGA TTG GAC GTG G 3'

Wzx O145-R (Reverse) – 5' CCC AAA ACT TCT AGG CCC G 3'

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A4 5C.1.2 Probe Sequences

Stx1 Probe 5' 56-FAM-CTG GAT GAT/zen/CTC AGT GGG CGT TCT TAT GTA A-3IABkFQ 3'
Stx2 Probe 5' 56-FAM-TCG TCA GGC/zen/ACT GTC TGA AAC TGC TCC-3IABkFQ 3'
Eae Probe 5' 5MAXN-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC-IABkFQ 3'
16S rRNA Probe 5' 5TYE665-GTG GGG TAA CGG CTC ACC TAG GCG AC-3IABrQSp 3'
Wzx O26-P – 5' 56-FAM-TGG TTC GGT TGG ATT GTC CAT AAG AGG G-3BHQ_1 3'
Wzx O45-P – 5' 56-FAM- ATT TTT TGC TGC AAG TGG GCT GTC CA-3BHQ_1 3'
Wzx O103-P – 5' 5MAXN- AGG CTT ATC TGG CTG TTC TTA CTA CGG C-3IABkFQ 3'
WbdI O111-P – 5' 5MAXN - TGA AGG CGA GGC AAC ACA TTA TAT AGT GC- 3IABkFQ 3'
Wzx O121-P – 5' 5MAXN - CGC TAT CAT GGC GGG ACA ATG ACA GTG C- 3IABkFQ 3'
Wzx O145-P – 5' 56-FAM- TGC TAA TTG CAG CCC TTG CAC TAC GAG GC -3BHQ_1 3'

A4 5C.2 Real-time PCR Assay Reagent Concentrations

The Real-time PCR assay described in this method has been optimized and validated specifically for the ABI® 7500 FAST. Use of other Real-time PCR platforms may require optimization with other probe quencher and reporter dyes. Additionally, the ABI® Environmental Master Mix has been validated and optimized for use on the ABI® 7500 FAST and would require additional optimization with reagent volumes and assay cycling parameters if using other Real-time PCR platforms. If different stock concentrations of any reagents are desired, it will be necessary to recalculate the volume of that reagent in the master mix preparation. Note: PCR assays were validated and are to be performed using Standard Chemistry on the ABI® 7500 FAST, not the Fast Chemistry. The ABI® 7500 FAST PCR assay parameters for all reactions (*stx/eae* and O antigen gene cluster) are as follows:

1 cycle

95°C for 10 minutes

45 cycles

95°C for 15 seconds

59°C for 1 minute

A4 5C.2.1 PCR Assay for *stx* and *eae* Genes

Master mix volume per well is 20.0 µl, and the DNA template volume for each PCR assay is 5.0 µl.

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Desired Reaction vol (µL) 25
 Desired number of rxns 1
 total final volume (µL) 25

	µL	[Final]	Units	[Stock]
ABI® Environmental Master Mix	12.50	1	x	2
Primer 16S rRNA F	0.20	0.16	µM	20
Primer 16S rRNA R	0.20	0.16	µM	20
Primer Eae-F	0.50	1	µM	50
Primer Eae-R	0.50	1	µM	50
Primer Stx F	0.63	1.25	µM	50
Primer Stx R	0.63	1.25	µM	50
Probe 16S rRNA P	0.50	0.1	µM	5
Probe Eae P	1.00	0.2	µM	5
Probe Stx1 P	1.25	0.25	µM	5
Probe Stx2 P	1.25	0.25	µM	5
dH2O	0.85			

A4 5C.2.2 PCR Assay for Serogroup-specific Genes from O antigen Gene Cluster

DNA template volume for each PCR assay is 5.0 µL.

A4 5C.2.2.1 Serogroup-specific PCR Assay for O26 and O111

	µL	[Final]	Units	[Stock]
ABI® Environmental Master Mix	12.50	1	x	2
Primer 16S rRNA F	0.20	0.16	µM	20
Primer 16S rRNA R	0.20	0.16	µM	20
Primer Wzx O26 F	0.31	0.25	µM	20
Primer Wzx O26 R	0.31	0.25	µM	20
Primer Wbdl O111 F	0.31	0.25	µM	20
Primer Wbdl O111 R	0.31	0.25	µM	20
Probe 16S rRNA P	0.50	0.1	µM	5
Probe Wzx O26 P	1.88	0.15	µM	2
Probe Wbdl O111 P	1.00	0.2	µM	5
dH2O	2.48			

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A4 5C.2.2.2 Serogroup-specific PCR Assay for O45 and O121

	μL	[Final]	Units	[Stock]
ABI® Environmental Master Mix	12.50	1	x	2
Primer 16S rRNA F	0.20	0.16	μM	20
Primer 16S rRNA R	0.20	0.16	μM	20
Primer Wzx O45 F	0.31	0.25	μM	20
Primer Wzx O45 R	0.31	0.25	μM	20
Primer Wzx O121 F	0.31	0.25	μM	20
Primer Wzx O121 R	0.31	0.25	μM	20
Probe 16S rRNA P	0.50	0.1	μM	5
Probe Wzx O45 P	2.34	0.1875	μM	2
Probe O121 P	1.00	0.2	μM	5
dH2O	2.01			

A4 5C.2.2.3 Serogroup-specific PCR Assay for O103 and O145

	μL	[Final]	Units	[Stock]
ABI® Environmental Master Mix	12.50	1	x	2
Primer 16S rRNA F	0.20	0.16	μM	20
Primer 16S rRNA R	0.20	0.16	μM	20
Primer Wzx O103 F	0.31	0.25	μM	20
Primer Wzx O103 R	0.31	0.25	μM	20
Primer Wzx O145 F	0.31	0.25	μM	20
Primer Wzx O145 R	0.31	0.25	μM	20
Probe 16S rRNA P	0.50	0.1	μM	5
Probe Wzx O103 P	1.00	0.2	μM	5
Probe Wzx O145 P	2.50	0.2	μM	2
dH2O	1.85			

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**A4 5C.3 Reference Tables for Component Volumes of Real-time PCR Primers/Probes
Master Mix for Use in FSIS Field Service Laboratories**

Table 1. Calculation Table for stx/eah Master Mix

Calculation Table for stx/eah Master Mix							
# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)	# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)
5	62.50	33.25	4.25	30	375.00	199.50	25.50
6	75.00	39.90	5.10	31	387.50	206.15	26.35
7	87.50	46.55	5.95	32	400.00	212.80	27.20
8	100.00	53.20	6.80	33	412.50	219.45	28.05
9	112.50	59.85	7.65	34	425.00	226.10	28.90
10	125.00	66.50	8.50	35	437.50	232.75	29.75
11	137.50	73.15	9.35	36	450.00	239.40	30.60
12	150.00	79.80	10.20	37	462.50	246.05	31.45
13	162.50	86.45	11.05	38	475.00	252.70	32.30
14	175.00	93.10	11.90	39	487.50	259.35	33.15
15	187.50	99.75	12.75	40	500.00	266.00	34.00
16	200.00	106.40	13.60	41	512.50	272.65	34.85
17	212.50	113.05	14.45	42	525.00	279.30	35.70
18	225.00	119.70	15.30	43	537.50	285.95	36.55
19	237.50	126.35	16.15	44	550.00	292.60	37.40
20	250.00	133.00	17.00	45	562.50	299.25	38.25
21	262.50	139.65	17.85	46	575.00	305.90	39.10
22	275.00	146.30	18.70	47	587.50	312.55	39.95
23	287.50	152.95	19.55	48	600.00	319.20	40.80
24	300.00	159.60	20.40	49	612.50	325.85	41.65
25	312.50	166.25	21.25	50	625.00	332.50	42.50
26	325.00	172.90	22.10	51	637.50	339.15	43.35
27	337.50	179.55	22.95	52	650.00	345.80	44.20
28	350.00	186.20	23.80	53	662.50	352.45	45.05
29	362.50	192.85	24.65	54	675.00	359.10	45.90

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Table 2. Calculation Table for O26/O111 Master Mix

Calculation Table for O26/O111 Master Mix							
# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)	# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)
5	62.50	25.13	12.38	30	375.00	150.75	74.25
6	75.00	30.15	14.85	31	387.50	155.78	76.73
7	87.50	35.18	17.33	32	400.00	160.80	79.20
8	100.00	40.20	19.80	33	412.50	165.83	81.68
9	112.50	45.23	22.28	34	425.00	170.85	84.15
10	125.00	50.25	24.75	35	437.50	175.88	86.63
11	137.50	55.28	27.23	36	450.00	180.90	89.10
12	150.00	60.30	29.70	37	462.50	185.93	91.57
13	162.50	65.33	32.18	38	475.00	190.95	94.05
14	175.00	70.35	34.65	39	487.50	195.98	96.52
15	187.50	75.38	37.13	40	500.00	201.00	99.00
16	200.00	80.40	39.60	41	512.50	206.03	101.48
17	212.50	85.43	42.08	42	525.00	211.05	103.95
18	225.00	90.45	44.55	43	537.50	216.08	106.43
19	237.50	95.48	47.03	44	550.00	221.10	108.90
20	250.00	100.50	49.50	45	562.50	226.13	111.38
21	262.50	105.53	51.98	46	575.00	231.15	113.85
22	275.00	110.55	54.45	47	587.50	236.18	116.33
23	287.50	115.58	56.93	48	600.00	241.20	118.80
24	300.00	120.60	59.40	49	612.50	246.23	121.28
25	312.50	125.63	61.88	50	625.00	251.25	123.75
26	325.00	130.65	64.35	51	637.50	256.28	126.23
27	337.50	135.68	66.83	52	650.00	261.30	128.70
28	350.00	140.70	69.30	53	662.50	266.33	131.18
29	362.50	145.73	71.78	54	675.00	271.35	133.65

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Table 3. Calculation Table for O45/O121 Master Mix

Calculation Table for O45/O121 Master Mix							
# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)	# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)
5	62.50	27.47	10.03	30	375.00	164.81	60.19
6	75.00	32.96	12.04	31	387.50	170.31	62.19
7	87.50	38.46	14.04	32	400.00	175.80	64.20
8	100.00	43.95	16.05	33	412.50	181.29	66.21
9	112.50	49.44	18.06	34	425.00	186.79	68.21
10	125.00	54.94	20.06	35	437.50	192.28	70.22
11	137.50	60.43	22.07	36	450.00	197.78	72.22
12	150.00	65.93	24.08	37	462.50	203.27	74.23
13	162.50	71.42	26.08	38	475.00	208.76	76.24
14	175.00	76.91	28.09	39	487.50	214.26	78.24
15	187.50	82.41	30.09	40	500.00	219.75	80.25
16	200.00	87.90	32.10	41	512.50	225.24	82.26
17	212.50	93.39	34.11	42	525.00	230.74	84.26
18	225.00	98.89	36.11	43	537.50	236.23	86.27
19	237.50	104.38	38.12	44	550.00	241.73	88.28
20	250.00	109.88	40.13	45	562.50	247.22	90.28
21	262.50	115.37	42.13	46	575.00	252.71	92.29
22	275.00	120.86	44.14	47	587.50	258.21	94.29
23	287.50	126.36	46.14	48	600.00	263.70	96.30
24	300.00	131.85	48.15	49	612.50	269.19	98.31
25	312.50	137.34	50.16	50	625.00	274.69	100.31
26	325.00	142.84	52.16	51	637.50	280.18	102.32
27	337.50	148.33	54.17	52	650.00	285.68	104.33
28	350.00	153.83	56.18	53	662.50	291.17	106.33
29	362.50	159.32	58.18	54	675.00	296.66	108.34

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Table 4. Calculation Table for O103/O145 Master Mix

Calculation Table for O103/O145 Master Mix							
# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)	# of Reactions	Volume of environmental master mix (µl)	Volume of primer/probe mix (µl)	Volume of water (µl)
5	62.50	28.25	9.25	30	375.00	169.50	55.50
6	75.00	33.90	11.10	31	387.50	175.15	57.35
7	87.50	39.55	12.95	32	400.00	180.80	59.20
8	100.00	45.20	14.80	33	412.50	186.45	61.05
9	112.50	50.85	16.65	34	425.00	192.10	62.90
10	125.00	56.50	18.50	35	437.50	197.75	64.75
11	137.50	62.15	20.35	36	450.00	203.40	66.60
12	150.00	67.80	22.20	37	462.50	209.05	68.45
13	162.50	73.45	24.05	38	475.00	214.70	70.30
14	175.00	79.10	25.90	39	487.50	220.35	72.15
15	187.50	84.75	27.75	40	500.00	226.00	74.00
16	200.00	90.40	29.60	41	512.50	231.65	75.85
17	212.50	96.05	31.45	42	525.00	237.30	77.70
18	225.0	101.7	33.3	43	537.50	242.95	79.55
19	237.5	107.4	35.2	44	550.00	248.60	81.40
20	250.0	113.0	37.0	45	562.50	254.25	83.25
21	262.5	118.7	38.9	46	575.00	259.90	85.10
22	275.0	124.3	40.7	47	587.50	265.55	86.95
23	287.5	130.0	42.6	48	600.00	271.20	88.80
24	300.0	135.6	44.4	49	612.50	276.85	90.65
25	312.5	141.3	46.3	50	625.00	282.50	92.50
26	325.00	146.90	48.10	51	637.50	288.15	94.35
27	337.50	152.55	49.95	52	650.00	293.80	96.20
28	350.00	158.20	51.80	53	662.50	299.45	98.05
29	362.50	163.85	53.65	54	675.00	305.10	99.90