



Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG 5B Appendix 1.01

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

Effective Date: 6/4/12

Description and purpose of change(s):

The following information was incorporated:

- a) The name of the gene target for the primers and probes for *E. coli* O111 has been corrected from *wzx* to *wbdI*. However, no changes were made in the sequences for the primers or probe.
- b) The method's required PCR platform was described in more detail.
- c) Reference tables for Component Volumes of Real-time PCR Master Mix using pre-mixed primers and probes were added for use in the FSIS Field Service Laboratories.

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use. Method validation is necessary to demonstrate the equivalence of alternative tests. FSIS provides guidance at: http://www.fsis.usda.gov/PDF/Validation_Studies_Pathogen_Detection_Methods.pdf

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Procedure Outline

- A5B.1 Sequences for non-O157 STEC Primers and Probes for Use on ABI® 7500 FAST
 - A5B.1.1 Primer Sequences
 - A5B.1.2 Probe Sequences
- A5B.2 Real-time PCR Assay Reagent Concentrations
 - A5B.2.1 PCR Assay for *stx* and *eae* Genes
 - A5B.2.2 PCR Assay for Serogroup-specific Genes from O antigen Gene Cluster
 - A5B.2.2.1 Serogroup-specific PCR Assay for O26 and O111
 - A5B.2.2.2 Serogroup-specific PCR Assay for O45 and O121
 - A5B.2.2.3 Serogroup-specific PCR Assay for O103 and O145
- A5B.3 Reference Tables for Component Volumes of Real-time PCR Primers/Probes Master Mixes for Use in FSIS Field Service Laboratories

A5B.1 Sequences for non-O157 STEC Primers and Probes for Use on ABI® 7500 FAST

A5B.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'

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

A5B.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'

A5B.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

A5B.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 | | | |

A5B.2.2 PCR Assay for Serogroup-specific Genes from O antigen Gene Cluster

DNA template volume for each PCR assay is 5.0 μL.

A5B.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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A5B.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 | | | |

A5B.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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A5B.3 Reference Tables for Component Volumes of Real-time PCR Master Mix for Use in FSIS Field Service Laboratories

Table A1. Calculation Table for stx/ae Master Mix

| Calculation Table for stx/ae Master Mix | | | | | | | |
|---|---|---------------------------------|----------------------|----------------|---|---------------------------------|----------------------|
| # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) | # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) |
| 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 A2. Calculation Table for O26/O111 Master Mix

| Calculation Table for O26/O111 Master Mix | | | | | | | |
|---|---|---------------------------------|----------------------|----------------|---|---------------------------------|----------------------|
| # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) | # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) |
| 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 A3. Calculation Table for O45/O121 Master Mix

| Calculation Table for O45/O121 Master Mix | | | | | | | |
|---|---|---------------------------------|----------------------|----------------|---|---------------------------------|----------------------|
| # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) | # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) |
| 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 A4. Calculation Table for O103/O145 Master Mix

| Calculation Table for O103/O145 Master Mix | | | | | | | |
|--|---|---------------------------------|----------------------|----------------|---|---------------------------------|----------------------|
| # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) | # of Reactions | Volume of environmental master mix (ul) | Volume of primer/probe mix (ul) | Volume of water (ul) |
| 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 |