Multiplex real-time PCR assay for detection of *Escherichia coli* O157:H7 and screening for non-O157 Shiga toxin-producing *E. coli*

Baoguang Li¹*, Huanli Liu² and Weimin Wang¹

**Abstract**

**Background:** Shiga toxin-producing *Escherichia coli* (STEC), including *E. coli* O157:H7, are responsible for numerous foodborne outbreaks annually worldwide. *E. coli* O157:H7, as well as pathogenic non-O157:H7 STECs, can cause life-threatening complications, such as bloody diarrhea (hemolytic colitis) and hemolytic-uremic syndrome (HUS). Previously, we developed a real-time PCR assay to detect *E. coli* O157:H7 in foods by targeting a unique putative fimbriae protein Z3276. To extend the detection spectrum of the assay, we report a multiplex real-time PCR assay to specifically detect *E. coli* O157:H7 and screen for non-O157 STEC by targeting Z3276 and Shiga toxin genes (stx1 and stx2). Also, an internal amplification control (IAC) was incorporated into the assay to monitor the amplification efficiency.

**Methods:** The multiplex real-time PCR assay was developed using the Life Technology ABI 7500 System platform and the standard chemistry. The optimal amplification mixture of the assay contains 12.5 μl of 2 × Universal Master Mix (Life Technology), 200 nM forward and reverse primers, appropriate concentrations of four probes ([Z3276 (80 nM), stx1 (80 nM), stx2 (20 nM), and IAC (40 nM)], 2 μl of template DNA, and water (to make up to 25 μl in total volume). The amplification conditions of the assay were set as follows: activation of TaqMan at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 60 s.

**Results:** The multiplex assay was optimized for amplification conditions. The limit of detection (LOD) for the multiplex assay was determined to be 200 fg of bacterial DNA, which is equivalent to 40 CFU per reaction which is similar to the LOD generated in single targeted PCRs. Inclusivity and exclusivity determinants were performed with 196 bacterial strains. All *E. coli* O157:H7 (n = 135) were detected as positive and all STEC strains (n = 33) were positive for stx1, or stx2, or stx1 and stx2 (Table 1). No cross reactivity was detected with *Salmonella enterica*, *Shigella* strains, or any other pathogenic strains tested.

**Conclusions:** A multiplex real-time PCR assay that can rapidly and simultaneously detect *E. coli* O157:H7 and screen for non-O157 STEC strains has been developed and assessed for efficacy. The inclusivity and exclusivity tests demonstrated high sensitivity and specificity of the multiplex real-time PCR assay. In addition, this multiplex assay was shown to be effective for the detection of *E. coli* O157:H7 from two common food matrices, beef and spinach, and may be applied for detection of *E. coli* O157:H7 and screening for non-O157 STEC strains from other food matrices as well.

**Keywords:** Multiplex real-time PCR, *Escherichia coli* O157:H7, Shiga toxins (stx1, stx2), Shiga toxin-producing *E. coli* (STEC), non-O157, Limit of detection (LOD), Pathogen detection, Sensitivity

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*Correspondence: baoguang.li@fda.hhs.gov
1Division of Molecular Biology, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD 20708, USA

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Table 1 Results of *E. coli* O157:H7 and non-O157 STEC strains detected by the multiplex real-time PCR assay

| Strain     | Serotype | Source                        | Target gene |
|------------|----------|-------------------------------|-------------|
|            |          |                               | Z3276  | stx1 | stx2 |
| EC1275     | O157:H7  | CDC EDL933, hamburger meat    | +   | +   | +   |
| EC1225     | O157:H7  | WA, 1993                      | +   | +   | +   |
| EC1759     | O157:H7  | USA (MI), 2003                | +   | +   | +   |
| EC1429     | O157:H7  | Denmark, 1987                 | +   | -   | +   |
| EC4420     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4421     | O157:H7  | White Onions, NY, 12/2006     | +   | +   | +   |
| EC4422     | O157:H7  | White Onions, NY, 12/2006     | +   | +   | +   |
| EC4504     | O157:H7  | MN, 12/2006                   | +   | +   | +   |
| EC1428     | O157:H7  | Argentina, 1977               | +   | -   | +   |
| EC1738     | O157:H7  | Food isolate                  | +   | -   | +   |
| EC1530     | O157:H7  | Thailand, 1994                | +   | -   | +   |
| EC4440     | O157:H7  | CDC                           | +   | +   | +   |
| EC4163     | O157:H7  | USA (IL)                      | +   | -   | +   |
| EC4438     | O157:H7  | CDC                           | +   | -   | +   |
| EC4431     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4452     | O157:H7  | USA (NJ), 2006                | +   | -   | +   |
| EC1709     | O157:H7  | USA (MI), 2002                | +   | -   | +   |
| EC1760     | O157:H7  | USA (MI), 2004                | +   | -   | +   |
| EC1431     | O157:H7  | Japan, 1987                   | +   | -   | +   |
| EC4115     | O157:H7  | Sep-06                        | +   | -   | +   |
| EC1734     | O157:H7  | MA, 2009                      | +   | -   | +   |
| EC4302     | O157:H7  | NY, 12/2006                   | +   | +   | +   |
| EC4301     | O157:H7  | NY, 12/2006                   | +   | +   | +   |
| EC4429     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4428     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4437     | O157:H7  | NJ, 12/2006                   | +   | -   | +   |
| EC4436     | O157:H7  | NJ, 12/2006                   | +   | -   | +   |
| EC4434     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4433     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4432     | O157:H7  | NY, 12/2006                   | +   | -   | +   |
| EC4449     | O157:H7  | NJ, 12/2006                   | +   | -   | +   |
| EC4448     | O157:H7  | WI, 12/2006                   | +   | -   | +   |
| EC4447     | O157:H7  | CA, 12/2006                   | +   | +   | +   |
| EC4201     | O157:H7  | USA (CA), 2006                | +   | -   | +   |
| EC4456     | O157:H7  | USA (NJ), 2006                | +   | -   | +   |
| EC4461     | O157:H7  | USA (NJ), 2006                | +   | -   | +   |
| EC4502     | O157:H7  | USA (NJ), 2006                | +   | +   | +   |
| EC4503     | O157:H7  | USA (NJ), 2006                | +   | +   | +   |
| EC4505     | O157:H7  | USA (NJ), 2006                | +   | +   | +   |
| EC4511     | O157:H7  | USA (NJ), 2006                | +   | +   | +   |
| EC4200     | O157:H7  | USA (CA), 2006                | +   | -   | +   |
| EC1267     | O157:H7  | USA, (NH), 1991               | +   | +   | +   |
| EC1268     | O157:H7  | USA (MT), 1991                | +   | +   | +   |
| Strain  | Serotype | Source                  | Target gene |
|---------|----------|-------------------------|-------------|
|         |          |                         | Z3276 | stx1 | stx2 |
| EC4194  | O157:H7  | USA (CA), 2006          | +     | -    | +    |
| EC1590  | O157:H7  | USA (WA)                | +     | +    | +    |
| EC4458  | O157:H7  | USA (NJ), 2006          | +     | -    | +    |
| EC4197  | O157:H7  | USA (CA), 2006          | +     | -    | +    |
| EC1231  | O157:H7  | USA (WA), 1995          | +     | +    | +    |
| EC4470  | O157:H7  | USA (NJ), 2006          | +     | -    | +    |
| EC4471  | O157:H7  | USA (NJ), 2006          | +     | -    | +    |
| EC1244  | O157:H7  | USA (CA), 1992          | +     | +    | +    |
| EC4199  | O157:H7  | USA (CA), 2006          | +     | -    | +    |
| EC1260  | O157:H7  | USA (GA), 1993          | +     | -    | +    |
| EC4167  | O157:H7  | USA (WI), 2006          | +     | -    | +    |
| EC4168  | O157:H7  | USA (WI), 2006          | +     | -    | +    |
| EC4193  | O157:H7  | USA (OH), 2006          | +     | -    | +    |
| EC1265  | O157:H7  | USA (CA), 1993          | +     | +    | +    |
| EC4184  | O157:H7  | USA (IL), 2006          | +     | -    | +    |
| EC4204  | O157:H7  | USA (CA), 2006          | +     | -    | +    |
| EC4171  | O157:H7  | USA (WI), 2006          | +     | -    | +    |
| EC4205  | O157:H7  | USA (CA), 2006          | +     | -    | +    |
| EC4206  | O157:H7  | USA (CA), 2006          | +     | -    | +    |
| EC1593  | O157:H7  | USA (MI), 2003          | +     | +    | +    |
| EC1239  | O157:H7  | USA (NE), 1993          | +     | -    | +    |
| EC4174  | O157:H7  | USA (IL), 2006          | +     | -    | +    |
| EC4187  | O157:H7  | USA (CT), 2006          | +     | -    | +    |
| EC4188  | O157:H7  | USA (CT), 2006          | +     | -    | +    |
| EC4191  | O157:H7  | Spinach, USA (IL), 2006 | +     | -    | +    |
| EC1276  | O157:H7  | Japan, 1996             | +     | +    | +    |
| EC4501  | O157:H7  | MN, 12/2006             | +     | +    | +    |
| EC558   | O157:H7  | Patient raw milk        | +     | +    | +    |
| EC867   | O157:H7  | USDA-FSIS-380-94        | +     | +    | +    |
| EC874   | O157:H7  | Apple cider             | +     | +    | +    |
| EC4162  | O157:H7  | Feces (New Jersey)      | +     | -    | +    |
| EC506   | O157:H7  | Feces                   | +     | -    | +    |
| EC507   | O157:H7  | Feces                   | +     | +    | +    |
| EC4443  | O157:H7  | CDC                     | +     | +    | +    |
| EC4442  | O157:H7  | CDC                     | +     | +    | +    |
| EC4441  | O157:H7  | CDC                     | +     | +    | +    |
| EC4451  | O157:H7  | NJ, 12/2006             | +     | -    | +    |
| EC4445  | O157:H7  | NJ, 12/2006             | +     | +    | +    |
| EC4446  | O157:H7  | NJ, 12/2006             | +     | +    | +    |
| EC1601  | O157:H7  | USA (MI), 2002          | +     | +    | +    |
| EC1727  | O157:H7  | MI, 2002                | +     | +    | +    |
| EC1426  | O157:H7  | Canada, 1988            | +     | -    | +    |
| EC4419  | O157:H7  | NY, 12/2006             | +     | -    | +    |
Table 1 Results of *E. coli* O157:H7 and non-O157 STEC strains detected by the multiplex real-time PCR assay (Continued)

| Strain   | Serotype | Source         | Target gene |
|----------|----------|----------------|-------------|
|          |          |                | Z5276 | stx1 | stx2 |
| EC4418   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4417   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4416   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4423   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4424   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4427   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4426   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4435   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4425   | O157:H7  | NY, 12/2006    | +     | -    | +    |
| EC4164   | O157:H7  | USA (WI), 2006 | +     | -    | +    |
| EC4439   | O157:H7  | MS, 12/2006    | +     | -    | +    |
| EC4450   | O157:H7  | NJ, 12/2006    | +     | -    | +    |
| EC1217   | O157:H7  | 2003           | +     | +    | +    |
| EC1577   | O157:H7  | USA (WA), 1995 | +     | +    | +    |
| EC4201   | O157:H7  | USA (CA), 2006 | +     | -    | +    |
| EC4207   | O157:H7  | USA (IL), 2006 | +     | -    | +    |
| EC4208   | O157:H7  | Spinach, USA (IL), 2006 | + | - | + |
| EC1245   | O157:H7  | USA (GA), 1995 | +     | +    | +    |
| EC4506   | O157:H7  | USA (MN), 2006 | +     | +    | +    |
| EC4507   | O157:H7  | USA (MN), 2006 | +     | +    | +    |
| EC4508   | O157:H7  | USA (MN), 2006 | +     | +    | +    |
| EC4509   | O157:H7  | USA (MN), 2006 | +     | +    | +    |
| EC4510   | O157:H7  | USA (MN), 2006 | +     | +    | +    |
| EC1590   | O157:H7  | USA (WA)       | +     | +    | +    |
| EC1597   | O157:H7  | USA (CT), 1996 | +     | +    | +    |
| EC1225   | O157:H7  | USA (WA), 1993 | +     | +    | +    |
| EC1236   | O157:H7  | Food, USA (CA), 1993 | + | + | + |
| EC1240   | O157:H7  | USA (OH), 1993 | +     | +    | +    |
| EC1241   | O157:H7  | Food, USA (OR), 1995 | + | + | + |
| EC4472   | O157:H7  | USA, (NJ), 2006 | +     | -    | +    |
| EC1242   | O157:H7  | USA (GA), 1992 | +     | -    | +    |
| EC1243   | O157:H7  | USA (GA), 1992 | +     | +    | +    |
| EC4463   | O157:H7  | USA (NJ), 2006 | +     | -    | +    |
| EC4165   | O157:H7  | USA (WI), 2006 | +     | -    | +    |
| EC4166   | O157:H7  | USA (WI), 2006 | +     | -    | +    |
| EC4195   | O157:H7  | Spinach, USA (OH), 2006 | + | - | + |
| EC4192   | O157:H7  | USA (CA), 2006 | +     | -    | +    |
| EC4182   | O157:H7  | USA (IL), 2006 | +     | -    | +    |
| EC4469   | O157:H7  | USA (NJ), 2006 | +     | -    | +    |
| EC4465   | O157:H7  | USA (NJ), 2006 | +     | -    | +    |
| EC4170   | O157:H7  | USA (WI), 2006 | +     | -    | +    |
| EC4183   | O157:H7  | USA (IL), 2006 | +     | -    | +    |
| EC4186   | O157:H7  | USA (IL), 2006 | +     | -    | +    |
**Table 1** Results of *E. coli* O157:H7 and non-O157 STEC strains detected by the multiplex real-time PCR assay (Continued)

| Strain | Serotype | Source | Target gene |
|--------|----------|--------|-------------|
| Z3276  | +        | -      | +           |
| EC4169 | O157:H7  | USA (W0), 2006 | + | - | + |
| EC4189 | O157:H7  | USA (CT), 2006 | + | - | + |
| EC4175 | O157:H7  | USA (IL), 2006 | + | - | + |
| EC4176 | O157:H7  | USA (IL), 2006 | + | - | + |
| EC4444 | O157:H7  | USA, NJ, 2006 | + | + | + |
| EC4173 | O157:H7  | USA (IL), 2006 | + | - | + |
| EC1892 | O104:H4  | STEC, Republic of Georgia, 2011 | - | - | + |
| EC1893 | O104:H4  | STEC, Republic of Georgia, 2011 | - | - | + |
| EC1891 | O104:H4  | STEC, Germany, 2011 | - | - | + |
| EC1894 | O104:H4  | STEC, Germany, 2011 | - | - | + |
| EC1769 | O26      | STEC | - | + | - |
| EC1770 | O26      | STEC | - | + | - |
| EC1771 | O26      | STEC | - | + | - |
| EC1773 | O26:H11  | STEC | - | + | - |
| EC1775 | O26:H11  | STEC | - | + | - |
| EC1768 | O26:H2   | STEC | - | + | - |
| EC1786 | O111:NM  | STEC | - | + | + |
| EC1787 | O111:H8  | STEC | - | + | + |
| EC1788 | O111:NM  | STEC | - | + | - |
| EC1791 | O145:H25 | STEC | - | - | + |
| EC1794 | O145:NM  | STEC | - | + | + |
| EC1801 | O103:H2  | STEC | - | + | - |
| EC1802 | O103:H25 | STEC | - | + | - |
| EC1803 | O103:H11 | STEC | - | + | + |
| EC1806 | O121:H19 | STEC | - | - | + |
| EC1807 | O121:H19 | STEC | - | - | + |
| EC1808 | O121:H19 | STEC | - | + | + |
| EC331  | O26      | STEC | - | + | + |
| EC400  | O26:H11  | STEC | - | + | - |
| EC521  | O26:H11  | STEC | - | - | + |
| EC540  | O26:H-   | STEC | - | + | + |
| EC550  | O26:H-   | STEC | - | + | + |
| EC1232 | O55:H7   | STEC | - | + | + |
| EC1235 | O55:H7   | STEC | - | + | + |
| EC1668 | O111:H8  | STEC | - | + | - |
| EC1669 | O118:H16 | STEC | - | + | - |
| EC1631 | O111:H8  | STEC | - | - | + |
| EC1655 | O111:H8  | STEC | - | + | - |
| K12    | Negative *E. coli* strain control | - | - | - |
| NTC    | -        | -      | -           |

**Background**

Shiga toxin-producing *Escherichia coli* (STEC), including *E. coli* O157:H7, is a heterogeneous group of enteric pathogens responsible for numerous sporadic infections and large outbreaks annually worldwide [1]. Besides *E. coli* O157:H7, non-O157 STEC strains are important
foodborne pathogens estimated to cause over 112,752 cases illnesses each year in the United States [2]. Shiga toxin (Stx) production, especially Stx2, has been implicated as an important factor in causing severe disease and hemolytic uremic syndrome (HUS) [3–5].

E. coli serotype O157:H7 was first recognized as a causative agent of food contamination outbreaks in 1982 in Oregon and Michigan [6]. Since then, numerous E. coli O157:H7 outbreaks have been reported worldwide [7]. In the United States alone during 2003 - 2012, 390 outbreaks of E. coli O157:H7 infections were documented, which resulted in 4,928 illnesses, 1,272 hospitalizations, and 33 deaths [8]. The typical symptoms caused by E. coli O157:H7 include abdominal pain, watery diarrhea and potential progression to bloody diarrhea (hemorrhagic colitis) [9]. The pathological features of hemorrhagic colitis are attributed to the production of Shiga like cytotoxins (Stx1 and Stx2), which consists of a 32-kDa A subunit and five identical 7.7 kDa B subunits. These toxins can bind to receptors located on membranes of eukaryotic cell and cause tissue damage resulting in pathological outcomes [4, 5, 10, 11].

Non-O157 STEC strains are foodborne pathogens and have been responsible for sporadic cases of infections and outbreaks. Although O157:H7 has garnered more attention, primarily based on historical record, recent attention has recognized the significance of non-O157 STEC strains as a pathogen [9, 12–20]. Considerable attention is now drawn to non-O157 STEC strains particularly after the occurrence of a severe foodborne outbreak happened in 2011 in Germany caused by consumption of sprouts contaminated by STEC O104:H4 [21].

The clinical significance and economic burden associated with outbreaks caused by E. coli O157:H7 and non-O157 STEC have led to development of a variety of detection methods. These include the application of conventional bacteriological methods using selective media or chromogenic agar, which usually take several days to complete [1, 9, 22], and molecular-based assays such as PCR-based methods [23–25], microarray [25–27], and whole genomic sequencing (WGS) [25, 28, 29]. Of these molecular methods, real-time PCR is a commonly used method [9]. More importantly, real-time PCR enables detection to be coincided with the amplification process by the introduction of fluorogenic probes [23], and multiplex real-time PCR allows multiple genes to be simultaneously amplified either from one template [9] or multiple templates by using different primer pairs [24].

Multiplex real-time PCR has gained more acceptance and use due to its ability to its differentiation potential and reliability [11, 30]. Various target genes have been used in PCR detection scheme for E.coli O157:H7, including the Shiga toxin genes (stx1 and stx2) [9, 12, 13], eae [31, 32], fimA [33], rfbE [34], uidA [24, 35], and Z3276 [23, 36, 37]. Of the target genes, uidA is most commonly used. Specificity with this gene is based on a highly conserved point mutation at position 93 of the β-glucuronidase gene [38, 39]. However, when a uidA-based commercial kit was used for identification of a large number of E. coli O157:H7 isolates (n = 391), numerus strains (n = 21) did not generate an amplified product. This prompted us to search for a more specific and reliable gene target for detection of E. coli O157:H7 [23]. As a result, a real-time PCR-based on Z3276 gene, a putative unique fimbriae gene in E. coli O157:H7 [40], was developed. All the 391 isolates, including the 21 strains that were “negative” by the uidA-based commercial kit, that were tested were positively identified [23].

The primary focus of our previous study was to search for a unique genetic marker and the development of a real-time PCR assay for the detection of E.coli O157:H7, and the Shiga toxin genes were not included in that assay [23]. Since the annual number of episodes of domestically acquired foodborne illnesses caused by non-O157 STEC (112,752) is almost doubled that of E. coli O157:H7 (63,153) in the United States [2], the inclusion of the stx genes can be used as the primary characteristic for STEC detection within the heterogeneous STEC group [41]. Thus, in the present study, we incorporated in a multiplex real-time PCR assay the Z3276, stx1, and stx2 genes, as well as an internal amplification control to for detection of Escherichia coli O157:H7 and screening for non-O157 STEC. In addition, the assay was assessed with two common food matrices, beef and spinach, for specific detection of E. coli O157:H7 and non-O157 STEC strains.

Methods
Bacterial strains and growth conditions
All the E. coli O157:H7 and non-O157 STEC strains used in this study are listed in Table 1. EDL933 (ATCC 43985) was used as the E. coli O157:H7 reference strain. Strains of E. coli O157:H7 (n = 135) and non-O157 STEC (n = 33) were used for inclusivity determination. Salmonella enterica strains, Shigella strains and other pathogenic strains were used for the exclusivity test (Table 2). These strains are all from the strain collections of Division of Molecular Biology, Food and Drug Administration (FDA).

Bacterial DNA preparation
Bacteria were grown at 37 °C in Luria-Bertani (LB) broth with agitation at 180 rpm, or on LB agar placed in a gravity convection incubator. Bacterial growth was measured by monitoring the turbidity at 600 nm (OD600) using a DU530 spectrophotometer (Beckman, CA). To enumerate bacterial cells, cultures were diluted serially in 10-fold increments with medium and plated on LB
agar plates at 37 °C overnight. DNA preparation from bacterial cultures was made with a Puregene cell and tissue kit (Gentra, Minneapolis, MN) as described previously [23]. Briefly, cell pellets from 1 ml of overnight culture were suspended in 3 ml of cell lysis solution and heated to 80 °C for 5 min, followed by addition of 15 μl of RNase A and incubation at 37 °C for 60 min. To remove protein and cell debris, the cell lysate was further mixed with 1 ml of protein precipitation solution, vortexed and centrifuged at 3000 × g. DNA in the supernatant was precipitated by the addition of 2-propanol, centrifuged as above, washed with 70% ethanol, and

| Genus       | Species       | Pathotype | Strain name/Serotype | Target gene | Z3276 | stx1 | stx2 |
|-------------|---------------|-----------|----------------------|-------------|-------|------|------|
| Escherichia | coli          | EHEC      | EDL933/O157:H7       | +           | +     | +    |      |
| Escherichia | coli          | ETEC      | EC1775/O26:H11       | 1           | 1     |      |      |
| Escherichia | coli          | STEC      | EC1803/O103:H11      | 1           | +     |      |      |
| Escherichia | coli          | STEC      | EC1807/O121:H19      | 1           |      | +    |      |
| Escherichia | coli          | ETEC      | EC1801               | 1           | +     |      |      |
| Escherichia | coli          | EPEC      | EC1501               | 1           |       |      |      |
| Escherichia | coli          | EIEC      | EC1513               | 1           |       |      |      |
| Escherichia | coli          | EDC       | DECSA                | 1           |       |      |      |
| Escherichia | coli          |          | K12/MG1655           | 1           |       |      |      |
| Salmonella  | enterica      |          | SL192/Typhi          | 1           |       |      |      |
| Salmonella  | enterica      |          | SL317/Newport        | 1           |       |      |      |
| Salmonella  | enterica      |          | SL35/Typhimurim      | 1           |       |      |      |
| Shigella    | sonnei        |          | SH20145              | 1           |       |      |      |
| Shigella    | dysenteriae   |          | SH20152              | 1           |       |      |      |
| Shigella    | flexneri      |          | SH20155              | 1           |       |      |      |
| Shigella    | boydii        |          | SH20140              | 1           |       |      |      |
| Staphylococcus | aureus    |          | ATCC25923             | 1           |       |      |      |
| Staphylococcus | epidermidis |          | ATCC12228             | 1           |       |      |      |
| Staphylococcus | pyogenes   |          | ATCC19615             | 1           |       |      |      |
| Vibrio      | alginoptyca   |          | ATCC17749             | 1           |       |      |      |
| Vibrio      | parahemolyticus |          | ATCC17802            | 1           |       |      |      |
| Vibrio      | vulasiensis   |          | ATCC27562             | 1           |       |      |      |
| Enterobacter | cloacae      |          | ATCC23355             | 1           |       |      |      |
| Enterobacter | cloacae      |          | ATCC13047             | 1           |       |      |      |
| Enterobacter | cloacae      |          | ATCC13048             | 1           |       |      |      |
| Citrobacter | freundii     |          | ATCC8090              | 1           |       |      |      |
| Klebsiella  | pneumoniae    |          | ATCC13883             | 1           |       |      |      |
| Pseudomonas | aeruginosa    |          | ATCC27853             | 1           |       |      |      |
dissolved in 500 μl of rehydration solution. The concentration of DNA extraction was determined by measuring the optical density (OD260) using a NanoDrop spectrophotometer (NanoDrop Technology, Wilmington, DE).

Primers and probes for the multiplex real-time PCR assay

All the primers, probes, and sequence information are listed in Table 3. The primers and labeled TaqMan probes in this study were designed using Primer Express 3.0 software (Life Technology, Foster City, CA) and synthesized by Life Technology. The primers and probe for *E. coli* O157:H7 specific gene open reading frame (ORF) ORFZ3276 were described previously [23], the primers and probes for *stx1*, and *stx2* were designed in this study, and the primers and probe for internal amplification control (IAC) were selected based on the DNA sequence of plasmid pUC19 as previously reported [42].

The IAC was incorporated into the multiplex real-time PCR assay to ensure the amplification is free of inhibitory factors from examined food samples.

Development of the multiplex real-time PCR assay

The multiplex real-time PCR assay was developed using the Life Technology ABI 7500 System platform and the standard chemistry. The concentrations of primers and probes for each target gene were adjusted to achieve optimal amplification condition. The reaction mixture contains 12.5 μl of 2 × Universal Master Mix (Life Technology), 200 nM forward and reverse primers, appropriate concentrations of four probes [[Z3276 (80 nM), stx1 (80 nM), stx2 (20 nM), and IAC (40 nM)] and 2 μl of template DNA. Water was added to make a final reaction volume of 25 μl. The amplification conditions for the multiplex assay were set as follows: activation of TaqMan at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 60 s.

To compare the efficiency of the multiplex assay with simplex assay, each of the target genes Z3276, *stx1*, and *stx2* was amplified by three individual simplex assays. For the simplex assays, three individual reaction mixtures each contains 12.5 μl of 2 × Universal Master Mix (Life Technology), corresponding forward and reverse primers (200 nM) and probe (100 nM). An equal amount of template DNA (2 μl) was used for the simplex assays, and water was added to make a final reaction volume of 25 μl. The amplification conditions for simplex assays were the same as the multiplex assay.

Sensitivity test and the limit of detection (LOD) of the multiplex qPCR assay

To determine the sensitivity of the multiplex real-time PCR, standard curves of Z3276, *stx1*, and *stx2* in the multiplex real-time PCR were generated. A serial 10-fold dilution from 10 ng to 10 fg/μl of genomic DNA of *E. coli* O157:H7 strain (EDL933) was prepared and 2.0 μl of each dilution was used as template for PCR amplification. The real-time PCR assay was performed using the conditions described as above. The amplification efficiency of the assay was determined using the formula $E = (10^{1/slope} - 1)^{-1} \times 100$ [43, 44].

Inclusivity and exclusivity tests

The inclusivity test for the multiplex real-time PCR was performed with the optimized concentrations for probes Z3276, *stx1* and *stx2* on the genomic DNA of *E. coli* O157:H7 strains (n = 135) and non-O157 STEC strains (n = 21) (Table 1). The exclusivity test was performed on various pathogenic strains including strains of EIEC, EPEC, *Shigella*, and *Salmonella* (n = 27) (Table 2). DNA

### Table 3 Primers and probes used in the multiplex real-time PCR assay

| Target gene | Primer/Probe | Sequence (5’ – 3’) | Amplicon length (bp) | Reference |
|-------------|-------------|--------------------|---------------------|-----------|
| Z3276       | Z3276 forward | TATTCGGCGATGCTTGTTTTT | 130 | Li and Chen. 2012 |
|             | Z3276 reverse | ATTATCTCACCACCAACTGGCGG |                |           |
|             | Z3276 probe  | FAM-CCCGCAATCTTCTTCCMGBNFQ |              |           |
| *stx1*      | *stx1* forward | GGATTTCGTACAACACTGTGATGAT | 67 | This study |
|             | *stx1* reverse | ATCCACATCTTCCAGTCATTACA |                |           |
|             | *stx1* probe  | TAMRA-CAGTGGGCGTTCTTMCMBNFQ |              |           |
| *stx2*      | *stx2* forward | GGGCAGTTATTGTGTGTGAT | 59 | This study |
|             | *stx2* reverse | GTCAAAAGGGCCCTGAT |                |           |
|             | *stx2* probe  | JOE-ACGAGGCTTGTATGTCMGBNFQ |              |           |
| IAC         | IAC forward | CAGGATTAGCAGAGCCGAGTGAT | 65 | Fricker et al. 2007 |
|             | IAC reverse | CGTAGTTAGCCACACCTCAG |                |           |
|             | IAC probe    | CY5-AGGCGGTGCTACAGAG-MGBNFQ |              |           |
samples were diluted with nuclease-free water to concentration of 50 pg/μl and 2 μl of DNA dilute was used for amplification of target genes. Furthermore, 2 μl of nuclease free water was used to substitute DNA in no template control in the triplex real-time PCR.

**Application of the multiplex real-time PCR assay to detect *E. coli* O157:H7 from spiked spinach and beef**

Fresh spinach and beef were purchased from a local retail source and used as food matrices to assess the multiplex real-time PCR assay. These samples were first confirmed to be free of *E. coli* O157:H7 and non-O157 STEC by standard FDA BAM method [45], and subsequently used for the spiking experiments. One for beef (set 1) spinach spiking (set 2). Each set contained six replicates (25 g of beef or spinach), and were inoculated with 80 and 800 CFU/g O157:H7 (EDL933) cells, respectively. Each sample was mixed with 225 ml of LB medium and homogenized for 2 min using a stomacher (Seward, England). The samples were incubated at 37 °C with shaking at 180 rpm for 24 h.

Two ml of the enriched culture was sampled at 0, 4, 8, 12 and 24 h. At these times, the samples were centrifuged at 600 × g for 1 min to remove fat tissues (for beef) or leaf (for spinach) from the samples. The supernatants were transferred to 2-ml microtubes and centrifuged again at 3000 × g for 5 min to collect bacterial cells. The cell pellets were used for DNA extraction with PreMan Ultra Sample Preparation Reagent (Life Technologies), following the instruction of the manufacturer. Two μl of the DNA

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**Fig. 1** Comparison of the sensitivity of simultaneously detecting the targeting genes Z3276, stx1, and stx2 in *E. coli* O157:H7 by the multiplex real-time PCR assay. The standard curves of Z3276, stx1, and stx2 in *E. coli* O157:H7 by the multiplex real-time PCR assay (a); the standard curves of Z3276, stx1, and stx2 generated by three individual simplex real-time PCR assays (b). The Ct values represent the average of six replicates from two independent tests ±SD.
extraction was used in the multiplex real-time PCR, and roughly 200 copies of plasmid pUC19 DNA (Promega, Madison, WI) was added as template for IAC.

**Results**

**Designation and optimization of multiplex real-time PCR Z3276** is a unique target gene that was used as the basis for a multiplex real-time PCR assay for the detection of E. coli O157:H7. In addition to the Z3276 marker, three additional targets stx1, stx2, and IAC, were optimized for amplification and detection (data not shown). In order to minimize the interference among probes during the amplification process, the concentration of each probe was titrated and tested with a fixed amount of DNA (1 ng/reaction). The optimal concentrations for probes Z3276, stx1, stx2, IAC were determined as 80 nM, 80 nM, 20 nM, and 40 nM, respectively.

We further assessed whether the sensitivity of the probes was affected in the multiplex real-time PCR by comparing with those from the corresponding simplex assays. The C_T values for the target genes Z3276, stx1, and stx2 were determined to be 23.86, 22.23, and 21.22, respectively in the multiplex real-time PCR (Fig. 1a), whereas the C_T values for Z3276, stx1, and stx2 were 21.62, 21.38, and 20.63, respectively in simplex real-time PCR (Fig. 1b). These results demonstrated that at a specified amount of target DNA, the sensitivity of the multiplex PCR assay is comparable with that of each respective simplex assay.

**Comparison of sensitivity and specificity of the multiplex and simplex real-time PCR assays**

Using a serial 10-fold dilution of genomic DNA from E. coli O157:H7 (EDL933) as template, the limit of detection (LOD) in the multiplex real-time PCR was determined to be 200 fg of DNA per reaction with average C_T values of 38.63, 36.21, and 34.04 for Z3276, stx1, and stx2, respectively. The value of 200 fg of DNA per reaction is equivalent to 40 CFU of DNA. Three standard curves with slopes of -3.60, -3.49, and -3.36, for the target genes Z3276, for stx1, and stx2, respectively, were generated and the amplification efficiencies for the three target genes differed slightly ranging from 90%-98% (Fig. 1). These data indicated the sensitivity of the multiplex real-time PCR was robust and reliable.

**Inclusivity and exclusivity of the multiplex real-time PCR**

The multiplex real-time PCR positively identified all the E. coli O157:H7 strains (n = 135) (Table 1). This collection of 135 positively identified E. coli O157:H7 strains included the 21 strains that were not amplified in a real-time PCR assay targeting the uidA gene [23]. The Shiga toxin profiles (stx1 and stx2) of these strains were also found to be perfectly matched with those previously determined by conventional PCR and uidA-based real-time PCR methods. No cross-reaction was observed from the E. coli O157:H7 specific probe Z3276 on all the non-O157 STEC strains, Salmonella strains, Shigella strains, and other pathogenic strains; while the stx probes positively identified the non-O157 STEC strains with presence of either stx1 or stx2, or both (Table 1).

**Detection of E. coli O157:H7 from spiked food matrices by the multiplex real-time PCR assay**

Beef and spinach samples were initially inoculated with 80 CFU/g E. coli O157:H7 cells. At 0 h, none of the three detection target genes (Z3276, stx1, and stx2) were detected by multiplex real-time PCR assay, but the IAC was positive. However, after 4-h enrichment, all the three detection target genes (Z3276, stx1, and stx2) from both food matrices were amplified in the multiplex real-time PCR assay (Table 4).

**Discussion**

PCR technology is widely used for pathogen detection from clinical, food, and environment samples. Real-time PCR methods are used for their enhanced sensitivity and specificity. Several PCR-based methods are available for the detection of E. coli O157:H7 and non-O157 STEC (4, 7, 9, 10) by amplifying various target genes [23]. Target genes such as stx1, stx2, IAC were determined as 80 nM, 80 nM, 20 nM, and 40 nM, respectively.

Selection of Z3276 as a unique genetic marker for detection of E. coli O157:H7 was the basis for the development of a multiplex PCR assay. Simultaneous detection of multiple genes in a single reaction may increase specificity and reliability for the detection of E. coli O157:H7, since the amplification of different target genes can corroborate the final conclusion. More importantly, the inclusion of the Shiga toxin genes enables the assay to detect not only E. coli O157:H7, but also screen for non-O157 STEC strains, the latter often underestimated [47-53].

Multiplex real-time PCR can provide better detection efficiency. However, interference among probes and competition among primers for supplies during amplification may compromise the sensitivity and increase the background. Therefore, it is necessary to fine tune the parameters of the multiplex reaction to achieve the optimal conditions for each target gene. In this study, by optimizing the concentration of each probe in the assay
were able to achieve robust sensitivity in the multiplex assay, and positively identified all the *E. coli* O157:H7 strains (n = 135), demonstrating the multiplex assay is compatible to the simplex assays.

Nowadays, WGS has been explored for a more efficient and more comprehensive approach for STEC detection. Although WGS potential with STEC characterization and surveillance is apparent, STEC detection will likely continue to rely on a combination of culture and non-culture methods, the latter including real-time PCR [49]. The multiplex real-time PCR developed in this study not only can detect *E. coli* O157:H7 and its profile of the Shiga toxin genes, but also detect non-O157 STEC strains. The capability for simultaneous detection of the Shiga toxin genes and the differentiation of *E. coli* O157:H7 from non-O157 STEC strains offers several advantages: i) determination of the presence or absence of Shiga toxin genes can be used to verify the detection results of *E. coli* O157:H7, because almost all *E. coli* O157:H7 strains possess *stx1* and/or *stx2* gene(s); ii) profile of the Shiga toxin genes of *E. coli* O157:H7 provides genetic markers for differentiating isolates from outbreaks; iii) differentiation of *stx1* and *stx2* harboring *E. coli* O157:H7 strains may help health care providers manage HUS patients caused by *E. coli* O157:H7 [49]; and iv) identification of *stx* gene harboring isolates can serve as a useful clue for detection of STEC, and then more comprehensive and sophistical analytical analyses, such as cultural biological tests, toxin detection, serotyping, genotyping, and WGS, can be performed to confirm the final detection result.

Multiplex real-time PCR targeting *uidA*, *stx1*, and *stx2* genes for detection of *E. coli* O157:H7 and non-O157 STECs has become a routine test for preliminary screening in clinical laboratories as the Centers for Disease Control and Prevention recommended [54]. Although WGS is not yet a routine testing, future prediction would include this technology as a means to track the mobility of pathogenic microbes as the food market has become global. The multiplex real-time PCR developed in this study has been demonstrated to be a reliable, efficient, and sensitive assay, and may serve as a useful method for the detection of *E. coli* O157:H7 and non-O157 STEC in epidemiological surveillance programs as well as in food analytical laboratories. The multiplex real-time PCR assay was successfully tested in this study for the detection of *E. coli* O157:H7 from spiked food matrices, i.e., beef and spinach, and more than likely applicable to other food matrices.

It is worth noting that on the one hand, in the development of the multiplex real-time PCR assay, great efforts were made toward getting high sensitivity and specificity by optimizing the amplification conditions and by minimizing the interferences among probes, primers, and target genes to reduce the false negative

### Table 4 Detection results of the multiplex real-time PCR on the spiked food samples that inoculated with different concentrations of *E. coli* O157:H7 and enriched with different incubation time

| Target gene | Food matrice | Incubation time (h) |
|-------------|--------------|---------------------|
|             | CFU/gram     | 0 | 4 | 8 | 12 | 24 |
| **Z3276**   |              | C₇ ±SD | C₉ ±SD | C₉ ±SD | C₉ ±SD | C₉ ±SD |
|             | 80           | UD ① | 34.50 | 0.29 | 28.43 | 0.07 | 27.98 | 0.10 | 27.98 | 0.10 |
|             | 800          | UD   | 30.51 | 0.38 | 24.54 | 0.10 | 24.00 | 0.04 | 24.00 | 0.04 |
| **stx1**    |              | UD   | 27.54 | 0.19 | 21.73 | 0.22 | 20.84 | 0.25 | 19.06 | 0.36 |
|             | 80           | UD   | 24.45 | 0.22 | 18.51 | 0.14 | 17.46 | 0.03 | 18.48 | 0.02 |
| **stx2**    |              | UD   | 31.34 | 0.10 | 25.47 | 0.02 | 25.03 | 0.04 | 23.63 | 0.02 |
|             | 80           | UD   | 27.93 | 0.34 | 21.68 | 0.03 | 21.33 | 0.06 | 22.52 | 0.05 |
| **IAC**     | 200 copies ② | 28.88 | 27.72 | 21.96 | 21.09 | 19.59 |
|             |             | C₇ ±SD | C₉ ±SD | C₉ ±SD | C₉ ±SD | C₉ ±SD |
| **Z3276**   | 80           | UD   | 33.92 | 0.04 | 24.32 | 0.26 | 22.86 | 0.15 | 24.26 | 0.09 |
|             | 800          | UD   | 30.91 | 0.05 | 23.04 | 0.11 | 23.08 | 0.08 | 23.12 | 0.07 |
| **stx1**    | 80           | UD   | 27.63 | 0.10 | 17.32 | 0.15 | 15.74 | 0.13 | 16.87 | 0.11 |
|             | 800          | UD   | 25.04 | 0.06 | 16.57 | 0.12 | 16.25 | 0.15 | 16.37 | 0.10 |
| **stx2**    | 80           | UD   | 30.88 | 0.06 | 21.21 | 0.07 | 20.10 | 0.06 | 21.64 | 0.07 |
|             | 800          | UD   | 28.19 | 0.14 | 20.05 | 0.15 | 20.58 | 0.05 | 20.74 | 0.04 |
| **IAC**     | 200 copies ② | 24.94 | 23.82 | 24.77 | 23.83 | 23.69 |

① Data were shown as average of two independent experiment
② UD refers to "Undetermined", a negative detection result
③ About 200 copies of pUC19 plasmid DNA was added as template for IAC to each multiplex real-time PCR reaction
rate in detection; on the other hand, in the use of the assay, precaution is needed in interpretation of the positive results from certain strains that free phages might harbor stx gene [55–57] or even some Shigella strains acquired stx genes [58–61].

Conclusions
A multiplex real-time PCR assay that can rapidly and simultaneously detect E. coli O157:H7 and screen for non-O157 STEC strains has been developed and assessed for efficacy. The inclusivity and exclusivity tests demonstrated high sensitivity and specificity of the multiplex real-time PCR assay. In addition, this multiplex assay was shown to be effective for the detection of E. coli O157:H7 from two common food matrices, beef and spinach, and may be applied for detection of E. coli O157:H7 and screening for non-O157 STEC strains from other food matrices as well.

Abbreviations
HUS: hemolytic-uremic syndrome; IAC: internal amplification control; ORF: open reading frame; STEC: Shiga toxin-producing Escherichia coli

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
BL conceived the project. BL and HL designed the study. BL, HL, and WW performed the experiments and analyzed the results. BL and HL wrote the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

Author details
1 Division of Molecular Biology, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD 20708, USA. 2 Branch of Microbiology, Office of Regulatory Affairs, Food and Drug Administration, Jefferson, AR 72079, USA.

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