Design of Novel Real-Time PCR Assays which Distinguish Between *E. coli* O157:H7 and Other *E. coli* Serotypes Including O55:H7

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Abstract

Escherichia coli O157:H7 poses a major threat to public safety, as evidenced by recent outbreaks of illnesses stemming from contaminated food supplies including ground beef and spinach. *E. coli* O157:H7 causes hemorrhagic colitis and can lead to severe complications that are potentially fatal. There is an apparent need for a rapid, reliable, and specific assay to detect and identify the O157:H7 serotype. The aim of the study was to develop an assay to detect O157:H7 while avoiding detection of other serotypes of *E. coli*, including O55:H7 which is genetically very closely related to O157:H7. Using publicly available sequences of O157:H7 and other *E. coli* serotypes, putative O157:H7 specific sequences were identified. However, genomic information for O55:H7 was extremely limited. To distinguish between O55:H7 and O157:H7, the genome sequence of O55:H7 was determined using the SOLiD™ system sequencing approach and compared to O157:H7. Whole genome comparison of O157:H7 and O55:H7 sequences yielded genome segments unique to O157:H7. These O157:H7 specific sequences were used as potential targets to design TaqMan® real-time PCR assays. The computational assay design pipeline minimized mismatches to O157:H7 sequences while maximizing mismatches to all other exclusion targets. The TaqMan assays were determined to be specific for the O157:H7 serotype by testing a large panel of *E. coli* of various serotypes and related pathogens such as *Shigella*. The assays exhibited no cross-reactivity against overnight cultures of different food matrices which confirmed that the assays did not detect other microorganisms that might be present in the background flora of the samples. High sensitivity of the assays in conjunction with novel sample prep solutions allowed detection of 1 to 3 cfu of *E. coli* O157:H7 in 65 g of cultured ground beef during an 8 hour sample-to-result workflow period. In summary, this study demonstrated that *E. coli* O157:H7 possesses unique sequences that can be targeted for sensitive detection and accurate differentiation of O157:H7 from other closely related *E. coli* serotypes including O55:H7.

E. coli O157:H7-specific sequences

Based on genomic comparisons, we designed several TaqMan® real-time PCR assays against the serotype *E. coli* O157:H7 and avoided detection of other serotypes. *E. coli* O55:H7, the nearest neighbor of O157:H7, is extremely close in sequence to O157:H7. Because genomic sequence of O55:H7 was not readily available for genomic comparison prior to our design process, we generated its genomic sequence using Applied Biosystems' SOLiD technology to compare with an O157:H7 reference sequence. Genomic regions that were specific to O157:H7—either different or absent in O55:H7—were identified as putative target sequences for the O157:H7 TaqMan primers and probes.

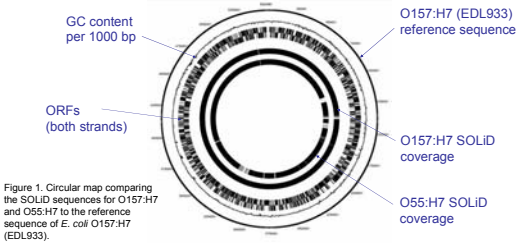


Figure 1. Circular map comparing the SOLiD sequences for O157:H7 and O55:H7 to the reference sequence of *E. coli* O157:H7 (EDL933).

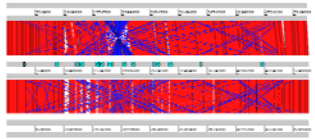


Figure 2. Linear map comparing SOLiD sequences of O157:H7 Sakai and O55:H7 to O157:H7 (EDL933). Red and blue blocks between genomes indicate conservation on the same and opposite strands, respectively.

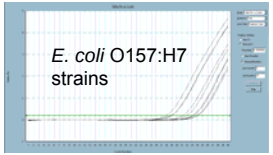
TaqMan® and the assay design process

Figure 3. TaqMan® assay development pipeline for *E. coli* O157:H7 targets. A TaqMan® assay consists of 2 primers and a dye-labeled probe and non-fluorescent quencher.

1. Generate potential target sequences based on multiple alignments of inclusion organisms
2. Criteria include Tm, nucleotide distribution, dimerization, polyN, amplicon size
3. Assess and minimize cross-hybridization to exclusion organisms
4. Select highest scoring primer and probe assay designs
5. Primer and probe synthesis
6. Perform experimental test on organism/DNA

Specificity

# Strain ID	Bacterial strain	Assay 1, Ct	Assay 2, Ct	
1 PE30	*E. coli* O157:H7	33.2	35.3	+
2 PE677	*E. coli* O157:H7	29.0	29.5	+
3 PE678	*E. coli* O157:H7	28.9	32.1	+
4 PE679	*E. coli* O157:H7	31.1	33.8	+
5 PE700	*E. coli* O157:H7	33.1	34.9	+
6 PE701	*E. coli* O157:H7	32.0	33.0	+
7 PE702	*E. coli* O157:H7	34.0	35.0	+
8 PE703	*E. coli* O157:H7	34.0	35.5	+
9 PE40	*E. coli* O157:HNM	28.0	29.4	+
10 PE674	*E. coli* O55:H7	No signal	No signal	-
11 PE704	*E. coli* O55:H7	No signal	No signal	-
12 PE705	*E. coli* O55:H7	No signal	No signal	-
13 PE706	*E. coli* O55:H7	No signal	No signal	-
14 PE735	*E. coli* O55:H9	No signal	No signal	-
15 PE684	*E. coli* O137:H41	No signal	No signal	-
16 PE37	*E. coli* O145:NM	No signal	No signal	-
17 PE826	*E. coli* O154:H25	No signal	No signal	-
18 PE827	*E. coli* O156:H8	No signal	No signal	-
19 PE731	*E. coli* O26:H32	No signal	No signal	-
20 PE688	*E. coli* O28:H35	No signal	No signal	-
21 PE687	*E. coli* O48:H21	No signal	No signal	-
22 PE673	*E. coli* O5:NM	No signal	No signal	-
23 PE38	*E. coli* O78:K80:H12	No signal	No signal	-
24 PE1199	*Shigella flexneri*	No signal	No signal	-
25 PE1200	*Shigella boydii*	No signal	No signal	-
26 PE1201	*Shigella sonnei*	No signal	No signal	-



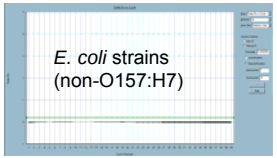
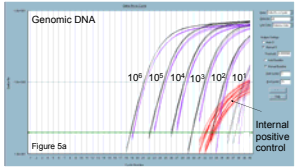


Figure 4. TaqMan results when 2 different *E. coli* O157:H7 assays were tested on a panel of *E. coli* O157:H7 and non-O157:H7 strains. Ct (cycle threshold) values are indicated. Non-O157:H7 strains including O55:H7 did not show any cross-reactivity with the assays. The reactions were run on the AB 7500 Fast Sequence Detection System.

Sensitivity



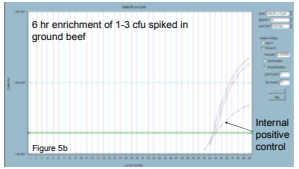


Figure 5. Fig 5a. Assay efficiencies close to 100% were determined for 2 assays by testing the assays over a dilution series of *E. coli* O157:H7 purified genomic DNA. 10 copies were consistently detected by both assays. The internal positive control appears in red. Fig. 5b. 1 to 3 cfu of *E. coli* O157:H7 was spiked in 65 g ground beef, enriched for 6 hr, and detected by the 2 assays post-sample prep.

Sample-to-result workflow

Figure 6a

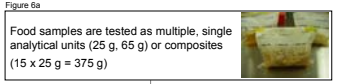
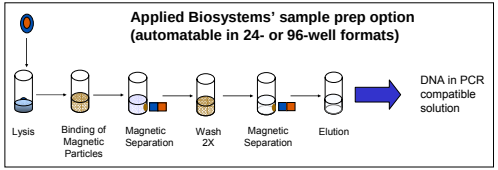


Figure 6. Sample prep is a crucial step in detection of *E. coli* O157:H7 within various food matrices. Foods are complex matrices and, without the appropriate sample prep, can often inhibit PCR. Avoiding inhibition is especially critical in order to enable detection of low levels of bacteria in a food sample. Fig. 6a outlines the steps involved in testing various food matrices for pathogens. Using Applied Biosystems' sample prep and Real-Time detection system, *E. coli* O157:H7 can be enriched in a food sample, prepared, and run on TaqMan® with results within an 8-hour workshift. Fig 6b. depicts one of Applied Biosystems' novel sample prep options. Bacteria in a food matrix is lysed, its nucleic acids separated using magnetic particles, and then eluted in a buffer compatible with PCR.

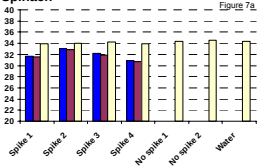
Figure 6b



Spiked and unspiked food matrices

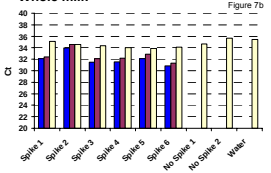
Spinach

Figure 7a



Whole milk

Figure 7b



Mayonnaise

Figure 7c

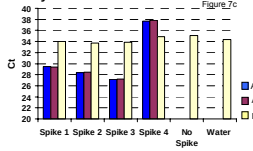


Figure 7. Spinach, whole milk, and mayonnaise were spiked with 1-3 cfu *E. coli* O157:H7. The cultures were enriched overnight in this study. Following sample prep, two *E. coli* O157:H7 assays were run on each sample (signals shown in blue and purple). Unspiked controls were included. Absence of signal from unspiked samples indicates no cross-reactivity of the assays to the food matrices. The internal positive control (yellow) is an inhibition indicator. None of the reactions in this study was significantly inhibited. The water sample is a TaqMan® control reaction and was run in the absence of the food matrix. Overnight enrichment of 1-3 cfu *E. coli* O157:H7 was detected by both assays in almost all cases. Only in figure 7c. spike #4 sample did not enrich well for *E. coli* O157:H7 and generated high Cts for both assays.

Conclusions

- Highly specific TaqMan® assays for the detection of *E. coli* O157:H7 were designed using the sequenced genomes of O157:H7 and O55:H7 and applying Applied Biosystems' assay design pipeline. The assays were able to distinguish between *E. coli* O157:H7 and its nearest neighbor O55:H7 in experimental tests.
- The sensitivity of the assays permits reliable detection of 10 copies of *E. coli* O157:H7 genomic DNA. It also detects 1-3 cfu of *E. coli* O157:H7 after enrichment in food matrices such as ground beef, whole milk, and mayonnaise.
- AB sample prep removes PCR inhibitors and permits detection of low level of *E. coli* O157:H7 enriched in complex food matrices.
- The assays do not cross-react with ground beef, spinach, whole milk, or mayonnaise.
- We have developed an 8 hr sample-to-result workflow for ground beef.