# Genotyping *Escherichia coli* O157:H7 for Its Ability to Cause Disease in Humans



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## ABSTRACT

*Escherichia coli* are ubiquitous in the world, and for the most part are non-pathogenic and part of the normal lower gastrointestinal tract in mammals. However, some pathogenic isolates can cause severe disease that range from meningitis to hemorrhagic colitis (HC). In recent years, Shiga toxin-containing *E. coli* (STEC) have been a major cause of food borne and environmental cases of HC and hemolytic uremic syndrome. One STEC serotype, O157:H7, has been responsible for numerous food-associated outbreaks and recalls worldwide. The protocols in this unit will allow the reader to use real-time polymerase chain reaction genotyping to identify isolates that are more likely to cause disease in humans. The genotyping assay targets a single-nucleotide polymorphism (SNP) in the *tir* gene. The *tir* gene is located in a virulence operon called the locus for enterocyte effacement and functions as a receptor for the tight adherence of *E. coli* O157:H7 to epithelial cells. As more genomes are sequenced, informative SNPs that associate with phenotypes will be identified. Identifying isolates not only by their genus and species, but also by using other informative genomic traits will increase the general knowledge about their genetic diversity. *Curr. Protoc. Microbiol.* 14:5A.3.1-5A.3.10. © 2009 by John Wiley & Sons, Inc.

Keywords: *Escherichia coli* • O157:H7 • real-time PCR • genotyping • disease

## INTRODUCTION

The capacity to sequence the genomes of bacteria has increased steadily because of new technologies that allow more sequences to be generated in less time and at a reduced cost (Mardis, 2008; Schuster, 2008). As a result, comparative sequencing of genes and genomes has discovered a plethora of genetic variation, some of which is associated with phenotypic traits (Bono et al., 2007). One form of genetic variation is single-nucleotide polymorphisms (SNP). There are several methodologies available to interrogate SNPs in bacterial populations, including sequencing, mass spectroscopy, and microarrays. This unit focuses on a methodology using real-time polymerase chain reaction (PCR) to genotype a SNP in Shiga toxigenic *Escherichia coli* O157:H7 that associates with an isolate's ability to cause disease in humans. The genotyping assay targets an A-T nucleotide polymorphism in a gene named *tir*, with the A allele isolates responsible for only 2% of human disease. This unit describes the isolation of DNA from *E. coli* O157:H7 isolates and a TaqMan-based assay that uses a minor groove–binding fluorescent probe to identify alleles.

*CAUTION: E. coli* O157:H7 is a Biosafety Level 2 (BSL-2) organism and caution should be used when working with this organism. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Check the guidelines of your local or Institutional Biosafety Committee (IBC) for more detailed regulations and approval, if necessary.

*IMPORTANT NOTE:* Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the



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name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

*NOTE:* Use sterile or molecular-grade water in all steps and solutions.

***BASIC PROTOCOL***

## REAL-TIME PCR GENOTYPING ASSAY FOR DISEASE-CAUSING ALLELE IN *E. COLI* O157:H7

Real-time PCR is a valuable technique as a rapid and sensitive method for identifying PCR amplicons and quantifying the amount of starting template. This system is based on the detection and quantification of a fluorescence probe (Lee et al., 1993; Livak et al., 1995; Shipley, 2006). One of the fluorescence probe types is a TaqMan probe. TaqMan assay uses PCR to amplify a targeted region of DNA. TaqMan probes are designed to hybridize to a portion of the amplified product. These probes are labeled on opposite ends with a fluorescent dye and a non-fluorescent quencher. When not hybridized to DNA, the close proximity of the non-fluorescent quencher to the fluorescent dye prevents emission of the signal when excited. When hybridized to DNA, the 5′ nuclease activity of *Taq* polymerase removes the fluorescent dye from the probe as it is extending, allowing the dye to emit when excited. Allelic discrimination has a unique problem because a probe is asked to differentiate sequences by 1-base difference. To overcome this problem, TaqMan probes can have a minor groove binding (MGB) protein attached to the 3′ end of the probe. The MGB increases the melting temperature ($T_m$) of probes, allowing the use of shorter probes, which exhibit greater differences in $T_m$ values between matched and mismatched probes. With a TaqMan allelic discrimination assay, the removal of the fluorescent dye from the probe by the nuclease activity of *Taq* is measured at the end of every cycle by excitation. The amount of fluorescence is proportional to the number of unbound fluorescent dye molecules. When the level of fluorescence of the dye and the cycle number are graphed after each cycle, a visual representation of the PCR reaction can be viewed in real-time. Once a baseline fluorescence threshold is determined, any fluorescence signal that crosses the threshold is given a cycle threshold number. For allelic discrimination assays, a cycle threshold ($C_T$) number will be given for one of the two alleles if homozygous or for both alleles if heterozygous.

### *Materials*

TaqMan Universal PCR Master Mix with UNG AmpErase
Sense primer: 5′- TGGCGGCGTCTGAGATAAC -3′
Antisense primer: 5′- GAGTATCGAGCGGACCATGATC -3′
*tir* 255 T allele probe: VIC-ACTGAATGATGGATTTG-MGB/NFQ
*tir* 255 A allele probe: FAM-CTGAATGAAGGATTTG-MGB/NFQ
Molecular-grade water
Genomic DNA extracts (Support Protocol 1 or Support Protocol 2)

Optical PCR tube, strip, or plate
Optical caps or optical adhesive cover for plates
Vortex
Real-time thermal cycler with the ability to detect amplified DNA after or during each thermal cycling period

Additional reagents and equipment for preparing genomic DNA extracts (Support Protocol 1 or Support Protocol 2)

1. Prepare real-time PCR genotyping assay mix on ice. The recipe is for one reaction, so multiply the number of samples to determine the final volume needed. Include in the samples two positive controls (one for each allele) and two negative controls (reactions with no DNA). Prepare the following mix:

10 µl 2× TaqMan Universal PCR Master Mix
0.5 µl 40× TaqMan probes (0.9 µM) and primer (0.2 µM)
8.5 µl molecular-grade water
1.0 µl genomic DNA extract (see Support Protocol 1 or Support Protocol 2).

2. Briefly vortex the tubes to mix the contents.

3. Pulse-centrifuge the tubes to bring the contents of the tube to the bottom.

4. Set up the real-time PCR thermal cycler with the following thermal cycling conditions:

| | | | |
|---|---|---|---|
| 1 cycle: | 2 min | 50°C | (UNG incubation) |
| 1 cycle: | 10 min | 96°C | (AmpliTaq gold activation) |
| 40 cycles: | 15 sec | 95°C | (denaturation) |
| | 1 min | 60°C | (anneal/extend). |

Read fluorescence.

*The software package that comes with the different real-time thermal cyclers sets up the thermal cycling and well arrangements differently. Familiarize yourself with the instructions before running the machine for the first time.*

5. Save the setting and start the thermal cycling.

***Analyze the genotyping results***

6. When the thermal cycling is over, set $C_T$ (Fig. 5A.3.1) line by manually moving the line above the negative control into the exponential part of the curve. Alternatively, the $C_T$ line can be set to a user-defined multiple of the standard deviation above the mean fluorescence across the cycle range specified by the user.

7. View the data by either using the real-time PCR's software or export the data to a spreadsheet and analyze separately.

*Exporting the spreadsheet from some software packages will allow the data to be arranged for better presentation and analysis.*

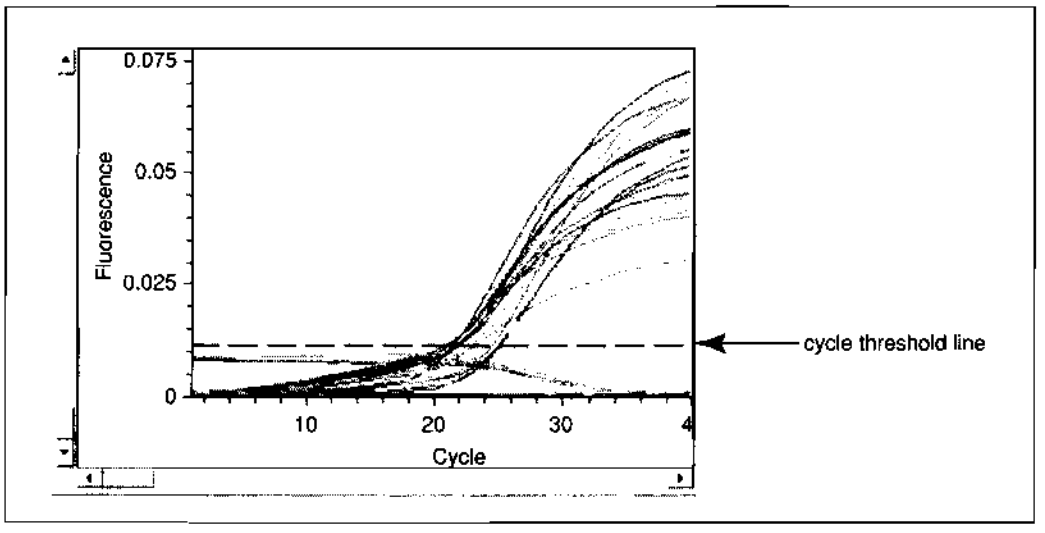


**Figure 5A.3.1** Real-time genotyping assay for *E. coli* O157:H7 *tir* 255T allele. The fluorescence curves are generated by graphing fluorescence intensity versus cycle number. The cycle threshold line is indicated by an arrow on the right side of the graph. For color version of this figure go to *http://www.currentprotocols.com/protocol/mc05a03.*

8. The allele with the lower $C_T$ is the positive allele.

*When genotyping bacterial cells, the results are usually homozygous for one allele. A heterozygous result indicates a duplicated gene elsewhere in the genome.*

***SUPPORT PROTOCOL 1***

## EXTRACTION OF DNA FROM *E. COLI* O157:H7 CULTURES USING THE QIAGEN GENOMIC DNA PURIFICATION PROCEDURE

DNA suitable for PCR can be extracted from *E. coli* O157:H7 by a number of different methods. This protocol describes a variation to the QIAGEN Genomic DNA purification procedure that allows for reliable and consistent extraction of DNA from low-passage *E. coli* O157:H7 isolates. Almost all of the isolates we have are less than two passages from the time they were isolated and give low yield and variable results when extracting DNA using the original QIAGEN procedure. Three modifications were made to the procedure that lysed the cells more efficiently. First, an incubation step is added at 70°C after resuspending the bacteria pellet in Buffer B1 with RNase A. Second, lysozyme is added and incubated for 10 min before adding proteinase K, followed by a 30-min incubation. The original procedure adds the lysozyme and proteinase K together followed by a 30-min incubation. Third, an equal volume of QBT buffer (4.7 ml) is added to the lysate and vortexed at least 10 sec before adding to the column. Currently, this procedure is expensive and it will cost about $8 an isolate. However, the resulting DNA is of high quality, can be used for other application without further purification, and will store for a very long time.

### *Materials*

LB medium containing 10 g NaCl per liter (see recipe)
*E. coli* O157:H7 (ATCC)
Ice
Qiagen Genomic DNA Buffer set containing:
- Buffer B1
- Buffer B2
- Buffer QBT
- Buffer QC
- Buffer QF

100 mg/ml RNase A (Qiagen)
100 mg/ml lysozyme (Qiagen)
50 mg/ml Proteinase K
Isopropanol, room temperature
70% (v/v) ethanol
95% (v/v) ethanol
TE buffer (*APPENDIX 2A*)
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

Shaking incubator set at 37°C
Centrifuge tubes
Centrifuge (1.5- and 5-ml samples)
Vortex
37°C, 50°C, and 70°C water baths
Genomic-tip 100/G (Qiagen)
Bunsen burner
Glass Pasteur pipets
Spectrophotometer (260 and 280 nm absorbance)

Additional reagents and equipment for streaking *E. coli* cultures on a plate and growing colonies (*APPENDIX 4A*)

***Prepare overnight cultures of E. coli O157:H7***

1. Inoculate 5 ml of LB medium containing 10 g NaCl per liter with an *E. coli* O157:H7 colony.

   *Qiagen recommends using LB containing 10 g NaCl per liter for their genomic DNA extraction kits.*

   *The colony can be taken from any agar plate on which E. coli O157:H7 will grow. The colony should be from a recently streaked plate. Avoid using colonies that are more than a month old.*

2. Incubate the culture overnight at 37°C while shaking.

***Extract DNA from culture***

3. Remove culture from the incubator, transfer culture to an appropriate centrifuge tube, and incubate on ice for 5 min.
4. Centrifuge sample 15 min at 3000 × *g*, 4°C.
5. Discard supernatant and ensure that all liquid is completely removed.
6. Resuspend the pellet completely by vortexing in 3.5 ml of Buffer B1 containing 7 µl of a 100-mg/ml solution of RNase A.
7. Incubate 10 min in a 70°C water bath.
8. Vortex on high for 10 to 15 sec.
9. Transfer to a 37°C water bath and equilibrate for 3 min.
10. Add 80 µl of lysozyme (100 mg/ml).
11. Incubate 10 min at 37°C.
12. Add 50 µl of Proteinase K (50 mg/ml).
13. Incubate 30 min at 37°C.

    *Lysate should be clear at this point.*

14. Transfer tube to a 50°C water bath and equilibrate for 5 min.
15. Add 1.2 ml of Buffer B2 and vortex 2 to 3 sec.
16. Incubate 30 min at 50°C.
17. At a time point 5 min before the end of the incubation, equilibrate a 100/G tip with 4 ml of Buffer QBT.
18. Add 4.7 ml of Buffer QBT to the tube.
19. Vortex the tube on maximum setting for 10 sec.

    *This step is very important. The column will plug more frequently if this step is skipped or shortened.*

20. Add sample to the 100/G tip.
21. Wash the 100/G tip twice, each time with 7.5 ml of Buffer QC.
22. Prewarm elution Buffer QF to 50°C.
23. Elute genomic DNA with 5 ml of prewarmed Buffer QF.
24. Precipitate the DNA by adding 3.5 ml of room-temperature isopropanol and gently inverting the tube 10 to 20 times.

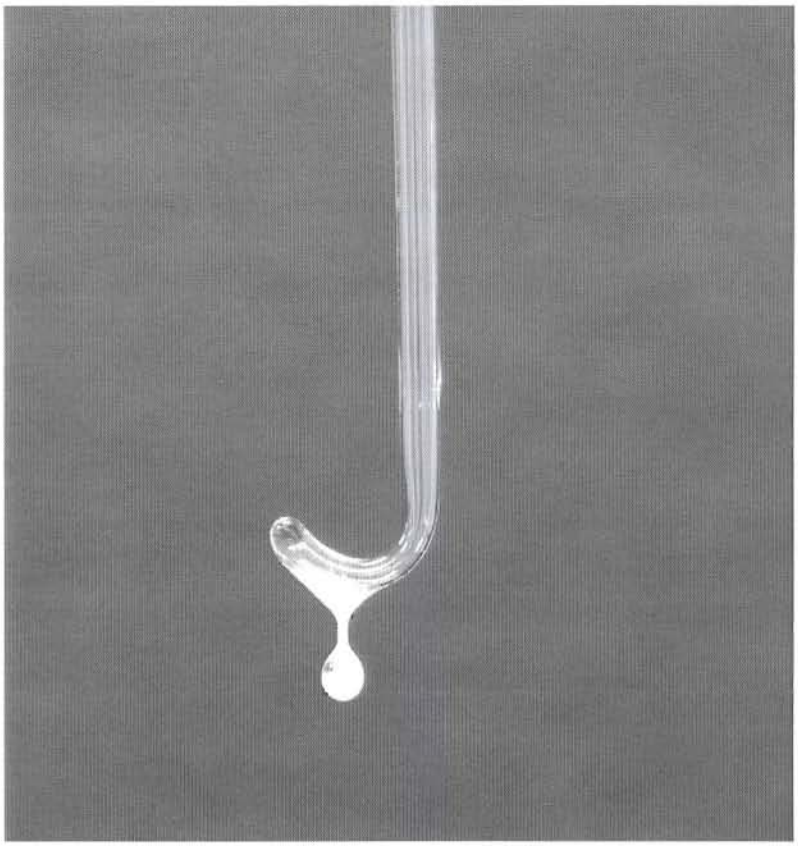

**Figure 5A.3.2** DNA hanging from a hooked Pasteur pipet prior to washing in ethanol.

25. Using an open flame from a Bunsen burner, seal the end of the Pasteur pipet and create a hook.

26. Use the hook to remove the DNA from the tube (Fig. 5A.3.2).

27. Wash the DNA in 1 ml of room-temperature 70% (v/v) ethanol by dipping the DNA up and down for 15 sec.

28. Wash the DNA in 1 ml of room-temperature 95% (v/v) ethanol by dipping the DNA up and down for 15 sec.

29. Air dry until the DNA no longer looks wet.

    *This takes 10 to 15 sec.*

30. Resuspend the DNA in 250 µl of TE buffer.

    *Alternatively, the DNA can be pelleted by centrifuging 15 min at >5000 × g, 4°C. The pellet should be washed with 2 ml of cold 70% ethanol, followed by a second centrifugation for 10 min at >5000 × g, 4°C. Resuspend the DNA in 250 µl of TE buffer.*

31. Dissolve the DNA by incubating 1 hr at 50°C, followed by rocking on a shaker overnight at room temperature.

***Determine DNA yield and purity***

32. Measure absorbance readings at 260 and 280 nm.

33. Determine yield of DNA by reading the absorbance at 260 nm using the Beer-Lambert equation, A = scl, where A is absorbance, s is the specific absorption

coefficient (0.020 $(\mu g/ml)^{-1}$ $cm^{-1}$), c is the concentration of the solute, and l is the length of the light path in solution measured in centimeters (usually 1 cm).

*260 nm Absorbance reading of 0.5 is calculated as:*

$0.5 = (0.02\ \mu g/ml^{-1}\ cm^{-1})(c)(1\ cm)$

$c = 0.5/0.02\ \mu g/ml^{-1}$

$c = 25\ \mu g/ml$ *or* $25\ ng/\mu l$.

34. Determine purity of DNA by dividing the 260-nm reading by the 280-nm reading.

    *A good ratio should be between 1.8 and 2.0.*

35. Dilute the DNA to a final concentration of 1 ng/µl with 10 mM Tris·Cl, pH 8.0.

## DNA EXTRACTION FROM *E. COLI* O157:H7 BY BOILING

***SUPPORT PROTOCOL 2***

A very simple method for extracting suitable DNA is placing the tube with bacteria in a boiling water bath followed by centrifugation to remove the cellular debris. This method provides DNA that does not store very well and has to be cleaned up in order to be used in other methods. If too much cellular debris is present, it can cause inhibition of the PCR and attenuate the fluorescent signal from the probe. Commercial companies sell reagents in which bacterial cells can be suspended to help lyse the cell during boiling and stabilize the DNA for longer storage. Several of these reagents are InstaGene matrix (BioRad Laboratories), PrepMan Ultra (Applied Biosystems), and ZenLyse (Anzen Biosciences). For the purpose of this protocol, the focus will be on using TE buffer or 10 mM Tris·Cl to suspend the bacteria pellets.

### *Materials*

LB medium containing 5 g NaCl per liter (see recipe)
*E. coli* O157:H7 (ATCC)
TE buffer (*APPENDIX 2A*) *or* 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Ice

Shaking incubator set at 37°C
1.5- *or* 2.0-ml microcentrifuge tubes
Centrifuge (1.5-ml samples)
Boiling water bath

Additional reagents and equipment for streaking *E. coli* cultures on a plate and growing colonies (*APPENDIX 4A*)

### *Prepare overnight cultures of E. coli O157:H7*

1. Inoculate 5 ml of LB containing 5 g NaCl per liter with an *E. coli* O157:H7 colony.

   *For extracting DNA by boiling, 5 g NaCl per liter of LB is used to grow overnight cultures.*

   *The colony can be taken from any agar plates that E. coli O157:H7 will grow on. The colony should be from a recently streaked plate. Avoid using colonies that are more than a month old.*

2. Incubate the culture overnight at 37°C while shaking.

### *Perform DNA extraction*

3. Add 1 ml of the overnight culture to a 1.5-ml microcentrifuge tube.

4. Centrifuge 5 min at 15,000 × *g*, 4°C, and then discard the supernatant.

5. Add 100 µl of TE buffer or 10 mM Tris·Cl, pH 8.0.

6. Place tube in a boiling water bath for 5 min.

7. Place tube on ice for 5 min.

8. Centrifuge 5 min at 15,000 × *g*, 4°C, and then transfer the supernatant into a new 1.5-ml microcentrifuge tube.

9. Use 1 µl in PCR reactions.

*Sometimes the DNA prep will need to be diluted for optimal PCR to take place.*

## REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

### *LB medium containing 10 g/liter NaCl*

Dissolve 10 g trypotone, 5 g yeast extract, and 10 g NaCl in 800 ml of distilled water. Adjust the pH to 7.0 with 1 M NaOH and increase the volume to 1 liter with distilled water. Store up to 6 months at room temperature.

*For the LB medium used in Support Protocol 2, use 5 g of NaCl.*

## COMMENTARY

### Background Information

*E. coli* O157:H7 is the major enterohemorrhagic *E. coli* (EHEC) serotype associated with infections in humans in the United States (Corrigan and Boineau, 2001). In the United States from 1982 to 2002 there were 350 *E. coli* O157:H7 outbreaks with two or more cases having a common epidemiologic exposure. The outbreaks occurred in 49 states and comprised over 8500 cases that resulted in 1500 hospitalizations and 40 deaths (Rangel et al., 2005). Outbreaks of *E. coli* O157:H7 were the result of contaminated hamburger, vegetables, water, person-to-person and animal contact, and unknown attributes (Rangel et al., 2005). *E. coli* O157:H7 infections in humans can range from a mild self-limiting diarrhea to a more severe disease including hemorrhagic colitis and hemolytic uremic syndrome (HUS; Nataro and Kaper, 1998; Paton and Paton, 1998). HUS is mainly seen in younger children and is the leading cause of renal failure for children under the age of five (Corrigan and Boineau, 2001). Diagnosis is by plating feces on sorbitol-MacConkey agar. *E. coli* O157:H7 colonies will appear as colorless or neutral/gray with a smoky center because of its inability to ferment sorbitol. Colonies are picked and they are tested using a commercial *E. coli* O157:H7 kit. Treatment is usually supportive to prevent dehydration caused by the diarrhea. Treatment with antibiotics is controversial, but usually not given. Antibiotics are thought to cause the release of Shiga toxin and increase the chance of developing HUS. Cattle are a dominant North American STEC O157 zoonotic reservoir (Elder et al., 2000) and contact with infected livestock and ingestion of contaminated meat are frequent human infection routes (CDC report, 2001, 2002; Crump et al., 2002; Heuvelink et al., 2002).

The locus of enterocyte effacement is an STEC O157 pathogenicity island (McDaniel and Kaper, 1997) encoding two genes, *tir* and *eae*, which are responsible for the tight bacterial association with the intestinal mucosa. The *eae*-encoded ligand protein intimin is located on the bacterial outer membrane; however, the intimin receptor protein Tir is translocated into the epithelial cell by type III secretion and integrated in the host cell membrane (Kenny et al., 1997; Frankel et al., 1998). An SNP at nucleotide position 255 in the *tir* gene (*tir* 255 T>A) was identified that is significantly overrepresented in human isolates relative to isolates with the other allele (99.1% versus 0.9%, $p < 0.0001$; Bono et al., 2007). The reason for this host difference in 255 T>A allele frequency is unknown. A real-time PCR genotyping assay was developed to screen *E. coli* O157:H7 isolates for their allele at *tir* 255 T>A.

### Critical Parameters and Troubleshooting

Before beginning an experiment using this protocol, familiarize yourself with the DNA extraction methodology and on how to operate the real-time PCR thermal cycler. The protocol that is presented here uses a commercially available PCR master mix that is optimized for TaqMan assays. Optimization of PCR master mix components will be needed if a master mix using individually purchased components

is made. The thermal cycling conditions will work on Applied Biosystems 7900HT, 7700, 7000, or 5700 Sequence Detection Systems and the BioRad Opticon Continuous Fluorescence detector, but may need to be optimized on other real-time PCR thermal cyclers. A positive result should be a low $C_T$ for one allele or the other. All *E. coli* O157:H7 have the *tir* gene and a negative result for both alleles indicates that the assay did not work. Possible problems could be: inappropriate reaction conditions, incorrect fluorescent dye chosen, a component of the reaction master mix omitted, primer or probe are the wrong sequence, degraded or no template added, or a PCR inhibitor is present. If a heterozygous result occurs or the no-template negative controls were positive, contamination of the assay with amplicons from a previous assay is probably the problem. Primers for this assay amplify a 69 base pair amplicon, which can aerosolize and contaminate equipment and reagents in the laboratory. There are several ways to avoid contaminating the laboratory. First, never remove the cover from the tubes or plate after the thermal cycling is finished. Second, the TaqMan Universal Master Mix can be purchased with UNG AmpErase. UNG AmpErase is a system that replaces dTTP with dUTP as a dNTP substrate for PCR. Uracil N-glycosylase (UNG) will remove the uracil from the double-stranded DNA leaving a gap in the sequence. After a 2-min incubation at 50°C, the amplicon from a previous real-time assay that used dUTP as a dNTP is degraded because of the gaps produced by UNG removing the uracil. However, the recently added DNA template is not affected by the UNG activity. The UNG activity is greatly reduced by the next step, a 10-min incubation at 95°C.

## Anticipated Results

A low $C_T$ value indicates which of the two possible alleles are present in the isolate tested. Since all *E. coli* O157:H7 have the *tir* gene, a positive result should be obtained from one of the two probes.

## Time Considerations

Depending upon what DNA extraction protocol is used, the whole procedure can be completed in 2 or 3 days. The modified Qiagen DNA extraction method (Support Protocol 1) takes 3 days. On day 1, an overnight culture is started. The following day, the extraction will take 5 to 7 hr to complete and the DNA will take overnight to resuspend. The day after the extraction, the real-time PCR can be set up and run. The set up takes 10 to 30 min, depending upon the number of samples being assayed and the thermal cycling run lasts ~2 hr. The boiling method (Support Protocol 2), will take 2 days to run. The culture is started the first day, and on the second day the DNA is extracted using the boiling method which takes ~45 min to complete, after which the real-time PCR can be set up and run.

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## Internet Resources

http://ecoli.cas.psu.edu/

*Gastroenteric Disease Center is one of the largest repositories of Escherichia coli strains in America and performs serotyping and diagnostics for E. coli.*

http://www.shigatox.net/cgi-bin/stec/index

*The STEC Center is designed to facilitate research on the Shiga toxin-producing E. coli by providing a standard reference collection of well-characterized strains and central online accessible databases.*

http://www.dorak.info/genetics/realtime.html

*A good reference for learning about real-time PCR and the different applications associated with this technique.*