TITLE: **Detection of Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7/NM by Multiplex Real-time PCR using the Cepheid SmartCycler II Instrument**

SOURCE: Food and Drug Laboratory Branch (FDLB)
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EFFECTIVE DATE: _____ INITIALS: _____

RETIRED DATE: _____ INITIALS: _____

REVISIONS:

No.	What has been revised?	Pages	Initials/Date
2.0	Reaction mix assembly	6 & 7	

Sign off sheet for: Procedures, Equipment, Test Methods, Work Instructions

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1. Introduction

1.1 Purpose

The purpose of this procedure is to rapidly identify the presence and absence of Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7/H$^-$ from food and environmental samples by detection of *stx1*, *stx2* and the *uidA* mutation.

1.2 Principle and Background

Shiga toxin-producing *E. coli* (STEC) have been implicated in food and water borne outbreaks of diarrhea worldwide. Among the STEC, enterohemorrhagic *E. coli* (EHEC) causes severe bloody diarrhea, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in children, the elderly, and immunocompromised individuals. There are several different serotypes of classified as EHEC. To date, the *E. coli* O157:H7 serotype is the serotype most often identified EHEC in Northern America, Japan and the United Kingdom. More than 30% of cattle carry STEC and/or *E. coli* O157:H7 in their gut without signs of infection. The organisms are introduced into the environment through manure. Direct or indirect contacts with cattle manure, such as consumption of contaminated undercooked meat, lettuce, sprouts, and apple juice, visiting petting zoos and farms, or from recreational water

exposure, have been linked to *E. coli* O157:H7 outbreaks in the US. The organism possesses viral (phage) and chromosomal virulence genes including *stx1*, *stx2,* and *eae* that play important roles in pathogenesis.

This is a multiplex real-time PCR method developed by *Jinneman et al*, 2003, for detection and amplification of *stx1*, *stx2* and the *uidA* mutation. All STEC may carry the genes for stx 1 and/or stx2. Only *E coli* O157:H7 and O157:NM carry the mutation in the β-glucuronidase gene uidA. The method was optimized in pure cultures with 100% specificity and more than 98.6% sensitivity. The multiplex format utilizes a fluorogenic 5' nuclease assay with fluorogenic probes for simultaneous detection of all 3 genes. There are 3 different reporter dyes labeled to different probes: FAM (6-carboxy-fluorescein), Texas Red (Sulforhodamine 101) and TET (Tetrachloro-6-carboxyfluorescein). Fluorogenic probes are labeled with a fluorescent dye at the 5' end and a quencher dye at the 3' end. Fluorescent signal is not detected when reporter dye and quencher dye are in close proximity while binding to the single strand target DNA. During the extension phase of polymerase chain reaction, *Taq* DNA polymerase with exonuclease activity degrades the probe, releasing reporter dye from quencher dye, and the fluorescent reporter dye becomes detectable. Real-time PCR instruments collect fluorescence data from PCR cycles where fluorescence accumulates in proportion to the formation of PCR products. Data are recorded as C_T value (threshold cycle number), which is the first PCR cycle when fluorescence signal increases above background or cut off value (threshold line).

2. Safety

E. coli O157:H7/NM is a human pathogen with a low infectious dose: ingestion of 10 – 100 cells can cause disease. The use of gloves and eye protection is mandatory and all work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must follow CDC guidelines for manipulating Biosafety Level II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis are kept in the appropriate laboratory areas. The personnel who will handle the materials should read all MSDS sheets. All workers in the laboratory should abide by all federal, state, local and laboratory safety guidelines and rules where they apply when using this method.

3. Quality Control

3.1 Control Cultures

3.1.1 The ATCC #43888 strain of *E. coli* O157:H7 is used in this procedure as a positive growth control. This strain does not contain stx 1 or 2 genes, but does contain the uidA mutation, as do other strains of *E. coli* O157.

3.1.2 Non-infectious DNA from the ATCC strain #43894 of *E. coli* O157:H7 is used as a positive control for the *stx1* and *stx2* genes, as well as *uidAm*. The culture for this organism is never handled in the diagnostic laboratory, and is stored securely. Nucleic acid is extracted as per 5.0 below. Working control stock is diluted to maintain a C_T value of 17 – 23.

3.1.3 Negative Template Control: PCR-grade water is used for template (instead of nucleic acid).

3.1.4 Reagent Quality Control: Each new lot of primer, probe and PCR reaction kit is tested for specificity and sensitivity in parallel with old reagent lot before use. All reagents are dispensed in small aliquots and required not to exceed 10 freeze-thaw cycles.

3.2 PCR Quality Control

In order to prevent carryover and aerosol contamination of PCR reactions it is necessary to be vigilant at all times, and maintain strict segregation of steps and functions throughout the process. Dedicated rooms, equipment, lab coats, tube racks, ice trays, even pens and markers, are assigned to each of three areas, and should never be transferred between areas. Color-coding (with colored tape) of each dedicated, transportable item is advised. Run sheets and other documents may be transferred from the cleanest to the "dirtiest" area, but never in reverse.

3.2.1 Pre-PCR reagent preparation room (clean room)

The cleanest space in the process, no nucleic acid, nor any article that has possibly come in contact with nucleic acid, should be brought into this space. Precautions taken in this space are to protect the space from contamination by the operator, from nucleases in perspiration and on hands, extraneous nucleic acids picked up on hands and shoes in other locations, and aerosol contamination from saliva and respiratory secretions. Wash hands immediately upon entering and put on gloves before touching counters, drawers, pipettors, etc. An acrylic, enclosed PCR workstation with UV decontamination capability is preferred. Decontaminate station and racks with 15-20 min UV light after each use. Decontaminate pipettors and equipment by wiping down with isopropanol (IPA), preferred, or 70% ethanol after each use.

3.2.2 Nucleic acid extraction room

The dirtiest space, in which multiple organisms and cultures are rendered non-infectious through extraction, so care must be taken to wipe down and decontaminate benches and equipment with **each use** with IPA or 70% ethanol. Dilute solutions of sodium hypochlorite are used if infectious material is spilled, but then must be rinsed off and removed to prevent corrosion.

3.2.3 PCR assembly room

Intermediate between the other two spaces, reagents prepared in the clean room are dispensed into Cepheid reaction tubes in this space, and nucleic acid is added to

each tube. All work should be done in a BSC with UV decontamination capability. Work surfaces and pipettors should be decontaminated following each use with IPA or ethanol, then UV irradiated for 15-20 min.

3.2.4 Post-PCR room

Real-time PCR does not involve subsequent handling of PCR products generated, and post-PCR space is not required. Care must be taken, however, when removing reaction tubes from the cycler that the caps do not pop open creating aerosols of PCR products and contaminating the surfaces.

4. Equipment, Materials, Supplies, Reagents, Control Cultures

4.1 Equipment

4.1.1 SmartCycler II (Cepheid, Sunnyvale, CA) real-time PCR instrument.

4.1.2 Labnet minicentrifuge for SmartCycler tubes (provided with purchase of thermocycler).

4.1.3 Ice machine

4.1.4 Dedicated equipment for <u>nucleic acid extraction</u>:

- 4.1.4.1 Micropipettors (Gilson or equivalent) to deliver 20 – 1000 µl; dedicated set of 20, 200, and 1000 µl.
- 4.1.4.2 Microcentrifuge with rotor for 1.5 ml eppendorf tubes (Eppendorf 5415 D or 5415 R or equivalent).
- 4.1.4.3 Vortex
- 4.1.4.4 Microtube racks to accommodate 1.5 ml eppendorf tubes.
- 4.1.4.5 Heating block, capable of 94 – 95°C.
- 4.1.4.6 Freezer storage space, -20 – 30°C.
- 4.1.4.7 Ice trays

4.1.5 Dedicated equipment for <u>pre-PCR reagent preparation</u>:

- 4.1.5.1 Micropipettors (Gilson or equivalent) to deliver 1 – 1000 µl; dedicated set of 10 µl, 20 µl, 200 µl and 1000 µl...
- 4.1.5.2 Enclosed acrylic PCR work station with UV light for decontamination.
- 4.1.5.3 Vortex.
- 4.1.5.4 Minicentrifuge for 1.5 ml eppendorf tubes.
- 4.1.5.5 Refrigerator, 4°C.
- 4.1.5.6 Freezer, -20°C.
- 4.1.5.7 Ice trays
- 4.1.5.8 Dedicated microtube racks to accommodate 1.5 ml eppendorf tubes.

4.1.6 Dedicated equipment for <u>PCR assembly</u>

- 4.1.6.1 Micropipettors (Gilson or equivalent) to deliver 1 – 200 µl; dedicated set of 10 µl and 200 µl.
- 4.1.6.2 Biological Safety Cabinet (BSC) with UV decontamination capability.
- 4.1.6.3 Ice trays
- 4.1.6.4 Racks to accommodate Cepheid PCR reaction tubes (provided by manufacturer).

4.2 Materials and Supplies

4.2.1 Sterile, lock-seal (stable for boiling) 1.5 ml eppendorf tubes, in pre-PCR and nucleic acid extraction rooms.
4.2.2 Sterile aerosol-resistant micropipette tips, dedicated for each preparation area.
4.2.3 Sterile Cepheid SmartCycler PCR reaction tubes for PCR Assembly room.
4.2.4 Dedicated latex powder-free disposable examination gloves for each area.
4.2.5 Dedicated pens, markers, scissors, and pencils for each area.
4.2.6 Dedicated lab coats for each area.
4.2.7 Sterile 1 ml pipettes for the nucleic acid extraction area.
4.2.8 Anti-aerosol micro tip disposal bags and stands for each area.
4.2.9 Kimwipes (large) for each area.
4.2.10 PCR-quality water, aliquotted, for each area.
4.2.11 IPA or 70% ethanol, for each area.

4.3 Reagents

4.3.1 For PCR Reaction Mix assembly

4.3.1.1 **Primers and probes designs** *(Jinneman et al, 2003).*

Primers:
stx1F934: 5'-GTG GCA TTA ATA CTG AAT TGT CAT CA-3'
stx1R1042: 5'-GCG TAA TCC CAC GGA CTC TTC-3'

stx2F1218: 5'-GAT GTT TAT GGC GGT TTT ATT TGC-3'
stx2R1300: 5'-TGG AAA ACT CAA TTT TAC CTT TAG CA-3'

uidAF241: 5'-CAG TCT GGA TCG CGA AAA CTG-3'
uidAR383: 5'-ACC AGA CGT TGC CCA CAT AAT T-3'

Probes:
stx1P990T: 5'-TxRd-TGA TGA GTT TCC TTC TAT GTG TCC GGC AGA T-BHQ2-3'
stx2P1249: 5'-FAM-TCT GTT AAT GCA ATG GCG GCG GAT T-BHQ1-3'
uidAP266: 5'-TET-ATT GAG CAG CGT TGG-MGB/NFQ-3'

Prepare 50 µM stock solutions of each and freeze at -20°C.

4.3.1.2 Primer Pool

Prepare 10 µM of each primer in a pool of all 6 primers. Aliquot and freeze at -20°C.

4.3.1.3 2X Real-time PCR enzyme and buffer kit (TaKaRa BioUSA, Madison, WI,), *PreMix ExTaq*™ DNA polymerase, RR039A or equivalent. Freeze at -20°C.

5.0 Nucleic acid extraction

DNA may be extracted from pre-enrichment culture for screening PCR, or bacterial culture for confirmation PCR.

5.1 Screening PCR: Remove 1 ml pre-enrichment broth and centrifuge 1 ml culture at 13,000 x g for 2 minutes. Discard supernate, resuspend pellet with 100 µl PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystem, Foster city, CA, Part # 4318930) and vortex.

5.2 Confirmation PCR: From solid media: Resuspend 1 isolated colony in 1 ml PBS or Butterfield's buffer. From liquid media: Remove 1 ml of broth and treat as above. Vortex, and centrifuge at 13,000 x g for 2 minutes. Discard supernate, resuspend pellet with 100 µl PrepMan™ Ultra Sample Preparation Reagent, and vortex.

5.3 Place the aliquot in 95°C heat block for 15 minutes and then on ice for 5 minutes. Centrifuge the aliquots at 12,000 rpm for 2 minutes. Transfer supernates to clean tubes and store tubes at –20 ^{o}C.

6.0 Real-time PCR Assembly

6.1 Reaction Mix Assembly

PCR Reaction Mix Assembly, per 25 µl reaction volume
Prepare a PCR Run Sheet with unique identifying run number. Record lot numbers and expiration dates of all reagents, analyst name and date. Multiply the following amounts times the number of reactions desired for samples plus three controls, plus 10% excess. All frozen reagents, must be thawed, vortexed to mix, and centrifuged briefly to consolidate. Hold all reagents and assembled reaction mix on ice during preparation and aliquotting.

	/1 Rx (µl)
PCR grade water	7.0625
2X *Premix Ex Taq* ™	12.5
Primer pool, 10 µM	3.75
Probes:	
stx1P990T, 10 µM	0.375
stx2P1249, 10 µM	0.0625
uidAP266, 10 µM	0.25
DNA template	1 µl

6.2 PCR Assembly

6.2.1 Transfer reaction mix tube and extracted nucleic acid samples to ice trays in the BSC of the PCR assembly room. Spin briefly if contents have been disturbed.

6.2.2 Label Cepheid reaction tubes and place in Cepheid tube racks on ice. Aliquot 24 µl of reaction mix per tube.

6.2.3 Add 1 µl of each extracted sample and each control, closing each tube after template addition. Transfer racks to PCR equipment room.

6.2.4 Spin each reaction tube briefly in the Labnet minicentrifuge to consolidate contents in the reaction tube window area.
6.2.5 Transfer to Cepheid SmartCycler blocks.
6.2.6 Controls:
6.2.6.1 ATCC 43888 growth control extracted DNA, 1 µl.
6.2.6.2 ATCC 43894 DNA control, 1 µl.
6.2.6.3 PCR-quality water, negative control, 1 µl.

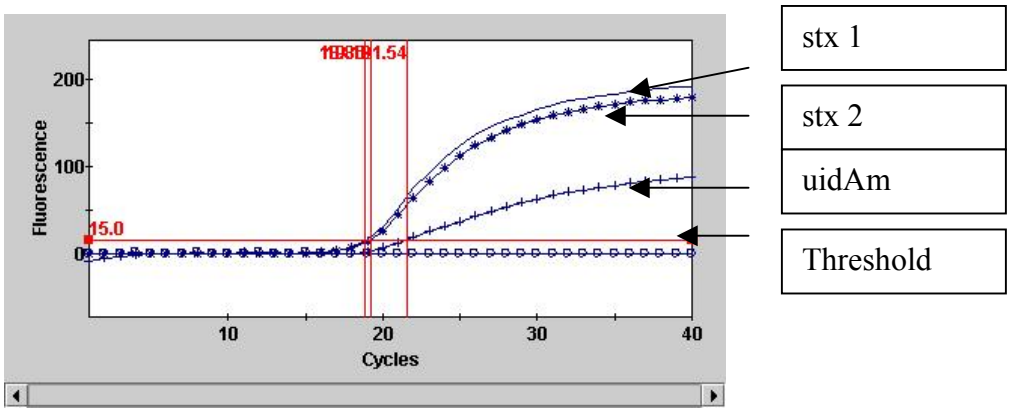
7. Instrument Settings

Select Run number. Use all default analysis setting parameters from version 1.2d except setting Manual threshold fluorescence units to 15. Select dye set FTTC25. Select "EHEC" protocol. The EHEC Protocol is: hold at 95 °C, 60 sec. then 40 cycles of 94 °C, 10 sec and 63 °C, 40 sec.

8. Interpretation

These following conditions need to be achieved:

a. There should not be any curve on the negative template control.
b. The positive DNA control should exhibit 3 curves for all 3 channels (Texas Red for *stx1*, FAM for *stx2* and TET for *uidA* mutation) that cross the threshold line.

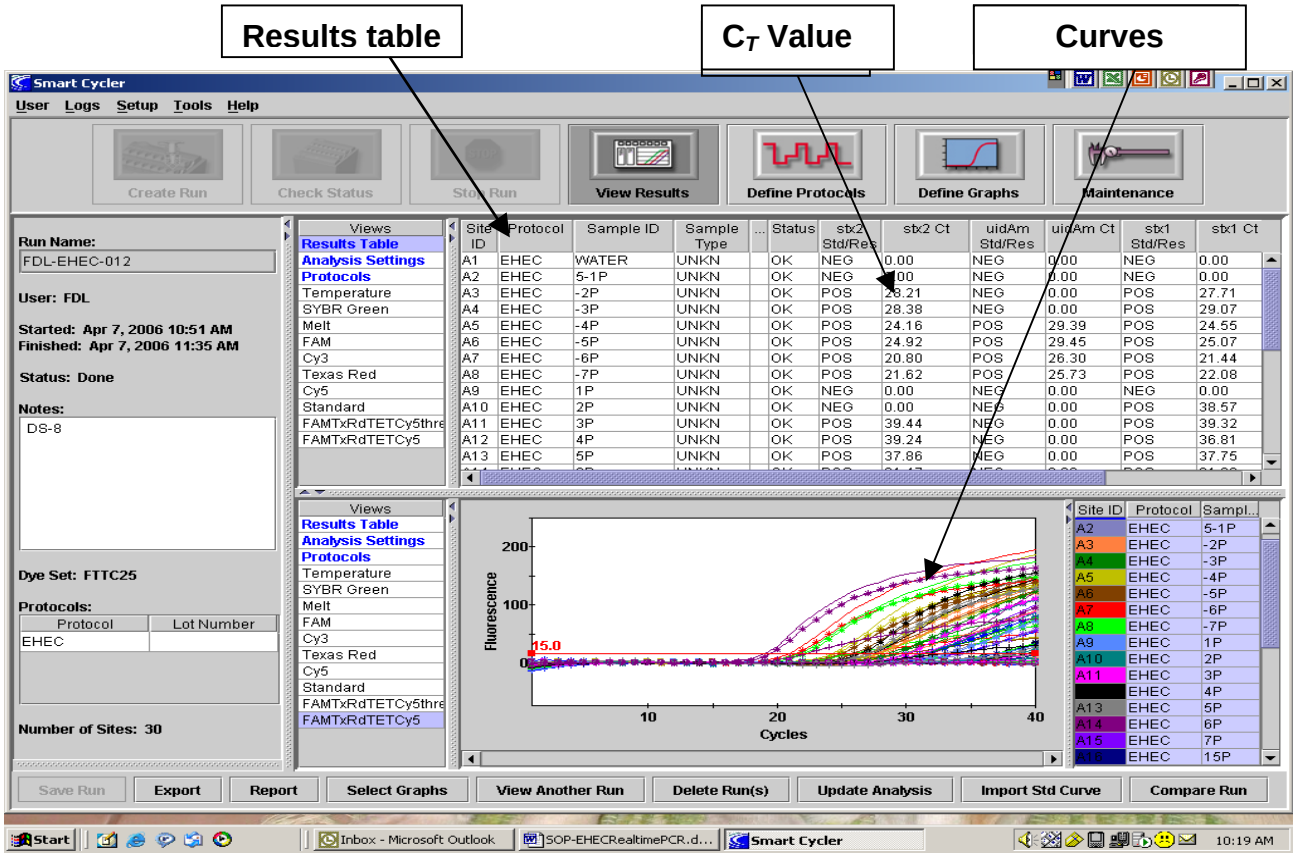


c. When both controls meet the stated requirement, a sample is considered positive for the *E. coli* O157:H7/H$^-$ if growth curves cross the threshold line within 40 cycles for *uidA* mutation and/or *stx1* and/or *stx2*. C_T values of 39 or less are recorded in result table by the instrument (see below).
d. When both controls meet the stated requirement, a sample is considered positive for the Shiga toxin-producing *E. coli* (STEC) but negative for *E. coli* O157 if curves DO NOT cross the threshold line within 40 cycles for *uidA* mutation BUT cross the threshold line for either or both *stx1* and/or *stx2* genes.
e. When both controls meet the stated requirement, a sample is considered negative for the *E. coli* O157:H7/H$^-$ if growth curves DO NOT cross the threshold line within 40 cycles for all 3 genes,

uidA mutation, *stx1* and *stx2*. C_T values of "0" are recorded in result table by the instrument (see below).

9.0 Report

Results are entered onto a Real-time PCR results form. Data are recorded as either positive or negative for all 3 genes individually, *stx1*, *stx2* and *uidAm*. Final result reads: "*E. coli* O157 detected" (positive) or "*E. coli* O157 not detected" (negative) based on detection of *uidAm* and one or more *stx* genes. The report is signed by analyst and supervisor, released by the Section Chief, and put on file.



9. References

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