Laboratory Procedure

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IDENTIFICATION OF *ESCHERICHIA COLI* O157:H7 AND VEROTOXIN-PRODUCING *ESCHERICHIA COLI* 0157:NM BY THE WARNEX™ REAL-TIME POLYMERASE CHAIN REACTION SYSTEM

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1. APPLICATION

The method is applicable to the rapid identification of *Escherichia coli* O157:H7 (*E. coli* O157:H7) strains and verotoxin-producing *Escherichia coli* O157:NM strains (*E. coli* O157:NM) isolated from raw ground beef using the standard *E. coli* O157:H7 culture technique, MFLP-80 (8.1), other HPFB methodologies, or the Warnex 8-hour enrichment methodology to detect *E. coli* O157:H7. It can be applied to the presumptive identification of *E. coli* O157:H7/NM from enrichment broth cultures. When product-based compliance action is anticipated, and where stipulated HPFB methodology, MFLP-80 (8.1) or equivalent is used for final confirmation of real-time polymerase chain reaction (PCR) positive colonies.

2. PRINCIPLE

Following the sample enrichment procedure for raw ground beef, the broth is subjected to a real-time PCR procedure in which the target pathogen DNA is amplified and detected using specific primers and molecular beacons. Molecular beacons consist of unique sequence probes that allow for the identification of the pathogen with a high level of specificity. Once bound to their target, the molecular beacons emit a fluorescent signal that is proportional to the amount of amplified pathogenic DNA. In the absence of target bacteria in food samples, no fluorescent signal is detected.

For the detection of *E. coli* O157:H7, two markers are used. The first detects the presence of *E. coli* O157 and the second confirms the presence of *E. coli* O157:H7. *E. coli* O157:NM (non-motile) strains that are verotoxin-producers, and thus pathogenic, will also be detected. The O157:NM strains that do not produce verotoxins will not. Taken together, positive results from these two markers will confirm the presence of *E. coli* O157:H7 or verotoxin-producing *E. coli* O157:NM contamination in the food sample.

The entire procedure, after the enrichment, identifies presumptive positive samples within 3 hours, and can replace the usual screening tests thus providing considerable savings on time, labour and cost of the analysis. The real-time PCR technique has proven to be a specific and sensitive method for the presumptive identification of *E. coli* O157:H7 and verotoxin-producing *E. coli* O157:NM in raw ground beef

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samples.

The Warnex™ Real-Time PCR Rapid Pathogen Detection System for *E. coli* O157:H7/NM has been AOAC-RI validated and was granted *Performance Tested Method*℠ status in 2004, Certificate No. 010409.

Warnex is a trademark of Warnex Diagnostics Inc., 3885 Industriel Blvd., Laval (Quebec), Canada, H7L 4S3. Ph: (450) 663-6724, Fax: (450) 669-2784, website: www.warnex.ca

3. DEFINITION OF TERMS

See Appendix A of Volume 3

4. COLLECTION OF SAMPLES

See Appendix B of Volume 3.

5. SPECIALIZED PRIMERS, REAGENTS, BUFFERS, MATERIALS AND EQUIPMENT

Note: The laboratory Supervisor must ensure that completion of the analysis described in this method is done in accordance with the International Standards reference "ISO/IEC 17025:1999 (or latest versioon): General Requirements for the Competence of Testing and Calibration Laboratories".

5.1. Materials and Equipment

5.1.1 Materials and special equipment provided

Extraction buffer (EX-1)
Extraction reagent, lyophilized (EX-2)
Extraction microplates
Extraction microplate seals
Detection buffer (DT-1)
Detection reagent, lyophilized (DT-2)
Detection microplates or PCR strip tubes (Flexiwells) containing pre-dispensed PCR reagents and primers
Flexiwell support and cover (for Flexiwell format only)
PCR optical-grade seals

5.1.2 Additional materials and special equipment required

Warnex-validated Real-Time PCR thermocycler *

* Thermocycler specifications: 96-well (or 48-well) low profile microplate or 96 (48) x 0.2 mL low profile strip tube sample capacity, ability to excite fluorophores with a peak excitation range of 485 to 520 nm and ability to detect fluorophores with a peak emission range of 500 to 600 nm

Vortex (with platform head adaptors and replacement inserts)
Centrifuge (with rotor and microplate adaptor)
Stomacher
Stomacher filter bags
Pipettors to cover range of volumes (0.5-1000 µL) with sterile plugged pipette tips

5.2 PCR Primers, Temperaure Cycling Program, Buffers and Reagents

5.2.1 PCR primers

The oligonucleotide primers and molecular beacon probes are supplied in the kit and their specificity for *E. coli* O157:H7/NM has been verified (8.2).

5.2.2 Temperature cycling programs

The temperature cycling programs for the PCR include an automated 15-minute extraction cycle, resulting in the lysis of the bacterial cells, followed by an automated PCR detection process. The thermal cycler program for the detection (amplification) process is preset for the following sequence of cycling parameters:

Amplification:

1 cycle of: hot start, 15 mins, 95°C

40 cycles of: denaturation, 15 secs, 94°C
annealing, 15 secs, 55°C
microplate read
extension, 15 secs, 72°C

5.2.3 Buffers and reagents

All buffers and reagents are provided by Warnex Diagnostics Inc. with each kit. These include the extraction reagents (EX-1, EX-2) and the detection reagents (DT-1, DT-2). All other detection buffers and reagents are pre-dispensed in the wells of the detection microplate or Flexiwell. All water, pipettes, pipette tips and other materials coming in contact with samples or PCR reagents should be sterile and DNase free and/or autoclaved prior to use to remove any DNase and/or other contamination.

6. PROCEDURE

6.1 Handling of Samples

Prepare and enrich samples according to the standard culture methodology for isolation of *E. coli* O157:H7 (8.1), other HPFB methodologies, or the Warnex 8-hour sample enrichment methodology, as per the manufacturer's instructions. Remove a portion of the culture enrichment and subject it to the PCR technique as indicated below. Positive control wells and negative control wells are also processed with each set of samples.

6.2 DNA Extraction

6.2.1. From the kit, dispense the contents from EX-1 (squeeze bottle) into the EX-2 vial to obtain reconstituted EX-2.

6.2.2 Recap the EX-2 vial and mix by inversion to ensure complete dissolution of desiccated reagent.

6.2.3 Pour reconstituted EX-2 into a multi-channel pipettor basin.

6.2.4 Aliquot 90 µL of reconstituted EX-2 into each well of the Extraction microplate (one well per extraction).

6.2.5 Transfer 10 µL of cell suspension from 6.1 into the Extraction microplate according to the sample assignment layout using a single-channel pipettor.

6.2.6 Seal the wells of the Extraction microplate with the domed microplate cap strips provided.

6.2.7 Place the Extraction microplate into the thermocycler and close the lid.

6.2.8 Begin the DNA extraction program for the thermocycler.

6.2.9 When the Extraction program has been completed, take out the Extraction microplate. All organisms are now lysed and inactivated.

6.2.10 Centrifuge the Extraction microplate for 5 minutes at 1,800 x g. The supernatant can now be used in the PCR amplification method.

6.3 PCR Amplification Method

6.3.1 From the kit, dispense the contents from DT-1 (squeeze bottle) into the DT-2 vial to obtain reconstituted DT-2.

6.3.2 Recap the DT-2 vial and mix by inversion to ensure complete dissolution of desiccated reagent.

6.3.3 Pour reconstituted DT-2 into a multi-channel pipettor basin.

6.3.4 From the kit, take out a Detection microplate or Flexiwell from its pouch. Discard the empty microplate pouch or reseal the Flexiwell pouch, if unused Flexiwells remain.

6.3.5 Aliquot 15 µL of reconstituted DT-2 into the wells of the Detection microplate or Flexiwell using a multi-channel pipettor.

6.3.6 Transfer 10 µL of extracted DNA from the Extraction microplate into the wells of the Detection microplate or Flexiwell.

6.3.7 Seal the Extraction microplate using the provided microplate seals and store at 4°C until the analysis is completed.

6.3.8 Seal the Detection microplate or Flexiwell with the optical-grade seals provided.

6.3.9 Vortex the Detection microplate or Flexiwells (in the Flexiwell support) for 1 minute.

6.3.10 Centrifuge the Detection microplate or Flexiwells (in the Flexiwell support) for 1 minute at 1,800 x g. This is to ensure that all the PCR mixture, including the sample's DNA extract, is in the bottom of the wells of the PCR microplate.

6.3.11 Secure the Detection microplate or Flexiwells (in the Flexiwell support) tightly in the PCR instrument. Make sure well A1 is in the upper left corner. Close the PCR lid and click on "Start Detection" to initialize the PCR protocol.

6.3.12 When the Detection program has been completed, remove and discard the Detection microplate or Flexiwells.

7. INTERPRETATION OF RESULTS

The amplicons (PCR products) generated from the *E. coli* O157/O157:H7/NM target sequences by this PCR method are double-stranded DNA fragments. A positive PCR test will result in a fluorescence response higher than the baseline produced by the negative controls within 40 cycles of the PCR amplification. The baseline cut-off fluorescence value is determined and set by the system. A negative PCR test will normally not produce visible fluorescence. If fluorescence occurs above the baseline cut-off value in the negative controls, the results of the test are invalidated and the analysis must be repeated with precautions taken to eliminate possible sources of error. Any sample found to be positive by the Warnex PCR technique must be confirmed with the required biochemical tests prescribed in the HPFB, MFLP-80 (8.1) or equivalent methods.

8. REFERENCES

8.1 Warburton, D. And D. Christensen. 2006. MFLP-80. Isolation of *E. coli* O157:H7 or NM in Foods. In: Volume 3. Compendium of Analytical Methods. Available at the Health Canada website at: http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/index_e.html.

8.2 Warnex Research Inc. 2005. Validation of the Warnex Rapid Pathogen Detection System for *Escherichia coli* O157:H7/NM 8-Hour Enrichment in Raw Ground Beef (in-house unpublished data and AOAC data).