AOAC

Duopath® Verotoxins

GLISA-Rapid Test (Gold Labelled ImmunoSorbent Assay) for the qualitative detection of Verotoxins from Verotoxinogenic *E. coli* isolated from food enrichments.

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 020402

Intended Purpose

The Duopath® Verotoxins GLISA test is an immunochromatographic rapid test intended to be used in food-analysing laboratories for the qualitative detection of Verotoxins (Shiga-like toxins) 1 and 2 from Verotoxinogenic (including *E. coli O157:H7*) isolated from food enrichments using FDA, USDA or other food enrichment methods. This test has been validated and received AOAC approval for detection of Verotoxins 1 and 2 from isolated Verotoxin-producing *E.coli* (including *E. coli O157:H7*). Duopath® Verotoxin is also intended to be used in clinical laboratories for the qualitative identification of Verotoxins 1 and 2 (Shiga-like toxins 1 and 2) produced by *E. coli* isolated in cultures derived from clinical stool specimens. The identification aids in the diagnosis of diseases caused by enterohemorrhagic *E. coli* infections.

Introduction

Among the *E. coli* human pathogens, Verotoxin (Shiga-like toxin) forming strains (VTEC) have gained in importance in recent years. The group of enterohaemorrhagic *E. coli* (EHEC) with its highly pathogenic serovars 0157:H7, 026, 0103, 0111, 0145, and other strains are of particular concern. Production of Verotoxins is the most common criteria for the detection of this group of bacteria. Verotoxins can be classified into two main categories Verotoxin 1 (VT1, SLT1, Stx1) and Verotoxin 2 (VT2, SLT2, Stx2). EHEC strains may produce either VT1 or VT2 only or both VT1 and VT2 simultaneously. EHEC are capable of initiating life threatening illnesses, particularly in those with immune deficiency, young children and the elderly. The main sources of infection are contaminated, raw or insufficiently heated foods of animal origin, e.g. meat and dairy products. The reservoir for EHEC is the feces of cattle, sheep and goats. These microorganisms can enter food during the processing of meat and dairy products if hygienic conditions are inadequate. The drastic increase in the incidence of food infection caused by E. coli 0157 demands reliable and rapid methods of detection. In addition to traditional culture methods, immunological techniques are becoming more useful due to their improved specificity and sensitivity. Duopath® Verotoxins is an immunological screening test based on the immune flow principle.

Typical Composition

Package contents:

25 test devices individually packaged in aluminum foil. Each device consists of a plastic housing with 2 ports which encloses and protects the pads containing test reagents.

Reagent components of the test device:

1. Membrane associated Mouse monoclonal anti-VT1 antibody
2. Membrane-associated mouse monoclonal anti-VT2 antibody
3. Membrane-associated goat polyclonal anti-mouse antibody
4. Gold-labeled mouse monoclonal anti-VT1 and anti-VT2 antibodies
5. Buffer
6. Blocking agents

Storage and shelf-life after first opening

Duopath© Verotoxins is stable until the expiry date printed on the box when stored at +2 to +8 °C. The test device should be used with 2 hr after removal from the sealed foil pouch. Tests should not be used if the foil pouch is torn or has been previously opened. If no red line appears in the poitive control zone C during a test the test device is not working properly and should be discarded.

Specimen Collection and Handling

Appropriate stool specimens may be held at controlled room temperature for up to 4 h prior to preparing cultures. Stool specimens that cannot be cultured within 4 hours should be placed at 2 - 8 °C and cultured within 24 hours. If the specimens cannot be cultured within 24 h they should be frozen at -70 °C as soon as possible after receipt.

Sample Preparation

Stool

Using a swab inoculate stool samples onto Sorbitol-MacConkey agar plates containing no tellurite or cefixime. Incubate for 18 - 24 h at 35 °C. Using a swab, sweep a few times across the confluent growth area of the plate avoiding mucoid colonies. Mucoid colonies may interfere with migration of the sample. Dacron swabs are preferred to cotton swabs since less liquid is retained by the Dacron swabs for subsequent testing. Suspend the swab in 0.5 ml distilled water containing 50 µg/ml polymyxin B to enhance the release of Verotoxins from VTEC. Incubate the mixture for 30 minutes at 35 °C.

Isolated bacteria from food

Pick 1 - 5 colonies from SMAC, CT-SMAC or Brain Heart Infusion (BHI) agar and dispense it in 1 ml CAYE broth containing CAYE broth supplement C.

Incubate for 6 h at +37 °C.

Pipette 180 µl CAYE-culture into a Eppendorf Cup.

Dissolve powder in Polymyxin B vial with 1 ml sterile distilled H_2O, the add 20 µl of Polymyxin B solution (concentration: 5 mg/ml) to the 180 µl CAYE culture and mix.

Incubate the Eppendorf Cup for 10 min. at 35 - 37 °C

Allow to cool to room temperature.

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Isolation of E. coli O157 from foods

Mix 25 g solid sample or 25 ml liquid sample with 225 ml enrichment medium 1 and homogenise with a Stomacher if necessary.

Incubate for 18 - 24 h at +42 °C (mEC + N) or at +35 - 37 °C (mTSB + N).

Inoculate CT-SMAC Agar with an aliquot from the enrichment broth.

Incubate for 18 - 24 h at +35 to +37°C.

Pick up 1 - 5 typical colonies from the CT-SMAC Agar and dispense it in 1 ml of the CAYE broth containing CAYE broth supplement C.

Incubate for 6 h at +37 °C.

Pipette 180 µl CAYE-culture into a Eppendorf Cup.

Add 20 µl Polymyxin B solution (preparation see above) and mix.

Incubate the Eppendorf Cup for 10 min. at 35-37°C.

Allow to cool to room temperature.

For dairy products, mTSB + Novobiocin selective enrichment broth (MERCK 1.09205.) is recommended.

For meat and meat products, mEC + Novobiocin selective enrichment broth (MERCK 1.14582.) should be used.

Only *E.coli O157* serovars are able to grow on CT-SMAC Agar. To detect Verotoxins of other serovars, CT-SMAC agar must be replaced by SMAC or BHI agar.

Test Procedure

Stool

Prior to use allow the enriched sample and the required number of foil sealed test devices to reach room temperature (+15 to +25 °C).

Remove the foil pouches from the required number of Duopath® Verotoxins devices.

Place the test device(s) on a flat surface and label with appropriate sample identification. Note: Perform the test within 2 h of opening.

Gently swirl the polymyxin B sample to mix.

Using a micropipetter and disposable pipette tip, draw up 200 µl and dispense it into the circular sample port on the test device. Alternatively using a disposable transfer pipette, squeeze the pipette bulb, insert the stem into the sample tube and release pressure on the bulb. This will draw sample up into the pipette. Dispense six (6) free falling drops into the circular sample port on the test device (about 190 µl). Incubate at room temperature for 10 min and read immediately.

Food

Remove the foil pouches from the required number of Duopath® Verotoxins devices. Place the test device(s) on a flat surface and label with appropriate sample identification. (Note: Perform the tests within a period of 2 hours after opening!).

Briefly stir the Eppendorf Cup with a Vortex mixer.

Using a micro pipette and disposable pipette tip, draw up 150 µl and pipette it into the circular sample port on the test device.

Alternatively using a disposable transfer pipette, squeeze the pipette bulb, insert the stem into the Eppendorf Cup and release pressure on bulb. This will draw sample up into the pipette. Dispense five (5) free falling drops (about 150 µl) into the circular sample port on the test device.

Incubate the test at room temperature (+15 to 25 °C) and observe the test result immediately 20 minutes after applying the sample to the device.

Methodology

Principle of the method

Duopath® Verotoxins (1.04144.) is an immunochromatographic rapid test utilizing monoclonal antibodies which are labeled by red-colored gold particles. The test device has a circular sample port and an oval shaped test (VT1, VT2) and control (C) window.

1. The sample is applied to the chromatography paper via the circular sample port.
2. The sample is absorbed through the pad to the reaction zone containing colloidal, gold labeled antibodies specific to Verotoxins.
3. Any Verotoxin (VT1 and VT2) antigen present complexes with the gold-labeled antibody and migrates through the pad until it encounters the binding zones in the test (VT1, VT2) area.
4. The binding zones (VT1 and VT2) contain another anti VT1 or VT2 antibody, which immobilizes any Verotoxin antibody complex present. Due to the gold labeling, a distinct red line is then formed.
5. The remainder of the sample continues to migrate to another binding reagent zone within the control (C) zone, and also forms a further distinct red line (positive control). Regardless of whether any Verotoxin is present or not, a distinct red line is always formed in the control (C) zone and confirms that the test is working correctly.

Interpretation of Results

The test is working correctly if a distinct red line appears in the control zone (C) within the read time of 10 - 20 min. A sample can be considered POSITIVE if at or prior to the read time, red lines appear on test (VT1 and/or VT2) and control (C) zones. A sample can be considered NEGATIVE if no red line appears in the test (VT1and VT2) zones but does appear distinctly in the control (C) zone within 10 - 20 min after application of sample to the device. The result line must be red to be considered Positive. A dark line which is not red should be considered Negative. A positive result indicates that Verotoxin 1 and/or Verotoxin 2 (Shiga-like toxins) from *E. coli* were detected in the sample. A negative result indicates that neither Verotoxin 1 nor Verotoxin 2 was detected in the sample.

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Detection Limit

One colony is the lower detection limit. The lower limits of detection are 25 ng/mL for VT1 and 62.5 ng/mL for VT2.

Interferences

Detection of Verotoxins from *E. coli O157* isolated from foods has been successfully tested in different laboratories with food samples such as raw ground beef and pasteurised whole milk when the above described protocol was used. Results obtained to date with numerous *E. coli* isolates indicate that there is no interference of Duopath® Verotoxins with non-Verotoxinogenic *E. coli* or food ingredients. Duopath® Verotoxin has been validated and AOAC approved for use of bacteria isolated from food enrichments using FDA, USDA or other food enrichment methods.

The test has been developed based on using CAYE medium from MERCK. Interference from other types of CAYE medium and other brands cannot be excluded. In particular use of broth of red-brown colour could potentially mask weak signals due to background coloration of the test zone.

Limitations of the Procedure

- The Duopath® Verotoxins assay detects the presence of Shiga-like toxin from culture isolates. The level of toxin has not been shown to be correlated with either the presence or severity of disease.
- The performance of this assay has not been evaluated with direct stools and enrichment broth testing.
- A positive result does not preclude the presence of other infectious organisms.
- Overgrowth of normal fecal flora could mask sorbitol negative colonies.
- Toxin expression may be lost upon serial passage. Colony sweeps may increase the likelihood of detecting Shiga toxin producing organisms.
- Enteric media other than Sorbitol/MacConkey (SMAC) has not been tested for use with this assay.

Trouble-shooting

Problem	Action
No line appears in Control zone after 10 minutes test period.	Re-run sample

Performance Characteristics

Stool specimen

REPRODUCIBILITY:

Three independent laboratories tested three samples in triplicate, on each of three different times in one day (intra-assay variability) and on three different days (inter-assay variability). Samples consisted of three negative, three low positive and three strong positive. The Duopath® Verotoxins produced 100% reproducibility including control lines.

SPECIFICITY:

The specifictiy of Duopath® Verotoxins was tested with the following clincial isolates which were inoculated onto SMAC plates and followed by polymyxin B extractions.

Microorganisms (number of strains tested)

Pseudomonas aeruginosa (10)

Klebsiella pneumoniae (10)

Enterobacter species (10)

Proteus species (10)

Non-Stx-producing *E. coli* (10)

Aeromonas species (3)

Serratia marcescens (5)

Shigella species (3)

None of the above isolates cross-react with the Duopath© Verotoxins

ASSAY SENSITIVITY (WITH STOCK CULTURES):

The following 40 STEC stock cultures were cultivated on SMAC plates and followed by the polymyxin extraction.

Number of Strains Tested	Serotype
32	O157:H7
1	O96:H9
1	O111:NM
2	O26:H11
1	O103:H2
1	O145:NM
1	O45:H2
1	O45:NM

All of the above isolates produced positive reactions on Duopath® Verotoxins Test Devices.

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Performance Data

The Duopath® Verotoxin test was evaluated in the United States and the tested specimens included 249 fresh stools and 41 Shiga-toxin positive frozen stools.

DUOPATH® VEROTOXIN
Fresh Stool Specimen

Reference Method*	Positive	Negative	Totals
Positive	5	0	5
Negative	1**	243	244
Totals	6	243	249
% agreement +	100 % 41/41		
% agreement -	99.6% 243/244		

DUOPATH® VEROTOXIN
Frozen Stool Specimen

Reference Method*	Positive	Negative	Totals
Positive	41	0	41
Negative	0	0	0
Totals	41	0	41
% agreement +	100 % 41/41		
% agreement -	No negative results		

*Premier EHEC (Meridian Bioscience, Inc.)

**_E. coli O157:H7_ was recovered from the culture but was not detected by the reference method.

Foods

Sensitivity	VT1	>99 %	VT2	>99 %
Specificity	VT1	>99 %	VT2	>99 %

Precautions

E. coli O157 (including H7) isolates have been shown to be infective at very low dosage (<50 bacteria). Users of Duopath® Verotoxin must be familiar with the appropriate aseptic techniques for the isolation and identification of _E. coli O157_ (including H7). Extreme care must be kept in handling samples, enriched culture media and devices.

Disposal

Decontaminate Duopath® devices, tubes, pipettes, and culture media by autoclave, bleach, etc. in accordance with local, state, and federal regulations.

Technical Assistance

For technical assistance, please contact your local Merck representative or Merck KGaA 64271 Darmstadt, Germany.
Tel : +49-6151-720, Fax : +49-6151-72 20 00,
Email: service@merck.de.

Literature

C.H. Park, H.J. Kim, D.L. Hixon, and A. Bubert; Evaluation of the Duopath® Verotoxin Test for Detection of Shiga Toxins in Cultures of Human Stools; Journal of Clinical Microbiology 41, June 2003, p. 2650-2653

Ordering Information

Product	Ordering No.	Pack size
Duopath® Verotoxins	1.04144.0001	25 tests
CAYE Broth mod. acc.to Evans	1.00060.0100	100 g
CAYE Broth Supplement C	1.00051.0001	16 vials
mEC Selective Enrichment Broth w/ Novobiocin	1.14582.0500	500 g
mTSB Selective Enrichment Broth w/ Novobiocin	1.09205.0500	500 g
Sorbitol-MacConkey (SMAC) Agar	1.09207.0500	500 g
CT-Supplement	1.09202.0001	16 vials

Additionally required materials and instrumentation

- Polymyxin Solution: 5 mg Polymyxin B sulfate (1.09875.0001 Bacillus Cereus Selective Supplement) in 1 ml of deionized water
- Stomacher/Stomacher bags
- Incubators +35 °C and +42 °C
- Distilled or deionized water
- Autoclave
- Disposable plastic transfer pipettes and/or appropriate micro pipettes and disposable tips for dispensing 200 µl
- Disposable inoculation loops