Version 15-04/24

User's Manual

E. Coli Verotoxin 1+2 Ag ELISA Kit

REF **DEIA1966**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The EHEC test is a rapid in vitro microwell ELISA for the detection of Shiga Toxins I and II (Verotoxins) in stool specimens, broth cultures, from individual colonies or colony sweeps of agar plates. The EHEC test is intended for use as an aid in the research of enterohemorrhagic E. coli (EHEC) infections.

General Description

Enterohemorrhagic E. coli (EHEC) have been isolated from patients who have hemorrhagic colitis and hemolytic-uremic syndrome (HUS). To date, O157:H7 is the most frequently encountered EHEC strain in stools from these patients. This is probably due to the fact that conventional diagnostic strategies such as O157 latex agglutination assays rely on the unique sorbitol negative fermentation property of this strain. The major weakness in this approach is that at least 50 other EHEC serotypes have been reported to be associated with the development of hemorrhagic colitis and HUS. One virulence trait of all EHEC strains is the ability to produce cytotoxin(s) called Shiga toxin (ST) or verotoxin (VT) ST-I and ST-II are the two most common toxins and individual EHEC strains have the ability to produce both or either, in varying quantities. Therefore, ST production and not individual (O157:H7) serotype identification is a better diagnostic strategy for the determination of EHEC associated disease. Cytotoxin can be identified by a specific cytotoxin assay described by Karmali. The cytotoxin assay, however, is labor intensive, requires tissue culture facilities, has not been standardized and may take up to 72 hours to confirm the presence of cytotoxin. Exploiting the EHEC attribute to produce these toxins, EHEC was developed for the direct detection of ST producing strains from stool specimens or culture systems.

Principles of Testing

The EHEC test utilizes monoclonal anti-Shiga toxin capture antibodies absorbed to microwells.Diluted samples are added to the wells and incubated at room temperature. A wash is performed to remove unbound material. Polyclonal anti-Shiga-like toxin antibodies are added for detection and incubated at room temperature. Another wash is used to remove unbound antibody. Enzyme conjugated anti-IgG polyclonal antibody is added and incubated at room temperature. If toxin is present, a reactive antibody-enzyme complex is formed. After washing to remove unbound conjugate, substrate is added and incubated for 10 minutes at room temperature. Color develops in the presence of bound enzyme. Stop Solution is added and the results are interpreted visually or spectrophotometrically.

Reagents And Materials Provided

The maximum number of tests obtained from this kit is listed on the outer box.

1. **EHEC Antibody Coated Microwells** – Breakaway plastic wells coated with monoclonal antibodies specific for E. coli Shiga toxin I and II.
2. **EHEC Positive Control** – Inactivated Shiga toxin in a buffered protein solution with preservative.
3. **EHEC Negative Control** – Buffered solution with a preservative.
4. **EHEC Sample Diluent** – Buffered Protein solution with a preservative.

5. **20× Wash Buffer II** – Concentrated wash buffer with a preservative.
6. **EHEC Detection Antibody** – Rabbit antibodies specific for Shiga toxins in buffered protein solution containing preservative.
7. **EHEC Enzyme Conjugate** – Goat anti-rabbit antibody conjugated to horseradish peroxidase in buffered protein solution containing preservative.
8. **Substrate II** – Buffered solution containing urea peroxide.
9. **Stop Solution II** – 2N Sulfuric Acid. **CAUTION:** Avoid contact with skin. Flush with water if contact occurs.
10. **Transfer pipettes** - Each pipette is marked to indicate 50 µL, 100 µL, 200 µL and 300 µL volumes.
11. **Microwell strip holder**
12. **Microwell strip sealers**

Materials Required But Not Supplied

1. Wooden applicator sticks
2. Test tubes (12 × 75 mm) for dilution of sample
3. Distilled or deionized water
4. Squirt bottle
5. Graduated cylinder for making 1× Wash Buffer
6. ELISA plate reader capable of reading absorbance of 450 nm or 450/630 nm*
7. STAT Fax™ 2200 plate shaker or equivalent (Optional – Broth Method Only)*

NOTE: It is the operator's responsibility to validate readers and plate shakers prior to their use with this product.

Storage

The expiration date is indicated on the kit label. Store the kit at 2-8°C and return the kit promptly to the refrigerator after each use.

Specimen Collection And Preparation

1. CDC Recommendations for Stool Specimen Storage, Isolation and Identification of EHEC Organisms:

   Raw stool specimens should be examined as soon as they are received in the laboratory. If not processed immediately, they should be placed at 2-8°C or frozen at ≤ -70°C.

   Refrigerated raw stool specimens should be examined within 1-2 hours. If stools cannot be examined within this time, they should be placed in a Cary-Blair-based transport medium. All rectal swabs should be placed immediately in a Cary-Blair-based transport medium.

   If specimens in transport medium will be examined in 2-3 days, they should be refrigerated. If specimens in transport medium are not examined in 2-3 days, they should be frozen immediately at ≤ -70°C. Specimens in transport medium should not be refrigerated for days, then frozen or left for any time at room temperature.
2. Stool and Broth Storage Prior to EHEC Testing: Stool specimens and broths may be held up to seven days

at 2-8°C before testing in the ELISA. If testing is not performed within this time period, the stool and/or broth should be frozen at (≤ -70°C). Repeated freeze-thaws should be avoided.

A. Direct Stool:

1. Add 200 µL of Sample Diluent to a clean 12 × 75 mm test tube. (NOTE: Third marking from tip on transfer pipette represents 200 µL.)

2. Mix stool as thoroughly as possible prior to pipetting.

a. Liquid, semi-solid or stools in transport medium: Using a transfer pipette, draw stool to the 50 µL calibration point (first mark from the tip of the pipette). Dispense the stool into the Sample Diluent. Using the same pipette, gently withdraw and expel the stool suspension several times, then vortex 15 seconds. Leave transfer pipette in tube for later use.

b. Non-pipetable stools: Using a wooden applicator stick, transfer a small "BB" sized portion (3-4 mm diameter) of thoroughly mixed stool into Sample Diluent. Emulsify stool using the wooden applicator stick, then vortex 15 seconds. Place a transfer pipette in the tube.

B. Plate Method:

1. Add 20 µL of stool or specimen to MacConkey or Sorbitol/MacConkey plate and spread with an inoculating loop.

2. Incubate 16-24 hours at 37°C.

3. Individual colonies or colony sweeps can be removed with a loop and placed in 200 µL of Sample Diluent for ELISA testing.

C. Broth Method:

1. Add 10-50 µL of stool to 5 mL of Mac onkey's Broth, or GN Broth. Vortex 10-15 seconds.

2. Incubate 16-24 hours at 37°C.

3. Add 50 µL of growth to 200 µL Sample Diluent for ELISA testing.

Reagent Preparation

1. Bring the entire kit, including microwell pouch, to room temperature before use.
2. Prepare 1× Wash Buffer as needed. For example: 4.0 mL of 20× Wash Buffer II + 76.0 mL of distilled or deionized water is sufficient to wash one strip. Place in a clean squirt bottle. The 1× Wash Buffer can be stored at room temperature for up to three months.

Assay Procedure

NOTE: With large numbers of specimens, repetitive or multichannel pipettes may be used for dispensing the reagents.

1. After the pouch has reached temperature, break off the required number of microwells (1 well for each specimen plus 1 positive and 1 negative control well per batch). Place the microwells in the microwell strip holder and record the location of all wells. Unused microwells must be resealed in the pouch immediately.
2. Add 100 µL of diluted specimen (second mark from the tip of the pipette) to the appropriate well (place pipette tip halfway into well and let sample slowly run down side of well).

3. Add 2 free-falling drops of Positive and Negative Control to the appropriate wells. Mix wells by firmly shaking/swirling the plate for 30 seconds.
4. Cut plate sealer to size and press firmly onto top of microwells to seal. Incubate the plate for 1 hour at room temperature (22-27°C). Broth Method Only: Alternatively, laboratories equipped with a heated plate shaker (STAT Fax™-2200) can incubate and rotate the plate for 30 minutes at 25°C at 1000 rpm (setting 5).
5. Carefully, remove the plate sealer and wash wells:

   a. Dump plate contents firmly into a biohazard receptacle.

   b. Bang the inverted plate on a clean stack of paper towels.

   c. Fill all wells with 1× Wash Buffer. Use of a squirt bottle is recommended.

   d. Repeat washing cycle (dump, bang on fresh towels, fill) 4 additional times. After the last fill, dump and bang plates on fresh towels hard enough to remove as much excess Wash Buffer as possible but do not allow wells to completely dry at any time.
6. Add 2 free-falling drops of Detection Antibody to each well. Firmly shake/swirl the plate for 30 seconds.
7. Press plate sealer firmly onto top of microwells to seal. Incubate the plate for 30 minutes at room temperature (22-27°C). Broth Method Only: Alternatively, incubate and rotate the plate for 15 minutes on the STAT Fax™ at 25°C and at 1000 rpm.
8. Repeat wash procedure (Step #5)
9. Add 2 free-falling drops of Enzyme Conjugate to each well. Firmly shake/swirl the plate for 30 seconds.
10. Press plate sealer firmly onto top of microwells to seal. Incubate the plate for 30 minutes at room temperature (22-27°C). Broth Method Only: Alternatively, incubate and rotate the plate for 15 minutes on the STAT Fax™ at 25°C and at 1000 rpm.
11. Repeat wash procedure (Step #5)
12. Clean the underside of all wells with a lint free tissue.
13. Add 2 free-falling drops of Substrate Solution II to each well. Firmly shake/swirl the plate for 30 seconds. Incubate for 10 minutes at room temperature.
14. Add 2 free-falling drops of Stop Solution II to each well. Firmly shake/swirl the plate for 30 seconds. **NOTE:** Initial color of positive reaction is blue, which changes to yellow upon addition of Stop Solution II.
15. Observe Reactions:

    a. Visual Determination – Read within 15 minutes after adding Stop Solution II.

    b. Spectrophotometric Determination – Zero ELISA reader on air. Wipe underside of wells with a lint free tissue. Read absorbance at 450 nm or 450/630 nm within 30 minutes of adding Stop Solution II.

Quality Control

This test should be performed per applicable local, state, or federal regulations or accrediting agencies.

1. The Positive and Negative Controls must be used with each assay run to provide quality assurance of the reagents.
2. The Positive Control should read> 0.500 at either 450 nm or 450/630 nm. The Positive Control should be a definite yellow color when read visually.
3. The Negative Control should read < 0.180 at 450 nm and <0.150 at 450/630 nm but greater than 0.00. If the Negative Control is < 0.00, re-blank the plate reader to air and re-read the plate. The Negative Control

should be colorless when read visually.

4. Any positive well without visible color should be repositioned, wiped on the underside of the well and re-read.
5. If the frequency of low positive results (OD between 0.150 and 0.200 with dual wavelength or OD between 0.180 and 0.230 with single wavelength) is greater than 5% of specimens tested, this indicates insufficient washing. More vigorous washing or increasing the washes to seven washes in step 5 of the Procedure is recommended.
6. If the expected control reactions are not observed and the reagents are still within their expiration date, repeat the control tests as the first step in determining the root cause of the failure.
7. At the time of each use, kit components should be visually examined for obvious signs of microbial contamination, freezing or leakage.
8. It is suggested that results of each quality control check be recorded in an appropriate log book to maintain high quality testing records.

Interpretation Of Results

1. Visual Reading

Negative – colorless

Positive = definite yellow color

A faint yellow color must be evaluated spectrophotometrically

NOTE: In view of the epidemiological importance of obtaining bacterial isolates it is recommended that all toxin positive samples be submitted for isolation of Shiga toxin positive organisms. We suggest that individual microbiology laboratories coordinate bacterial isolation with their local state health laboratories.

2. Spectrophotometric Single Wavelength (450 nm)

Negative = OD450 < 0.180

Positive = OD450 ≥ 0.180

3. Spectrophotometric Dual Wavelength (450/630 nm)

Negative = OD450/630 < 0.150

Positive = OD450/630 ≥ 0.150

NOTE:

1. A positive result indicates the presence of Shiga toxins. A negative result indicates the absence of Shiga toxins, or that the level of toxin is below that which can be detected by the test.

2. See Quality Control section regarding low positive results. Extremely strong positive reactions may yield a purple precipitate within a few minutes of stopping the reaction.

Performance Characteristics

The EHEC test was evaluated at three major medical centers and one state health laboratory in the United States. Each study site tested diarrheal stools submitted for routine culture of enteric pathogens. Each stool was tested directly and by the broth method in the EHEC assay. A specific ST cytotoxin neutralization assay

was performed at CD on these specimens. The results obtained for the direct stool assay and the broth assay are summarized in the following table.

	CD EHEC			
	Direct Stool		Broth Assay	
Cytotoxin	Positive	Negative	Positive	Negative
Positive	15	4	22	0
Negative	17	391	5	236
Indeterminate	2	17	0	3

Relative Sensitivity = 78.9% (56.6-91.5%)* 100% (85.1-100%)

Relative Specificity = 95.8% (93.4-97.4%) 97.9% (95.2-99.1%)

Relative Agreement = 95.1% (92.6-96.8%) 98.1% (95.6-99.2%)

ST producing EHEC were isolated from two of the 17 EHEC positive/cytotoxin negative direct stool specimens: one O157:H7 and one O6:H untypeable. In addition, PCR** confirmed the presence of the ST gene in two EHEC positive/cytotoxin negative and one positive/cytotoxin indeterminate stool specimens. The 15 EHEC positive/cytotoxin positive direct stool specimens were comprised of: nine – O157:H7, two – O6:H untypeable, one – O untypeable: H12, 41w or 51, and two – O111:NM EHEC strains. An isolate could not be obtained from the remaining stool specimen.

ST producing EHEC were isolated from two of five EHEC positive/cytotoxin negative broths: one O157:H7 and one O111:NM. The 22 EHEC positive/cytotoxin positive samples were comprised of 13 O157:H7, three O6:H untypeable, one O untypeable H:12, 41w or 51, and one O111:NM ST producing strains of EHEC. No isolates could be found in the remaining four broth specimens.

*95% confidence intervals calculated by the normal method as indicated in parenthesis.

**PCR is a research tool and not intended for in vitro diagnostic use.

Various E. coli ST producing organisms were tested in the ELISA by both the Plate and Broth Methods (Specimen Collection And Preparation). Each strain is a clinical isolate and each was tested by a cytotoxin assay and by a polymerase chain reaction (PCR) to confirm the presence of the ST gene. All organisms generated positive results when tested in this manner. The number in parenthesis represents the number of different strains tested in that group type. Also, the type of toxin produced by each strain is indicated.

Strain Type	ST Type	Strain Type	ST Type
O157:H7 (3)	I	O91:H21 (1)	II
O157:H7 (7)	II	O146:H21 (1)	I
O157:H7 (9)	I & II	O137:H41 (1)	I & II
O111:NM (3)	I & II	O111:H8 (1)	I & II
OX3:H21 (1)	II	O50:H7 (1)	I
O4:NM (1)	I & II	O145:NM (2)	II
O165:H25 (1)	II	O103:H2 (1)	I
O165:H25 (1)	I & II	O125:NM (2)	I
O45:H2 (1)	I	O26:H11 (1)	I
O126:H27 (1)	I	O5:NM (3)	I
O121:H19 (1)	I & II	O171:NM (1)	II
O121:H19 (1)	II	O83:H1 (1)	I & II

Detection Limit

The limit of detection for ST-I and ST-II are approximately 7 and 15 pg/well respectively.

Specificity

The EHEC ELISA was tested for specificity using the clinical isolates (CI) or ATCC strains listed. Each strain was tested directly in the ELISA by the Plate method using the colony isolate procedure (Specimen Collection And Preparation). Every strain was negative when tested in this manner. In an additional study, the bacterial strains were spiked into either a single positive EHEC stool or single negative EHEC stool at a concentration of approximately 2.4×10^8 cfu/mL. The following clinical isolates (CI) or ATCC strains were all found to be negative after direct testing. In addition, the following strains did not alter the expected results when spiked into an EHEC positive or negative stool.

Description		Description	
Campylobacter coli	CI	*Prevotella bivia*	ATCC 29303
Campylobacter fetus	CI	*Proteus vulgaris*	ATCC 6380
Campylobacter jejuni	CI	*Providencia alcalifaciens*	ATCC 9886
Campylobacter lari	CI	*Providencia stuartii*	ATCC 33672
Candida albicans	ATCC 10231	*Pseudomonas aeruginosa*	ATCC 9027
Citrobacter freundii	ATCC 8090	*Pseudomonas fluorescens*	ATCC 6570
Clostridium perfringens	ATCC 3624	*Pseudomonas fluorescens*	ATCC 6571
Clostridium perfringens	ATCC 14810	*Salmonella* Group B	CI
Entercoccus faecalis	CI	*Salmonella hilversum* (Grp N)	ATCC 15784
Enterobacter cloacae	ATCC 13048	*Salmonella minnesota*	ATCC 9700
Escherichia coli	ATCC 8739	*Serratia liquefaciens*	CI
Escherichia coli	ATCC 9637	*Shigella dysenteriae*	ATCC 11456
Escherichia fergusonii	ATCC 35469	*Shigella flexneri*	CI
Escherichia hermanii	ATCC 33650	*Shigella sonnei*	CI
Gardnerella vaginalis	CI	*Staphylococcus aureus*	ATCC 6538
Helicobacter pylori	CI	*Staphylococcus aureus* (Cowan I)	ATCC 12598
Klebsiella pneumoniae	ATCC 13883	*Staphylococcus epidermidis*	ATCC 12228
Lactobacillus acidophilus	ATCC 4356	*Streptococcus agalactiae*	CI
Mycobacterium smegmatis	CI	*Streptococcus faecalis*	CI
Neisseria gonorrhorae	CI	*Yersinia enterocolitica*	CI
Nocardia asteroides	ATCC 3308	*Yersinia enterocolitica* O:9	ATCC 55075

Reproducibility

Intra-assay Variability – Ten replicates of two known positive broth specimens and two known stool positive specimens were tested in one assay.

Sample	Mean $A_{450/630}$	SD	% CV
Broth #1	0.362	0.013	3.5
Broth #2	1.920	0.094	4.9
Stool #1	0.317	0.010	3.1
Stool #2	0.774	0.032	4.2

Inter-assay Variability – Ten replicates of two known positive broth specimens and one negative broth specimen were run on three separate days. This protocol was repeated using one negative and two positive stool specimens.

Sample	Mean $A_{450/630}$	SD	% CV
Broth #1	0.369	0.032	8.7
Broth #2	1.546	0.293	18.9
Broth #3	0.074	0.007	9.2
Stool #1	0.335	0.027	8.1
Stool #2	0.818	0.079	9.6
Stool #3	0.058	0.007	11.4

Precautions

1. All reagents are for reaearch use only.
2. Patient specimens may contain infectious agents and should be handled at Biosafety Level 2 as recommended in the CDC/NIH manual, "Biosafety in Microbiology and Biomedical Laboratories".
3. All reagents should be mixed gently before use.

4. Do not interchange Microwells, Detection Antibody, Enzyme Conjugate or Positive Control Reagent between different kits. 20× Wash Buffer II, Sample Diluent, Substrate II and Stop Solution II can be interchanged provided they are within their expiration dates at the time of testing.
5. Allow reagents to warm to room temperature before use.
6. Hold reagent vials vertically at suitable distance above the well to insure proper drop size and delivery.
7. Do not use kit components beyond labeled expiration date.
8. Replace colored caps on correct vials.
9. Dispose of used wash buffer and all test materials in appropriate container. Treat as potential biohazard.
10. The Positive Control regent contains inactivated Shiga toxin. It should be handled, however, as a potentially hazardous material.
11. Avoid skin contact with Stop Solution II (2N Sulfuric Acid). Flush with water immediately if contact occurs.
12. Do not reuse microwells.
13. Unused microwells must be placed back inside resealable pouch. It is important to protect strips from moisture.
14. Transfer pipettes provided must be used for specimen preparation and transfer. Use one per specimen.
15. Avoid splashing when dispensing diluted samples into microwell by placing transfer pipette tip about halfway down the well and dispensing slowly down the side of well.
16. Microwell washing is to be performed precisely as directed in assay procedure. **Inadequate washing will cause elevated background and false positive results.**
17. All reagents except the 20× Wash Buffer II are provided already diluted to the proper concentration.
18. Any deviation below or above set incubation times may affect sensitivity and specificity and should be avoided.
19. Do not use microwells with pouches that have been damaged (ie, show holes or punctures).

Limitations

1. The EHEC test detects the presence of Shiga toxin from stool, broths or culture isolates. The level of toxin has not been shown to be correlated with either the presence or severity of disease. The kit is for research use only.
2. Shiga toxin I and the toxin produced by Shigella dysenteriae type 1 strains (shiga toxin) are nearly identical. Therefore, EHEC will generate a positive signal when Shiga toxin is present in the specimen of other infectious organisms.
3. A positive result does not preclude the presence of other infectious organisms.
4. Toxin expression may be lost upon serial passage. Colony sweeps may increase the likelihood of detecting ST producing organisms.