



REF 6006

March 20, 2008

# Verotoxin Antigen

- 96 determinations -



IVD *In vitro* diagnostic device

Enzyme immunoassay for the determination of Verotoxin 1 + 2 (Shiga toxin 1 + 2) in fecal specimens

<b>REF</b>	Catalogue number	<b>LOT</b>	Batch code
	Consult accompanying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction		Biological risk

## INTENDED USE

Verotoxin Antigen is used for the qualitative detection of verotoxin 1 and verotoxin 2 (shiga toxin 1 and shiga toxin 2) in fecal specimens.

Invasive and toxigenic *Escherichia coli*-strains cause diarrhea in infants and adults. Among pathogenic *E. coli* strains the group of enterohemorrhagic *E. coli* (EHEC) can cause life threatening hemorrhagic colitis and hemolytic uremic syndrome (HUS) leading to acute renal failure and hemolytic anemia with thrombocytopenia (1,2,3). Strains like *E. coli* O:157; O:26; O:111 and other serovars are characterized by the production of cytotoxins (verotoxin 1 and 2 or shiga-toxin 1 and 2, shiga-toxin variants). The diagnosis of an EHEC infection is initially done by the detection of the shiga-toxins. Diagnostic methods can be direct toxin detection by cytotoxicity test on vero-cells and subsequent neutralization test or the detection of the encoding genes with probes or polymerase chain reaction. These methods are time consuming and not suited for a routine diagnostic laboratory. Immunological methods like enzyme immunoassay enable a fast and specific shiga-toxin detection in stool specimens. It is commonly recommended to enrich the EHEC bacteria in selective broth media prior to the test run to enhance the sensitivity of the method (4, 5, 6).

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2. Bockemühl, J., Karch, H. und Tschäpe, H.: Infektionen des Menschen durch enterohämorrhagische *Escherichia coli* (EHEC) in Deutschland, 1996. Bundesgesundheitsbl. 6 (1997): 194-197
3. Stock, I. und Wiedemann, B.: Infektionen durch enterohämorrhagische *Escherichia coli* (EHEC-) Stämme. MMP, 20. Jahrgang, Heft 3 (1997):58-65
4. Gerritzen, A.: Vergleichender Verotoxin-Nachweis im Stuhl mit zwei Enzymimmunoassays und dem Zytotoxizitätstest auf Verozellen. Lab. Med. 1998; 22 (12): 704-712
5. Reissbrodt, R.: Enterohemorrhagic *Escherichia coli*: isolation and identification. Biotest Bulletin 6: 65-74 (1998)
6. Fruth, A. et al.: Zur Verbesserung der gegenwärtigen bakteriologischen Diagnostik von enterohämorrhagischen *Escherichia coli* (EHEC). Bundesgesundheitsbl-Gesundheitsforsch-Gesundheitsschutz 4, 310-317 (2000)

## PRINCIPLE OF THE TEST

Verotoxin Antigen is a fast enzymometric immunoassay for the qualitative determination of both verotoxin 1 and verotoxin 2 employing polyclonal solid phase immobilized and monoclonal biotinylated antibodies to verotoxin 1 and verotoxin 2.

Verotoxin of specimens and the positive control reacts with anti-verotoxin antibodies coated on the solid phase of the microplate. After incubation for 60 minutes at 22-25° non-bound material is removed by a wash step.

Subsequently bound verotoxin reacts specifically with anti-verotoxin biotinylated antibodies. After an incubation period of 30 min at 22-25°C non-bound components are separated from the solid-phase immune complexes formed by the following wash step.

During the next incubation of 30 min at 22-25°C streptavidin conjugated to horseradish peroxidase (HRP) recognizes the bound biotinylated antibodies. Non-bound components are separated by the following wash step.

HRP converts the colorless substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) added into a blue product. The enzyme reaction is stopped by dispensing an acidic solution into the wells after 15 min at 22-25°C turning the solution from blue to yellow.

The optical density (OD) of the solution read at 450 nm is directly proportional to the amount of verotoxin bound. For optimal results a reference filter (620 nm wavelength) should be used. Considering the cut-off value results are interpreted as positive or negative.



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## SAMPLE PREPARATION

### Specimen collection and storage

Store samples at 2 - 8 °C immediately after collection and test them within 24 hours or store them frozen at -20 °C in case immediate testing is not possible. Repeated freezing and thawing of samples should be avoided. In case of direct verotoxin detection from stool suspension in sample diluent immediate testing after the sample has arrived in the laboratory is strongly recommended.

Stool specimens for enrichment culture should be transferred to the enrichment broth within 1-2 hours after the sample has arrived in the laboratory.

#### Preparation for direct testing from stool specimens

- Quickly thaw frozen stool specimens and mix them well. Samples treated with transport media should also be mixed before testing.
- Pipette 500 µl of sample diluent into a clean tube.
- Transfer 200 mg (diameter about 4-5 mm) or 200 µl stool specimen into the tube with 500 µl sample diluent and mix thoroughly.

**Caution:** the direct investigation of stool specimens by ELISA without prior enrichment should only serve as screening method for a fast first result. A subsequent additional investigation of the concerning sample after enrichment is absolutely necessary to reach a sufficient sensitivity.

**A negative ELISA result does not exclude an infection with EHEC when stool samples are tested without pre-enrichment culture.**

#### Preparation for testing from enrichment culture

- Transfer about 1 g or 1 ml of stool sample into 10 ml of enrichment broth (e. g. mTSB with 20 mg/l Novobiocin) or commercially available EHEC-enrichment broth (Heipha Heidelberg, Germany) and incubate for 18 to 24 h at 37°C with shaking.

After culture sediment floating particles by centrifugation at maximum speed for 10 min if necessary.

- Test the culture supernatant without further dilution (100 µl/well).

## TEST COMPONENTS FOR 96 WELLS

<b>A</b> <b>Ag</b> <b>96</b>	<b>Microtiter plate</b> , 12 breakable strips per 8 wells coated with polyclonal antibodies to Verotoxin 1 and 2 (sheep)	1 vacuum sealed with desiccant
<b>B</b> <b>BUF</b> <b>WASH</b> <b>10x</b>	<b>Concentrated wash buffer</b> sufficient for 1000 ml solution	100 ml concentrate capped white
<b>C</b> <b>DIL</b>	<b>Sample diluent</b>	100 ml ready for use capped black
<b>D</b> <b>A-VT</b>	<b>Anti-VT</b> biotinylated monoclonal anti-verotoxin 1 and 2 antibodies (mouse)	15 ml ready for use capped white
<b>E</b> <b>CONJ</b>	<b>Conjugate</b> containing streptavidin coupled with HRP	15 ml ready for use capped brown
<b>F</b> <b>SOLN</b> <b>TMB</b>	<b>Substrate</b> 3,3',5,5'-tetramethylbenzidine in citrate buffer containing hydrogen peroxide	15 ml ready for use capped blue
<b>G</b> <b>H2SO4</b> <b>0.25 M</b>	<b>Stop solution</b> 0.25 sulfuric acid	15 ml ready for use capped yellow
<b>P</b> <b>CONTROL</b>	<b>Positive control</b> Verotoxin positive Specimen (inactivated) <span style="float: right; border: 1px solid black; padding: 2px;">+</span>	2.0 ml ready for use capped red
<b>N</b> <b>CONTROL</b>	<b>Negative</b> Verotoxin negative Specimen <span style="float: right; border: 1px solid black; padding: 2px;">-</span>	2.0 ml ready for use capped green

## Materials required but not provided

- micropipettes
- multi-channel pipette or multi-pipette trough for multi-channel pipette
- 8-channel wash comb with vacuum pump and waste bottle or microplate washer
- mTSB broth with 20 mg/l Novobiocin, commercially available EHEC-enrichment broth or EHEC-Direct Medium (Heipha, Heidelberg, Germany)
- distilled or de-ionized water
- glassware

## Size and storage

Verotoxin Antigen has been designed for 96 determinations.

The expiry date of each component is reported on its respective label that of the complete kit on the box labels.

Upon receipt, all components of the Verotoxin Antigen have to be kept at 2 - 8 °C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.

## Preparation before use

Allow all components to reach room temperature prior to use in the assay.

The microtiter plate is vacuum-sealed in a foil with desiccant. The plate consists of a frame and strips with breakable wells. Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original cover carefully resealed.

Prepare a sufficient amount of wash solution by diluting the concentrated wash buffer 10 times with de-ionized or distilled water. For example, dilute 5 ml of the concentrate with 45 ml of distilled water per strip. The wash solution prepared is stable at 2 - 8 °C up to 30 days.

Make sure the soak time of the wash buffer in the wells is at least 5 seconds per wash cycle.

Avoid exposure of the TMB substrate solution to light!

## ASSAY PROCEDURE

- **Dilute samples with sample diluent (C) 1 + 2.5 (w/v), e.g. 200 mg stool + 500 µl sample diluent (C)**
- **Avoid any time shift during pipetting of reagents and samples.**

1. Bring all reagents to room temperature (20-25°C) before use. Mix gently without causing foam.
2. Dispense  
**3 drops** negative control (N)  
**3 drops** positive control (P)  
**100 µl** diluted samples or undiluted culture supernatant
3. Seal plate, incubate **60 min** at 22-25°C.
4. Decant, then wash each well **five** times using **300 µl** wash solution (made of B).
5. Dispense **3 drops** of antibody-biotin conjugate (D) into the respective wells
6. Seal plate, incubate **30 min** at 22-25°C.
7. Decant, then wash each well **five** times using **300 µl** wash solution (made of B).
8. Dispense **3 drops** of conjugate (E) into the respective wells
9. Seal plate, incubate **30 min** at 22-25°C.
10. Decant, then wash each well **five** times using **300 µl** wash solution (made of B).
11. Add **3 drops** of substrate (F) to each well.
12. Incubate **15 min protected from light** at 22-25°C.
13. Add **3 drops** of stop solution (G) to each well and mix gently.
14. Read the OD at **450 nm** versus 620 or 690 nm within **30 min** after adding the stop solution.

## DATA PROCESSING

### Qualitative evaluation

### Cut-off determination

OD of the negative control + 0.2 OD units

## REFERENCE VALUES

Verotoxin Antigen	
Negative	≤ cut-off
Positive	> cut-off

### Example of typical assay results

Wells	OD (a)	OD (b)	OD (mean)
Negative control	0.088	0.094	0.091
Positive control	2.916	2.934	2.925
Positive	> 0.091 + 0.200		= 0.291
Specimen 1	2.022	2.086	2.054 – positive
Specimen 2	0.218	0.226	0.222 – negative

### Test validity

The test run is valid if:

- the mean OD of the negative control is ≤ 0.20
- the mean OD of the positive control is ≥ 1.00

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier.

### Limitations of the method

The qualitative detection of verotoxin 1 and 2 by ELISA does not allow to correlate the measured OD to the severity of the disease. Sample ODs may not be correlated with the OD of the positive control. Cross-contamination of reagents and samples can produce false results. Incorrect dilutions, not sufficiently homogenized samples and floating particles may cause false results. Samples with pH-value < 5 after dilution may cause false negative results.

A negative test result in the Verotoxin Antigen ELISA does not necessarily exclude an EHEC infection: due to the usually low toxin concentrations in stool samples, the selective enrichment of EHEC bacteria in special culture media is of decisive influence on the sensitivity of the toxin detection with ELISA.

Clinical findings have to be considered for a final interpretation of the test results as well.

## CHARACTERISTIC ASSAY DATA

### Precision

Intraassay variation in the Verotoxin Antigen ELISA calculated from 12fold determinations of the samples:

verotoxin 2 concentration (pg/ml)	OD 450nm	standard deviation	coefficient of variation (%)
3125	2.268	0.051	2.3
800	0.799	0.037	4.7
200	0.262	0.013	5.1
0	0.056	0.006	11.3

Interassay variation in the Verotoxin Antigen ELISA in 11 different test runs (samples run in triplicate):

verotoxin 2 concentration (pg/ml)	OD 450nm	standard deviation	coefficient of variation (%)
3125	2.026	0.057	2.8
800	0.752	0.055	7.3
200	0.241	0.022	9.3
0	0.048	0.007	15.2

### Lower detection limit

The lower detection limit of the Verotoxin Antigen ELISA was determined by separate titration of verotoxin 1 and verotoxin 2. The lower detection limit is < 100 pg/ml (< 10 pg/well).

### Clinical evaluation

A total of 825 stool specimens was investigated in parallel with the vero-cell cytotoxicity assay and the Verotoxin Antigen ELISA. The sample material consisted of 795 specimens sent for TPE-group diagnosis and 30 specimens that were already characterized by shiga-toxin gene PCR and culture and stored at -20 °C until testing. Enrichment culture of all samples was carried out prior to ELISA testing.

The results concerning specificity and sensitivity of the ELISA in comparison to the cytotoxicity assay are summarized:

	cytotoxicity assay positive	cytotoxicity assay negative
ELISA positive	0	2
ELISA negative	0	793

specificity: 98.8 %

	cytotoxicity assay positive	cytotoxicity assay negative
ELISA positive	11	0
ELISA negative	2	17

sensitivity: 84.6 %

### Cross reactivity

The routine diagnosis for TPE-group bacteria in this study revealed a positive pathogen detection in 141 samples. These pathogens belonged to 12 different bacteria species. The following table shows that non of these pathogens caused false positive reactions in the Verotoxin Antigen ELISA.

Investigation of stool-culture supernatants with different pathogens in the Verotoxin Antigen ELISA:

pathogen	no	OD 450nm
<i>Staphylococcus aureus</i> , non toxigenic	48	0.053
<i>Staphylococcus aureus</i> , enterotoxin producer	16	0.057
Clostridium-difficile-toxin positive	29	0.054
<i>Pseudomonas aeruginosa</i>	3	0.087
<i>Salmonella typhimurium</i>	7	0.113
<i>Salmonella enteritidis</i>	22	0.077
<i>Salmonella spec.</i>	4	0.085
<i>Aeromonas hydrophila</i>	8	0.044
<i>Aeromonas caviae</i>	1	0.031
<i>Campylobacter spec.</i>	1	0.069
<i>Hafnia alvei</i>	1	0.043
<i>Yersinia enterocolitica</i> O:3	1	0.065

## INCUBATION SCHEME

# Verotoxin Antigen (6006)

<b>Dilute patients sample</b>	<b>Direct detection: 200 mg/200 µl sample + 500 µl sample diluent (C)</b> <b>Enrichment culture: undiluted culture supernatant is used</b>
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1	<b>Bring all reagents to room temperature (20-25°C)</b>	
2	Dispense      Negative control (N) Positive control (P) 1 + 2.5 (w/v) prediluted samples or undiluted culture supernatant	3 drops 3 drops 100 µl
3	Seal plate and incubate	60 min, at 22-25°C
4	Wash	Decant, 5 x 300 µl wash solution (made of B)
5	Dispense antibody-biotin conjugate (D)	3 drops
6	Seal plate and incubate	30 min, at 22-25°C
7	Wash	Decant, 5 x 300 µl wash solution (made of B)
8	Dispense conjugate (E)	3 drops
9	Seal plate and incubate	30 min, at 22-25°C
10	Wash	Decant, 5 x 300 µl wash solution (made of B)
11	Dispense substrate (F)	3 drops
12	Incubate protected from light	15 min, at 22-25°C
13	Dispense stop solution (G)	3 drops
14	Read at 450 nm against 620 (690) nm within 30 min.	

## SAFETY PRECAUTIONS

- **This kit is for in vitro use only.** Follow the working instructions carefully. GA GENERIC ASSAYS GmbH and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated. The kit should be performed by trained technical staff only.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts of Thimerosal (< 0.1 % w/v) and Kathon (1.0 % v/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
  - Do not smoke, eat or drink while handling kit material,
  - Always use protective gloves,
  - Never pipette material by mouth,
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.