DRG

DRG® E. coli Verotoxin 1+2 Ag ELISA (EIA-4452)

CE

As of July 2005

USA: RUO

1 INTRODUCTION

Invasive and toxigenic Escherichia coli strains cause dirrhoea in infants and adults. Among pathogenic E. coli strains the group of enterohaemorrhagic E. coli (EHEC) can cause lifethreatening haemorrhagic colitis and haemolytic uraemic syndrome (HUS) leading to acute renal failure and haemolytic anaemia 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 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 (PCR). 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).

References:

1. Beutin, L.: Infektionen mit enterohämorrhagischen Escherichia coli (EHEC). Bundesgesundheitsbl. 39, 11 (1996): 426-429
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 enterohömorrhagische 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. J. 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)

2 INTENDED USE

The ***DRG E. coli Verotoxin 1+2 Ag ELISA*** is an in vitro device for direct detection of verotoxin 1 and 2 (shiga-toxin 1 and 2) in faecal specimens and stool culture supernatants.

3 PRINCIPLE OF THE TEST

The *DRG E. coli Verotoxin 1+2 Ag ELISA* is an indirect two-site-immunoassay for the qualitative determination of verotoxin 1 and 2 based on polyclonal and monoclonal antibodies.
Verotoxin 1 and/or 2 of specimens and the positive control react with polyclonal anti-verotoxin 1 and 2 antibodies coated on the solid phase of the microplate. After incubation for 60 minutes at 22-25°C non-bound material is removed by a washing step.
Subsequently bound toxins specifically react with biotinylated monoclonal anti-verotoxin 1 and anti-verotoxin 2 antibodies during a second incubation period of 30 min at 22-25°C. Non-bound material is separated from the solid-phase immune complexes by a subsequent washing step.
During the next incubation period of 30 min at 22-25°C horseradish peroxidase (HRP) conjugated streptavidin reacts with the bound biotinylated antibodies. Unbound conjugate is removed by a washing step.

HRP converts the subsequently added colorless substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) into a blue product. The enzyme reaction is terminated by sulphuric acid dispensed into the wells after 15 min incubation at 22-25°C turning the solution from blue to yellow.
The optical density (OD) of the solution read at 450/620 nm is directly proportional to the specifically bound amount of verotoxin 1 and/or 2.
Considering the cut-off value results are interpreted as positive or negative.

4 PREPARATION AND STORAGE OF SAMPLES

4.1 Collection and storage

Stool samples should be stored at 2-8°C immediately after collection and processed within 48 hours. Longer storage is possible at -20°C. Repeated freezing and thawing of samples should be avoided.
In case of direct verotoxin detection from stool suspension in sample diluent, testing immediately after the sample has arrived in the laboratory should be preferred.
Stool specimens for enrichment culture should be transferred to the enrichment broth within 1-2 hours after the sample has arrived.

4.2 Preparation

4.2.1 Sample preparation for direct testing from diluted 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 into the tube with the 500 µl of sample diluent and mix thoroughly.
Caution: the direct investigation of stool specimens with ELISA without prior enrichment should only serve as screening method for a fast preliminary 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 necessarily exclude an infection with EHEC when stool samples are tested without enrichment culture.**

4.2.2 Sample preparation for testing from enrichment culture

Transfer about 200 mg or 200 µl of stool sample into a tube with 4 ml enrichment broth , e.g. EHEC-Direktmedium (Haipha) or mTSB (Mast) containing 50 ng/ml Mitomycin C and
incubate for 18 to 20 hours at 37°C. If possible, use a shaker during sample incubation.
Subsequently allow floating particles to sediment or if necessary sediment floating particles by a centrifugation step.
Test the culture supernatant without further dilution (100 µl/well).

5 TEST COMPONENTS FOR 96 WELLS

Microtiter wells	**Microtitration plate, 12 single breakable 8-well strips (in all 96 wells) coated with polyclonal anti-Verotoxin 1+2 antibodies (sheep)**	**1 vacuum sealed with desiccant**
Wash Buffer 10X	Wash buffer, 10-fold for 1000 ml solution	100 ml concentrate white cap
Sample Diluent	Sample diluent	100 ml ready to use black cap
Positive Control	Positive control; Inactivated Verotoxin positive culture supernatant	2.0 ml ready to use red cap
Negative Control	Negative control; Verotoxin negative sample	2.0 ml ready to use green cap
Biotin Conjugate	Biotin-conjugate Biotinylated, monoclonal anti-Verotoxin 1 and 2 antibodies (mouse)	15 ml ready to use white cap
Streptavidin Conjugate	Streptavidin-poly-HRP-conjugate	15 ml ready to use brown cap
TMB Substrate Solution	Substrate 3,3',5,5'-Tetramethylbenzidine and hydrogen peroxide	15 ml ready to use blue cap
Stop Solution	Stop solution 0.25 M Sulphuric acid	15 ml ready to use yellow cap

6 MATERIALS REQUIRED BUT NOT PROVIDED

- adjustable one-channel micropipettes and pipette tips
- adjustable multi-channel pipette or multi-pipette and pipette tips
- Reagent container for multi-channel pipette
- 8-channel wash comb with vacuum pump and waste bottle or microplate washer
- microplate reader with optical filters for 450 nm and 620 nm or 690 nm
- distilled or de-ionized water
- glassware
- tubes (1 ml) for sample preparation
- enrichment broth, e. g. EHEC-Direktmedium (Haipha) or mTSB
- (Mast) containing 50 ng/ml Mitomycin C

7 PREPARATION AND STORAGE OF REAGENTS

7.1 Kit size and expiry

One kit is designed for 96 determinations.
The expiry date of each component is reported on its respective label, of the complete kit on the outer box label.
Upon receipt, all test components 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.

7.2 Reagent preparation

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

The microtitration plate is vacuum-sealed in a foil with desiccant. The plate consists of a frame and strips with breakable wells. Allow the sealed plate 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 (1 + 9) with distilled or de-ionized water.
For Example: 10 ml *Wash Buffer* concentrate + 90 ml distilled water.
This ready to use wash buffer solution is stable for at least 30 days when stored at 2-8°C.

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

Avoid light exposure of the TMB substrate solution!

8 ASSAY PROCEDURE

- Dilute samples with *Sample Diluent* **1 + 2.5**; e.g. 200 mg or 200 µl stool + 0.5 ml sample diluent

For enrichment culture:

- Transfer 200 mg or 200 µl sample to 4 ml enrichment broth (e.g. EHEC-Direktmedium or mTSB + 50 ng/ml Mitomycin C) and incubate for 18 – 20 hours at 37°C if possible on a shaker. Use 100 µl/well of culture supernatant for ELISA testing without further dilution.

Avoid any time shift during dispensing of reagents and samples.
For larger sample series dispensing of reagents from reagent containers by using a multichannel-pipette is recommended.

8.1 Working steps

1. Warm all reagents to room temperature (20-25°C) before use. Mix gently without causing foam.
2. Dispense:
   3 drops (or 120 µl) ***Positive Control***
   3 drops (or 120 µl) ***Negative Control***
   100 µl diluted **specimen** or **culture supernatant**
3. Cover plate and incubate for **60 min** at 22-25°C.
4. Decant, then wash each well **5x** with **300 µl** wash solution.

5. Dispense **3 drops (or 120 µl)** ***Biotin Conjugate*** per well
6. Cover plate and incubate for **30 min** at 22-25°C.
7. Decant, then wash each well **5x** with **300 µl** wash solution.
8. Dispense **3 drops (or 120 µl)** ***Streptavidin Conjugate*** per well.
9. Cover plate and incubate for **30 min** at 22-25°C.
10. Decant, then wash each well **5x** with **300 µl** wash solution.
11. Dispense **3 drops (or 120 µl)** ***TMB Substrate Solution*** per well.
12. Incubate for **15 min** at 22-25°C protected from light.
13. Dispense **3 drops (or 120 µl)** ***Stop Solution***, mix gently.
14. Read OD at **450 nm** (reference filter 620 or 690 nm) with a microplate reader within 30 min after reaction stop.

9 RESULT INTERPRETATION

Qualitative evaluation

Cut-off determination: OD negative control + 0.20

Samples with absorbances higher than the cut-off value are considered **positive**,

samples with absorbances equal to or below the cut-off value are considered **negative** for Verotoxin 1 and/or 2 antigen

10 REFERENCE VALUES

E. coli Verotoxin 1+2

Negative	≤ Cut-off
Positive	> Cut-off

It is recommended that each laboratory establishes its own normal and pathological reference ranges as usually done for other diagnostic parameters too. Therefore, the above mentioned reference values provide a guide only to values which might be expected.

10.1 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.

10.2 Limitations of the procedure

There is no correlation between measured absorbance and seriousness of the infection.
It is also not allowed to correlate absorbances of the samples with that of the positive control.

Cross contamination of reagents and samples can produce false positive results. Incorrect dilutions, not sufficiently homogenized samples or solid particles after centrifugation of the suspension can cause false results. Fermented samples with pH values below 5 after resuspension may produce false negative results.
A negative test result in the *DRG E. coli Verotoxin 1+2 Ag 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 (6).
The overall interpretation of the ELISA results should always consider the microbiological examination as well as clinical findings.

11 PERFORMANCE CHARACTERISTICS

11.1 Precision

Intra-assay coefficient of variation (CV) in the *E. coli Verotoxin 1+2 Ag ELISA* calculated from 12fold determination of samples:

Verotoxin 2 (pg/ml)	Mean OD	Standard deviation	CV (%)
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

Inter-assay coefficient of variation (CV) in the *E. coli Verotoxin 1+2 Ag ELISA* from 11 different test runs from 3fold determination of samples:

Verotoxin 2 (pg/ml)	Mean OD	Standard deviation	CV (%)
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	15.1

11.2 Lower detection limit

The lower detection limit of the *DRG E. coli Verotoxin 1+2 Ag ELISA* was determined with < 100 pg/ml (< 10 pg/well) by separate titration of verotoxin 1 and 2.

11.3 Specificity and Sensitivity

A total of 825 stool specimens was tested in parallel with the vero-cell cytotoxicity assay and the *DRG E. coli Verotoxin 1+2 Ag ELISA*.

The sample material consisted of 795 specimens sent for TPE-group diagnosis and 30 specimens that were already characterized by shiga-toxin gen PCR and culture and stored at – 20 °C until testing.
Enrichment culture (mTSB; 18-20 hours at 37 °C) of all samples was carried out prior to ELISA testing.

	Vero-cell assay positive	Vero-cell assay negative
DRG ELISA positive	0	2
DRG ELISA negative	0	793

Specificity: 98.8 %

	Vero-cell assay positive	Vero-cell assay negative
DRG ELISA positive	11	0
DRG ELISA negative	2	17

Sensitivity: 84.6 %

11.4 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 bacterial species:

Staphylococcus aureus, enterotoxin non-forming*; Staphylococcus aureus,* enterotoxin forming*;* EHEC*; Pseudomonas aeruginosa; Salmonella typhimurium; Salmonella enteritidis; Salmonella spec. Aeromonas hydrophila; Aeromonas caviae; Campylobacter spec.; Hafnia alvei; Yersinia enterocolitica* O:3.
None of them caused false positive reactions in the *DRG E. coli Verotoxin 1+2 Ag ELISA*.

12 COMMON ADVICES AND PRECAUTIONS

This kit is for in vitro use only. Follow the working instructions carefully. 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 except for sample diluent, wash buffer, TMB/substrate solution and stop solution.
Do not use reagents from other manufacturers.
Avoid time shift during dispensing of reagents.
All reagents should be kept at 2-8°C before use.
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 mucous membranes.
Handle all components and all patient samples as if potentially hazardous.

Since the kit contains potentially hazardous materials, the following precautions should generally be observed:
- Do not smoke, eat or drink while handling kit material,
- Always use protective gloves,
- Never pipette material by mouth,

– Note safety precautions of the single test components.

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SYMBOLS USED WITH DRG ELISA´S

Symbol	English	Deutsch	Francais	Espanol	Italiano
	User´s Manual	Arbeitsanleitung	Mode d'emploi	Instrucciones de empleo	Istruzioni d'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic	In-vitro-Diagnostikum	Diagnostic in vitro	Diagnóstico in vitro	Diagnostica in vitro
REF	Catalogue number	Katalog-Nr.	Référence	No de catálogo	No. di Cat.
LOT	Lot. No.	Chargen-Nr.	No. de lot	Número de lote	Lotto no
Σ	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Temperature de conservation	Temperatura de conservacion	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Distributeur	Distributeur	Distribuidor	Distributtore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità
Microtiterwells	Microtiterwells	Mikrotiterwells	Barrettes de microtitration	Pocillos de la Microplaca	Micropozzetti
Stop Solution	Stop Solution	Stopplösung	Solution d'arret	Solución de paro	Soluzione d´arresto
Sample Diluent	Sample Diluent	Probenverdünnungs-medium			Diluente dei campioni

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Instruções de uso	Brugermanual	Användar manual	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
Σ		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevaringstemperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..

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Microtiterwells	Alvéolos de microtitulação	Mikrotiterbrønde	Brunnar i Mikrotiterplatta	Πηγαδάκια Μικροτιτλοδοτήσεως
Stop Solution	Solução de paragem	Stopopløsning	Stopp lösning	Διάλυμα τερματισμού
Sample Diluent				