DRG® Clostridium difficile Toxin A+B Ag (EIA-4448)

Revised 20 Sept. 2013 cc (Vers. 6.0) **RUO in the USA**

Please use only the valid version of the package insert provided with the kit.

1 INTRODUCTION

Clostridium difficile is a bacterium causing nosocomial diarrhea in adults during or after the treatment with antibiotics such as 3rd generation cephalosporines (1). Although 2-3% of healthy adults and 20-50% of healthy children are colonized with Clostridium difficile, the infection is usually of exogenous origin and results from the contact either to hospital staff or to Clostridium difficile spores which may contaminate toilets, bed clothes etc.
Both exotoxins A and B of this spore-forming bacteria cause the depolymerisation of actin filaments due to the intracellular enzymatic modification of rho-proteins. Consequently, the permeability of cell membrane is raised and neutrophiles may invade leading to expression of the clinical picture of the so-called Clostridium difficile-associated diarrhea and colitis or finally the pseudomembraneous colitis (PMC) (1).
As the production of toxins and the outbreak of disease is correlated, diagnosis of Clostridium difficile infection is based mainly on a direct detection of the toxins in stool specimens. To date the cytotoxicity test has been considered as the gold standard for detection of Clostridium difficile toxins. Recently it has been replaced to a large extent by immunological tests such as enzyme immunoassay (2).

2 INTENDED USE

The ***Clostridium difficile Toxin A+B Ag ELISA*** is an in vitro diagnostic device for direct detection of the toxins A and B of Clostridium difficile in stool specimens and culture suspensions.

3 PRINCIPLE OF THE TEST

The *Clostridium difficile Toxin A + B ELISA* is an indirect two-site-immunoassay for the qualitative determination of both Clostridium difficile toxins A and B based on polyclonal and monoclonal antibodies.
Clostridium difficile toxins of stool specimens or culture suspensions and the positive control react with monoclonal anti-toxin A and polyclonal anti-toxin B antibodies coated on the solid phase of the microplate. After incubation non-bound material is removed by a washing step.
Subsequently bound toxins specifically react with biotinylated polyclonal anti-toxin A and monoclonal anti-toxin B antibodies during a second incubation period. Non-bound material is separated from the solid-phase immune complexes by a subsequent washing step.
During the next incubation period horseradish peroxidase (HRP) conjugated streptavidin reacts with the bound biotinylated antibodies. Unbound conjugate is removed by a washing step.
HRP converts the subsequently added colourless substrate solution of 3,3',5,5'-tetra¬methylbenzidine (TMB) into a blue product. The enzyme reaction is terminated by sulphuric acid dispensed into the wells 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 Clostridium difficile toxin A and B.
After consideration of the cut-off value, results are interpreted as positive or negative.

4 TEST COMPONENTS FOR 96 WELLS

1	WELLS	**Microtitration plate** coated with monoclonal anti-Toxin A- (mouse) and polyclonal anti-Toxin B-antibodies (rabbit)	12 single breakable 8-well strips colour coding red vacuum-sealed with desiccant
2	WASHBUF CONC 10x	**Wash buffer** 10-fold	100 mL concentrate for 1000 mL solution white cap
3	DIL	**Sample diluent**	100 mL · ready to use coloured yellow black cap
4	CONTROL +	**Positive control** *C. difficile* Toxin reactive sample	2.0 mL · ready to use coloured blue red cap
5	CONTROL –	**Negative control** *C. difficile* Toxin negative sample	2.0 mL · ready to use coloured blue green cap
6/1	CONJ BIOTIN	**Biotin-conjugate** Biotinylated, polyclonal anti-Toxin A- (rabbit) and monoclonal anti-Toxin B-antibodies (mouse)	15 mL · ready to use coloured green white cap
6/2	CONJ STREPT	**Streptavidin-HRP-conjugate**	15 mL · ready to use coloured red brown cap
7	SUBSTR TMB	**Substrate** 3,3',5,5'-Tetramethylbenzidine and hydrogen peroxide	15 mL · ready to use blue cap
8	STOP	**Stop solution** 0.25 M sulphuric acid	15 mL · ready to use yellow cap

5 PREPARATION AND STORAGE OF SAMPLES

5.1 Toxin detection from stool specimens

Collection and storage

Stool samples should be stored at 2 °C - 8 °C immediately after collection and processed within 72 hours or frozen at -80 °C. Storage at -20 °C as well as repeated freezing and thawing of samples should be avoided.
Formalin-preserved stool samples should not be used in this assay.

Stool samples already diluted with sample diluent can be stored for up to 72 h at 2 °C - 8 °C and tested on the following day.

Preparation
Warm samples to room temperature and mix well.
Pipette **1000 µL** of sample diluent into a clean tube.
Using a disposable stirring rod transfer about **200 mg** (diameter about 2-3 mm) of faeces if solid or pipette **200 µL** if liquid into the tube and suspend thoroughly.
If necessary spin down floating particles in a micro centrifuge at maximum speed for 1 minute.

5.2 Toxin detection from culture suspensions (toxigenic culture)

Colonies of Clostridium difficile grown on blood or CCFA agar for 48 hours can be tested directly in the Clostridium difficile Toxin A+B ELISA.
Prepare a bacterial suspension according to Mc Farland standard 1 (OD value at 600 nm: 0.20 - 0.25 after zero compensation to the yellow coloured sample diluent):
Pipette **1000 µL** of sample diluent into a clean tube,
Transfer **2 - 4 inoculating loops** of a C. difficile culture into the sample diluent and suspend on a vortex mixer.
Read OD value at 600 nm as described above where required.
Use 100 µL for ELISA testing.
If selective culture media are used the detectable amount of toxins may be reduced due to inhibitory components of such media resulting in decreased OD values in the ELISA. Therefore using selective media for toxigenic culture requires the preparation of a bacterial suspension at least according to Mc Farland standard 4 (OD 600 nm > 1.0 after zero compensation to the yellow coloured sample diluent). In this case the Clostridium difficile colonies of at least half of a densely grown agar plate have to be harvested. Where required the recommendations and instructions of the medium manufacturers are to be observed.

6 MATERIALS REQUIRED BUT NOT PROVIDED

- Micropipettes
- Multi-channel pipette or multi-pipette
- Reagent container for multi-channel pipette
- 8-channel wash comb with vacuum pump and waste bottle or microplate washer or 8-channel pipette
- Microplate reader with optical filters for 450 nm for measurement and ≥ 620 nm for reference.
- Distilled or de-ionized water
- Glassware
- Tubes (2 mL) for sample preparation
- Orbital shaker for performance of test variant 2

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 °C - 8 °C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.
This ready to use wash solution is stable for at least one month when stored at 2 °C - 8 °C.

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 10-fold concentrated *Wash Buffer* **1 + 9** with distilled or deionized water.
For Example: 10 mL *Wash Buffer* concentrate + 90 mL distilled or deionized water.

8 ASSAY PROCEDURE

The *Clostridium difficile Toxin A+B ELISA* can be performed in two ways:

1. Incubation without shaking;
   complete test duration 2 hours and 15 minutes
2. Incubation with shaking;
   complete test duration 1 hour and 15 minutes

- Dilute samples with sample diluent (3) 1 : 6 e.g. 200 mg or 200 µL stool + 1.0 mL sample diluent (3)
  - or -
  Transfer 2-4 inoculation loops of a C. difficile colony into a tube with 1.0 mL sample diluent (3) and mix thoroughly on a vortex.
- Avoid any time shift during dispensing of reagents and samples.
- Make sure the soak time of the wash buffer in the wells is at least 5 seconds per wash cycle.
- Avoid direct light exposure of the TMB substrate solution!

8.1 Working steps variant 1: without shaking

1. Warm all reagents to room temperature (RT) before use. Mix gently without causing foam.
2. Pipette:
   100 µL CONTROL + positive control (4)
   100 µL CONTROL - negative control (5)
   100 µL diluted stool specimen or culture suspension
3. Cover plate and incubate for 60 min at RT.
4. Decant, then wash each well 5x with **300 µL** wash solution (diluted from (2)) and tap dry onto absorbent paper.
5. Dispense **3 drops (or 120 µL)** CONJ BIOTIN biotin-conjugate (6/1) per well.
6. Cover plate and incubate for 30 min at RT.
7. Decant, then wash each well 5x with **300 µL** wash solution (diluted from (2)) and tap dry onto absorbent paper.
8. Dispense **3 drops (or 120 µL)** CONJ STREPT streptavidin-HRP-conjugate (6/2) per well.

9. Cover plate and incubate for 30 min at RT
10. Decant, then wash each well **5x with 300 µL** wash solution (diluted from (2)) and tap dry onto absorbent paper.
11. Dispense **3 drops (or 120 µL)** SUBSTR TMB substrate (7) per well.
12. Incubate for 15 min at RT protected from light.
13. Dispense **3 drops (or 120 µL)** STOP stop solution (8) per well, mix gently.
14. Read OD at 450 nm / ≥ 620 nm with a microplate reader within 30 min after reaction stop.

8.2 Working steps variant 2: with shaking

1. Warm all reagents to room temperature (RT) before use. Mix gently without causing foam.
2. Pipette:
   100 µL CONTROL + positive control (4)
   100 µL CONTROL – negative control (5)
   100 µL diluted stool specimen or culture suspension.
3. Cover plate and incubate for 30 min at RT on an orbital shaker with a frequency of 500-700/min.
4. Decant, then wash each well **5x with 300 µL** wash solution (diluted from (2)) and tap dry onto absorbent paper.
5. Dispense **3 drops (or 120 µL)** CONJ BIOTIN biotin-conjugate (6/1) per well.
6. Cover plate and incubate for 15 min at RT on an orbital shaker with a frequency of 500-700/min.
7. Decant, then wash each well 5x with 300 µL wash solution (diluted from (2)) and tap dry onto absorbent paper.
8. Dispense **3 drops (or 120 µL)** CONJ STREPT streptavidin-HRP-conjugate (6/2) per well.
9. Cover plate and incubate for 15 min at RT on an orbital shaker with a frequency of 500-700/min.
10. Decant, then wash each well **5x with 300 µL** wash solution (diluted from (2)) and tap dry onto absorbent paper.
11. Dispense **3 drops (or 120 µL)** SUBSTR TMB substrate (7) per well.
12. Incubate for 15 min at RT without shaking protected from light.
13. Dispense **3 drops (or 120 µL)** STOP stop solution (8) per well, mix gently.
14. Read OD at 450 nm / ≥ 620 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 10% below the cut-off value are considered **negative** for Clostridium difficile toxin A and B antigen.

Samples within 10 % below the cut-off up to the cut-off value have to be considered **borderline** and should be repeatedly tested. In case of repeated borderline result a second sample of the corresponding patient should be investigated.

10 REFERENCE VALUES

Clostridium difficile Toxin A+B

Positive	> Cut-off
Borderline	0.9 x Cut-off – cut-off
Negative	≤ 0.9 x 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 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 (manual performance)
  ≤ 0.30 (automatic performance)
- 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 negative as well as false positive results. Fermented samples with pH values below 5 after resuspension may produce false negative results. Formalin treated samples may cause false positive results.
A negative test result in the Clostridium difficile Toxin A+B ELISA does not exclude an infection:
The overall interpretation of the ELISA results should always consider the microbiological examination as well as clinical findings.

10.3 Automatic Processing

Performing the *Clostridium difficile Toxin A+B ELISA* on fully automated microplate processors (e.g. DS2, DSX) may cause elevated absorbances in comparison to the manual procedure due to individual differences concerning wash procedures and general technical specifications of the equipment. In these cases a maximum value of 0.3 absorbance units is permissible for the negative control.
It is recommended to use a wash procedure including 10 seconds soak time per strip and wash step followed by a wash step with distilled or deionized water with 10 seconds of soak time after the final wash step of each wash cycle. If necessary the number of washing steps can be enhanced from 5x to 7x-8x.

Correlation:
Manual – automatic processing

A panel of 125 stool specimens was investigated in parallel by manual and automatic processing method (DS2, Dynex Technologies) resp. The correlation was calculated with r = 0.976.

11 PERFORMANCE CHARACTERISTICS

11.1 Precision

Intra-assay coefficient of variation (CV) in the *Clostridium difficile Toxin A+B Ag ELISA* calculated from 8fold determination of samples:

sample	mean OD	standard deviation	CV (%)
I	1.386	0.042	3.0
II	0.506	0.017	3.3
III	0.332	0.028	8.5

Inter-assay coefficient of variation (CV) in the *Clostridium difficile Toxin A+B Ag ELISA* in 5 different test runs on 2 different days from 8fold determination of samples:

sample	mean OD	standard deviation	CV (%)
I	1.321	0.102	7.7
II	0.485	0.034	6.9
III	0.345	0.037	10.8

11.2 Specificity and Sensitivity

A total of 154 stool specimens were tested in parallel with the *Clostridium difficile Toxin A+B Ag ELISA* and another commercially available ELISA.

	comparative ELISA positive	comparative ELISA negative
DRG ELISA positive	103	4
DRG ELISA negative	2	45

Specificity: 91.8 %
Sensitivity: 98.0 %

11.3 Cross reactivity

Faecal samples positive for one of the following intestinal bacteria did not show any cross reaction in the *Clostridium difficile Toxin A+B Ag ELISA*:
Staphylococcus aureus, enterotoxin negative*;*
Staphylococcus aureus, enterotoxin positive*;*
EHEC*; Pseudomonas aeruginosa; Salmonella typhimurium; Salmonella enteritidis; Salmonella spec. Aeromonas hydrophila; Aeromonas caviae; Campylobacter spec.; Hafnia alvei; Yersinia enterocolitica* O:3.

Negative stool specimens have been spiked with ≥ 10^8 colony forming units of the following microorganisms and tested negative with the ELISA (OD 450/620 nm < Cut-Off):

Aeromonas hydrophila	**(ATCC 7966)**
Bacillus cereus	(ATCC 11778)
Bacillus subtilis	(ATCC 6633)
Bacteroides fragilis	(ATCC 25285)
Candida albicans	(ATCC 10231)
Campylobacter coli	(ATCC 33559)
Campylobacter jejuni	(ATCC 33291)
Citrobacter freundii	(ATCC 8090)
Clostridium sordellii	(ATCC 9714)
Enterobacter aerogenes	(ATCC 13048)
Enterobacter cloacae	(ATCC 13047)
Enterococcus faecalis	(ATCC 29212)
Escherichia coli	(ATCC 25922)
Klebsiella pneumoniae	(ATCC 13883)
Peptostreptococcus anaerobius	(ATCC 27337)
Proteus vulgaris	(ATCC 8427)
Pseudomonas aeruginosa	(ATCC 10145)
Salmonella enterica Serovar enteritidis	(ATCC 13076)
Salmonella enterica Serovar typhimurium	(ATCC 14028)
Shigella flexneri	(ATCC 12022)
Shigella sonnei	(ATCC 25931)
Staphylococcus aureus	(ATCC 25923)
Staphylococcus epidermidis	(ATCC 12228)
Vibrio parahaemolyticus	(ATCC 17802)

The C. sordellii strain ATCC 9714 did not cross react in the Clostridium difficile Toxin A+B ELISA although some publications describe cross reactivities of toxins of some C. sordellii strains with anti- C. difficile toxin antibodies.

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.
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 °C - 8 °C before use.
Some of the reagents (2, 3, 4,5,6,7) contain small amounts of Thimerosal (0.01 % 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.

References:

1. Rambaud J-C., LaMont J-T. (Hrsg.): "Ökosystem Darm Special- Updates on Clostridium difficile" Springer Verlag 1995
2. Wilkins T.D. and Lyerly D.M. (2003): „Clostridium difficile Testing: after 20 Years, Still Challenging" Journal Of Clinical Microbiology, Vol. 41, No. 2, p. 531-534

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SYMBOLS USED WITH DRG ASSAYS

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konformitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
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	Température de conservation	Temperatura de conservación	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	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

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