INSTRUCTION MANUAL

REF 6007

May 22, 2007

Clostridium difficile Antigen

- 96 determinations -

CE

IVD *In vitro* diagnostic device

Enzyme immunoassay for the determination of Clostridium difficile Toxin A and B in fecal specimens

REF	Catalogue number	**LOT**	Batch code
	Consult accompanying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction		Biological risk

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INTENDED USE

Clostridium difficile Antigen is used for the qualitative detection of Clostridium difficile Toxin A and B in fecal specimens.

Clostridium difficile is a bacterium causing nosocomial diarrhea in adults during or after the treatment with antibiotics such as 3rd generation cephalosporins (1). Although 2-3% of healthy adults and 20-50% of healthy children are colonized with Clostridium difficile, the infection is usually of exogeneous 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 the cell leading to expression of the clinical picture of the so-called Clostridium difficile-associated diarrhea and colitis or finally the pseudomembranous 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. Todate 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).

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

PRINCIPLE OF THE TEST

Clostridium difficile Antigen is a fast enzymometric two-step immunoassay for the qualitative determination of both Clostridium difficile toxins A and B based on polyclonal and monoclonal antibodies against the two toxins.

Clostridium difficile toxins of specimens and the positive control reacts with monoclonal anti-toxin A and B antibodies coated on the solid phase of the microplate. After incubation of 60 minutes at 22-25°C non-bound material is removed by a wash step.

Subsequently bound toxins react specifically with biotinylated polyclonal anti-toxin A and B 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 following wash step.

During the next incubation period of 30 min at 22-25°C horseradish peroxidase (HRP)-dextran conjugated streptavidin reacts with the bound biotinylated antibodies. Unbound conjugate is separated by a wash step again.

HRP converts the colorless substrate solution of 3,3',5,5'-tetra-methylbenzidine (TMB) added into a blue product. The enzyme reaction is terminated by an acidic solution 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 nm is directly proportional to the amount of Clostridium difficile toxin A and B 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.

SAMPLE PREPARATION

Specimen collection and storage

The 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. Fermented samples with pH values below 5 in suspended condition may produce false results and should be discarded. Formalin-preserved stool samples should not be used in this assay.

Sample preparation

- Bring samples to room temperature and mix well.
- Pipette 500 µl of sample diluent into a clean tube.
- With a one-way stirring rod transfer about 100 mg (diameter about 2-3 mm) of faeces if solid or pipette 100 µl if liquid into the tube and suspend thoroughly.
- If necessary, sediment floating particles by a centrifugation step.

TEST COMPONENTS FOR 96 WELLS

A Ag 96	**Microtiter plate**, 12 breakable strips per 8 wells coated with mono-clonal antibodies to C.difficile toxin A and B (mouse)		1 vacuum sealed with desiccant
B BUF WASH	**Concentrated wash buffer** sufficient for 1000 ml solution 10x		100 ml concentrate capped white
C DIL	**Sample diluent**		100 ml ready for use capped black
D A-CT	**Anti-CT** biotinylated polyclonal anti-C.difficile toxin A and B antibodies (rabbit)		15 ml ready for use capped white
E CONJ	**Conjugate** containing streptavidin labelled with HRP		15 ml ready for use capped brown
F SOLN TMB	**Substrate** 3,3',5,5'-tetramethylbenzidine in citrate buffer containing hydrogen peroxide		15 ml ready for use capped blue
G H2SO4	**Stop solution** 0.25 sulfuric acid 0.25 M		15 ml ready for use capped yellow
P CONTROL	**Positive control** C.difficile toxin culture supernatant containing toxin A and B (inactivated)	+	2.0 ml ready for use capped red
N CONTROL	**Negative control** C.difficile toxin negative sample	–	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
- microplate reader with optical filters for 450 nm and 620 nm or 690 nm
- distilled or de-ionized water
- glassware

Size and storage

Clostridium difficile 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 Clostridium difficile 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 (1 + 9) with distilled or de-ionized water.
For example, dilute 8 ml of the concentrate with 72 ml of distilled water. 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 + 5 (w/v), e.g. 100 mg stool + 0.5 ml sample diluent (C)**
- **Avoid any time shift during pipetting of reagents and samples.**
- **Alternatively the test can be processed on an orbital shaker (frequency 500-700 per min). In this case incubation times are shortened to 30-15-15-15 min.**

1. Bring all reagents to room temperature (20-25°C) before use. Mix gently without causing foam.
2. Dispense
   100 µ of negative control (N)
   100 µ of positive control (P)
   100 µl of diluted samples or undiluted culture supernatant
3. Seal plate, incubate **60 min** at 22-25 °C
   (alternatively 30 min while shaking at 500-700/min).
4. Decant, then wash each well **five** times using **300 µl** wash solution (made of B).
5. Dispense **3 drops** (or 100 µl) of anti-CT (antibody-biotin conjugate) (D) into the respective wells
6. Seal plate, incubate **30 min** at 22-25 °C
   (alternatively 15 min while shaking at 500-700/min).
7. Decant, then wash each well **five** times using **300 µl** wash solution (made of B).
8. Dispense **3 drops** (or 100 µl) of streptavidin-HRP conjugate (E) into the respective wells
9. Seal plate, incubate **30 min** at 22-25 °C
   (alternatively 15 min while shaking at 500-700/min).
10. Decant, then wash each well **five** times using **300 µl** wash solution (made of B).
11. Add **3 drops** (or 100 µl) of substrate (F) to each well.
12. Incubate **15 min protected from light** at 22-25°C (do not shake).
13. Add **3 drops** (or 100 µl) 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 + 0.20 OD units

Samples with absorbance values higher than the cut-off value are considered positive, samples with absorbance values 10% below the cut-off value or lower are considered to be negative for Clostridium difficile toxin A and B antigen.

REFERENCE VALUES

Clostridium difficile	
negative	**< cut-off x 0.9**
borderline	**Cut-off x 0.9 – cut-off**
positive	**> cut-off**

Example of typical assay results

Wells	OD (a)	OD (b)	OD (mean)
negative control	0.087	0.094	0.090
positive control	1.916	1.934	1.925
positive		> (0.090 + 0.20) = 0.290	
negative		< (0.090 + 0.20) x 0.9 = 0.261	
specimen 1	2.012	2.076	2.044 – positive
specimen 2	0.118	0.126	0.122 – negative
specimen 3	0.279	0.274	0.276 – borderline

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.

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

There is no correlation between measured absorbance and seriousness of the infection. It is also not allowed to correlate absorbance 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.

A negative result in the Clostridium difficile Toxin A and B Antigen does not rule out an infection with Clostridium difficile.

The overall interpretation of the ELISA results should always consider the microbiological examination as well as clinical findings.

CHARACTERISTIC ASSAY DATA

Precision

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

Sample	Mean OD	SD	C.V. (%)
I	1.386	0.042	3.0
II	0.506	0.017	3.3
III	0.332	0.028	8.5

Interassay variation in the Clostridium difficile Antigen ELISA in 10 different test runs (samples run in triplicate):

Sample	Mean OD	SD	C.V. (%)
I	1.321	0.102	7.7
II	0.486	0.034	6.9
III	0.345	0.037	10.8

Clinical evaluation

340 stool samples were examined in parallel in a cytotoxicity test, a competitor's commercial ELISA test and in the Clostridium difficile Antigen ELISA.

	Cytotoxicity test positive	Cytotoxicity test negative
GA ELISA positive	72	0
GA ELISA negative	5	263

Specificity: 100 %
Sensitivity: 93.5 %

	competitor's ELISA positive	competitor's ELISA negative
GA ELISA positive	71	1
GA ELISA negative	0	268

Specificity: 99.6 %
Sensitivity: 100 %

In comparison to another competitor's commercial ELISA test based on the evaluation of 154 stool samples the following data were obtained:

	competitor's ELISA positive	competitor's ELISA negative
GA ELISA positive	103	4
GA ELISA negative	2	45

Specificity: 91.8 %
Sensitivity 98.1 %

Cross reactivity

Faecal samples positive for one of the following intestinal bacteria did not show any cross reaction in the Clostridium difficile Antigen ELISA:

Staphylococcus aureus, EHEC*; Pseudomonas aeruginosa; Salmonella typhimurium; Salmonella enteritidis; Salmonella spec. Aeromonas hydrophila; Aeromonas caviae; Campylobacter spec.; Hafnia alvei; Yersinia enterocolitica* O:3*.*

INCUBATION SCHEME

Clostridium difficile Antigen (6007)

Dilute patients sample	**100 mg sample + 0.5 ml sample diluent (C)**

1	**Bring all reagents to room temperature (20-25°C)**		
2	Dispense	Negative control (N) Positive control (P) 1 + 5 (w/v) prediluted samples	100 µl 100 µl 100 µl
3	Seal plate and incubate		60 min, at 22-25°C (alternatively 30 min while shaking at 500-700/min)
4	Wash		Decant, 5 x 300 µl wash solution (made of B)
5	Dispense anti-CT (D)		3 drops (or 100 µl)
6	Seal plate and incubate		30 min, at 22-25°C (alternatively 15 min while shaking at 500-700/min)
7	Wash		Decant, 5 x 300 µl wash solution (made of B)
8	Dispense conjugate (E)		3 drops (or 100 µl)
9	Seal plate and incubate		30 min, at 22-25°C (alternatively 15 min while shaking at 500-700/min)
10	Wash		Decant, 5 x 300 µl wash solution (made of B)
11	Dispense substrate (F)		3 drops (or 100 µl)
12	Incubate protected from light		15 min, at 22-25°C (do not shake!)
13	Dispense stop solution (G)		3 drops (or 100 µl)
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 (except: diluent, washing buffer, substrate and stop solution).
- 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.
- 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.