RIDASCREEN® Clostridium difficile Toxin A/B

Article no.: C0801

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® *Clostridium difficile* Toxin A/B is an enzyme immunoassay for qualitative determination of the *Clostridium difficile* toxins A and B in human stool specimens and from cultures of toxin-producing *Clostridium difficile* strains that were previously cultured from stool specimens.

2. Summary and explanation of the test

Diarrhea illnesses are a relatively frequent side effect of antibiotic therapies. Particularly since the introduction of clindamycin early in the 1970s, there has been a more frequent occurrence of antibiotic associated colitis (AAC) in more severe forms, with signs and symptoms that can build up to a massive pseudomembranous colitis (PMC). The pathogen *Clostridium difficile* has been found to cause pseudomembranous colitis (PMC) and to be one of the causes of AAC and AAD. Production of the toxins A and B by toxigenic strains of *Clostridium difficile* plays a significant role in the etiology of clinical manifestations of the illness. There are differences in the functions and immunology of these proteins with molar masses greater than 200 kDA. Toxin A is an enterotoxin, toxin B is a cytotoxin; and both toxins exhibit synergy in their *in vivo* effects. Considering that some strains of *Clostridium difficile* do not produce the toxins and that approximately 2–8 % of healthy adults and up to 50 % of children younger than two years of age may be carrying *Clostridium difficile,* the determination of these toxins appears to be more important in diagnostic practice than to determine the pathogenic microorganism.
The RIDASCREEN® Clostridium difficile Toxin A/B ELISA is an enzyme immunoassay that can specifically determine toxin A and toxin B simultaneously in patient stool specimens with the help of monoclonal antibodies. Reliable examination results are available already after two hours, making it possible to initiate effective therapeutic measures at an early stage.

3. Test principle

The RIDASCREEN® Clostridium difficile Toxin A/B Test employs monoclonal antibodies in a sandwich-type method. These monoclonal antibodies to *Clostridium difficile* toxins A and B are attached to the well surface of the microwell plate.
A pipette is used to place a suspension of the stool sample to be examined as well as control specimens into the well of the microwell plate together with biotinylated anti-toxin A/B antibodies (conjugate 1) for incubation at room temperature (20–25 °C). After a wash step, streptavidin poly-peroxidase conjugate (Conjugate 2) is added and it is incubated again at room temperature (20–25 °C). With the presence of toxins in a specimen, immobilized antibodies, toxins, and conjugated antibodies form a sandwich complex. Another wash step removes the unattached streptavidin poly-peroxidase conjugate. After adding the substrate, the attached enzyme changes the colour of the previously colourless solution in the wells of the microwell plate to blue if the test is positive. Addition of a stop reagent changes the color from blue to yellow. The extinction is proportional to the concentration of toxins A and B found in the specimen.

4. Reagents provided

The reagents in the kit are sufficient for 96 determinations.

Plate	96 tests	Microwell plate, 12 microwell strips (which can be divided) in the strip holder; coated with specific monoclonal antibodies to the toxins A and B of *Clostridium difficile*
Diluent | 1	100 ml	Sample dilution buffer, protein-buffered NaCl solution; ready for use; blue colored
Wash	100 ml	Wash buffer, phosphate buffered NaCl solution (concentrated 10-fold); contains 0.1% thimerosal
Control | +	2 ml	Positive control; inactivated toxin; ready for use
Control | -	2 ml	Negative control (sample dilution buffer); ready for use
Conjugate | 1	13 ml	Biotin-conjugated antibodies to *Clostridium difficile* toxins A and B in stabilized protein solution; ready for use; blue colored
Conjugate | 2	13 ml	Streptavidin poly-peroxidase conjugate in stabilized protein solution; ready for use; orange colored
Substrate	13 ml	Hydrogen peroxide/TMB; ready for use
Stop	12 ml	Stop reagent; 1 N sulphuric acid; ready for use

5. Reagents and their storage

All reagents must be stored at 2–8 °C and can be used until the date printed on the label. Stored at 2–8 °C, the diluted wash buffer can be used for a maximum of 4 weeks. Microbial contamination must be prevented.
After the expiry date, the quality guarantee is no longer valid.
The aluminium bag must be opened with scissors in such a way that the clip seal is not torn off. Any microwell strips which are not required must be placed in the aluminium bag immediately and stored at 2 – 8 °C.
The colorless substrate must also be protected from direct light to prevent it from decomposing or turning blue due to auto-oxidation. Once the substrate has turned blue, it must not be used.

6. Additional necessary reagents – and necessary equipment

6.1. Reagents

- Distilled or deionized water

6.2. Equipment

- Test tubes
- Disposable pipettes (Article no.: Z0001)

- Vortex mixer (optional, see 9.3.)
- Micropipette for 50–100 µl and 1 ml volumes
- Measuring cylinder (1,000 ml)
- Timer
- Washing device for microwell plates or multichannel pipettes (300 µl)
- Photometer for microwell plates (450 nm and reference filter 620 - 650 nm)
- Filter paper (laboratory towels)
- Waste container with a 0.5 % hypochlorite solution

7. Precaution for users

For *in vitro* diagnostic only.

This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories must be followed. Always adhere strictly to the user instructions for this test.
Specimens or reagents must not be pipetted by mouth, and contact with injured skin or mucous membranes must be prevented. Wear personal safety gear (suitable gloves, laboratory coat , safety glasses) when handling the specimens, and wash hands after finishing the test. Do not smoke, eat, or drink in areas where samples are being processed.
For more details, refer to Material Safety Data Sheets (MSDS) at www.r-biopharm.com .

The positive control included in the kit contains inactivated *Clostridium difficile* toxin that showed positive reaction before inactivation in the cytotoxicity test. The toxins and stool samples must be treated as potentially infectious material and handled in accordance with the national safety regulations.

The wash buffer contains 0.1 % thimerosal as preservative. This substance must not be allowed to come into contact with skin or mucous membranes.

Ensure the proper and responsible disposal of all reagents and materials after their use. For disposal, please adhere to national regulations.

8. Specimen collection and storage

Stool samples must be taken as soon as possible within three days after occurrence of the initial symptoms of diarrhea.
Until it is used, store the test material at 2 – 8 °C. If the material cannot be used for a test within three days, we recommend storage at –20 °C or colder. Avoid freezing and thawing the specimen repeatedly. After diluting a stool sample in sample dilution buffer 1:11, it can be stored at 4 °C for use within three days.
Stool samples should not be collected in transport containers which contain transport media with preservatives, animal sera, metal ions, oxidizing agents, or detergents since these may interfere with the RIDASCREEN® Clostridium difficile Toxin A/B Test.

If rectal smears are used, make sure that the volume of stool material is sufficient (approx. 100 mg) for the test.
Contact tracing should include stool samples taken from contact persons who do not exhibit clinical symptoms, in order to identify asymptomatic carriers.

9. Test procedures

9.1. General information

All reagents and the plate of the microwell plate must be brought to room temperature (20 - 25 °C) before use. The microwell strips must not be removed from the aluminium bag until they have reached room temperature. The reagents must be thoroughly mixed immediately before use. After use, the microwell strips (placed in sealed bags) and the reagents must be stored at 2 - 8 °C. Once used, the microwell strips must not be used again. The reagents and microwell strips must not be used if the packaging is damaged or the vials are leaking.

In order to prevent cross contamination, the samples must be prevented from coming into direct contact with the kit components. The test must not be carried out in direct sunlight. We recommend covering the microwell plate or sealing with plastic wrap to prevent evaporation losses.

9.2. Preparing the wash buffer

Mix 1 part wash buffer concentrate Wash with 9 parts distilled water. Any crystals present in the concentrate must be warmed beforehand (in a water bath at 37 °C) to dissolve them.

9.3. Preparing the samples

Take a labelled test tube and fill 1 ml RIDASCREEN® sample dilution buffer Diluent | 1 in it. Use a disposable pipette (Article no. Z0001) to aspirate a sample of thin stool (approx. 100 µl) to just above the second marking and add to buffer in the test tube to make a suspension. To make a suspension with a solid stool sample, add an equivalent amount (approx. 50 - 100 mg) with a spatula or disposable inoculation loop.
Homogenize the stool suspension by aspiration into and ejection from a disposable pipette or, alternatively, blend in a Vortex mixer. Let the suspension stand a short period of time (10 minutes) for the coarse stool particles to settle, and this clarified supernatant of the stool suspension can be used directly in the test. If the test procedure is carried out in an automated ELISA system, the supernatant must be particle-free. In this case, it is advisable to centrifuge the sample at 2,500 G for 5 minutes.

Note:
Stool samples diluted in Diluent | 1 can be tested in all RIDASCREEN® ELISA for which Diluent | 1 is used.

9.4. Testing the toxins from *Clostridium difficile* cultures

To examine colonies after culture in solid substrates (CCFA or Schaedler agar) use an inocula-

tion loop to remove them from the agar plate; suspend in 1 ml of the sample dilution buffer Diluent |1 and blend well. In this case, it is advisable to centrifuge the suspension (2,500 G for 5 minutes). The clarified supernatant can be used directly in the test.
To examine liquid cultures, suspend 100 µl of the sample in 1 ml of the sample dilution buffer Diluent |1 and blend well. In this case, it is advisable to centrifuge the suspension (2500 G for 5 minutes). The clarified supernatant can be used directly in the test.

Note:

It is only possible to determine toxins in a culture of Clostridium cells once sporulation has begun. The sporulation and associated excretion of toxins only occur in the late, stationary phase of pathogen growth, when all nutrients have been consumed.

9.5. First incubation

After filling a sufficient number of wells in the strip holder, add 100 µl of the positive control | +, the negative control | - or the stool sample suspension (or, if available, the supernatant of the colony suspension) to the wells. Subsequently add 100 µl of the biotin-conjugated antibody Conjugate | 1 and blend (by tapping lightly on the side of the plate); then incubate for 60 minutes at room temperature (20 - 25 °C).

9.6. Washing

Careful washing is important in order to achieve the correct results and should therefore proceed strictly according to the instructions. The incubated substance in the wells must be emptied into a waste container for disposal in accordance with local regulations. Subsequently turn the plate over onto absorbent paper and tap to remove residual moisture. Then wash the plate five times using 300 µl wash buffer each time. Make sure that the wells are emptied completely by knocking them out after each wash on a part of the absorbent paper which is still dry and unused.
If you use a microplate washer or fully automated ELISA, make sure that the machine is correctly adjusted; request settings from the manufacturer, if necessary.
Appliances delivered by R-Biopharm are already programmed with validated settings and work protocols. To avoid blocking the wash needles, stool suspensions should never be filled in that are not particle-free (see Item 9.3., Preparing the samples). Also make sure that all of the liquid is sucked away during each wash step.

9.7. Second incubation

Use a pipette to fill 100 µl streptavidin poly-peroxidase conjugate Conjugate | 2 into the wells, then incubate for 30 minutes at room temperature (20–25 °C).

9.8. Washing

Wash as described in Item 9.6.

9.9. Third incubation

Fill all wells with 100 µl substrate Substrate. Then incubate the plate for 15 minutes in darkness at room temperature (20 - 25 °C). Subsequently fill all wells with 50 µl stop reagent Stop in order to stop the reaction. After blending cautiously by tapping lightly on the side of the plate, measure the extinction at 450 nm (optional: 450/620 nm). Adjust the zero point in the air, which is without the microwell plate.

Note:

High-positive patient samples may cause black-colored precipitates of the substrate.

9.9 Abridged test protocol

The incubation times described under Items 9.5, 9.7, and 9.9 can be significantly shortened, if the plate is incubated at 37 °C and a vibration frequency of 20–25 Hz. (DSX®, Fa. Dynex). The incubation times change to the following:

Incubation 1: 30 min
Incubation 2: 15 min
Incubation 3: 15 min

Separate microwell plate shakers are also suitable, such as the Thermomixer by Eppendorf (frequency setting: 850 rpm) or DTS-2 by LTF Labortechnik (frequency setting 800 rpm).

10. Quality control – indications of reagent expiry

For quality control purposes, positive and negative controls must be used each time the test is carried out, to ensure that the reagents are stable and that the test is conducted correctly. The test has been carried out correctly if the extinction rate (O.D.) for the negative control is less than 0.2 at 450 nm (less than 0.160 at 450/620 nm) and the measured value for the positive control is greater than 0.8 at 450 nm or at 450/620 nm. A value greater than 0.2 (0.160) for the negative control may indicate that washing was insufficient. Deviation from the required values, just like a turbid or blue coloring of the colorless substrate before it is filled into the wells, may indicate that the reagents have expired.

If the stipulated values are not met, the following points must be checked before repeating the test:

- Expiry date of the reagents used
- Functionality of the equipment being used (e.g. calibration)
- Correct test procedure
- Visual inspection of the kit components for contamination or leaks – a substrate solution which has turned blue must not be used.

If the conditions are still not fulfilled after repeating the test, please consult the manufacturer or your local R-Biopharm distributor.

11. Assessment and interpretation

11.1. Calculating the cut-off

In order to establish the cut-off, 0.15 extinction units are added to the measured extinction for the negative control.

Cut-off = extinction for the negative control + 0.15

11.2. Test results

Assessment of the specimen is positive if the extinction rate is more than 10 % higher than the calculated cut-off value.

Assessment of the specimen is marginal if the extinction rate ranges from 10 % less to 10 % greater than the cut-off value. If the repeat examination with a fresh stool sample again falls within the gray zone, assessment of the sample is negative.

Samples with extinctions more than 10 % below the calculated cut-off must be considered negative.

12. Limitations of the method

The RIDASCREEN® Clostridium difficile Toxin A/B Test shows presence of *Clostridium difficile* toxins A and B in stool samples. It is not possible to associate the determined level of extinction to the occurrence or severity of clinical symptoms. The results obtained must always be interpreted in combination with the clinical picture.

A positive result does not rule out the presence of other infectious pathogens.

A negative result does not rule out the possibility of *Clostridium difficile*infection.

The result may be caused by proteolytic breakdown of toxins in the specimen due to unsatisfactory storage conditions. If the patient anamnesis supports a suspicion of *Clostridium difficile* infection, the examination should be repeated with another stool sample.

A marginal result may be due to non-homogeneous distribution of toxins in the stool sample. In this case, examination should either be repeated with a second suspension from the same sample or another stool sample should be requested.

Negative test results for toxins in a *Clostridium difficile* culture from a stool sample may indicate the presence of non-toxigenic strains of *Clostridium difficile*. To clarify such a result, the possible production of toxins in suspicious colonies should be examined by way of ELISA.

13. Performance characteristics

13.1. Test quality

In a prospective study conducted at a large clinic in Germany, 502 stool samples from patients with suspicion of CDAD (*Clostridium difficile* associated diarrhea) were examined by

RIDASCREEN® ELISA and other commercial ELISAs and subsequently compared to the cytotoxicity test as gold standard. The RIDASCREEN® ELISA proved to coincide best with the gold standard. The results of that study are summarized in Table 1.

Table 1: Comparison of RIDASCREEN® Clostridium difficile Toxin A/B with gold standard (cytotoxicity test with neutralization)

		RIDASCREEN® Clostridium difficile Toxin A/B	
		pos	neg
Gold standard	positive	61	7
	negative	14	420

Sensitivity:	89.7 %
Specificity:	96.8 %
Positive predictability:	81.3 %
Negative predictability:	98.4 %

13.2. Cross reactivity

A variety of pathogenic microorganisms from the intestinal tract were examined with the RIDASCREEN® Clostridium difficile Toxin A/B ELISA and showed no cross reactivity. These tests were conducted with bacterial suspensions (10^6 to 10^9 cfu/ml), with parasite cultures (10^7 to 10^9 organisms/ml) and with cell culture supernatants from virus infected cells. The results of that study are summarized in Table 2.

Table 2: Cross reactivity with pathogenic microorganisms from the intestinal tract

Organism	**Result [OD 450]**
Adenovirus	0.060
Aeromonas hydrophila	0.057
Astrovirus	0.055
Bacillus cereus	0.053
Bacteroides fragilis	0.058
Campylobacter coli	0.061
Campylobacter jejuni	0.059
Candida albicans	0.066
Citrobacter freundii	0.055
Cryptosporidium muris	0.061
Cryptosporidium parvum	0.054
E. coli (EPEC)	0.060
E. coli (ETEC)	0.054

E. coli (STEC)	0.053
Enterobacter cloacae	0.059
Enterococcus faecalis	0.054
Giardia lamblia	0.056
Klebsiella oxytoca	0.054
Proteus vulgaris	0.055
Pseudomonas aeruginosa	0.055
Rotavirus	0.056
Salmonella enteritidis	0.063
Salmonella typhimurium	0.062
Serratia liquefaciens	0.053
Shigella flexneri	0.067
Staphylococcus aureus	0.065
Staphylococcus epidermidis	0.055
Vibrio parahaemolyticus	0.054
Yersinia enterocolitica	0.053

3.2.1. Strain specificity:

The following Clostridium strains tested negative in RIDASCREEN® Clostridium difficile Toxin A/B ELISA: *C. bifermentans, C. butyricum*, non-toxigenic *C. difficile* (3 strains), *C. perfringens, C. sordellii, C. sporogenes, C. tetani.*

13.3. Precision

The RIDASCREEN® Clostridium difficile Toxin A/B ELISA reproducibility was tested with six references representing the complete measurement range from negative to high-positive. To determine the intra-assay reproducibility, 40 replicates of these references were assayed. The mean values and the variation coefficients (VC) were determined for three lots of the kits. For the inter-assay reproducibility, references from ten sequential working days were assayed in duplicates, with two runs per day. The measurements were determined by three technicians for three lots of the kits. The inter-lot reproducibility was determined for all three lots of the kits.

Reference Mean value / VC	Intra-assay			Inter-assay			Inter-lot
	Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1–3
1	1,181 / 5,09%	1,307 / 6,13%	1,175 / 4,28%	1,256 / 13,93%	1,218 / 12,86%	1,211 / 11,33%	1,228 / 12,78%
2	0,867 / 6,25%	0,895 / 4,59%	0,879 / 4,81%	0,947 / 15,51%	0,900 / 14,41%	0,897 / 12,47%	0,915 / 14,24%
3	0,659/ 6,01%	0,665 / 5,16%	0,616 / 7,61%	0,620 / 17,87%	0,596 / 16,85%	0,601 / 12,45%	0,605 / 15,94%

4	0,428 / 9,64%	0,461 / 4,74%	0,462 / 3,30%	0,471 / 20,13%	0,445 / 18,24%	0,482 / 18,32%	0,466 / 18,98%
5	0,320 / 8,42%	0,337 / 5,69%	0,300 / 4,71%	0,319 / 24,51%	0,293 / 19,98%	0,319 / 14,19%	0,310 / 20,14%
6	0,046 / 5,60%	0,049 / 6,91%	0,053 / 15,40%	0,058 / 24,16%	0,058 / 20,54%	0,059 / 15,37%	0,058 / 20,33%

13.4 Analytical sensitivity

The analytical detection limits of Clostridium difficile toxin A and toxin B were determined separately. The limit of blank (LoB) was determined with 270 assays of negative samples and for toxins A and B the limits of detection (LoD) were analyzed in 90 assays each. The results of that study are summarized in Table 1.

Table 1: LoB and LoD

	OD (450 nm)	Analytical concentration in the sample (ng/ml)	Analytical concentration in sample suspension (1+10 in diluent) (ng/ml)
LoB	0.073	-	-
LoD (toxin A)	0.132	1.56	0.156
LoD (toxin B)	0.117	1.56	0.156

14. Interfering substances

The following list of substances showed no effects on the test results when they were blended into *C. difficile* toxin A/B positive and *C. difficile* toxin A/B negative stool samples in the described concentrations**:** barium sulfate (5 % w/w), loperamide (antidiarrheal drug; 5 % w/w), Pepto-Bismol (antidiarrheal drug, 5 % v/w), mucins (5 % w/w), sodium cyclamate (artificial sweetener, 5 % v/w), human blood (5 % v/w), stearic acid / palmitinic acid (mixture 1:1, 40 % w/w), metronidazole (0.5) (antibiotic 5 % v/w), diclofenac (0.00263 % v/w).

Appendix

Test specific symbols:

Plate	Microwell plate
Diluent | 1	Sample dilution buffer
Wash	Wash buffer
Control | +	Positive control
Control | -	Negative control
Conjugate | 1	Conjugate 1
Conjugate | 2	Conjugate 2
Substrate	Substrate
Stop	Stop reagent

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