

RIDASCREEN[®] Clostridium perfringens Enterotoxin

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1. Intended use

For *in vitro* diagnostic use. The enzyme immunoassay RIDASCREEN® Clostridium perfringens Enterotoxin provides the qualitative identification of *Clostridium perfringens enterotoxins* in human stool samples.

2. Summary and explanation of the test

Clostridium perfringens bacteria, like all other Clostridia, are ubiquitous, spore-forming, Gram-positive, anaerobic bacteria which are regularly found among the flora of the large intestine in humans. These differ from other Clostridia, because they lack the peritrichous flagella. Five strains of *Clostridium perfringens* are distinguished as Types A to E. The Type A strain has the greatest significance for humans, along with Type C, the cause of necrotic enteritis, under certain conditions (diet, trypsin deficiency), while all five types are seen in animals.

These groups are classified according to the production of four major toxins (Alpha, Beta, Epsilon, Iota), all of which have cytotoxic and necrotizing effects. Besides these four major toxins, certain strains of *C. perfringens* produce a number of other toxins, among which the enterotoxin (a polypeptide 35 kDa in size) is of pathogenic significance for humans.

With the consumption of improperly stored foods, and then pre-cooked foods in particular, *C. perfringens* may be imbibed in large amounts. They, in turn, form large amounts of *C. perfringens* enterotoxins (CPE) and these are the cause of intestinal diseases with diarrhea and stomach cramps. The signs and symptoms begin about 8–24 hours after the intake of contaminated food, and they recede after another 24 hours. Vomiting, fever, and headache are only seldomly seen to accompany the illness. In addition to food poisoning, *C. perfringens* causes about 10% of all cases of antibiotic-associated diarrhea (AAD) as well as 5–20% of the cases of sporadic diarrhea (SPOR) that are not caused by food poisoning. This form of diarrhea ranges as far as pseudomembranous colitis (PMC) with courses of illness that are much more severe and longer in duration (10–30 days), often with findings of blood and mucous in the stool. On histological examination of biopsies from lower sections of the large intestine and the rectum, one finds edematous areas stemming from the lesions caused by CPE.

Recent investigations have shown that the enterotoxin code may be chromosomal (food poisoning) and it may be episomal (in AAD and SPOR).

The RIDASCREEN® Clostridium perfringens Enterotoxin Test is available parallel to the RIDASCREEN® Clostridium difficile Toxin A/B Test, providing a significant identification system for clarification of the causes of antibiotic-associated diarrhea and sporadic diarrhea. Allowing fast and reliable diagnosis, this tool is a valuable aid in the treatment decision process.

3. Test principle

The RIDASCREEN® Clostridium perfringens Enterotoxin Test employs specific antibodies in a sandwich-type method. Monoclonal antibodies to epitopes of the *C. perfringens* enterotoxin 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-enterotoxin 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 presence of the enterotoxin in a specimen, immobilized antibodies, enterotoxin, 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 CPE found in the specimen.

4. Reagents provided

The reagents in the kit are sufficient for 96 determinations.

Plate	96	Microwell plate, 12 microwell strips (which can be divided) in the strip holder; coated with specific monoclonal antibodies to the enterotoxin of <i>Clostridium perfringens</i>
Diluent 1	100 ml	Sample dilution buffer, protein-buffered NaCl solution; ready to use, blue color
Wash	100 ml	Wash buffer, phosphate buffered NaCl solution (concentrated 10-fold); contains 0.1% thimerosal
Control +	2 ml	Positive control; inactivated enterotoxin, ready for use
Control -	2 ml	Negative control (sample dilution buffer); ready for use
Conjugate 1	13 ml	Biotin-conjugated antibodies to enterotoxin in stabilized protein solution; ready to 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 aluminum 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 aluminum 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

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 kit includes a positive control that contains the inactivated enterotoxin. Just like the stool samples, it must be treated as potentially infectious material and handled in accordance with the relevant 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

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 seven days.

Stool samples and rectal smears 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 perfringens Enterotoxin Test. Stool samples packed in the commonly marketed transport media (Cary Blair, Amies) can be used in the RIDASCREEN® Clostridium perfringens Enterotoxin Test. However, the here required pre-dilution of the sample must be taken into account. As far as possible, the end dilution of the stool sample in Diluent 1 should be precisely 1:11.

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 microwell **Plate** must be brought to room temperature (20–25 °C) before use. The microwell strips must not be removed from the aluminum 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 again 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 placing plastic wrap over it 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 dissolved beforehand by warming in a water bath at 37 °C.

9.3 Preparing the samples

Fill a labelled test tube with 1 ml RIDASCREEN® sample dilution buffer **Diluent 1**. 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 (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 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 used in any other RIDASCREEN® ELISA, provided that it also uses **Diluent | 1**.

9.4. First incubation

After inserting a sufficient number of wells in the strip holder, add 100 µl of the positive control **Control | +**, the negative control **Control | -**, or the stool sample 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).

9.5. 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 the official requirements. 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, only particle-free stool suspensions should be dispensed (see Item 9.3., Preparing the samples). Also make sure that all of the liquid is aspirated during each wash step.

9.6. Second incubation

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

9.7. Washing

Wash as described in Item 9.5.

9.8. 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, that is without the microwell plate.

Note:

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

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 test has been carried out correctly and that the reagents are stable. The test has been carried out correctly if the extinction rate (OD) 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.

$$\text{Cut-off} = \text{extinction for the negative control} + 0.15$$

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 perfringens Enterotoxin Test identifies the enterotoxin of *Clostridium perfringens* 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 signs and symptoms.

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

A negative result does not rule out the possibility of *Clostridium perfringens* infection. The result may be caused by proteolytic breakdown of enterotoxins in the specimen due to unsatisfactory storage conditions. If the patient history supports a suspicion of *Clostridium perfringens* infection, the examination should be repeated with another stool sample.

A marginal result may be due to inhomogeneous 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.

13. Performance characteristics

13.1. Comparison of methods for detection limit

To establish the detection limit, a stock solution of the enterotoxin (10 µg/ml) was titrated in each relevant sample solution buffer and then applied in a RIDASCREEN® Clostridium perfringens Enterotoxin Test as well as a competitor's commercially available ELISA. The concentrations of toxin in both ELISA results are shown in Table 1.

Table 1: Comparison of two ELISAs for identification of the *Clostridium perfringens* enterotoxin.

CPE (ng/ml)	10	5	2,5	1,25	0,625	0,312	0,156	0,078	0,039	0,019
RIDASCREEN®	+	+	+	+	+	+	+	+	+	-
other ELISA	+	+	+	-	-	-	-	-	-	-

The RIDASCREEN® Clostridium perfringens Enterotoxin ELISA can detect 0.04 ng/ml of pure enterotoxin and is thus 60 times more sensitive than the competitor's commercially available ELISA.

13.2. Cross reactivity

A variety of pathogenic microorganisms from the intestinal tract were examined with the

RIDASCREEN® Clostridium perfringens Enterotoxin 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 nm]
Adenovirus	0.050
<i>Aeromonas hydrophila</i>	0.046
<i>Bacillus cereus</i>	0.054
<i>Bacteroides fragilis</i>	0.053
<i>Campylobacter coli</i>	0.060
<i>Campylobacter fetus</i>	0.055
<i>Campylobacter jejuni</i>	0.053
<i>Campylobacter lari</i>	0.060
<i>Campylobacter upsaliensis</i>	0.051
<i>Candida albicans</i>	0.052
<i>Citrobacter freundii</i>	0.052
<i>Clostridium bifermentans</i>	0.044
<i>Clostridium difficile</i>	0.044
<i>Clostridium novyi</i>	0.049
<i>Clostridium perfringens</i>	0.046
<i>Clostridium septicum</i>	0.050
<i>Clostridium sordellii</i>	0.049
<i>Clostridium sporogenes</i>	0.060
<i>Cryptosporidium parvum</i>	0.053
<i>E. coli</i> (O26:H-)	0.049
<i>E. coli</i> (O6)	0.045
<i>E. coli</i> (O157:H7)	0.044
<i>Enterobacter cloacae</i>	0.051
<i>Enterococcus faecalis</i>	0.050
<i>Giardia lamblia</i>	0.052
<i>Klebsiella oxytoca</i>	0.046
<i>Proteus vulgaris</i>	0.046
<i>Pseudomonas aeruginosa</i>	0.051
Rotavirus	0.080
<i>Salmonella enteritidis</i>	0.044
<i>Salmonella typhimurium</i>	0.045
<i>Serratia liquefaciens</i>	0.044
<i>Shigella flexneri</i>	0.043

<i>Staphylococcus aureus</i>	0.054
<i>Staphylococcus epidermidis</i>	0.043
<i>Vibrio parahaemolyticus</i>	0.044
<i>Yersinia enterocolitica</i>	0.046

13.3. Precision

The reproducibility of the RIDASCREEN® *Clostridium perfringens* Enterotoxin Test was investigated with six references representing the complete measurement range from weak 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 kit lots. For the inter-assay reproducibility, references from ten different working days were assayed in duplicates with two runs per day. The measurements were determined by two technicians in three kit lots. 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	1.966 / 7.55%	1.941 / 5.38%	1.882 / 6.66%	2.199 / 13.16%	1.917 / 12.10%	1.735 / 12.11%	1.951 / 17.21%
2	0.948 / 5.40%	0.927 / 7.37%	0.813 / 7.77%	1.114 / 14.72%	0.966 / 11.98%	0.843 / 14.31%	0.974 / 19.46%
3	0.494 / 9.34%	0.484 / 7.56%	0.459 / 6.20%	0.551 / 13.55%	0.495 / 11.04%	0.416 / 15.15%	0.487 / 19.09%
4	0.361 / 9.94%	0.405 / 8.33%	0.320 / 6.43%	0.423 / 23.05%	0.382 / 13.94%	0.322 / 15.50%	0.376 / 22.79%
5	0.184 / 11.44%	0.197 / 5.88%	0.143 / 11.60%	0.199 / 16.92%	0.183 / 14.92%	0.143 / 15.20%	0.175 / 22.55%
6	0.046 / 8.79%	0.054 / 6.69%	0.048 / 17.25%	0.054 / 20.07%	0.053 / 16.00%	0.050 / 7.88%	0.052 / 15.68%

14 Interfering substances

The following list of substances showed no effects on the test results when they were blended into *Clostridium perfringens* enterotoxin-positive and *Clostridium perfringens* enterotoxin-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), *mucines* (5% w/w), *cyclamate* (artificial sweetener, 5% v/w), *human blood* (5% v/w), *stearic acid and palmitinic acid* (mixture 1:1, 40% w/w), *metronidazole* (0.5) (antibiotic drug 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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