RIDASCREEN® Clostridium difficile GDH

REF C0701

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Clostridium difficile GDH is an enzyme immunoassay for the qualitative detection of *Clostridium difficile*-specific glutamate dehydrogenase in human stool samples.

2. Summary and explanation of the test

Clostridium difficile are strictly anaerobic, spore-forming, rod-shaped bacteria that are part of the normal stool flora in humans. A proposal was made in 2016 to reclassify the bacterium as *Clostridioides difficile*. Both names are acceptable in usage today. Under normal conditions, the colonization rate of these mostly harmless pathogens is very high in the earliest years of childhood, ranging up to 80 %. This rate decreases steadily during the course of life and averages no more than 2 % to 10 % in adulthood. Under certain conditions, however, for example during a hospital stay, it can slightly exceed 30 %. Decisive for a *Clostridium difficile* infection (CDI) is the formation of high-molecular-weight toxins A (enterotoxin) and B (cytotoxin). Considering that some strains of *Clostridium difficile* do not produce the toxins and that approximately 2 % to 8 % of healthy adults and up to 80 % of children younger than two years of age may be colonized with *Clostridium difficile*, the detection of toxins A and B alone is of pathognomonic significance in the case of suspected *C. difficile*-associated diarrhea (CDAD). However, that depends on successful colonization of the large intestine with *C. difficile* bacteria capable of sufficient toxin formation. A reduction in the protective intestinal flora promotes colonization. Imbalance due to antibiotic therapy, for example, or other factors capable of impairing intestinal immunity can allow especially the strains of *C. difficile*, which have a broad and increasing resistance toward various antibiotic drugs to spread. Other virulence factors such as reinforced toxin production resulting from the regulation defects of several newly defined strains have made *C. difficile* a “re-emerging germ” with a pathogenicity that is no longer limited only to people who develop a *Clostridium difficile* infection (CDI) resulting from antibiotic treatment, but increasingly also includes persons who have not undergone treatment and were not hospitalized. The growing significance of this microorganism, in particular as a nosocomial pathogen, has led to new treatment approaches and, most importantly, to new algorithms for the diagnosis of *C. difficile* infections. The main objective is to detect *C. difficile* in order to prevent transmission to hospitalized patients, regardless of whether the strains of *C. difficile* are toxigenic or non-toxigenic. The enzyme glutamate dehydrogenase (GDH), which is widespread among many organisms and found in high numbers, has proven to be a sensitive screening marker. Since many of the intestinal bacteria also have this enzyme, detection systems must be able to detect GDH specific to *C. difficile* with reliable specificity and high sensitivity.

The present RIDASCREEN® Clostridium difficile GDH enzyme immunoassay meets both of these requirements to a high degree. While this EIA cannot replace the obligatory detection of the toxins A and B as proof of a CDI, it improves the reliable detection of this very significant nosocomial pathogen when conducted either sequentially prior to or in parallel with the RIDASCREEN® Clostridium difficile Toxin A/B ELISA. Only the specific symptoms of the disease and identification of the toxins A and B make the CDI diagnosis and an adequate treatment decision possible.

3. Test principle

The RIDASCREEN® Clostridium difficile GDH test employs monoclonal antibodies in a sandwich-type method. These monoclonal antibodies against the glutamate dehydrogenase of *Clostridium difficile* are attached to the well surface of the microtiter plate.
A pipette is used to place a suspension of the stool sample to be examined as well as controls into the well of the microtiter plate together with biotinylated anti-GDH antibodies (Conjugate 1) for incubation at room temperature (20 to 25 °C). After a washing stage, streptavidin poly-peroxidase conjugate (Conjugate 2) is added and incubated again at room temperature (20 to 25 °C). Any GDH specific to *Clostridium difficile* present in the specimen forms a sandwich complex consisting of immobilized antibodies, GDH, and conjugated antibodies. A further washing stage removes the unattached streptavidin poly-peroxidase conjugate. In positive samples, after the addition of a substrate, the bound enzyme changes the colorless solution in the wells of the microtiter plate to a blue solution. The addition of a stop reagent changes the color from blue to yellow. The extinction is proportional to the concentration of the GDH of *Clostridium difficile* found in the specimen.

4. Reagents provided

The reagents in the kit are sufficient for 96 determinations.

Table 1: Reagents provided

Plate	96 assays	Microtiter plate, 12 microtiter strips (breakable) in strip holder; coated with specific antibodies (mouse) against *Clostridium difficile*-specific glutamate dehydrogenase.
Diluent | 1	100 ml	Sample dilution buffer 1, protein-buffered NaCl solution, ready to use, blue colored
Wash buffer	100 ml	Wash buffer, phosphate-buffered NaCl solution (concentrated 10-fold); contains 0.1 % thimerosal
Control | +	2 ml	Positive control; inactivated GDH protein; ready to use
Control | -	2 ml	Negative control (sample dilution buffer); ready to use
Conjugate | 1	13 ml	Biotin-conjugated antibodies (mouse) against *Clostridium difficile*-specific glutamate dehydrogenase in stabilized protein solution; ready to use; green color
Conjugate | 2	13 ml	Streptavidin poly-peroxidase conjugate in stabilized protein solution; ready to use; orange colored
Substrate	13 ml	Hydrogen peroxide/TMB; ready to use
Stop	12 ml	Stop reagent; 1 N sulfuric acid; ready to use

Hazardous materials are indicated according to labeling obligations. For further details, see the safety data sheets (SDSs) at www.r-biopharm.com .

5. Storage instructions

All reagents must be stored at 2 to 8 °C and can be used until the date printed on the label. The diluted wash buffer has a shelf life of four weeks when stored at 2 to 8 °C and one week when stored at 20 to 25 °C. Microbial contamination must be prevented. After the expiration date, the quality guarantee is no longer valid.
When stored correctly at 2 to 8 °C, the opened kit can be used for at least six weeks.

The aluminum bag must be opened with scissors in such a way that the clip seal is not separated. Any microtiter strips that are not required must immediately be returned to the aluminum bag and stored at 2 to 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. Reagents required but not provided

6.1 Necessary reagents

The following reagents are needed to perform the RIDASCREEN® Clostridium difficile GDH test:

Reagents
Distilled or deionized water

6.2 Necessary laboratory equipment

The following equipment is needed to perform the RIDASCREEN® Clostridium difficile GDH test:

Equipment
Sample vials
Disposable pipettes (Article no.: Z0001)
Vortex mixer (optional, see 9.3.)
Micropipette for 50 to 100 µl and 1 ml volumes
Graduated cylinder (1000 ml)
Stopwatch
Washing device for microtiter plates or multichannel pipettes (300 µl)
Photometer for microtiter plates (450 nm, reference filter 620 to 650 nm)
Filter paper (lab wipes)

7. Warnings and precautions for the users

For *in vitro* diagnostic use only.

This test must be carried out only by trained laboratory personnel. The guidelines for working in medical laboratories must be followed. Always adhere strictly to the user instructions for carrying out 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 equipment (suitable gloves, gown, safety glasses) when handling the specimens, and wash hands after finishing the test. Do not smoke, eat, or drink in areas where samples are handled.

For further details, see the safety data sheets (SDSs) at www.r-biopharm.com .
The positive control provided in the kit contains recombinant GDH protein. It must be treated as potentially infectious material and handled in accordance with the national safety regulations, just like the patient specimens.

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

Users are responsible for proper disposal of all reagents and materials after use. For disposal, please adhere to national regulations.

8. Collection and storage of samples

Stool samples must be taken as soon as possible within three days after occurrence of symptoms of diarrhea. Until it is used, test material is to be stored at 2 to 8 °C. If the material cannot be used for a test within three days, we recommend storage at -20 °C or below. Avoid repeated freezing and thawing of the sample. A stool specimen diluted in the sample dilution buffer 1:11 can be stored at 2 to 8 °C for up to three days (Tab. 2).

Table 2: Specimen storage

Undiluted stool specimen		Diluted stool specimen
2 to 8 °C	≤ -20 °C	2 to 8 °C
≤ 3 days	> 3 days	≤ 3 days

Stool samples and rectal smears should not be collected in transport containers that have transport media containing preservatives, animal sera, metal ions, oxidizing agents, or detergents since they can interfere with the RIDASCREEN® Clostridium difficile GDH 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 procedure

9.1 General information

All reagents and the microtiter plate Plate must be brought to room temperature (20 to 25 °C) before use. Once they have reached room temperature, remove the microtiter strips from the aluminum bag. Mix the reagents well immediately before use. After use, the microtiter strips (placed in sealed bags) and the reagents must be stored again at 2 to 8 °C.
Once used, the microtiter strips must not be re-used. Do not use reagents or microtiter strips if the packaging is damaged or the containers are not tightly sealed.
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 microtiter plate or sealing with plastic wrap to prevent evaporation losses.

9.2 Preparing the wash buffer

Mix 1 part wash buffer concentrate Wash buffer with 9 parts distilled water. Any crystals present in the concentrate should be dissolved beforehand through heating (water bath at 37 °C).

9.3 Preparing the specimens

Fill a labeled test tube with 1 ml sample dilution buffer Diluent | 1. Use a disposable pipette (article no. Z0001) to suction 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.
In the case of solid stool samples, add an equivalent amount of the stool sample (approx. 50 to 100 mg) using a spatula or disposable inoculation loop and suspend.
Homogenize the stool suspension by suction into and ejection from a disposable pipette or, alternatively, by blending in a Vortex mixer. Let the suspension stand a short period of time (10 minutes) for the coarse stool particles to settle, and use this clarified supernatant of the stool suspension 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 2,500 g for 5 minutes.

Note: Stool samples diluted in Diluent | 1 can also be used in all other RIDASCREEN® ELISAs that likewise use Diluent | 1.

9.4 First incubation

After placing a sufficient number of wells in the holder, add 100 µl of the positive control Control | + , negative control Control | - , or stool sample suspension to the wells. Then add 100 µl of the biotin-conjugated antibody Conjugate | 1 , mix (by tapping lightly on the edge of the plate), and incubate for 60 minutes at room temperature (20 to 25 °C).

9.5 Washing

Careful washing is important in order to achieve the correct results and should therefore be carried out strictly according to the instructions. The incubated substance in the wells must be emptied into a waste container for disposal in accordance with official regulations. Next, tap the plate over absorbent paper to remove the remaining moisture. Then wash the plate five times using 300 µl diluted wash buffer each time Wash buffer . Make sure that the wells are emptied completely by tapping them out after each wash onto a part of the absorbent paper that is still dry and unused.
If a microplate washer or fully automated ELISA is used, ensure that the machine is correctly adjusted or request the 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 9.3., Preparing the samples). Also ensure that all of the liquid is aspirated during each washing stage.

9.6 Second incubation

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

9.7 Washing

Wash as described in 9.5.

9.8 Third incubation

Add 100 µl substrate Substrate to each well. Then incubate the plate for 15 minutes in darkness at room temperature (20 to 25 °C). Subsequently, stop the reaction by adding 50 µl stop reagent Stop to each well. After mixing carefully (by tapping lightly on the side of the plate), measure the extinction at 450 nm and reference wavelengths of 620 nm.

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

9.9 Abridged test protocol

The incubation times described in Sections 9.4, 9.6, and 9.8 can be significantly shortened if the plate is incubated at 37 °C and at a vibration frequency of 20 to 25 Hz (DSX-, manufactured by Dynex). The incubation times change as follows:

1st incubation: 30 min
2nd incubation: 15 min
3rd incubation: 15 min

Separate microtiter plate shakers also suitable for use include Thermomixer made by Eppendorf (frequency setting: 850 rpm) and DTS-2 made by LTF Labortechnik (frequency setting: 800 rpm).

10. Quality control – Indication of instability or deterioration of reagents

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 was done correctly when the extinction value (OD) of the negative control at 450/620 nm is less than 0.160 and the measured value of the positive control at 450/620 nm is greater than 0.8. A value greater than 0.160 for the negative control may indicate that there was insufficient washing. Deviation from the required values, just like a turbid or blue coloration 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:

- Expiration date of the reagents used
- Functional performance of the equipment used (e.g., calibration)
- Correct test procedure
- Visual inspection of the kit components for contamination or leaks; a substrate solution that 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. Evaluation and interpretation

11.1 Calculating the cut-off

In order to establish the cut-off, 0.10 extinction units are added to the measured extinction for the negative control.

Cut-off = extinction for the negative control + 0.10

11.2 Test result

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

Samples that are considered borderline and need to be retested are those that have an extinction rate ranging from ≤ 10 % above to ≤ 10 % below the cut-off. If the results of repeat testing of a newly prepared stool sample are within the gray zone again, the sample should be considered borderline.

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

12 Limitations of the method

The RIDASCREEN® Clostridium difficile GDH test detects the *Clostridium difficile*-specific glutamate dehydrogenase in stool samples. It is not possible to associate the determined level of extinction to the occurrence or severity of clinical symptoms. Only the additionally necessary RIDASCREEN® Clostridium difficile Toxin A/B ELISA to be performed as well as the clinical symptoms allow CDI to be diagnosed with high certainty.

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

A negative result does not rule out the possibility of *C. difficile* infection. Such a result may be due to intermittent excretion of the pathogen, or the amount of antigen in the sample may be too small. If the patient history supports a suspicion of CDI, another stool sample should be tested.

A borderline result may be due to non-homogeneous distribution of the antigens in the stool sample. In this case, a second suspension from the same sample should be tested or another stool sample should be requested for testing.

13 Performance characteristics

13.1 Test quality

A retrospective validation study with RIDASCREEN® Clostridium difficile GDH ELISA examined 92 stool samples. The thawed samples were homogenized and tested in a comparison of the RIDASCREEN® Clostridium difficile GDH ELISA and another commercial ELISA. The result of the study is summarized in Table 3.

Table 3: Comparison of RIDASCREEN® Clostridium difficile GDH ELISA to a commercial ELISA

		Competitor ELISA	
		+	-
RIDASCREEN® Clostridium difficile GDH	+	30	7
	-	0	55

Positive agreement: 89.6 %
Negative agreement: 94.0 %

13.2 Analytical sensitivity

To determine the analytical sensitivity of the RIDASCREEN® Clostridium difficile GDH ELISA, the limit of blank (LoB) was determined using 90 assays of negative stool samples. The limit of detection (LoD) was then determined using 72 assays of a recombinant glutamate dehydrogenase protein. The results are shown in Table 4.

Table 4: Analytical sensitivity results for the RIDASCREEN® Clostridium difficile GDH ELISA

	MV [OD 450/620]	ng/ml
LoB	0.018	-
LoD	-	0.024

13.3 Cross-reactivity

A variety of pathogenic micro-organisms from the intestinal tract were examined using the RIDASCREEN® Clostridium difficile GDH ELISA and demonstrated no cross-reactivity. These studies were conducted with bacterial suspensions shown to have concentrations of 10^6 to 10^9 organisms per ml. Virus culture supernatants and stool samples are listed accordingly. The results of that study are listed in Table 5.

Tab.5: Cross reactivity with pathogenic microorganisms

Organism	Origin	MV [OD 450/620]
Adenovirus	Culture supernatant	-0.010
Aeromonas hydrophila	Culture	-0.010
Astrovirus	Culture supernatant	-0.006
Bacillus cereus	Culture	-0.012
Bacteroides fragilis	Culture	-0.004
Campylobacter coli	Culture	-0.008
Campylobacter jejuni	Culture	-0.011
Candida albicans	Culture	-0.007
Citrobacter freundii	Culture	-0.006
Clostridium bifermentans	Culture	-0.004
Clostridium difficile	Culture	3.472
Clostridium novyi	Culture	-0.007
Clostridium perfringens	Culture	-0.007
Clostridium sordellii	Culture	-0.009
Clostridium sporogenes	Culture	-0.011
Cryptosporidium muris	Culture	-0.005
Cryptosporidium parvum	Culture	-0.010
E. coli (O157:H7)	Culture	-0.006
E. coli (O26:H-)	Culture	-0.006
E. coli (O6)	Culture	-0.001
Entamoeba histolytica	Stool	-0.001
Enterobacter cloacae	Culture	-0.001
Enterococcus faecalis	Culture	-0.004
Giardia lamblia	Stool	0.001
Klebsiella oxytoca	Culture	-0.005
Norovirus Ag Desert Shield Virus	Capsid	-0.004
Norovirus Ag Hawaii Virus	Capsid	-0.004
Norovirus Ag md 14S	Capsid	-0.008
Norovirus Ag Norwalk Virus	Capsid	0.000
Norovirus Ag Snow Mountain Virus	Capsid	-0.001
Norovirus Ag Toronto Virus	Capsid	-0.005
Proteus vulgaris	Culture	-0.009
Pseudomonas aeruginosa	Culture	-0.015

Rotavirus	Culture supernatant	-0.011
Salmonella enteritidis	Culture	-0.004
Salmonella typhimurium	Culture	-0.005
Serratia liquefaciens	Culture	-0.006
Shigella flexneri	Culture	-0.005
Staphylococcus aureus	Culture	-0.009
Staphylococcus epidermidis	Culture	-0.012
Vibrio parahaemolyticus	Culture	-0.009
Yersinia enterocolitica	Culture	-0.007

13.4 Precision

The reproducibility of the RIDASCREEN® Clostridium difficile GDH ELISA was tested using 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. For the inter-assay reproducibility, references from 10 different working days were assayed in duplicates, with 2 runs per day. The measurements were determined in 3 lots by 4 technicians. The inter-lot reproducibility was determined for all 3 lots. The results are shown in Table 6.

Table 6: Reproducibility/precision results for the RIDASCREEN® Clostridium difficile GDH ELISA

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	MV	2.333	2.192	2.438	2.504	2.411	2.376	2.430
	CV (%)	8.10 %	6.71 %	4.23 %	5.65 %	7.84 %	8.14 %	7.59 %
2	MV	1.804	1.593	1.741	1.935	1.907	1.871	1.904
	CV (%)	5.24 %	6.73 %	5.34 %	6.82 %	9.26 %	9.29 %	8.51 %
3	MV	1.477	1.312	1.436	1.531	1.561	1.481	1.525
	CV (%)	7.74 %	8.91 %	6.88 %	7.91 %	10.95 %	9.64 %	9.76 %
4	MV	1.125	0.888	0.951	1.139	1.183	1.117	1.146
	CV (%)	8.25 %	8.24 %	8.55 %	8.05 %	11.49 %	11.55 %	10.67 %
5	MV	0.697	0.590	0.714	0.729	0.776	0.708	0.738
	CV (%)	8.06 %	10.13 %	13.78 %	9.88 %	11.51 %	13.59 %	12.38 %
6	MV	-0.003	-0.003	-0.007	0.008	-0.001	-0.002	0.002
	CV (%)	n/a	n/a	n/a	n/a	n/a	n/a	n/a

13.5 Interfering substances

The substances listed below showed no effects on the test results when mixed into *C. difficile*-positive and -negative stool specimens in the specified concentrations:

Mucin	5.0 % w/w	Diclofenac	0.1 % v/w
Human blood	5.0 % v/w	Cyclamate	1.3 % v/w
Barium sulphate	18.5 % w/w	Metronidazole	3.0 % w/w
Loperamide	0.02 % w/w	Hemoglobin	5.0 % v/w
Pepto-Bismol	6.3 % v/w	Vancomycin	3.0 % w/w
Stearic acid/ palmitic acid	40 % w/w (1:1)		

14 Version history

Version number	Section and designation
2017-04-20	Previous version
2020-02-18	General revision 2 Summary and explanation of the test 4 Reagents provided 5 Storage instructions 8 Collection and storage of samples 9.2 Preparing the wash buffer 9.5 Washing 9.8 Third incubation 10 Quality control – Indication of instability or deterioration of reagents 11 Evaluation and interpretation 13 Performance characteristics 15 Explanation of symbols 16 References

15 Explanation of symbols

General symbols

IVD	For *in vitro* diagnostic use
	Consult instructions for use
LOT	Lot number
	Expiry
	Store at
REF	Article number
	Number of tests
	Date of manufacture
	Manufacturer

Test-specific symbols

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

16. References

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