RIDASCREEN® Cryptosporidium

Article no.: C1201

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Cryptosporidium is an enzyme immunoassay for the qualitative determination of *Cryptosporidium parvum* and *Cryptosporidium hominis* in human stool samples**.**

2. Summary and explanation of the test

Cryptosporidiosis is a protozoan infection caused by the *Cryptosporidium* genus. This parasite is found widespread in animals, and it has significant presence as a pathogenic microorganism in domestic animals, calves in particular.
In immune competent patients the disease manifests as a self-healing gastroenteritis. The diarrhea lasts between 3 and 10 days and may be accompanied by fever and gastrointestinal symptoms such as nausea and pain similar to that of giardiasis (lambliasis).
The symptoms and effects are substantially more severe in immune compromised patients, where the course of diarrhea is very severe and persistent. The infection may pass from an animal to the human through contaminated water, but it is also contagious on personal contact with a patient. In the past, the method most often used to diagnose cryptosporidiosis was microscopic determination of oocysts in the stool and/or microscopic examination of small intestinal bioptates, which required the availability of experienced personnel.
The here described Cryptosporidium ELISA for simultaneous determination of both pathogens in stool samples is an important alternative to the diagnosis by microscopic examination. Its sensitivity equals that of a microscopic examination, requires no personnel specifically trained in parasitology, is simple and can be conducted quickly, and it is not dependent on finding intact organisms (cysts or trophozoites) in the stool sample.

3. Test principle

The RIDASCREEN® Cryptosporidium Test employs specific antibodies in a sandwich-type method. These specific antibodies to *Cryptosporidium parvum* and *Cryptosporidium hominis* 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-Cryptosporidium 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 *Cryptosporidium* antigens in a specimen, immobilized antibodies, the *Cryptosporidium* antigen, and the conjugated antibody 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 *Cryptosporidium* antigens 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 antibodies to *Cryptosporidium parvum* and *Cryptosporidium hominis* antigens.
Diluent | 1	100 ml	Sample dilution buffer, protein-buffered NaCl solution; ready to use; blue colored
Wash	100 ml	Wash buffer, phosphate-buffered NaCI solution (concentrated 10-fold); contains 0.1 % thimerosal
Control | +	2 ml	Inactivated *Cryptosporidium* antigen; ready for use
Control | -	2 ml	Negative control (sample dilution buffer); ready for use
Conjugate | 1	13 ml	Biotin conjugated antibodies to *Cryptosporidium* specific antigens 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. Providing the diluted wash buffer is stored at 2 – 8 °C, it 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 (1000 ml)
- Timer
- Washing device for microwell plates or multichannel pipettes (300 µl)
- Photometer for microwell plate (450 nm, 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, 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 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 *Cryptosporidium* antigen. The control 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

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. 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® Cryptosporidium Test. If rectal swabs 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 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 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

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 bulge 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 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 2500 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. First incubation

After filling a sufficient number of wells in the strip holder, use a pipette to add 100 µl of the positive control Control | +, the negative control Control | -, or the stool 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 30 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 and discarded in accordance with local regulations. After this, knock out the plate onto absorbent paper in order to remove the residual moisture. Then wash the plate five times using 300 µl wash buffer each time. Make sure that the wells are emptied com-

pletely 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 which R-Biopharm delivers 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 streptavidin poly-peroxidase conjugate Conjugate | 2 into the wells, then incubate for 15 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 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 receive a negative assessment.

12. Limitations of the method

The RIDASCREEN® Cryptosporidium Test can determine antigens of *Cryptosporidium parvum* and *Cryptosporidium hominis*. 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 *Cryptosporidium* infection. Such a result may be due to intermittent excretion of the parasite, or the amount of antigen in the sample may be too small. If the patient anamnesis supports a suspicion of infection with *Cryptosporid*ium, the examination should be repeated with another stool sample.
A marginal result may be due to non-homogeneous distribution of the parasites 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 Analytical sensitivity

To determine the analytical sensitivity of the RIDASCREEN® Cryptosporidium ELISA the limit of blank (LoB) was analyzed in 270 measurements of Diluent 1, and the limit of detection (LoD) was analyzed in 90 measurements. The results of that study are shown in Table 1.

Table 1: Analytical sensitivity results for RIDASCREEN® Cryptosporidium ELISA

	MV [OD 450/620]	**Cysts / Reaction**
LoB	0,031	**-**
LoD	0,053	1.56×10^4

13.2. Clinical comparison study

A clinical investigation in Great Britain evaluated the RIDASCREEN® Cryptosporidium Test with a total of 240 stool samples (prospective and blinded retrospective studies). There was a comparison with established British methods of microscopy for *Cryptosporidia* (auramine-phenol and Ziehl-Neelsen stain) as well as a differentiating real-time PCR. The results of that study are summarized in Table 2.

Tab.2: Comparison of RIDASCREEN® Cryptosporidium ELISA with microscopy and real-time PCR

		Microscopy		Real-time PCR[#]	
		Positive	Negative	Positive	Negative
RIDASCREEN® Cryptosporidium	Positive	12	11	22	1
	Negative	1	216	1	214
Sensitivity (CI):		92 % (64 – 100)		96 % (78 – 100)	
Specificity (CI):		95 % (91 – 98)		100 % (97 – 100)	

[#]Insufficient amount of material in two stool samples

CI: Confidence interval in %

13.3. Cross reactivity

A variety of pathogenic microorganisms from the intestinal tract were examined with the RIDASCREEN® Cryptosporidium Test and except for *Campylobacter coli* they showed no cross reactivity. These studies were conducted with undiluted bacteria or virus suspensions shown to have concentrations of 10^6 to 10^9 organisms per ml. The results of that study are listed in Table 3.

Table 3: Cross reactivity with pathogenic microorganisms

Organism	**Origin**	**OD [450/620] Mean value**
Adenovirus	Cell culture supernatant	0,011
Aeromonas hydrophila	Culture	-0,001
Arcobacter butzleri	Culture	0,012
Astrovirus	Cell culture supernatant	-0,001
Bacillus cereus	Culture	0,006
Bacteroides fragilis	Culture	-0,005
Campylobacter coli	Culture	0,181
Campylobacter jejuni	Culture	0,034
Candida albicans	Culture	0,016
Citrobacter freundii	Culture	0,008
Clostridium difficile	Culture	0,009

Clostridium perfringens	Culture	0,016
Clostridium sordellii	Culture	0,003
Cryptosporidium muris	Culture	2,782
E. coli EPEC	Culture	0,031
E. coli ETEC	Culture	0,002
E. coli STEC	Culture	0,017
Entamoeba histolytica	Culture	-0,003
Enterobacter cloacae	Culture	0,007
Enterococcus faecalis	Culture	0,001
Giardia lamblia	Stool	-0,004
Klebsiella oxytoca	Culture	0,030
Proteus vulgaris	Culture	0,016
Pseudomonas aeruginosa	Culture	0,037
Rotavirus	Cell culture supernatant	0,009
Salmonella enteritidis	Culture	0,018
Salmonella typhimurium	Culture	0,010
Serratia liquefaciens	Culture	0,005
Shigella flexneri	Culture	0,013
Staphylococcus aureus	Culture	0,020
Staphylococcus epidermidis	Culture	0,018
Vibrio parahaemolyticus	Culture	0,019
Yersinia enterocolitica	Culture	0,015

13.4. Precision

To determine the intra-assay reproducibility, 40 replicates of 6 references were assayed, representing the complete measurement range from negative to high-positive. The mean values and the variation coefficients (VC) were determined for three lots of the kits. 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 three technicians for three lots of the kits. The inter-lot reproducibility was determined for all three lots of the kits. The results of that study are shown in Table 4.

Table 4: Reproducibility/precision of the RIDASCREEN® Cryptosporidium ELISA

Reference		**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 lot 1-3
1	MV [OD 450/620]	2,262	2,584	2,562	1,729	2,163	1,926	1,939
	VC (%)	6,00%	7,36%	4,75%	17,34%	15,94%	11,19%	18,47%
2	MV [OD 450/620]	1,391	1,636	1,508	1,000	1,275	1,158	1,144
	VC (%)	5,81%	6,94%	5,93%	14,94%	18,02%	11,26%	19,20%

3	MV [OD 450/620]	1,050	1,083	1,262	0,759	1,012	0,891	0,887
	VC (%)	8,17%	8,15%	7,67%	17,72%	18,02%	13,20%	22,23%
4	MV [OD 450/620]	0,675	0,706	0,766	0,413	0,562	0,511	0,495
	VC (%)	4,93%	7,33%	7,97%	18,15%	20,13%	13,03%	23,01%
5	MV [OD 450/620]	0,461	0,487	0,479	0,259	0,357	0,320	0,312
	VC (%)	5,60%	9,69%	7,49%	25,29%	26,51%	22,13%	29,03%
6	MV [OD 450/620]	0,228	0,287	0,268	0,148	0,214	0,194	0,185
	VC (%)	6,96%	9,71%	6,67%	26,68%	25,63%	20,87%	30,12%

13.5. Interfering substances

The following list of substances showed no effects on the test results when they were blended into the supernatants of positive and negative stool samples in the described concentrations:
barium sulfate (X-ray contrast medium, 18.5 % w/w), loperamide (antidiarrheal drug; 0.02 % w/w), Pepto-Bismol (antidiarrheal drug, 6.6 % v/w), sodium cyclamate (artificial sweetener, 1.3 % v/w), human blood (5.0 % v/w), stearic acid / palmitinic acid (fats in stool, mixture 1:1, 40.0 % w/w), diclofenac (analgesic drug, 0.1 % v/w).
In the case of metronidazole (0.5) (antibiotic, 3.0 % v/w) and mucins (5.0 % w/w), possible dose effect relationships were studied.
Investigation of serial dilution with metronidazole, however, did not show a relationship between the concentration and the OD values. The only exception is the highest concentration that was tested, but it is even higher than the "worst case" concentration that was tested already in the first analysis (three times the daily dose). Interference due to metronidazole can therefore be considered to be improbable.
Tests with mucins confirmed the first analysis, because OD values that were consistently far too low and altered assessments of the samples appeared repeatedly in all serial dilutions. Mucins are therefore seen to be interfering substances.

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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