

# RIDASCREEN<sup>®</sup> Cryptosporidium/Giardia Combi

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R-Biopharm AG, An der neuen Bergstraße 17, D-64297 Darmstadt, Germany

Phone: +49 (0) 61 51 81 02-0 / Fax: +49 (0) 61 51 81 02-20



## 1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Cryptosporidium/Giardia Combi is an enzyme immunoassay for the qualitative identification of *Cryptosporidium parvum*, *Cryptosporidium hominis*, and/or *Giardia lamblia* in human stool samples.

## 2. Summary and explanation of the test

*Giardia lamblia* are flagellate parasites that cause infections in the small intestines of humans after the oral uptake of giardia cysts. Because it is found worldwide, the *Giardia lamblia* pathogen is a significant cause of chronic diarrhea, in particular in medical cases related to travel. The infection occurs after ingestion of the cysts in contaminated food or water. In the USA, the *Giardia lamblia* pathogen is the most frequent cause of illness with diarrhea in connection with the intake of water. In underdeveloped countries, giardia infection is one of the most frequent causes of disease in children under the age of ten years, with a prevalence of 15–20%. Giardiasis (lambliasis) occurs as acute or chronic diarrhea, whereby asymptomatic persons can also excrete the cysts of the parasite. The incubation period ranges from 3 to 42 days.

The acute infection presents the following symptoms: sudden occurrence of watery diarrhea (frequent stools of yellow color, foamy consistence, and unpleasant smell, along with flatulence), loss of appetite, nausea, lethargy, and weight loss.

In the past, the method used most often to diagnose lambliasis was to examine stool samples under the microscope to detect the cysts, and this requires the availability of experienced staff. It is also necessary to conduct these tests in a sequence of multiple stool samples throughout a lengthy period of time, because the frequency of cyst excretion can vary largely.

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 incompetent 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 bioplates, which required the availability of experienced personnel.

An important alternative to microscopy is available in the here described Cryptosporidium/Giardia Combi ELISA, which allows simultaneous examination for both of these pathogens in stool samples. 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/Giardia Combi Test employs specific monoclonal antibodies in a sandwich-type method. The well surface of the microwell plate is coated with specific antibodies to the antigens of both the cryptosporidium and the giardia pathogen species. 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 and anti-giardia 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 and/or giardia antigens in a specimen, immobilized antibodies, the cryptosporidium and/or the giardia 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 and/or giardia 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 monoclonal antibodies to the antigens of <i>Cryptosporidium parvum</i> , <i>Cryptosporidium hominis</i> and <i>Giardia lamblia</i>
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	Inactivated cryptosporidium and giardia antigen; ready for use
Control   -	2 ml	Negative control (sample dilution buffer); ready for use
Conjugate   1	13 ml	Biotin-conjugated antibodies to cryptosporidium and giardia antigens in stabilized protein solution; ready for use; red color
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 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 returned to the aluminum bag and immediately 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 0.5% hypochlorite solution

## 7. Precaution for users

For *in vitro* diagnostic use 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](http://www.r-biopharm.com).

The kit includes a positive control that contains the inactivated cryptosporidium and giardia antigen. It must be treated as potentially infectious material and handled in accordance with the national safety regulations, just like the patient samples.

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 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® Cryptosporidium/Giardia Combi 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 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 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 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 bulge and add this to buffer in the test tube to make a suspension.

To suspend a solid stool sample, use an equivalent amount (approx. 50–100 mg) of the sample, handling it 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 that 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. 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 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 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 streptavidin poly-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 reagents are stable and that the test is conducted correctly. 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® Cryptosporidium/Giardia Combi Test can determine antigens of *Cryptosporidium parvum*, *Cryptosporidium hominis*, and/or *Giardia lamblia*. 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 possibilities of cryptosporidiosis or giardiasis. Such a result may be due to intermittent excretion of the pathogens, or the amount of antigen in the sample may be too small. If the patient history supports a suspicion of infection with either of the two pathogens, the examination should be repeated with another stool sample.

A marginal result may be due to non-homogeneous distribution of the antigens 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. Clinical comparison study

A clinical investigation in Great Britain evaluated the RIDASCREEN® Cryptosporidium/Giardia Combi Test with a total of 240 stool samples (prospective and blinded retrospective studies). There was a comparison with established British methods of microscopy for giardia (iodine stain in saline emulsion) and cryptosporidia (auramine-phenol and Ziehl-Neelsen stain) as well as a differentiating real-time PCR. The results of that study are summarized in Table 1.

Table 1: Comparison of the RIDASCREEN® Cryptosporidium/Giardia Combi ELISA with microscopy and real-time PCR

		Microscopy		real-time PCR	
		Positive	Negative	Positive	Negative
RIDASCREEN® Cryptosporidium / Giardia	Positive	25	24	47	2
	Negative	1	190	2	189

CI: Confidence interval

Sensitivity (CI):	96 % (80-100)	96 % (86-100)
Specificity (CI):	89 % (84-93)	99 % (96-100)

### 13.2 Analytical sensitivity

To determine the analytic sensitivity of the RIDASCREEN® Cryptosporidium/Giardia Combi ELISA, a linear dilution series from a sample with a known quantity of giardia cysts was

produced and then measured in triplicates. The limit of detection (LoD) is the last concentration to be evaluated as positive in all replicates. The results of those measurements are shown in Table 2.

Table 2: Analytical sensitivity results for RIDASCREEN® Cryptosporidium/Giardia Combi ELISA

<b>Cryptosporidium cysts/ Reaction</b>	<b>MV [OD 450/620]</b>	<b>Results</b>
5 x 10 <sup>5</sup>	1.728	Positive
2.5 x 10 <sup>5</sup>	0.740	Positive
1.25 x 10 <sup>5</sup>	0.449	Positive
6.25 x 10 <sup>4</sup>	0.216	Positive
3.12 x 10 <sup>4</sup>	0.104	Negative
1.56 x 10 <sup>4</sup>	0.053	Negative
7812	0.020	Negative
3906	0.016	Negative
<b>Giardia cysts/ Reaction</b>	<b>MV [OD 450/620]</b>	<b>Results</b>
1.25 x 10 <sup>5</sup>	1.630	Positive
6.25 x 10 <sup>4</sup>	0.803	Positive
3.12 x 10 <sup>4</sup>	0.440	Positive
1.56 x 10 <sup>4</sup>	0.191	Positive
7812	0.084	Negative
3906	0.049	Negative
1953	0.012	Negative

### 13.3. Cross reactivity

A variety of pathogenic microorganisms from the intestinal tract were examined with the RIDASCREEN® Cryptosporidium/Giardia Combi 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<sup>6</sup> to 10<sup>9</sup> organisms per ml. The results of that study are summarized in Table 3.

Table 3: Cross reactivity with pathogenic microorganisms

<b>Organism</b>	<b>Origin</b>	<b>Mean value [OD 450/620]</b>
<i>Adenovirus</i>	Cell culture supernatant	0.017
<i>Aeromonas hydrophila</i>	Culture	0.040
<i>Arcobacter butzleri</i>	Culture	0.030
<i>Astrovirus</i>	Cell culture supernatant	0.002
<i>Bacillus cereus</i>	Culture	0.011

<i>Bacteroides fragilis</i>	Culture	0.013
<i>Campylobacter coli</i>	Culture	0.231
<i>Campylobacter jejuni</i>	Culture	0.017
<i>Candida albicans</i>	Culture	0.014
<i>Citrobacter freundii</i>	Culture	0.018
<i>Clostridium difficile</i>	Culture	0.011
<i>Clostridium perfringens</i>	Culture	0.014
<i>Clostridium sordellii</i>	Culture	0.017
<i>E. coli</i> EPEC	Culture	0.015
<i>E. coli</i> ETEC	Culture	0.020
<i>E. coli</i> STEC	Culture	0.019
<i>Entamoeba histolytica</i>	Culture	0.004
<i>Enterobacter cloacae</i>	Culture	0.012
<i>Enterococcus faecalis</i>	Culture	0.013
<i>Klebsiella oxytoca</i>	Culture	0.017
<i>Proteus vulgaris</i>	Culture	0.017
<i>Pseudomonas aeruginosa</i>	Culture	0.016
<i>Rotavirus</i>	Cell culture supernatant	0.058
<i>Salmonella enteritidis</i>	Culture	0.016
<i>Salmonella typhimurium</i>	Culture	0.018
<i>Serratia liquefaciens</i>	Culture	0.015
<i>Shigella flexneri</i>	Culture	0.011
<i>Staphylococcus aureus</i>	Culture	0.035
<i>Staphylococcus epidermidis</i>	Culture	0.016
<i>Vibrio parahaemolyticus</i>	Culture	0.017
<i>Yersinia enterocolitica</i>	Culture	0.012

#### 13.4. Precision

To determine the intra-assay reproducibility, 40 replicates of these 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/Giardia Combi 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]	1.948	2.393	2.142	1.881	2.186	1.909	1.992
	VC (%)	8.42%	7.87%	6.74%	15.26%	8.79%	12.44%	14.58%

2	MV [OD 450/620]	1.653	2.033	1.584	1.543	1.778	1.551	1.624
	VC (%)	6.28%	6.65%	10.78%	15.94%	11.13%	13.04%	15.42%
3	MV [OD 450/620]	1.045	1.241	1.264	0.970	1.119	1.120	1.070
	VC (%)	7.08%	7.13%	6.99%	23.06%	16.75%	15.26%	19.58%
4	MV [OD 450/620]	0.839	0.918	0.863	0.721	0.848	0.768	0.779
	VC (%)	3.73%	6.03%	5.64%	22.42%	15.44%	15.77%	19.26%
5	MV [OD 450/620]	0.552	0.573	0.601	0.476	0.564	0.512	0.517
	VC (%)	10.42%	8.43%	4.55%	25.53%	17.20%	14.97%	20.72%
6	MV [OD 450/620]	0.285	0.348	0.298	0.302	0.347	0.302	0.317
	VC (%)	13.26%	6.97%	5.02%	23.43%	16.60%	22.27%	21.73%

### 13.5 Interfering substances

The following list of substances showed no effects on the test results when they were blended into cryptosporidium and giardia 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), cyclamate (artificial sweetener, 1.3% v/w), metronidazole 0.5 (antibiotic drug, 3.0% v/w) stearic acid / palmitinic acid (fats in stool, mixture 1:1, 40.0% w/w), diclofenac (analgesic drug, 0.1% v/w).

For human blood (5.0% v/w) and mucins (5.0% w/w), possible dose effect relationships were examined as described below.

Investigation of a serial dilution series with human blood did not show a direct relationship between the concentration and the OD values. The only exception is the highest concentration that was tested, and that was far higher than the normal physiological concentration. Interference due to human blood can therefore be considered to be improbable.

Tests with mucins confirmed the first analysis, with distinctly and consistently decreased OD values determined repeatedly in all serial dilutions of the cryptosporidium positive samples. That was not seen in the giardia positive stool samples. All the same, mucins must be considered as 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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