



CoproELISA

Giardia/Cryptosporidium

Enzyme-Linked Immunosorbent Assay (ELISA)
for the detection of *Giardia lamblia* and
Cryptosporidium spp. antigens in human feces

Instruction Manual

Test kit for 96 determinations
Catalog Number: 744-01

For *In Vitro* Diagnostic Use
Store at 2-8°C. **Do Not Freeze**



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Intended Use

Savyon's CoproELISA™ *Giardia/Cryptosporidium* test is an Enzyme-Linked Immunosorbent Assay (ELISA) for detection of *Giardia lamblia* (*G. lamblia*) and *Cryptosporidium* antigens in human fecal specimens collected from patients with gastrointestinal symptoms. The test can be used for fecal specimens submitted for routine clinical testing from adults or children.

For *In-Vitro* Diagnostic Use

Introduction

Giardia lamblia (*G. lamblia*) is one of the most common intestinal parasites in the world, with an estimated 3 million infections per year in humans, contributing to diarrhea and nutritional deficiencies in children in developing regions (1).

G. lamblia is a flagellated protozoan parasite that colonizes and reproduces in the small intestine, causing Giardiasis.

Giardiasis does not spread via the bloodstream, nor does it spread to other parts of the gastro-intestinal tract, but remains confined to the lumen of the small intestine (2).

Giardia trophozoites absorb their nutrients from the lumen of the small intestine, and are anaerobes. *Giardia* infection can occur through ingestion of dormant cysts in contaminated water, food, or by the faecal-oral route. In humans, infection is symptomatic only about 50% of the cases. Symptoms of infection include (in order of frequency) diarrhea, malaise, excessive gas, steatorrhea, epigastric pain, bloating, nausea, diminished interest in food, possible (but rare) vomiting which is often violent, and weight loss. People with recurring *Giardia* infections, particularly those with a lack of IgA, may develop chronic disease (3).

Giardia infection in humans is frequently misdiagnosed. Accurate diagnosis requires an antigen test or, if unavailable, an ova and parasite (O&P) examination of stool. Multiple stool examinations are recommended since the cysts and trophozoites are not shed consistently. *Giardia* infection is conventionally treated with metronidazole, tinidazole or nitazoxanide (4).

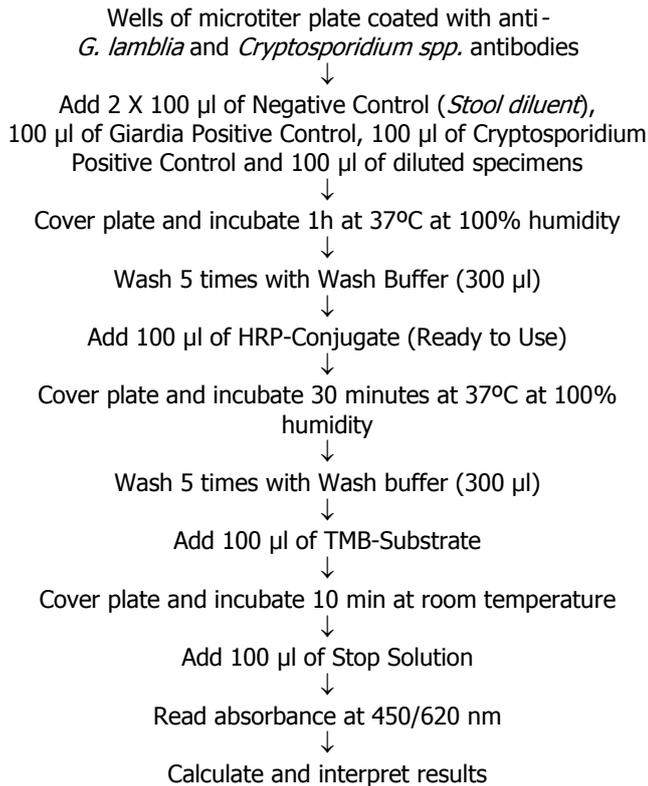
Cryptosporidiosis is a self-limited diarrheal disease that occurs in the community setting but can be chronic and potentially serious in immunocompromised patients (5). Cryptosporidiosis is caused by gastrointestinal infection with the protozoan parasite *Cryptosporidium spp.* Symptoms of cryptosporidiosis include watery diarrhea, stomach cramps, weight loss, nausea, and fever (6). This highly pathogenic parasite is transmitted in contaminated water and by the faecal-oral route. Prevalence rates of Cryptosporidiosis in symptomatic population at developed countries exceed 2-3% (5) and serological surveys indicate that the vast majority in the US has been exposed to this pathogen. In addition, this opportunistic pathogen is also highly prevalent in immunocompromised patients (e.g., 10-40% in HIV patients (7)).

Diagnosis of cryptosporidiosis is routinely performed by microscopic analysis of stool samples using organic dyes such as Ziehl-Neelsen stain or fast acid stain, or by immunostaining by direct fluorescent antibody [DFA] (8). Because detection of *Cryptosporidium* can be difficult, patients may be asked to submit several stool samples over several days. Several ELISA tests are also available for specific detection of oocyst antigens. DNA amplification techniques such as PCR or RT-PCR have been also reported, however, such tests are not commercially available yet. Nitazoxanide has been FDA-approved for treatment of diarrhea caused by *Cryptosporidium* in Immunocompetent patients (8).

Principle of the Test

- Plates are coated with specific antibodies directed against *G. lamblia* and *Cryptosporidium spp.* antigens.
- Fecal sample to be tested is diluted in stool diluent and incubated with the pre-coated plate. In this step *G. lamblia* and *Cryptosporidium spp.* antigens are bound to the immobilized antibodies.
- Non-specific antigens are removed by washing.
- Anti-*G. lamblia* and *Cryptosporidium spp.* monoclonal antibodies conjugated to horseradish peroxidase (HRP) are added and incubated. In this step the HRP-conjugate mixture is bound to the pre-bound antigen-antibody complex.
- Unbound conjugate is removed by washing.
- Upon the addition of TMB-substrate, the substrate is hydrolyzed by the peroxidase, yielding a blue solution of the reduced substrate.
- Upon the addition of the stop solution, the blue color turns yellow and should be read by an ELISA reader at a wavelength of 450/620 nm.
- Absorbance values are interpreted (see below). A positive result indicates the presence of *G. lamblia* and/or *Cryptosporidium spp.* antigen in the sample.

Summary of Procedure Manual/Automation*



*Automation procedure:

Sample incubation: 50 minutes at 35°C.

Conjugate incubation: 50 minutes at 35°C.

TMB incubation: 7 minutes at Room Temp.

Kit contents for Manual/Automation use

Test Kit for 96 determinations:

- Microtiter plate coated with anti – *G. lamblia* and *Cryptosporidium spp.* antibodies:**
96 break-apart wells (8x12) coated with *anti-G. lamblia* and *Cryptosporidium spp.* antibodies, packed in an aluminum pouch containing a desiccant card.
1 plate/1 plate
- Concentrated Wash Buffer (20x):** A PBS -Tween buffer.
1 bottle, 100 ml
- Stool Diluent (Blue):** A ready-to-use buffer solution. Contains less than 0.05% Proclin as preservative. The Diluent is also to be used as the negative control solution (see TEST PROCEDURE)
2 bottles, 50 ml
- HRP-Conjugate (Green):** A ready-to-use solution containing Horseradish peroxidase (HRP) conjugated to anti-*G. lamblia* and anti-*Cryptosporidium spp.* monoclonal antibodies.
1 bottle, 16ml
- Giardia Positive Control:** A ready to use solution containing *G. lamblia* antigen.
1 vial, 2.5 ml
- Cryptosporidium Positive Control:** A ready to use solution containing *Cryptosporidium spp.* antigen.
1 vial, 2.5 ml

- TMB-Substrate:** A ready to use solution contains 3,3',5,5' tetramethylbenzidine as a chromogen and peroxide as a substrate.
1 bottle, 16 ml
 - Stop Solution:** A ready to use solution. Contains 1M H₂SO₄.
1 bottle, 16 ml
 - Disposable pipettes:**
100 pc/none
 - Plate cover:**
1 unit/none
-

Materials Required But Not Supplied:

- Clean test tubes for dilution of patients' stool.
 - Adjustable micropipettes, or multichannel pipettes (50-200 and 200-1000µl ranges) and disposable tips.
 - Disposable plastic/wooden collectors or teaspoons.
 - One-liter volumetric flask.
 - One 50 ml volumetric cylinder.
 - Wash bottle.
 - Absorbent paper.
 - Vortex mixer.
 - A 37°C water bath with a lid, or a moisture chamber placed in a 37°C incubator.
 - ELISA-reader equipped with 450/620 nm filters.
 - Distilled or double de-ionized water.
 - For Automation use:** A centrifuge equipped with a rotor compatible with sample tubes to be used in the automation machine.
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Warnings and Precautions

- This kit's Controls contain *Cryptosporidium spp.* or *Giardia* antigens, which have been inactivated to avoid transmission of infection. Nevertheless, all the Controls' components supplied in this kit must be handled as potentially infectious agents, according to the recommendations published in the CDC/NIH manual "Biosafety in Micro Biological and Biomedical Laboratories, 1988".
- Reagents should be brought to room temperature before use.
- When handling assay wells, avoid scratching the bottom of the wells because this may result in elevated absorbance readings.
- Stool samples, microassay wells, micropipette tips and disposable stool collectors and tubes should be handled and disposed of as potentially biohazards after use. Wear gloves when doing the test.
- Unused microassay wells must be replaced in the re-sealable pouch with the desiccant to protect them from moisture.**
- TMB-Substrate solution is an irritant material to skin and mucous membranes. Avoid direct contact.
- Diluted sulfuric acid (1M H₂SO₄) is an irritant agent for the eyes and skin. In case of contact with eyes, immediately flush area with water and consult a physician).

Storage and Shelf-Life of Reagents

- The expiration date of the kit is given on the label. Expiration dates for each component are listed on individual labels. The kit should be stored between 2° and 8°C and should be returned to the refrigerator as soon as possible after use. Exposure of originally stoppered or sealed components to ambient temperature for a few hours will not cause damage to the reagents. **DO NOT FREEZE!**

- Unused strips must be resealed in the aluminum pouch with the desiccant card, by rolling the open end and sealing tightly with tape over the entire length of the opening.

Stool Collection

- Standard collection and handling procedures used in-house for fecal specimens for culture are appropriate.
- Preserved stool:** The test is compatible with specimens that were fixed in 10% formalin or in Sodium Acetate Formalin (SAF). Preserved samples can be stored at room temperature for up to 24 months. *The test is not compatible with stool specimens fixed in Polyvinyl Alcohol (PVA).*
- Unpreserved specimens:** Unpreserved specimens should be stored between 2° and 8°C and tested within 48 hours after collection. If testing cannot be performed within 48 hours store samples at -20°C, or lower.
- Freezing and thawing of the specimen, especially multiple times, may result in loss of activity due to degradation or proteolysis of the antigens.

Test Procedure for manual use

A. Preparation of Reagents

- Bring all components and clinical specimens to be tested to room temperature. Determine the total number of specimens to be tested. In addition to the specimens, the following must be included in each test: one well of Negative Control (Use Stool Diluent for this purpose), one well of Giardia Positive Control and one well of Cryptosporidium Positive Control.
- Withdraw the microtiter plate from its aluminum pouch by cutting one end near the seal. Leave the required number of strips (according to the number of specimens to be tested) in the 96 well frame.
- Dilute the Concentrated Wash Buffer 1/20 with double-deionized or distilled water. For example, in order to prepare one liter of Wash Buffer, add 50 ml of the Concentrated Wash Buffer to 950 ml of double-deionized or distilled water.

B. Sample Processing

- Set up one dilution tube for each specimen to be tested. 1.5 mL Eppendorf tubes are recommended for this purpose. Add 400 µL Stool Diluent to each tube. Label the tube.
- Thoroughly mix (vortex) the fecal specimen to ensure adequate sampling.**
- Formed samples:** Use a wooden collector or a disposable teaspoon to transfer the fecal specimen to the tube. Transfer approximately 0.1 to 0.15 g of specimen (about the size of a small pea) to the stool Diluent. Mix the collector in the *Stool Diluent* to remove as much sample as possible and squeeze the collector against the side of the tube to express any residual liquid.
Liquid samples: transfer 150 µL of specimen to the tube. Make sure the liquid specimens are evenly suspended (vortexed).
- Let the tube stand for at least 10 minutes but not more than 30 minutes until large particulate matter is precipitated (decantation). Use upper liquid phase for testing. **DO NOT USE CENTRIFUGE FOR THIS PURPOSE**

C. Incubation of stool samples and controls

- Pipette 100 µl of Giardia Positive control, 100 µl of Cryptosporidium Positive control and 2X100µl (duplicate) of Negative Control (i.e., Stool Diluent) into separate wells of the test strip.
- Dispense 100 µl of diluted stool samples into separate wells of the test strip using the provided disposable pipettes (the lowest mark on the pipette).
- Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
- Washing step:** Discard the liquid content of the wells. Fill each well with Wash Buffer up to the end of the well (300 µl). Repeat this step 4 times to a total of **FIVE** times. Automatic washing machine can be used.
- Dry the strips and frame by gently tapping them over clean absorbent paper.

D. Incubation with Conjugate

- Dispense 100µl of ready-to-use conjugate into each well.
- Cover the strips with a plate cover and incubate for 30 minutes at 37°C in a moisture chamber.
- Discard the liquid content and wash **FIVE** times as described in steps 7-8.

E. Incubation with TMB Substrate

- Dispense 100 µl of TMB-Substrate into each well, cover the strips with a plate cover and incubate at room temperature for **10 minutes**.
- Stop the reaction by adding 100µl of Stop Solution (1M H₂SO₄) into each well.

F. Determination of Results

- Determine the absorbance at 450/620 nm and record the results. Determination should not exceed 10 minutes following stopping of the chromogenic reaction.

Note: Any air bubbles should be removed before reading. The bottom of the ELISA plate should be carefully wiped

Test Procedure for automation use

A. Preparation of Reagents

- Bring all components and clinical specimens to be tested to room temperature. Determine the total number of specimens to be tested. In addition to the specimens, the following must be included in each test: one well of Negative Control (Use Stool Diluent for this purpose), one well of Giardia Positive Control and one well of Cryptosporidium Positive Control.
- Withdraw the microtiter plate from its aluminum pouch by cutting one end near the seal. Leave the required number of strips (according to the number of specimens to be tested) in the 96 well frame.
- Dilute the Concentrated Wash Buffer 1/20 with double-deionized or distilled water. For example, in order to prepare one liter of Wash Buffer, add 50 ml of the Concentrated Wash Buffer to 950 ml of double-deionized or distilled water.

B. Sample Processing

- Set up one sample's dilution tube for each specimen to be tested (use sample's tubes compatible with the available automation equipment). Add 800 µL Stool Diluent to each sample's tube. Label the tube.
- Thoroughly mix (vortex) the fecal specimen to ensure adequate sampling.**

- Formed samples:** Use a wooden collector or a disposable teaspoon to add the fecal specimen to the sample's tube. Transfer approximately 0.2 to 0.3 g of specimen (about the size of 2 small peas) to the sample's tube. Mix the collector in the *Stool Diluent* to remove as much sample as possible and squeeze the collector against the side of the tube to extract any residual liquid.

Liquid samples: transfer 400 µL of specimen to the tube. Make sure the liquid specimens are evenly suspended (vortexed).

- Let the tube stand for at least 10 minutes. Centrifuge the tubes at 1000 g for 30 sec. Ensure that the formed supernatant does not contain large particulate material.
- Transfer the sample's tubes to the corresponding rack at the automation machine.

C. Incubation of stool samples and controls

- Pipette 100 µl of Giardia Positive control, 100 µl of Cryptosporidium Positive control and 2X100µl (duplicate) of Negative Control (i.e., Stool Diluent) into separate wells of the test strip.
- Dispense 100 µl of diluted stool samples into separate wells of the test strip.
- Incubate for 50 minutes at 35°C±1°C.
- Eliminate assay drift caused by this operation.*
- Washing step:** Perform 5 X 500µl wash cycles using Savyon's Wash Buffer.
- "Perform 2 aspirate cycles no aspirate sweep. Partial plate mode: maintain full plate time"*

D. Incubation with Conjugate

- Dispense 100µl of Ready-to-Use HRP-conjugate into each well.
- Incubate for 50 minutes at 35°C±1°C.
- Wash as described in steps 13-14.

E. Incubation with TMB Substrate

- Dispense 100µl TMB-Substrate into each well and incubate at room temperature (22-28°C) for **7 minutes in the dark**. Time incubation is taken starting from dispensing the first vial, as indicated in the automation program: *"Time incubation from start of previous assay step"*
- Stop the reaction by adding 100µl of Stop Solution (1M H₂SO₄) to each well.

F. Determination of Results

- Determine the absorbance at 450/620 nm and record the results.

Please note that each automation machine has specific technical commands. Please implement Savyon's automation procedure for this kit on the operation protocol of your automation equipment.

Test Validation

For the test to be valid the following criteria must be met. If these criteria are not met the test should be considered invalid and should be repeated.

- Positive Control:** The absorbance value should be ≥ 1.0 at 450/620 nm.
- Negative Control:** The absorbance value should be ≤ 0.25 at 450/620 nm.

Determination of Cut-Off Value

The average absorbance value of the Negative Control run in duplicate should be calculated.

The cut-off value (COV) for fresh, frozen or preserved in formalin/SAF solution samples is determined according to the following formula:

$$\text{COV} = \text{OD Average of Negative control}_{450/620} + 0.25$$

Interpretation of Results

Absorbance (450/620nm)	Results
O.D < COV	Negative: no detectable <i>G. lamblia</i> or <i>Cryptosporidium spp.</i> antigen
O.D ≥ COV	Positive: relevant levels of <i>G. lamblia</i> and/or <i>Cryptosporidium spp.</i> antigen

Test Limitations

- The test is not compatible with stool specimens fixed in Polyvinyl Alcohol (PVA).
- Stool preservation in formalin/SAF solution (as performed at the physician's office) should yield a mixture containing up to 1:5 ratio (w:v) of stool in preservative solution.
- Positive result does not exclude the presence of other etiologies. It is therefore advised to take into account all clinical and laboratory data before making final diagnosis and decide upon appropriate patient management.

Performance Characteristics of the Test

In a study performed at a reference laboratory in the US, a total of 173 formalin, SAF, or total fixed stool samples were tested by CoproELISA™ Giardia/Cryptosporidium test. The presence of gastrointestinal parasites in these specimens was pre-determined by microscopic examination. The results of this evaluation are shown in Table 1:

CoproELISA™ Giardia/Cryptosporidium	Competitor ELISA	
	Positive	Negative
Positive	100	0
Negative	8	65

Sensitivity: 92.6% Specificity: 100%
 PPV: 100% NPV: 89%
 Agreement: 95%

Cross Reactivity and Interference by Mixed infections

The CoproELISA™ Giardia/Cryptosporidium test was evaluated using stool specimens defined as positive for a various gastrointestinal pathogens. No cross-reactivity or interference by mixed infection with any of the pathogens listed below:

E. histolytica, E. dispar, E. hartmanii, Blastocystis spp., D. fragilis, E. coli, E. nana, I. butschlii, Ascaris, Hookworm, T. trichiura, C. cayetanensis, Taenia spp. eggs, E. vermicularis eggs, S. stercoralis larvae, E. hartmanii D. latum. Also, no interference by white blood cells was observed.

Precision

Table 2: Intra-assay (within-run) precision of the CoproELISA™ Giardia/Cryptosporidium test is shown below:

Sample	No. of Replicates (G/C)*	Mean Value (G/C)*	CV% (G/C)*
Positive	8/8	1.53/1.1	5.7/5.1
Negative	8/8	0.06/0.06	12/10

*G: Giardia C: Cryptosporidium

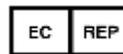
Table 3: Inter-assay (between-run) precision of the CoproELISA™ Giardia/Cryptosporidium test is shown below:

Sample	No. of Replicates (G/C)*	Mean Value (G/C)*	CV% (G/C)*
Positive	8/8	1.33/1.13	4.5/7.3
Negative	8/8	0.1/0.09	14/12

*G: Giardia C: Cryptosporidium

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	Temperature Limitation
	Consult instructions for use
	In Vitro Diagnostic Medical Device
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