CoproELISA™ *Cryptosporidium*

Enzyme-Linked Immunosorbent Assay (ELISA)
For the detection of *Cryptosporidium spp.* Antigens in human feces

Instruction Manual

Test kit for 96 determinations
Catalog Number: 734-01

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

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Intended Use

Savyon's CoproELISA™ *Cryptosporidium* test is an Enzyme-Linked Immunosorbent Assay (ELISA) for detection of *Cryptosporidium spp.* 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

Cryptosporidiosis is a self-limited diarrheal disease that occurs in the community setting but can be chronic and potentially serious in immunocompromised patients (1). 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 (2). 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% (1) 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 immuno-compromised patients (e.g., 10-40% in HIV patients (3)).

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 immuno-staining by direct fluorescent antibody [DFA] (4). 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 (4).

Principle of the Test

- Plates are coated with specific polyclonal antibodies directed against *Cryptosporidium spp.* antigens.
- Fecal sample to be tested is diluted in stool diluent and incubated with the pre-coated plate. In this step *Cryptosporidium spp.* antigens are bound to the immobilized antibodies.
- Non-specific antigens are removed by washing.
- Anti-*Cryptosporidium* monoclonal antibody conjugated to horseradish peroxidase (HRP) is added and incubated. In this step the HRP-conjugate 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.
- The absorbance is proportional to the number of *Cryptosporidium spp.* cells present in the sample.

Summary of Procedure Manual/Automation*

Wells of microtiter plate coated with anti-*Cryptospridium spp.* antibodies
↓
Add 2 X 100 µl of Negative Control (*Stool diluent*), 100 µl of Positive Control and 100 µl of diluted specimens
↓
Cover plate and incubate 1h at 37ºC at 100% humidity
↓
Wash 5 times with Wash Buffer (300 µl)
↓
Add 100 µl of HRP-Conjugate (Ready to Use)
↓
Cover plate and incubate 1 h at 37ºC at 100% humidity
↓
Wash 5 times with Wash buffer (300 µl)
↓
Add 100 µl of TMB-Substrate
↓
Cover plate and incubate 15 min at room temperature
↓
Add 100 µl of Stop Solution
↓
Read absorbance at 450/620 nm
↓
Calculate and interpret results

***Automation Procedure:**

- 50 minutes sample incubation
- Wash cycles volume: 500 µl /well
- 10 minutes substrate incubation

Kit contents for Manual/Automation use

Test Kit for 96 determinations:

1. **Microtiter plate coated with anti– *Cryptosporidium spp.* polyclonal antibodies:** 96 break-apart wells (8x12) coated with *anti-Cryptosporidium spp.* polyclonal antibodies, packed in an aluminum pouch containing a desiccant card.
   1 plate/1 plate
2. **Concentrated Wash Buffer (20x):** A PBS -Tween buffer.
   1 bottle, 100 ml/1 bottle, 100 ml
3. **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)
   1 bottle, 60 ml/2 bottles, 50 ml
4. **HRP-Conjugate (Green):** A ready-to-use solution containing Horseradish peroxidase (HRP) conjugated anti-*Cryptosporidium spp.* monoclonal antibody. Contains less than 0.05% Proclin as preservative.
   1 bottle, 12 ml/1 bottle, 16ml
5. **Positive Control:** A ready to use solution containing *Cryptosporidium spp.* antigen. Contains less than 0.05% Proclin as preservative.
   1 vial, 2.5 ml/1 vial, 2.5 ml
6. **TMB-Substrate:** A ready to use solution contains 3,3'5,5' tetramethylbenzidine as a chromogen and peroxide as a substrate.
   1 bottle, 14 ml/1 bottle, 16 ml
7. **Stop Solution:** A ready to use solution. Contains 1M H_2SO_4.
   1 bottle, 15 ml/1 bottle, 16 ml
8. **Disposable plastic pipettes: 100 pc/none**
9. **Plate cover: 1 unit/none**
10. **Instruction Manual: 1 unit/1 unit**

Materials Required But Not Supplied:

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

Warnings and Precautions

1. This kit's Control contains *Cryptosporidium spp*. antigen, which has been inactivated to avoid transmission of infection. Nevertheless, all the Control's 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".
2. Reagents should be brought to room temperature before use.
3. When handling assay wells, avoid scratching the bottom of the wells because this may result in elevated absorbance readings.
4. 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.
5. **Unused wells must be replaced in the re-sealable pouch with the desiccant to protect them from moisture.**
6. TMB-Substrate solution is an irritant material to skin and mucous membranes. Avoid direct contact.
7. Diluted sulfuric acid (1M H_2SO_4) 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

1. 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**!
2. 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

1. Standard collection and handling procedures used in-house for fecal specimens for culture are appropriate.
2. **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)*.
3. **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.
4. 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

1. 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: two wells of Negative Control (Use Stool Diluent for this purpose) and one well of Positive Control.
2. 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.
3. 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

4. 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.
5. **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.
6. **Thoroughly mix (vortex) the fecal specimen to ensure adequate sampling.**
7. 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

8. Pipette 100 µl of Positive control and 2X100µl (duplicate) of Negative Control (i.e., Stool Diluent) into separate wells of the test strip.
9. 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).
10. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
11. **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.
12. Dry the strips and frame by gently tapping them over clean absorbent paper.

D. Incubation with Conjugate

13. Dispense 100µl of ready-to-use conjugate into each well.
14. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
15. Discard the liquid content and wash **FIVE** times as described in steps 11-12.

E. Incubation with TMB Substrate

16. Dispense 100 µl of TMB-Substrate into each well, cover the strips with a plate cover and incubate at room temperature for **15 minutes**.
17. Stop the reaction by adding 100µl of Stop Solution (1M H_2SO_4) into each well.

F. Determination of Results

18. 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

1. 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: two wells of Negative Control (Use Stool Diluent for this purpose) and one well of Positive Control
2. 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.
3. 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

4. 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.
5. **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 0.30 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 300 µL of specimen to the tube. Make sure the liquid specimens are evenly suspended.
6. **Thoroughly mix (vortex) the fecal specimen to ensure adequate sampling.**
7. 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.
8. Transfer the sample's tubes to the corresponding rack at the automation machine.

C. Incubation of stool samples and controls

9. Pipette 100 µl of Positive control and 2X100µl (duplicate) of Negative Control (i.e., Stool Diluent) into separate wells of the test strip.
10. Dispense 100 µl of diluted stool samples into separate wells of the test strip.
11. Incubate the plate at 37°C for **50** minutes.
12. Perform 5 X **500 µl** wash cycles using the pre-diluted Wash Buffer.
13. Perform 2 aspirate cycles with aspirate sweep.

D. Incubation with Conjugate

14. Dispense 100µl of ready-to-use conjugate into each well.
15. Incubate for 1h at 37°C.
16. Repeat washing cycles as described in steps 12-13.

E. Incubation with TMB Substrate

17. Dispense 100 µl of TMB-Substrate into each well. Incubate at room temperature for **10 minutes**.
18. Stop the reaction by adding 100µl of Stop Solution (1M H_2SO_4) into each well.

F. Determination of Results

19. 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.

1. **Positive Control:** The absorbance value should be ≥ 1.0 at 450/620 nm.
2. **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) is determined according to the following formula:

COV = OD Negative control$_{450/620}$ + 0.3

Interpretation of Results

Absorbance (450/620nm)	Results
O.D < COV	**Negative:** no detectable cryptosporidial antigen
O.D ≥COV	**Positive:** relevant levels of cryptosporidial antigen

Test Limitations

1. The test is not compatible with stool specimens fixed in Polyvinyl Alcohol (PVA).
2. 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.
3. 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

An independent study performed at a reference laboratory in the US, a total of 120 formalin, SAF, or total fixed stool samples were tested by CoproELISA™ *Cryptosporidium* test. The presence of gastrointestinal parasites in these specimens was analyzed by microscopic examination. The results of this evaluation are shown in Table 1:

Table 1.

	Microscopy	
CoproELISA™ *Cryptosporidium*	Positive	Negative
Positive	60	0
Negative	0	60

Sensitivity: 100% Specificity: 100%
PPV: 100% NPV: 100%

Additional study which was performed in the US, The CoproELISA™ *Cryptosporidium* test was evaluated versus a FDA-cleared commercial ELISA test. 100% agreement was found between the two kits (Data is not shown).

Cross Reactivity and Interference by Mixed infections

The CoproELISA™ *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. G. lamblia, D. fragilis, E. coli, E. nana and *I. butschlii. Ascaris, Hookworm, T. trichiura, C. cayetanensis.* Also, no interference by white blood cells was observed.

Precision

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

Sample	No. of Replicates	Mean Value	CV%
Positive	8	1.48	4.02
Negative	8	0.03	5.99

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

Sample	No. of Replicates	Mean Value	CV%
Positive	8	1.47	7.7
Negative	8	0.025	15.6

Bibliography

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EC REP

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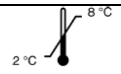	Temperature Limitation
	Consult instructions for use
IVD	In Vitro Diagnostic Medical Device
	Manufacturer
EC REP	Authorized European Representative