Cryptosporidium spp.
ELISA Kit

Catalog Number KA2094
96 assays
Version: 02

Intended for research use only
# Table of Contents

Introduction..................................................................................................................3
  Intended Use..................................................................................................................3
  Background....................................................................................................................3
  Principle of the Assay....................................................................................................3

General Information ......................................................................................................5
  Materials Supplied.........................................................................................................5
  Storage Instruction.........................................................................................................5
  Materials Required but Not Supplied............................................................................5
  Precautions for Use........................................................................................................6

Assay Protocol ................................................................................................................7
  Reagent Preparation.......................................................................................................7
  Sample Preparation........................................................................................................7
  Assay Procedure............................................................................................................8

Data Analysis ................................................................................................................10
  Calculation of Results...................................................................................................10
  Performance Characteristics.........................................................................................10

Resources.......................................................................................................................12
  References....................................................................................................................12
  Plate Layout..................................................................................................................13
Introduction

Intended Use

*Cryptosporidium* spp. ELISA Kit is an Enzyme-Linked Immunosorbent Assay (ELISA) for detection of *Cryptosporidium* spp. antigens in human fecal samples collected from individuals with gastrointestinal symptoms. The test can be used for fecal samples submitted for routine testing from adults or children.

Background

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 Assay

- 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**

Wells of microtiter plate coated with anti-*Cryptosporidium spp.* antibodies

Add 2 x 100 µL of Negative Control (Stool diluent), 100 µL of Positive Control and 100 µL of diluted samples

Cover plate and incubate 1h at 37°C at 100% humidity

Wash 5 times with Wash Buffer (300 µL)

Add 100 µL of HRP-Conjugate (Ready to Use)

Cover plate and incubate 1h at 37°C 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
General Information

Materials Supplied

List of component

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microtiter plate coated with anti-<em>Cryptosporidium</em> spp. polyclonal antibodies: 96 break-apart wells (8x12) coated with anti-<em>Cryptosporidium</em> spp. polyclonal antibodies, packed in an aluminum pouch containing a desiccant card.</td>
<td>96 (8x12) wells</td>
</tr>
<tr>
<td>Concentrated Wash Buffer (20x): A PBS - Tween buffer</td>
<td>100 mL</td>
</tr>
<tr>
<td>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 Assay Procedure)</td>
<td>50 mL x 2</td>
</tr>
<tr>
<td>HRP-Conjugate (green): A ready-to-use solution containing Horseradish peroxidase (HRP) conjugated anti-<em>Cryptosporidium</em> spp. monoclonal antibody. Contains less than 0.05% Proclin as preservative.</td>
<td>16 mL</td>
</tr>
<tr>
<td>Positive Control: A ready to use solution containing <em>Cryptosporidium</em> spp. antigen. Contains less than 0.05% Proclin as preservative.</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>TMB-Substrate: A ready to use solution contains 3,3',5,5' tetramethylbenzidine as a chromogen and peroxide as a substrate.</td>
<td>16 mL</td>
</tr>
<tr>
<td>Stop Solution: A ready to use solution. Contains 1 M H₂SO₄.</td>
<td>16 mL</td>
</tr>
<tr>
<td>Disposable plastic pipettes</td>
<td>100 pc</td>
</tr>
<tr>
<td>Plate cover</td>
<td>1 unit</td>
</tr>
</tbody>
</table>

Storage Instruction

✓ 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.

Materials Required but Not Supplied

✓ Clean test tubes for dilution of human samples.
✓ Adjustable micropipettes, or multichannel pipettes (50-200 and 200-1000 μL 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 with 450/620nm filter.
✓ Distilled or double deionized water.
✓ For Automation use: A centrifuge equipped with a rotor compatible with sample tubes to be used in the automation machine.

**Precautions for Use**

✓ Precautions
  • 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).

✓ Test Limitations
  • The test is not compatible with stool samples fixed in Polyvinyl Alcohol (PVA).
  • Stool preservation in formalin/SAF solution 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 conditions.
Assay Protocol

Reagent Preparation

1. Bring all components and samples to be tested to room temperature. Determine the total number of samples to be tested. In addition to the samples, the following must be included in each test: one well 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 samples 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.

Sample Preparation

✓ Stool Collection
1. Standard collection and handling procedures used in-house for fecal samples for culture are appropriate.
2. Preserved stool: The test is compatible with samples 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 samples fixed in Polyvinyl Alcohol (PVA).
3. Unpreserved samples: Unpreserved samples 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 sample, especially multiple times, may result in loss of activity due to degradation or proteolysis of the antigens.

✓ Sample Processing

• For Manual Use
1. Set up one dilution tube for each sample to be tested. 1.5 mL Eppendorf tubes are recommended for this purpose. Add 400 μL Stool Diluent to each tube. Label the tube.
2. Formed samples: Use a wooden collector or a disposable teaspoon to transfer the fecal sample to the tube. Transfer approximately 0.1 to 0.15 g of sample (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 sample to the tube. Make sure the liquid samples are evenly suspended.
3. Thoroughly mix (vortex) the fecal sample to ensure adequate sampling.
4. 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.
For Automation use
1. Set up one sample's dilution tube for each sample 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.
2. Formed samples: Use a wooden collector or a disposable teaspoon to add the fecal sample to the sample's tube. Transfer approximately 0.2 to 0.3 g of sample (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 sample to the tube. Make sure the liquid samples are evenly suspended.
3. Thoroughly mix (vortex) the fecal sample to ensure adequate sampling.
4. Let the tube stand or at least 10 minutes. Centrifuge the tubes at 1000 g for 30 sec. Ensure that the formed supernatant does not contain large particulate material.
5. Transfer the sample's tube to the corresponding rack at the automation machine.

Assay Procedure

✓ For Manual Use

• Incubation of stool samples and controls
1. Pipette 100 µL of Positive control and 2 x 100 µL (duplicate) of Negative Control (i.e., Stool Diluent) into separate wells of the test strip.
2. 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).
3. Cover the strips with a plate cover and incubate for 1 h at 37°C in a moisture chamber.
4. 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.
5. Dry the strips and frame by gently tapping them over clean absorbent paper.

• Incubation with Conjugate
6. Dispense 100 µL of ready-to-use conjugate into each well.
7. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
8. Discard the liquid content and wash FIVE times as described in in steps 4 and 5 in Incubation of stool samples and controls.

• Incubation with TMB Substrate
9. Dispense 100 µL of TMB-Substrate into each well, cover the strips with a plate cover, and incubate at room temperature for 15 minutes.
10. Stop the reaction by adding 100 µL of Stop Solution (1M H₂SO₄) into each well.

• Determination of Results
11. 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.*

✔ For Automation Use
- Incubation of stool samples and controls
  1. Pipette 100 µL of Positive control, and 2 x 100 µL (duplicate) of Negative Control (i.e., Stool Diluent) into separate wells of the test strip.
  2. Dispense 100 µL of diluted stool samples into separate wells of the test strip.
  3. Incubate the plate at 37°C for 50 minutes.
  4. Perform 5 X 500 µL wash cycles using the pre-diluted Wash Buffer.
  5. Perform 2 aspirate cycles with aspirate sweep.

- Incubation with Conjugate
  6. Dispense 100 µL of ready-to-use conjugate into each well.
  7. Incubate for 1h at 37°C.
  8. Repeat washing cycles as described in steps 4 and 5 in Automation Use.

- Incubation with TMB Substrate
  9. Dispense 100 µL of TMB-Substrate into each well. Incubate at room temperature for 10 minutes.
  10. Stop the reaction by adding 100 µL of Stop Solution (1 M H₂SO₄) into each well.

- Determination of Results
  11. Determine the absorbance at 450/620 nm and record the results.

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

Calculation of Results

✓ 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 ≤ 0.2 at 450/620 nm.

✓ Determination of Cut-Off Value

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

• The cut-off value (COV) is determined according to the following formula:
  \[ \text{COV} = \text{OD Negative control}_{450/620} + 0.3 \]

✓ Interpretation of Results

<table>
<thead>
<tr>
<th>Absorbance (450/620 nm)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.D &lt; COV</td>
<td>Negative: no detectable cryptosporidial antigen</td>
</tr>
<tr>
<td>O.D ≥ COV</td>
<td>Positive: relevant levels of cryptosporidial antigen</td>
</tr>
</tbody>
</table>

Performance Characteristics

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 this kit. The presence of gastrointestinal parasites in these samples was analyzed by microscopic examination. The results of this evaluation are shown in Table 1:

<table>
<thead>
<tr>
<th>Cryptosporidium spp. ELISA Kit</th>
<th>Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>60</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

Sensitivity: 100%,
Specificity: 100%
PPV: 100%
NPV: 100%
Cross Reactivity and Interference by Mixed infections

The *Cryptosporidium* spp. ELISA Kit was evaluated using stool samples 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. hartmannii, 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 *Cryptosporidium* spp. ELISA Kit is shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Replicates</th>
<th>Mean Value</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>8</td>
<td>1.48</td>
<td>4.02</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>0.03</td>
<td>5.99</td>
</tr>
</tbody>
</table>

Table 3: Inter-assay (between-run) precision of the *Cryptosporidium* spp. ELISA Kit is shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Replicates</th>
<th>Mean Value</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>8</td>
<td>1.47</td>
<td>7.7</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>0.025</td>
<td>15.6</td>
</tr>
</tbody>
</table>


Resources

References

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>