Cryptosporidium
ELISA [Enzyme-Linked ImmunoSorbent Assay]

Catalog # 7063

Qualitative assay for the determination of
Cryptosporidium antigen in human stool samples

September 2010

I. INTENDED USE

The Biomerica *Cryptosporidium* ELISA is intended for the qualitative detection of *Cryptosporidium*-specific antigen (CSA) in fecal specimens. This assay is intended for *in vitro* diagnostic use only.

II. SUMMARY AND EXPLANATION

Cryptosporidium is a protozoan parasite commonly found in animals. It is considered an important pathogenic organism in domestic farm animals – particularly in calves. Historically, *cryptosporidium* was thought to cause diarrhea in animals only, until the first case of human infection was reported in 1976. [1] Since that time, this parasite has been associated with diarrheal disease throughout the world. It is particularly prevalent in tropical developing countries and has been known to cause epidemics of diarrhea among children.[4] *Cryptosporidium* is often the cause of travelers' diarrhea.

Symptoms of cryptosporidiosis include mild to severe diarrhea, which may last from three to ten days, and possible abdominal pain, fever, nausea, vomiting and weight loss.[2,9,10,11,12,14] In immunocompetent (normal) patients, the disease is usually manifested as a self-healing gastroenteritis.[14] However, the infection in immunocompromised patients can be much more severe and may often be life threatening due to dehydration. Loss of water, from three to twelve liters per day, has been reported.[2,3,13,16]

The infection can be transmitted from animals to humans through contaminated water. The oocysts involved in transmission have been shown to be remarkably resistant to common disinfectants and routine chlorination of drinking water. The infection can also be passed human to human, between household members, schoolchildren, and members of high-risk groups such as homosexual men and those with HIV.[2,3,4,13,16]

In the past, diagnosis of *Cryptosporidium* infections was done by microscopic detection of oocysts in the stool, or the microscopic examination of intestinal biopsy samples. However, these methods can be time-consuming and rely on experienced technicians. Because of the historically low proficiency of correct microscopic examinations, alternative diagnostic methods have been investigated. [5,6,16,17]

One important alternative has been the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of CSA in stool specimens. These tests have been shown to have comparable sensitivity to experienced microscopic examinations, do not require personnel specially trained in parasitology, are fairly simple to perform and do not require the observation of intact organisms in the stool sample.[7,8]

III. PRINCIPLE OF THE TEST

The Biomerica *Cryptosporidium* test is an enzyme immunoassay which detects the presence of *Cryptosporidium*-specific antigen (CSA) in stool samples. Antibodies to CSA have been immobilized on breakaway microwells. Diluted patient specimens are added to the microwells along with horseradish peroxidase-conjugated antibodies to CSA. If CSA is present in the sample, it will bind to the detecting antibody and the immobilized antibody to form a complex, which will remain in the microwell after washing to remove unbound enzyme. After washing, a substrate/chromogen is added which develops a blue color in the presence of the enzyme complex. The stop solution ends the reaction and turns the blue color to yellow.

IV. KIT COMPONENTS

Kit Components	Description	Symbol
Test Strips	96 microtiter wells containing anti-*Cryptosporidium* antibodies in a test strip holder.	**PLA**
Enzyme Conjugate	One (1) bottle containing 11 ml of HRP- labeled anti-*Cryptosporidium* antibodies with thimerosal.	**CONJ ENZ**
Positive Control	One (1) vial containing 2 ml of a diluted *Cryptosporidium* positive stool supernatant in formalin.	**CTRL +**
Negative Control	One (1) vial containing 2 ml of buffered protein solution.	**CTRL -**
Substrate	One (1) bottle containing 11 ml of the chromogen tetramethylbenzidine (TMB) and peroxide.	**SUBS TMB**
Wash Concentrate (20X)	Two (2) bottles containing 25 ml of concentrated buffer with detergent and thimerosal.	**BUF WASH 20X**
Dilution Buffer	Four (4) bottles containing 30 ml of a buffered protein solution with thimerosal.	**SPEC DIL**
Stop Solution	One (1) bottle containing 11 ml of 5 % phosphoric acid solution.	**STOP**

MATERIAL AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- Microplate reader (Optional)
- Microplate washer [if washer is unavailable, manual washing may be acceptable].
- Micropipettes capable of delivering 50 µL and 100 µL.
- Transfer Pipettes.
- Wash Bottle.
- Absorbent Paper.
- Parafilm or cover for microwell plate
- Timer
- Reagent grade (DI) water.
- Waste container with disinfectant or biohazard bags.
- Sample Dilution Tubes.
- Applicator Stick (recommended) or swabs for sample preparation.

V. WARNINGS AND PRECAUTIONS FOR USERS

Do not deviate from the specified procedures when performing this assay. All specimen dilutions, incubation times/temperatures and washings have been optimized for the best performance. Deviations from the specified procedures may affect the sensitivity and specificity of the assay.

- All reagents are for *In Vitro* Diagnostic Use Only.
- Reagents from different kit lots should not be interchanged.
- Do not use reagents that are beyond their expiration dates.
- All reagents should be at room temperature before using.
- If using dropper bottles, hold them vertically to ensure proper drop size.
- Wear gloves when performing the test, and handle specimens and used microwells as if able to transmit infectious agents.
- Unused microwells should be stored in the resealable pouch with desiccant to protect them from moisture.
- Do not use solutions if they precipitate or become cloudy.
  Exception: Wash concentrate may precipitate during refrigerated storage, but will dissolve upon warming.
- Controls and some reagents contain thimerosal as a preservative, which may be irritating to skin, eyes and mucous membranes. In case of contact, flush eyes or rinse skin with large amounts of water.
- Treat all reagents and samples as potentially infectious materials. Use care to prevent aerosols and decontaminate any sample spills.
- Stop solution is a 5% solution of phosphoric acid in water. In case of contact with skin or mucous membranes, flush with water immediately.

VI. SAMPLE COLLECTION AND STORAGE

NOTE: All dilutions of stools must be made with the Dilution Buffer provided**.**

Standard collection techniques used for fecal specimens for culture can be employed. Stool samples may be used as unpreserved or frozen, in transport medium such as Cary-Blair, or in preservation media such as 10% formalin or Sodium Acetate Formalin (SAF). Unpreserved samples should be stored at 2-8°C and tested within 24 hours of collection. Samples that cannot be tested within this time frame should be frozen at -20°C or lower until used. Avoid multiple freeze/thaw cycles. Formalinized and SAF preserved samples may be stored at room temperature (15-25°C) or at 2-8°C and tested within 18 months of collection. **DO NOT FREEZE PRESERVED SAMPLES**. Samples in Cary-Blair should be kept at 2-8°C or -20°C and tested within 1 week of collection. Avoid multiple freeze/thaw cycles.

VII. REAGENT PREPARATION AND STORAGE

1. All reagents, with the exception of the Wash Concentrate are supplied ready-to-use. All reagents should be stored at 2-8° C. The Wash concentrate may precipitate during refrigerated storage but will dissolve upon warming.
2. Before use, bring all reagents and samples to room temperature (15-25°C) and mix.
3. Wash Concentrate: To prepare a 1X wash solution, add contents of one bottle of Wash Concentrate (25 ml) to 475 ml of distilled or deionized water and mix.

VIII. SAMPLE PREPARATION

1. Prepare sample dilutions in tubes using **700 µl** of Dilution Buffer and **0.1 g**, about the size of a small pea (~4 mm diameter), of fecal sample using an applicator stick. Mix thoroughly before using.
2. **IF USING SWABS**, add **1 ml** of dilution buffer to dilution tube. Coat the swab with a thin layer of specimen and mix into dilution buffer, expressing as much fluid as possible. Mix thoroughly before using.
3. **For watery unpreserved specimens**, mix contents then add **100 µl** of sample to **700 µl** of Dilution Buffer in dilution tubes. Mix thoroughly before using.
4. **For samples in SAF, 10% Formalin or Cary-Blair**, mix contents then add **200 µl** of sample to **300 µl** of Dilution Buffer in dilution tubes. Mix thoroughly before using.

NOTES:

- Ensure all samples and reagents are at room temperature (15-25°C) before use. Frozen samples **MUST** be thawed completely before use.
- If needed, prepared samples can be centrifuged at 2000-3000 g for 5-10 minutes. Ensure supernatant is clear before use.

IX. ASSAY PROCEDURE

NOTES:

- All incubations are at room temperature (15 - 25°C)
- When running the assay, try to avoid the formation of bubbles in the wells. Slapping the wells out on a clean absorbent towel after each wash step should help to minimize bubbles in the wells.

1. Break off number of wells needed (number of samples plus 2 for controls) and place in strip holder. Return any unused wells to foil pouch with desiccant. Reseal pouch tightly to exclude moisture.
2. Using a micropipette, add **100 µl** of negative control to well # 1.
3. Using a micropipette, add **100 µl** of positive control to well # 2.
4. Add **100 µl** of the diluted sample to the appropriate test wells.

NOTE: Place the opening of the transfer pipette just inside the well to avoid splashing into adjacent wells.

5. Cover the microwell plate with parafilm or an appropriate cover and incubate at room temperature for **60 minutes**. Begin timing after the addition of the last specimen.
6. Decant the contents of the assay wells.
7. Wash each well using the 1X wash solution in a squirt bottle with a fine-tipped nozzle, directing the wash solution to the bottom of the well with force. Fill the well, and then decant the wash solution out of the well. Repeat this step four more times for a total of five washes*.

NOTE: If using semi-automated or automated washing equipment, the specimens must be centrifuged (2000-3000g x 10 minutes) to remove any particulate matter prior to adding to plate. Add 350 µl of 1X wash solution to each well. Wash a total of five times.

8. After washing, completely remove any residue liquid in the wells by slapping the plate onto a dry paper towel until no liquid comes out.
9. Add **100 µl (or 2 drops)** of Enzyme Conjugate to each well.
10. Cover the microwell plate and incubate at room temperature for **30 minutes**.
11. Decant the contents of the wells and wash each well five times as in **Step 7-8***.
12. Add **100 µl (or 2 drops)** of Substrate Solution to each well.
13. Cover the microwell plate and incubate at room temperature for **10 minutes**. Protect plate from light. Place plate in a dark area or cover plate with aluminum foil.
14. Add **100 µl (or 2 drops)** of Stop Solution to each well. Gently tap the wells to mix for approximately **15 seconds**. Read reaction within **5 minutes** after adding stop solution.
15. Read results visually or with a spectrophotometer having a dual wavelength of 450/620-650 nm. Zero reader on air.

* ***Washings consist of vigorously filling each well to overflowing and decanting contents, banging the wells on a clean absorbent towel after each wash.***

X. CALCULATION OF RESULTS

Interpretation of Results – Visual Method

Positive: Any sample well that is obviously more yellow than the negative control well.

Negative: Any sample well that is not obviously more yellow than the negative control well.

NOTE: The negative control, as well as some samples, may show some slight color. A sample well must be obviously darker than the negative control well to be called a positive result.

Interpretation of Results - Spectrophotometric Method:

Zero spectrophotometer against air**. Read all wells at 450/620-650 nm.**

Positive: Absorbance reading of 0.08 O.D. units and above indicates the sample contains *Cryptosporidium* antigen.

Negative: Absorbance reading less than 0.08 O.D. units indicates the sample does not contain detectable levels of *Cryptosporidium* antigen.

XI. QUALITY CONTROL

Positive and negative controls must be included each time the assay is run. The use of a positive and negative control provides an easy validation of kit stability.

- Negative control should appear colorless when read visually and should read less than 0.08 O.D. when read at a dual wavelength of 450/620-650 nm.
- Positive control should be a clearly visible yellow color and read at greater than 0.5 O.D. when read at a dual wavelength of 450/620-650 nm.

XII. LIMITATION OF PROCEDURE

- Test results should be used as an aid in diagnosis and should not be interpreted as diagnostic by themselves.
- DO NOT concentrate stool samples. Assay will not give accurate results on a concentrated sample.
- Inadequate washings may lead to the negative control having excessive color development. Care should be taken to perform wash steps in the manner described.
- A negative result can occur from an antigen level lower than the detection limits of this assay. Multiple samples over time may be indicated for those patients that are suspected of being positive for *Cryptosporidium*.

XIII. PERFORMANCE CHARACTERISTICS

Clinical Evaluation

A total of 80 stool specimens were tested on the Biomerica Cryptosporidium ELISA and a Reference ELISA for comparison. The clinical profile for the Biomerica Cryptosporidium ELISA is shown below.

		Reference Crypto ELISA		
		Positive	Negative	Total
Biomerica Crypto ELISA	Positive	25	2	27
	Negative	0	53	53
	Total	25	55	80

Clinical Data	Result
Accuracy	98%
Sensitivity	100 %
Specificity	96%

Precision and Reproducibility

The precision (intra-assay variation) of the Biomerica Cryptosporidium ELISA test was calculated from 24 replicate determinations on each of the two control samples.

Intra Assay

Sample	Mean O.D	N	% CV
Control 1	1.6	24	5.4 %
Control 2	0.9	24	6.0 %

The reproducibility (inter-assay variation) of the Biomerica Cryptosporidium ELISA test was calculated from data on two control samples obtained in 12 different assays, by four technicians on two different lots of reagents.

Inter Assay

Sample	Mean O.D	N	% CV
Control 1	1.2	12	7.4 %
Control 2	0.7	12	9.9 %

Cross-Reactivity

The following bacterial strains, greater than 10^6 cell/mL, were spiked into a confirmed negative fecal specimen. No cross-reactivity to the below bacterial strains was observed in the Biomerica Cryptosporidium ELISA.

Bacterial Strain	ATCC #	OD_{450nm}-OD_{630nm}
Negative Sample - No Spike	N/A	0.003
Campylobacter coli 1114 - Spike	N/A	0.012
Campylobacter fetus - Spike	19438	0.008
Escherichia coli serotype 055:k59 (B5) - Spike	12014	0.005
Escherichia coli serotype 0111:K58 (B4) - Spike	33780	0.007
Escherichia coli serotype 0111:NM - Spike	43887	0.003
Escherichia coli serotype 0157.H7 - Spike	43890	0.007
Escherichia coli serotype 0124.NM - Spike	43893	0.007
Campylobacter jejuni - Spike	29428	0.008
Salmonella typhimurium - Spike	SA972229	0.007
Shigella sonneii - Spike	25931	0.016

Interference

The following bacterial strains, greater than 10^6 cell/mL, were spiked into a confirmed positive fecal specimen. No interference to the below bacterial strains was observed in the Biomerica Cryptosporidium ELISA, as all positive samples remained positive.

Bacterial Strain	ATCC #	OD_{450nm}-OD_{630nm}
Positive Sample - No Spike	N/A	2.062
Campylobacter coli 1114 - Spike	N/A	2.039
Campylobacter fetus - Spike	19438	1.98
Escherichia coli serotype 055:k59 (B5) - Spike	12014	1.887
Escherichia coli serotype 0111:K58 (B4) - Spike	33780	1.984
Escherichia coli serotype 0111:NM - Spike	43887	1.993
Escherichia coli serotype 0157.H7 - Spike	43890	1.778
Escherichia coli serotype 0124.NM - Spike	43893	1.936
Campylobacter jejuni - Spike	29428	2.021
Salmonella typhimurium - Spike	SA972229	1.871
Shigella sonneii - Spike	25931	1.935

XIV. REFERENCES

1. Meisel, J.L., Perera, D.R., Meligro, C., and Rubin, C.E. "Overwhelming watery diarrhea associated with *Cryptosporidium* in an immunosuppressed patient." 1976. Gasteroenterology 70: 1156-60.
2. Chapman, P.A. "Cryptosporidiosis: Recent Trends in Epidemiology, Diagnosis, and Treatment." Serodiag & Immunother Infect Dis #2, 1988, pp. 311-317.
3. Meyer, E.A. "Waterborne Giardia and Cryptosporidium." Parasit Today. Vol. 4, #7, 1988, pp. 200-201.4
4. Alpert, G., Bell, L.M., Kirkpatrick, C.E., *et. al.* "Cryptosporidiosis in a day-care centre." New Eng. J. Med. 311:860-1.
5. Stibbs, H., Ongerth, J. "Immunofluorescence Detection of Cryptosporidium Oocysts in Fecal Smears." J Clin Micro, Vol 24 #4, Oct. 1986, pp.517-521.
6. McLaughlin, J. et al. "Indentification of Cryptosporidium Oocysts by Monoclonal Antibody." Lancet, January 3, 1987, pp.51.
7. Ungar, B. "Enzyme-Linked Immunoassay for Detection of Cryptosporidium Antigens in Fecal Specimens." J Clin Micro, Vol. 28 #11, Nov 1990, pp. 2491-2495.
8. Anusz, K., et al. "Detection of Cryptosporidium parvum Oocysts in Bovine Feces by Monoclonal Antibody Capture Enzyme-Linked Immunosorbent Assay." J. Clin Micro, Vol. 28 #12, dec. 1990, pp. 2770-2774.
9. Jokipii, L., et al. "Cryptosporidium: A Frequent Finding In Patients With Gastrointestinal Symptoms." Lancet, August 13, 1983, pp. 358-360.
10. Shephard, R., et al. "Shedding of Oocysts of Cryptosporidium in Immunocompetent Patients." J Clin Pathol, Vol. 41, 1988, pp. 1104-1106.
11. Holten-Anderson, W., et al. "Prevelence of Cryptosporidium Among Patients with Acute Enteric Infection." J. Infect, Vol. 9, 1984, pp. 277-282.
12. Jokipii, L. and Jokipii, M. "Timing of Symptoms and Oocyst Excretion in Human Cryptosporidiosis." N Engl J Med, Vol. 315 #26, 1986, pp.1643-1647.
13. Egger, M., et al. "Symptoms and Transmission of Intestinal Cryptosporidiosis." Arch Dis Child, Vol 65, pp 445-447.
14. Hart, M., et al. "Acute Self-Limited Colitis Associated with Cryptosporidium in an Immunocompetent Patient." J Ped Gastro Nutr, Vol. 8, 1989, pp. 401-403.
15. Sloan, L.M., and Rosenblatt, J.E. "Evaluation of Enzyme-Linked Immunosorbent Assay for Detection of Cryptosporodium spp. in Stool Specimens." J Clin Micro, Vol. 31 #6, June 1993, pp. 1468-1471.
16. Current, W. and Garica, L. "Cryptosporidiosis." Clin Micro Rev, Vol. 4 #3, July 1991, pp. 325-358.
17. Weber, R. et al. "Threshold of Detection of Cryptosporidium Oocysts in Human Stool Specimens; Evidence for Low Sensitivity of Current Diagnostic Methods." J Clin Micro, Vol. 29 #7, July 1991, pp. 1323-1327.

XV. SYMBOLS

Symbol	Meaning
	Storage Temperature
LOT	Lot Code
	Expiration
	Manufacturer
EC REP	Authorized Representative
	Caution, see instructions
IVD	For in vitro diagnostic use
REF	Catalog No.

XVI. ORDERING INFORMATION

ORDERING: Send purchase order to:
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"Authorized Representative"
according to IVDD 98/79/ EC

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