C. difficile Toxin A+B

Cat # 8308-3

Intended Use

This ELISA is an in vitro immunoassay for the qualitative determination of C. difficile toxins A+B in feces. It is a double antibody (sandwich) ELISA using an anti-toxin A+B antibodies to capture the antigen from the stool supernatant. A second set of anti-toxin A+B antibodies are added which sandwiches the captured antigen. This reaction is visualized by the addition of an anti-second antibody conjugated to peroxidase and the chromogen tetramethylbenzidine (TMB). The resulting blue color development indicates the presence of C. difficile toxins A+B being bound by the antibodies.

Summary

Clostridium difficile may be part of the normal bacterial flora of the human intestinal tract, but can become an opportunistic pathogen when the intestinal tract has been compromised or altered, as with patients undergoing antibiotic therapy. Hall and O’Toole isolated the bacteria and described its toxigenic characteristics in 1935.1 Toxin-producing strains of c. difficile produce two toxins - toxin A, an enterotoxin, and toxin B, a cytotoxin. C. difficile was not considered an opportunistic pathogen until the late 1970’s when a correlation between the bacteria and pseudomembranous colitis (PMC) was established.2,3 PMC is an antibiotic-associated disease that progresses from diarrhea and mucosal inflammation to the formation of colonic pseudomembranes composed of fibrin, mucous, necrotic epithelial cells and leukocytes.4,5

Though up to 50% of infants are colonized by toxigenic c. difficile and exhibit high levels of toxin A and B, few develop PMC, instead remaining asymptomatic. Hypotheses for this phenomenon include colostrum’s ability to neutralize toxin A and B, a diminished sensitivity of toxin A by fetal intestinal cells, and the possible lack of toxin receptors.5 A less studied population exhibiting reduced susceptibility to PMC is cystic fibrosis patients.5 Rapid methods of isolation and identification of C. difficile or its toxin(s) are readily available. The most common clinical diagnostic procedures for C. difficile antibiotic-associated
Colitis are cell culture cytotoxicity and latex agglutination assays. The cell culture cytotoxicity assay (CTA) detects the presence of toxin B by the observation of cytopathic effect on cell culture. The assay is very sensitive (50 pg/ml toxin B) but requires a minimum of two days to complete. Latex agglutination is a common stool screening method for detection of proteins associated with C. difficile, though cross-reactivity and detection of nontoxigenic C. difficile has been reported. C. difficile EIA methods have been researched by a number of investigators, with a reported sensitivity to either toxin A or toxin B of 1-10 ng/mL.

**Principle of Procedure**

During the first incubation, C. difficile toxins A+B present in the stool supernatant are captured by antibodies attached to the wells. The second incubation adds additional anti-toxin A+B antibodies that "sandwiches" the antigen. The next incubation adds an anti-second antibody conjugated to peroxidase. After washings to remove unbound enzyme, a chromogen is added which develops a blue color in the presence of the enzyme complex and peroxide. The stop solution ends the reaction and turns the blue color to yellow.

**Reagents**

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Strips</td>
<td>Microwells containing anti-C. difficile toxin A+B polyclonal antibodies - 96 test wells in a test strip holder.</td>
<td>MT PLATE</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>One (1) bottle containing 6 ml of anti-toxin A+B polyclonal antibodies with blue dye and Thimerosal.</td>
<td>Ab</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>One (1) bottle containing 11 ml of a second antibody conjugated to peroxidase with red dye and Thimerosal.</td>
<td>CONJ</td>
</tr>
<tr>
<td>Positive Control</td>
<td>Two (2) vials containing 2 ml each of either toxin A or toxin B in a buffer.</td>
<td>CONTROL+</td>
</tr>
<tr>
<td>Negative Control</td>
<td>One (1) vial containing 2 ml of buffer.</td>
<td>CONTROL-</td>
</tr>
<tr>
<td>Chromogen</td>
<td>One (1) bottle containing 11 ml of the chromogen tetramethylbenzidine (TMB) and peroxide.</td>
<td>SUBS TMB</td>
</tr>
<tr>
<td>Dilution Buffer</td>
<td>Two (2) bottles containing 30 ml of buffered protein solution.</td>
<td>SPECM DIL</td>
</tr>
<tr>
<td>Wash Concentrate (20X)</td>
<td>One (1) bottle containing 25 ml of concentrated buffer and surfactant with Thimerosal.</td>
<td>WASH BUF</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>One (1) bottle containing 11 ml of 1 M phosphoric acid.</td>
<td>SOLN</td>
</tr>
</tbody>
</table>

**Precautions**

Do not use solutions if they precipitate or become cloudy.

Exception: Wash concentrate may precipitate during refrigerated storage, but will dissolve upon warming.

Do not add azides to the samples or any of the reagents.
Controls and some reagents contain Thimerosal as a preservative. Treat all reagents and samples as potentially infectious materials. Use care to prevent aerosols and decontaminate any spills of samples.

**Storage Conditions**

Reagents, strips and bottled components:
Store between 2 – 8 ºC.
Squeeze bottle containing diluted wash buffer may be stored at room temperature.

**Preparation**

Wash Buffer - Remove cap and add contents of one bottle of Wash Concentrate to a squeeze bottle containing 475 ml of DI water. Swirl to mix. Squeeze bottle should have a narrow tip to optimize washings.

**Collection of Stool (Feces) and Preparation**

No modification of collection techniques used for standard bacterial examinations is needed. Stool samples may be used as unpreserved or frozen. Make a 1:4 (1 part sample, about the size of a pea, and 3 parts dilution buffer) stool sample dilution using the dilution buffer provided.

Unpreserved samples should be kept at 2 – 8 ºC and tested within 24 hours of collection. Samples that cannot be tested within this time should be frozen at -20 ºC or lower until used. Freezing does not adversely affect the test.

All dilutions of samples must be made with the dilution buffer provided.

**Procedure**

**Materials Provided**

*C. difficile* Toxin A+B Stool Antigen Microwell ELISA Kit

**Materials Required But Not Provided**

Transfer Pipettes
Squeeze bottle for washing strips (narrow tip is recommended)
Graduated Cylinder
Reagent grade (DI) water

**Suggested Equipment**

ELISA plate reader with 450 and 620-650 nm filters

All incubations are at room temperature (15 to 25 ºC)

**Test Procedure**
1. Break off the number of wells needed (number of samples plus 3 for controls) and place in holder.
2. Add 2 drops (approximately 100 µl) of negative control to well # 1.
3. Add 2 drops (approximately 100 µl) of positive toxin A and toxin B to well # 2 and 3, respectively.
4. Add 2 drops of the sample stool supernatant to each test well.
5. Incubate for 30 minutes at room temperature (15-25 ºC), then wash.*
6. Add 1 drop of Reagent 1 (blue solution) then 2 drops of Reagent 2 (red solution) to each well. Mix for 15-30 seconds.**
7. Incubate for 10 minutes, then wash.
8. Add 2 drops of Chromogen to each well.
9. Incubate 10 minutes.
10. Add 2 drops of Stop Solution to each well. Mix wells by gently tapping the side of the strip holder with index finger.
11. Incubate 1 minute at room temperature then, read results visually or at 450/620-650 nm. Zero reader on air.

* Washings consist of vigorously filling each well to overflowing and decanting contents three separate times. Strip(s) should then be tapped out on a dry towel before next reagents are added.

** The two reagents must be mixed thoroughly. This can be performed by shaking or tapping the plate, for 10-20 seconds.

Controls must be included each time the kit is run.

**Interpretation of Results – Visual**

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

**Non-reactive:** 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 - ELISA Reader**

Zero reader on air. Read all wells at 450/620-650 nm.

**Reactive:** Absorbance reading of 0.15 OD units and above indicates the sample contains *C. difficile* toxin.

**Non-reactive:** Absorbance reading less than 0.15 OD units indicates the sample does not contain detectable levels of *C. difficile* toxin.

**Test Limitations**

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.
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 *C. difficile*.

**Expected Results**

A positive reaction indicates that the patient is shedding detectable amounts of *C. difficile* antigen. The frequency of *C. difficile* disease is dependent on various factors such as the type of institution, patient population and potential outbreak status. Asymptomatic carrier rates have been reported from a low of 2% in Sweden to a high of 15% in Japan. Hospitalized patients taking certain antibiotics are at high risk of acquiring *C. difficile* with infection rates of 21% being reported in one study. A recent article in *Journal of Clinical Microbiology* (ref. #18) provides a good overview of testing for *C. difficile*. Further information on *C. difficile* and antibiotic colitis can also be found in the *Manual of Clinical Microbiology*, ASM Press, 7th Edition.

**Specific Performance Characteristics**

**Study #1 (Toxin B Cell Culture)**

A total of 69 stools were tested against a cytotoxin B cell culture procedure. The following results were obtained.

<table>
<thead>
<tr>
<th></th>
<th>Cyto B +</th>
<th>Cyto B -</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAI ELISA +</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>DAI ELISA -</td>
<td>4</td>
<td>49</td>
</tr>
</tbody>
</table>

Sensitivity: 78% (14/18)  95% CI = 52% to 94%
Specificity: 96% (49/51)  95% CI = 87% to 100%

**Study #2 (In House Study)**

A total of 24 stools in Cary Blair transport media (5 positive, 19 negative) were tested in this ELISA. In addition, 14 fresh/frozen stools (6 positive, 8 negative) were diluted using the Quick’N’Easy Fecal Sample Prep device and tested in the ELISA. The following results were obtained.

<table>
<thead>
<tr>
<th></th>
<th>ELISA/OIA +</th>
<th>ELISA/OIA -</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAI ELISA +</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>DAI ELISA -</td>
<td>0</td>
<td>27</td>
</tr>
</tbody>
</table>

Positive Agreement = 100% (11/11)
Negative Agreement = 100% (27/27)
Study #3 (Mid-West Clinical Lab)

This study compared 53 fresh samples versus another commercial A+B ELISA. The DAI ELISA had a positive agreement of 100% (2/2) and a negative agreement of 98% (50/51).

Study #4 (Reference #17)

A total of 311 stools were tested against culture and another commercial toxin A+B ELISA kit. The following results were obtained.

<table>
<thead>
<tr>
<th></th>
<th>Culture +</th>
<th>Culture –</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAI ELISA +</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td>DAI ELISA -</td>
<td>33</td>
<td>221</td>
</tr>
</tbody>
</table>

Sensitivity: 60% (49/82) 95% CI = 48% to 70%
Specificity: 97% (221/229) 95% CI = 93% to 99%

The other commercial ELISA showed a sensitivity of 66% (54/82) and a specificity of 98% (225/229) on the same set of samples.
Between the two ELISA’s there was a positive agreement of 91% (49/54) and a negative agreement of 98% (221/225).

Study #5 (East Coast Hospital Lab)

This study compared 82 fresh or frozen samples versus another commercial Toxin A Only Optical Immunoassay. The DAI ELISA had a positive agreement of 79% (27/34) and a negative agreement of 94% (45/48).

Cross Reactivity Study

The following cultures were tested in the ELISA by seeding normal human stool to an approximate concentration of 10(8) CFU’s/ml. All cultures were negative in the assay.

Salmonella typhimurium, Proteus vulgaris, E. coli 43887, Campylobacter coli 1114, Salmonella enteritidis, Campylobacter fetus, Staphlococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Serratia liquefaciens, Enterobacter cloacae, Citrobacter braakii, Shigella flexneri, Shigella sonneii, Shigella dysenteria, E. hermanii, Campylobacter jejuni, Salmonella hadar, Salmonella infantis, Yersinia enterocolitica, Enterococcus fæcalis, Helicobacter cinaedi, Candida albicans, Bacteroides fragilis, Clostridium perfringens, Clostridium haemolyticum, Clostridium novyi, Clostridium septicum and Clostridium sporogenes.
An additional culture of Clostridium sordellii was tested in the assay since C. sordellii is known to produce toxins that are structurally and immunologically similar to C. difficile. This culture did produce a low positive reaction in the assay.

**Analytical Sensitivity**

This assay can detect approximately 2 nanograms/ml of toxin A and 3 nanograms/ml of toxin B. At these detection levels, an OD range of 0.15 to 0.20 can be expected.

**Precision**

Two sites performed the assay using the same lot of kits and the same samples three times over a three-day period. The following results were obtained:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>Std Dev</th>
<th>CV</th>
<th>Sample</th>
<th>Mean</th>
<th>Std Dev</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>0.03</td>
<td>0.02</td>
<td>0.62</td>
<td>Moderate Sample</td>
<td>1.0</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>Toxin A Control</td>
<td>2.40</td>
<td>0.28</td>
<td>0.12</td>
<td>Moderate Sample</td>
<td>0.75</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Toxin B Control</td>
<td>2.27</td>
<td>0.24</td>
<td>0.10</td>
<td>Moderate Sample</td>
<td>0.64</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Negative Sample</td>
<td>0.06</td>
<td>0.04</td>
<td>0.62</td>
<td>Moderate Sample</td>
<td>0.76</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Negative Sample</td>
<td>0.06</td>
<td>0.03</td>
<td>0.49</td>
<td>High Positive</td>
<td>2.34</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Borderline Sample</td>
<td>0.15</td>
<td>0.02</td>
<td>0.13</td>
<td>High Positive</td>
<td>2.52</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>Borderline Sample</td>
<td>0.15</td>
<td>0.02</td>
<td>0.13</td>
<td>Borderline Sample</td>
<td>0.17</td>
<td>0.01</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**CV Range - Inter Lab**

Lab 1 - Neg .42 to .72, Pos .02 to .15
Lab 2 - Neg .12, Pos .06 to .20
Lab 3 - Neg .07 to .34, Pos .05 to .21

**Quality Control**

The use of controls allows validation of kit stability. For a valid test, the positive control must have an absorbance of at least 0.5 OD units and the negative control must be less than 0.15 OD units. Should the value fall below this limit, the kit should not be used.
Troubleshooting

Negative control has excessive color after development.

Reason: inadequate washings
Correction: wash more vigorously. Remove excessive liquid from the wells by tapping against an Absorbent towel. Do not allow test wells to dry out.

References


<table>
<thead>
<tr>
<th>Date Adopted</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007-12-01</td>
<td>DA-C.difficile-2008</td>
</tr>
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</table>