



CoproELISA™ *C. difficile* ToxA/B

Enzyme-Linked Immunosorbent Assay (ELISA)
For the Detection of *Clostridium difficile* toxin A and
toxin B in human feces

Instruction Manual

Test kit for 96 determinations
Catalog Number: 794-01

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™ *C. difficile* ToxA/B is an Enzyme-Linked Immunosorbent Assay (ELISA) for detection of *C. difficile* toxin A and toxin B in human fecal specimens collected from patients suspected of having *C. difficile* disease. The test results together with the patients history is intended to aid in the diagnosis of *C. difficile* infection (CDI).

For *In-Vitro* Diagnostic Use

Introduction

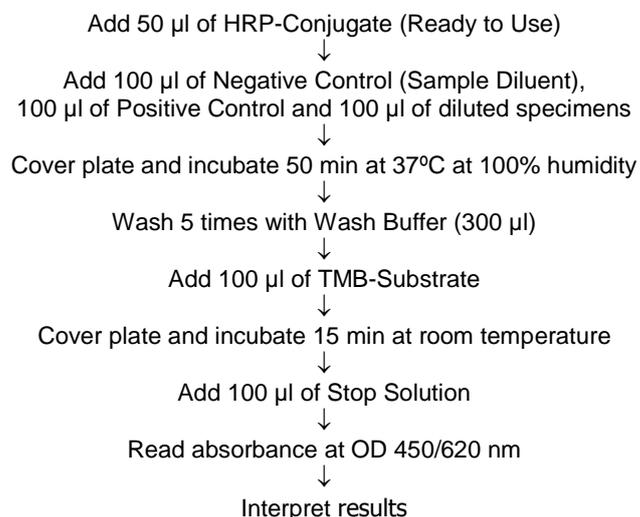
The gram-positive anaerobic bacillus *Clostridium difficile* is the leading causative agent of antibiotic-associated diarrhea and pseudomembranous colitis (1). This pathogen is capable of causing disease that could be severe or fatal if not diagnosed on time and treated. Exposure to antibiotics is the major risk factor for *C. difficile* infection. Infection can develop if the normal gastrointestinal flora is disrupted by antibiotic therapy and a person acquires toxin-producing *C. difficile*, typically via the fecal-oral route (2). *C. difficile*'s key virulence factors are toxin A and toxin B (3, 4). These toxins show high sequence and functional homology. Toxin A has been described as a tissue damaging enterotoxin which attracts neutrophils and monocytes and toxin B as a potent cytotoxin that degrades the colonic epithelial cells (5). Most virulent strains produce both toxins, however, strains that are toxin A negative and toxin B positive are also capable of causing (6, 7). Immunoassay detection of toxin A and toxin B in stool specimen is commonly used as a diagnostic aid.

Principle of the Test

CoproELISA™ *C. difficile* ToxA/B is an enzyme immunoassay for the detection of toxin A and toxin B in human feces.

- break-apart microwells are coated with *C. difficile* toxin-specific polyclonal antibodies.
- A set of horseradish peroxidase (HRP) conjugated polyclonal anti-toxin A and anti-toxin B antibodies are added to the antibody-coated microwells.
- Fecal samples are diluted in sample diluent and added to the microwells. In this step *C. difficile* toxins are specifically marked by the HRP conjugated antibodies and immobilized by the coated antibodies.
- 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 level of *C. difficile* toxins in the sample.

Summary of Procedure Manual/ Automation*



*Automation Procedure:

Please follow the following recommendations to ensure the high quality results of the test when using an automation procedure

- **Fill the plate in three sets** (up to 4 strips at a time). Add the HRP-Conjugate solution to the first set followed by adding the diluted samples before moving to the next set
- **30 minutes** sample incubation at 37°C

Kit contents for Manual / Automation use

Test Kit for 96 determinations:

1. **Microtiter plate coated with anti-toxin A and anti-toxin B polyclonal antibody:**
96 break-apart wells (8x12) coated with polyclonal antibodies specific for toxin A and B, packed in an aluminum pouch containing a desiccant card. **1 plate**
2. **Concentrated Wash Buffer (20x):** PBS -Tween buffer **1 bottle, 100 ml**

3. **Sample Diluent:** 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

4. **HRP-Conjugate (green):** A ready-to-use solution containing Horseradish peroxidase (HRP) conjugated anti-toxin A and toxin B polyclonal antibody. Contains less than 0.05% Proclin as preservative. **May accumulate protein precipitates (protein blocker) with time. Make sure to eliminate these participants by vortexing the bottle until they disappear.**

1 bottle, 7 ml

5. **Positive Control (blue):** A ready to use solution containing inactivated toxins A and B. Contains less than 0.05% Proclin as preservative.

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, 16 ml

7. **Stop Solution:** A ready to use solution. Contains 1M H₂SO₄.

1 bottle, 16 ml

8. **Disposable plastic pipettes:** **100 pc**

9. **Plate cover:** **1 unit**

10. **Instruction Manual:** **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. Reagents should be brought to room temperature before use.
2. When handling assay wells, avoid scratching the bottom of the wells because this may result in elevated absorbance readings.
3. 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.
4. **Unused microassay wells must be replaced in the re-sealable pouch with the desiccant to protect them from moisture.**
5. TMB-Substrate solution is an irritant material to skin and mucous membranes. Avoid direct contact.
6. 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

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 or culture are appropriate.
2. Preserved stool: The test is not compatible with specimens that were fixed in 10% formalin or in Sodium Acetate Formalin (SAF). The test is also not compatible with stool specimens fixed in Polyvinyl Alcohol (PVA).
3. Specimens should be kept 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. Minimize specimen freezing and thawing which may cause degradation/proteolysis of the toxins and result in loss of activity.

Test Procedure for manual use

A. Preparation of Reagents

1. Bring all components and clinical specimens to be tested to room temperature. Vortex the HRP-conjugate bottle for 15 sec. 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 Sample 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 200 µl Sample Diluent to each tube. Label the tube.
5. **Thoroughly mix (vortex) the fecal specimen to ensure adequate sampling.**
6. **Formed samples:** Use a wooden applicator stick or a disposable teaspoon to transfer the fecal specimen to the tube. Transfer approximately 0.05 to 0.1 g of specimen (about 3 mm in diameter) to the Sample Diluent. Mix the collector in the Sample 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 50 µl of specimen to the tube. Make sure the liquid specimens are evenly suspended (vortexed).

7. Thoroughly mix (vortex) the fecal specimen to ensure adequate sampling. Store the diluted samples between 2° to 8° C until test is performed.

C. Incubation of stool samples and controls

8. **Vortex the the HRP-conjugate*** bottle and dispense 50µl into each well.
9. Pipette 100 µl of Positive control (blue cap) and 100µl of Negative Control (i.e., Sample Diluent) into separate wells of the test strip.
10. 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).
11. Cover the strips with a plate cover and incubate for 50 min at 37°C in a moisture chamber.
12. **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.
13. Dry the strips and frame by gently tapping them over clean absorbent paper.

D. Incubation with TMB Substrate

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

F. Determination of Results**

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

Note: *The HRP-conjugate may accumulate protein precipitates (protein blocker) with time. Make sure to eliminate these participants by vortexing the bottle until they disappear.*

****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. Vortex the HRP- conjugate bottle for 15 sec, 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 Sample 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 Sample 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 0.3 g of specimen (about the size of 2 small peas) to the sample's tube. Mix the collector in the Sample 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 until large particulate matter is precipitated (decantation). Ensure that the formed supernatant does not contain large particulate material. In case and required centrifuge the tubes at 1000 g for 30 sec.
8. Transfer the sample's tubes to the corresponding rack at the automation machine.

C. Incubation of conjugate with stool samples and controls

Dispense ready-to-use conjugate and samples into each well in consecutive sets of up to 4 strips at a time as follows:

9. Dispense 50µl of ready-to-use conjugate into each well of up to 4 strips.
10. Pipette 100µl of Positive control (blue cap) and 100µl of Negative Control (i.e., Sample Diluent) into separate wells of the test strip (containing the ready-to-use conjugate).
11. Dispense 100µl of diluted stool samples into the test strip (from the 4 strip set containing the ready-to-use conjugate).
12. Repeat dispensing of ready-to-use conjugate and samples to next 4 strip sets as described in steps 9 and 11.
13. Incubate the plate at 37°C for **30 minutes**.
14. Perform 5 X 300µl wash cycles using the pre-diluted Wash Buffer.
15. Perform 2 aspirate cycles with aspirate sweep.

D. Incubation with TMB Substrate

16. Dispense 100µl of TMB-Substrate into each well. Incubate at room temperature for 15 minutes.
17. Stop the reaction by adding 100µl of Stop Solution (1M H₂SO₄) into each well.

E. Determination of Results

18. 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.

Negative Control:

The absorbance value should be ≤ **0.07** at 450/620 nm.

Positive Control:

The absorbance value should be ≥ **0.5** at 450/620 nm.

Interpretation of Results

Spectrophotometric Dual Wavelength at 450/620 nm

Negative = OD < 0.08
Positive = OD ≥ 0.08

Test Limitations

1. CoproELISA™ *C. difficile* toxA/B detects *C. difficile* toxins in fecal specimen. Detection of *C. difficile* toxins in stool should be taken into account by the physician in light of the patient's clinical history before making a final diagnosis. Inability to detect toxin A and toxin B in patient's fecal samples may not preclude actual disease but may be caused by other factors such as incorrect sampling handling or storage of stool. It is also possible that toxin levels are below the kit's limit detection. The CoproELISA™ *C. difficile* toxA/B detects in stool toxin A or B at levels of ≥ 3 ng/ml.
2. The stability of *C. difficile* toxins in stool samples may be affected especially at low concentrations. Therefore, it is important to keep samples at 2-8° C soon after collection. Samples that are not analyzed within 48 hours may be frozen and thawed.
3. Some samples may give low detection levels. This could be caused by a number of reasons such as the presence of a weakly toxinogenic strain, low level of bacteria producing toxins, or by factors in the feces that interfere with *C. difficile* toxins or the test. Under these conditions it is recommended to retest samples using fresh specimen.
4. Some *Clostridium sordellii* strains produce toxins that are similar to *C. difficile*'s toxin A and B, however, *Clostridium sordellii* has not been detected in patient stools with antibiotic-associated diarrhea.

Performance Characteristics of the Test

Study: Clinical stool specimens were evaluated by CoproELISA™ *C. difficile* toxA/B test. The study was performed in house on a total of 86 samples (Table 1) and externally in an Israeli medical center on a total of 56 samples (Table 2). The results below were compared to results of an FDA approved commercial reference ELISA kit.

Table 1:

		ref. kit		
		positive	negative	total
CoproELISA™ <i>C. difficile</i> toxA/B	positive	45	1	46
	negative	2	38	40
	total	47	39	86

Sensitivity: 95.7 %, Specificity: 97.4 %
PPV: 97.8%, NPV: 95%

Table 2:

		ref. kit		
		positive	negative	total
CoproELISA™ <i>C. difficile</i> toxA/B	positive	9	1	10
	negative	0	46	46
	total	9	47	56

Sensitivity: 100 %, Specificity: 97.9 %
PPV: 90%, NPV: 100%

Cross Reactivity and Interference by Mixed infections

The CoproELISA™ *C. difficile* toxA/B test was evaluated using microbial culture isolates and clinical stool specimens*.

No cross-reactivity was observed with any of the gastrointestinal pathogens and microbes listed below:
Blastocystis, *Campylobacter**, *Cryptosporidium parvum**, *Dientamoeba fragilis**, *Escherichia coli*, *Entamoeba histolitica**, *Enterococcus faecali*, *Enterococcus faesium*, *Enterococcus avium*, *Enterococcus aerogenes*, *Enterococcus cloacae*, *Enterococcus gallinarum*, *Enterococcus durans*, *Giardia lamblia**, *Helicobacter pylori**, *Klebsiella pneumonia*, *Salmonella enterica**, and *Shigella**.

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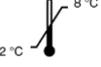
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	Temperature Limitation
	Consult instructions for use
	In Vitro Diagnostic Medical Device
	Manufacturer
	Authorized European Representative