Fecal C. Difficile Toxin A Qualitative ELISA Assay Kit

Catalog Number:
DFA35-K01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
v. 1.1 (10.26.17)

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INTENDED USE

This Eagle Biosciences Fecal C. Difficile Toxin A ELISA Assay Kit is a microplate-based ELISA (enzyme linked immunosorbent assay) and is intended for the qualitative detection of C. difficile Toxin A in feces. The assay is a useful tool as an aid of detection of C. difficile infection. This Eagle Biosciences Fecal C. Difficile Toxin A ELISA Assay Kit is for Research Use Only and is not intended for diagnostic or therapeutic purposes.

SUMMARY OF PHYSIOLOGY

Clostridium difficile is a common pathogen and a major cause of infectious diarrhea in hospitalized patients. While most strains produce both Toxin A and Toxin B, some only produce one or the other. These enterotoxins, which are both proinflamatory and cytotoxic, attack the mucosal lining of the intestines. If not identified and treated in a timely fashion, can result in permanent damage to the colon or colitis. This assay employs an antibody specific to Toxin A without cross-reactivity to toxin B.

ASSAY PRINCIPLE

This Eagle Biosciences Fecal C. Difficile Toxin A ELISA Assay Kit is a "sandwich" ELISA is designed, developed and produced for the qualitative measurement of Toxin A in stool specimen. The assay utilizes the microplate-based enzyme immunoassay technique by coating highly purified antibody onto the wall of microtiter wells. Controls and extracted fecal specimen are added to microtiter wells of microplate that was coated with a highly purified monoclonal anti-Toxin A on its wall. During the assay, the Toxin A Antibody will be bound to the antibody coated plate after an incubation period. The unbound material is washed away and another HRP-conjugated monoclonal antibody which specifically recognizes the protein of Toxin A is added for further immunoreactions. After an incubation period, the immunocomplex of "Anti-Toxin A Capture Antibody – C. Diff. Toxin A – HRP-conjugated Anti-Toxin A Tracer Antibody" is formed if Toxin A is present in the test sample. The unbound tracer antibody and other proteins in buffer matrix are removed in the subsequent washing step. HRP conjugated tracer antibody bound to the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the tracer antibody bound to Toxin A proteins captured on the wall of each microtiter well is directly proportional to the amount of Toxin A level in each test specimen.

REAGENTS

This Eagle Biosciences Fecal C. Difficile Toxin A ELISA Assay Kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Regents from different kit lot numbers should not be combined or interchanged.

1. **Toxin A Antibody Coated Microplate**
   One microplate with twelve by eight strips (96 wells total) coated with monoclonal anti-human Toxin A. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2 – 8 °C and is stable until the expiration date on the kit box.

2. **Anti-Toxin A Tracer Antibody**
   One vial containing 12 mL ready-to-use horseradish peroxidase (HRP) conjugated monoclonal Toxin A antibody in a stabilized protein matrix. This reagent should be stored at 2 – 8 °C and is stable until the expiration date on the kit box.

3. ELISA HRP Substrate
One bottle contains **12 mL** of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

4. ELISA Stop Solution
One bottle contains **12 mL** of 0.5 M sulfuric acid. This reagent should be stored at 2 – 8°C or room temperature and is stable until the expiration date on the kit box.

5. Toxin A Negative Control
One vial contains **1 mL** ready-to-use Toxin A negative control in a liquid bovine serum albumin based matrix with a non-azide preservative. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

6. Toxin A Positive Control
One vial contains **1 mL** ready-to-use Toxin A positive control in a liquid bovine serum albumin based matrix with a non-azide preservative. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

7. **Concentrated Fecal Sample Extraction Buffer**
One bottle containing 10 mL of **10-fold concentrated** fecal sample extraction buffer. This reagent should be diluted with 90 mL distilled water and mixed well. This yields as the fecal sample extraction buffer. The Fecal Sample Extraction Buffer may be stored at 2-8ºC and is stable until the expiration date on the kit box.

8. ELISA Wash Concentrate
One bottle contains **30** mL of 30-fold concentrate. Before use the content must be diluted with **870 mL** of distilled water and mixed well. Upon dilution this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide preservative. The diluted wash buffer should be stored at room temperature and is stable until the expiration date on the kit box.

STORAGE
Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in the sealed pouch to minimize exposure to air.

SAFETY PRECAUTIONS
The reagents must be used in research laboratory and are for research use only. Reagents containing bovine serum were derived in the contiguous 48 United States and have been obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases.

Wear gloves while performing this assay and handle these reagents as if they are potential infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. Upon contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision single channel pipettes capable of delivering 10 µL, 25 µL, 50 µl, 65 µL, 100 µL, and 1000 µL.
- Repeating dispenser suitable for delivering 100 µL.
- Disposable pipette tips suitable for above volume dispensing.
- Disposable 12 x 75 mm glass or plastic tubes.
- Disposable plastic 1000 mL bottle with cap.
- Aluminum foil.
- Plastic microtiter well cover or polyethylene film.
- ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
- Spectrophotometric microplate reader capable of reading absorbance at 450 nm.

SPECIMEN COLLECTION

Fresh fecal sample should be collected into a stool sample collections container. It is required to collect a minimum of 1-2 mL liquid stool sample or 1-2g solid sample. The collected fecal sample must be transported to the lab in a frozen condition (-20ºC). If the stool sample is collected and tested the same day, it is allowed to be stored at 2-8 ºC for up to 3 days. Avoid more than 3x freeze and thaw.

ASSAY PROCEDURE

1. Reagent Preparation

(1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
(2) ELISA Wash Concentrate must be diluted to working solution prior use. Please see REAGENTS section for details.
(3) Concentrated Fecal Extraction Buffer must be diluted to working solution prior use. Please see REAGENTS section for details.

2. Patient Sample Preparation

2.1. For manual weighing procedure only:

(1) Label a test tube (12x75 mm) or a 4 ml plastic vial.
(2) Patient samples need to be diluted **1:5** with working Fecal Extraction Buffer (1x) before being measured.

Following is a detailed sample extraction process.
(a) Label and tare an empty polypropylene tube together with an inoculation loop.
(b) Weigh 250 – 500 mg of stool using the inoculation loop by placing it into the pre-tared tube.
(c) Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube.
(d) Add diluted Extraction Buffer (4 parts of the stool volume, 1 g stool = 1mL) into the tube:

Fecal Sample Weight (mg)	Extraction Buffer Volume (mL)
225 – 274	1.25
275 – 324	1.50
325 – 374	1.75
375 – 424	2.00
425 - 474	2.25
475 -525	2.50

(3) Centrifuge the diluted fecal sample at 3000 rpm (800- 1500 g) for 5-10 minutes. The supernatant can be directly used in the assay.
Note: If the test procedure is performed on an automated ELISA system, the supernatant must be particle-free by centrifuging the sample**.**
(4) This extracted sample can be stored at 2-8 ºC up to twelve (12) days and below -20ºC for longer storage. Avoid more than 3x freeze and thaw cycle.
(5) The extracted samples must be mixed with vortex before re-using. Therefore, it is recommended to aliquote the supernatant in a clean test tube, capped, for future use.

2.2. Using Fecal Sample Collection Devices

(1) Label a Fecal Sample Collection tube
(2) Follow the instructions on the Sample Collection Tube insert.
(3) This sample can be stored at 2-8 ºC up to twelve (12) days and below -20 ºC for longer storage. Avoid more than 3x freeze and thaw cycle.
(4) Two drops of the extracted sample is equivalent to 100 µl.

3. Assay Procedure

(1) Place a sufficient number of Toxin A monoclonal antibody-coated microwell strips in a frame.
(2) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	NEG CTL	SAMPLE 3	SAMPLE 7
B	NEG CTL	SAMPLE 3	SAMPLE 7
C	POS CTL	SAMPLE 4	SAMPLE 8
D	POS CTL	SAMPLE 4	SAMPLE 8
E	SAMPLE 1	SAMPLE 5	SAMPLE 9
F	SAMPLE 1	SAMPLE 5	SAMPLE 9
G	SAMPLE 2	SAMPLE 6	SAMPLE 10
H	SAMPLE 2	SAMPLE 6	SAMPLE 10

(3) Add **100 µL** of controls and extracted patient stool samples into the designated microwell. Mix by gently tapping the plate. Cover the plate with one plate sealer. Cover with foil or other material to protect from light.

Note: *if the collection tubes are used, add two drops of extracted fecal sample into each well.*

(4) Incubate plate at room temperature, static, for **1 hour**.
(5) Remove the aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL to 400 µL of working wash solution into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
(6) Add **100 µL** of Toxin A Tracer Antibody to each well. Mix by gently tapping the plate.
(7) Cover the plate with one plate sealer and also with aluminum foil to avoid exposure to light.
(8) Incubate plate at room temperature, static, for **30 minutes**.
(9) Remove the plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL to 400 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
(10) Add **100 µL** of ELISA HRP Substrate into each of the wells.
(11) Cover the plate with a new plate sealer and also with aluminum foil to avoid exposure to light.
(12) Incubate plate at room temperature for **20 minutes**.
(13) Remove the aluminum foil and plate sealer. Add **100 µL** of ELISA Stop Solution into each of the wells. Mix gently.
(14) Read the absorbance at 450 nm.

PROCEDURAL NOTES

1. It is recommended that all control and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light sensitive reagents in the original amber bottles.
   Store any unused antibody coated strips in the foil zip-seal bag with desiccant to protect from moisture. Exposure of the plates to humidity drastically reduces the shelf life.
3. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
4. Incubation times or temperatures other than those stated in this insert may affect the results.
5. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate readings.
6. All reagents should be mixed gently and thoroughly prior use. Avoid foaming.

INTERPRETATION OF RESULTS

1. **Visual:**

    a. Positive or reactive: Any sample well that is obviously more yellow than the negative control well.
    b. Negative or non-reactive: Any sample well that is not obviously more yellow than the negative control well.

Note: The negative control, as well as some patient samples, may show some slight yellow color. A sample well must be obviously darker or more yellow than the negative control well, when it is interpreted as a positive result.

ELISA Reader:

1. Calculate the average absorbance for each pair of duplicate test results.
2. Calculate the cut-off
   The positive cut-off and the negative cut-off are established by using following formula.

   Positive Cut-Off = 1.1 x (mean extinction of negative control + 0.10)
   Negative Cut-Off = 0.9 x (mean extinction of negative control + 0.10)

3. Interpret test result
   - Positive: patient sample extinction is greater than the Positive Cut-Off
   - Negative: patient sample extinction is less than the Negative Cut-Off
   - Equivocal: patient sample extinction is between the Positive Cut-Off and the Negative Cut-Off.

4. Assay quality control
   1. Positive control must show an average OD reading greater than 0.6.
   2. Negative control should show an average OD reading less than 0.09.

EXAMPLE DATA AND CALCULATED CUT-OFF

A typical absorbance data and the resulting negative control and positive controls are represented. This absorbance must not be used in lieu of control values run with each assay.

ROW	STRIP 1	(OD 450 nm)
A	Neg. Ctr	0.049
B	Neg. Ctr	0.048
C	Pos. Ctr.	1.053
D	Pos. Ctr.	1.051
E	Sample 1	0.128
F	Sample 2	0.145
G	Sample 3	0.334
H	Sample 4	0.636

1. The OD of negative controls and positive control meet the Internal Quality Control Standard. The Assay is valid.

2. Calculate the Mean OD for negative control:

   $Mean_{neg.} = (0.049 + 0.048)/2 = 0.048$

3. Calculate the Positive and Negative Cut-Off Value:

   - Positive Cut-Off = 1.1 x (0.048 + 0.10) = 0.163
   - Negative Cut-Off = 0.9 x (0.048 + 0.10) = 0.133
   - Equivocal = 0.134 ~ 0.162

4. Interpret the Sample Result:

   Sample 1 = 0.128 ≤ Negative COV → Negative
   Sample 2 = 0.145 ≤Pos. COV; ≥ Neg COV → Equivocal
   Sample 3 = 0.334 ≥ Positive COV → Positive
   Sample 4 = 0.636 ≥ Positive COV → Positive

EXPECTED VALUES

Forty stool samples were collected from normal subjects agin 23 to 64 years old. Ten fecal samples from patients with C. difficile infection (also positive in C. difficile GDH by using Eagle Biosciences CDF35-K01 kit) were also collected. All fecal samples were prepared and assayed according to the procedures described in this manual. The results are summarized below.

Test Result	Normal Subjects (True Negative)	Patients (True Positive)
Negative	40	3
Positive	0	7
Total	40	10

The clinical sensitivity is **70%** (7/10 x 100% = 70%)
The clinical diagnostics specificity is **100%** 40/40 x 100% = 100%)

LIMITATION OF THE PROCEDURE

The results obtained with this Eagle Biosciences C. Difficile Toxin A ELISA Assay Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves without other clinical findings such as endoscopy and biopsy, etc.

QUALITY CONTROL

To assure the validity of the results each assay must include both negative and positive controls. 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 0.12 OD units. We also recommend that all assays include the laboratory's own controls in addition to those provided with this kit.

PERFORMANCE CHARACTERISTICS

Specificity

The assay does not cross react to the following:
Toxin B, Helicobacter pylori, glutamate dehydrogenase 1 (GDH) Cryptosoridium parvum, Giardia lamblia, rotavirus and adenovirus.

Reproducibility and Precision

The reproducibility of this assay is validated by measuring two samples both in a single assay of 16-replicate determinations (intra-assay) and in 10 different assays run on different dates (inter-assay).

Intra-Assay		
	Sample 1	Sample 2
Mean	0.632	1.184
Std Dev	0.045	0.086
%CV	7.2%	7.2%
Inter-Assay		
	Sample 1	Sample 2
Mean	0.605	1.067
StdDev	0.050	0.091
%CV	8.2%	8.5%

Interference

One positive sample is added with 5% volume of interference materials to reach a final concentration shown in the table below. All samples are tested in an assay in duplicate.

	Mean OD 450 nm		
	Additive	**Amt Added (mg/mL)**	**Sample**
1	Test Control	-	0.352
2	Bilirubin –L	0.4	0.373
3	Bilirubin - M	2.0	0.427
4	Bilirubin - H	10.0	0.319
5	Test Control	-	0.351
6	Hb - L	0.4	0.300
7	Hb – M	2.0	0.343
8	Hb – H	10.0	0.326
9	Lipid – L	8	0.304
10	Lipid - M	40	0.331
11	Lipid -H	200	0.365

REFERENCES

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4. Borriello, S.P., FE. Barclay, P.J. Reed, A.R. Welch, J.D. Brown, and O.W. Burden. Analysis of latex agglutination test for Clostridium difficile toxin A(D-1) and differentiation between Clostridium difficile toxins A and B, and latex reactive protein. J. Clin Path. 1987. 40:573-580.2. Kelly, C.P. Immune response to *Clostridium difficile* infection. Eur J Gastroenterol Hepatol. 1996; 8: 1048–1053

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