PRO-LAB DIAGNOSTICS

Prolisa™ *C. difficile* GDH EIA

(for *in vitro* diagnostic use)

PRODUCT CODE: PL.2002

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INTENDED USE

The Prolisa™ *C. difficile* GDH EIA is a microwell assay for the qualitative detection of *Clostridium difficile* glutamate dehydrogenase (GDH) in faecal specimens. The Prolisa™ *C. difficile* GDH EIA is intended for use as an aid in the diagnosis of *C. difficile* infections. This test detects GDH and will not differentiate between toxigenic and non-toxigenic strains of *C. difficile*. Like alternative *C. difficile* tests, results should be considered in conjunction with patient history and additional laboratory investigations.

SUMMARY AND EXPLANATION

Mechanism of Disease

Clostridium difficile is an anaerobic spore-forming bacillus that produces two clinically important toxins, called Toxin A and Toxin B, that act in the gut to produce local tissue damage that can progress to pseudomembranous colitis. Toxigenic *C. difficile* can be carried asymptomatically; however, serious sequelae sometimes follow overgrowth of *C. difficile* resulting from antimicrobial therapy. Institutional outbreaks of *C. difficile*–associated disease are frequently caused by ingestion of acid-resistant spores present in the environment. *Clostridium difficile* strains that do not produce Toxin A and Toxin B are considered non-pathogenic (1).

Diagnosis of Disease

Clostridium difficile-associated disease is diagnosed by a combination of clinical and microbiological findings. The gold standard for microbiological identification of toxigenic *C. difficile* infection is cytotoxigenic culture, a test in which *C. difficile* isolates from selective differential agar are enriched in broth and then tested for elaboration of Toxin B by cytotoxicity assay on cultured cells (2). Rapid immunoassays have been developed for detection of Toxin A and/or Toxin B in faecal specimens; however, these tests lack sensitivity (3). Immunoassays for GDH, a protein shared by toxigenic and non-toxigenic *C. difficile*, have been developed and incorporated into algorithms for identification of toxigenic *C. difficile*. It has been shown that toxigenic *C. difficile* can be more efficiently and economically identified by first testing for GDH and then Toxin A and/or Toxin B rather than by testing for the toxins alone (3).

PRINCIPLE OF THE TEST

The Prolisa™ *C. difficile* GDH EIA is a sandwich immunoassay that uses specific antibodies that recognize *C. difficile* GDH. The stripwells contain immobilized mouse monoclonal antibody, and the immunoconjugate contains rabbit polyclonal antibodies conjugated to horseradish peroxidase. To perform the test, a portion of a faecal specimen is first thoroughly suspended in diluent to create a sample suitable for testing. A portion of this sample and the immunoconjugate are then incubated simultaneously in a well containing immobilized monoclonal antibody. If GDH is present in the sample, an insoluble antibody-enzyme complex that cannot be easily washed from the wells is formed. After the wells are washed to remove unbound material, the bound enzyme is detected through the use of a chromogenic substrate.

MATERIALS PROVIDED

Component	Cat. No.	Per Kit	Description	Note
Coated and Stabilized Plate	PL.2115	1 plate / pouch	Mouse monoclonal antibody to GDH coated onto strip wells	Each pouch contains 1 plate with a sealer and 2 desiccants
Sample Diluent	PL.2113	2 x 30 ml	A protein-free solution with preservative	White bottle
Positive Control	PL.2112	1 x 2.5 ml	Recombinant GDH in a buffered protein solution with preservative	Dropper bottle with blue cap
Immunoconjugate	PL.2114	1 x 7 ml	Rabbit polyclonal anti-GDH antibody conjugated to horseradish peroxidase	Dropper bottle with red cap
20X Wash Buffer	PL.2110	2 x 25 ml	Concentrated buffer containing detergent and 0.1% thimerosal (w/v)	White bottle
Substrate Solution	PL.2104	1 x 14 ml	3,3′,5,5′-tetramethylbenzidine in a mildly acidic buffer	Amber bottle
Stop Solution	PL.2103	1 x 14 ml	0.2 N sulfuric acid	Dropper bottle with yellow cap
Plate Sealer	N/A	3	---	----
Transfer pipette	N/A	100 in 4 bags	---	----
Instructions for Use	N/A	1	---	----

MATERIALS REQUIRED BUT NOT PROVIDED

1. Wooden applicator sticks or loop
2. Timer
3. Pipette capable of delivering 50 µl to 1000 µl
4. Pipette tips
5. Test tubes (12 X 75 mm or other suitable size) for sample dilution
6. Distilled or deionized water
7. Wash bottle or a plate washer or an automated EIA system
8. Graduated cylinder
9. EIA plate reader with 450/630 nm absorbance-reading capability or an automated EIA system.
10. Vortex Mixer
11. Centrifuge

STABILITY AND STORAGE

The expiration date is indicated on the kit label. Store the kit at 2-8°C (20X wash buffer may be stored at room temperature). Return the kit promptly to 2-8°C storage after each use. 1X Wash Buffer may be stored at room temperature for up to 1 month.

PRECAUTIONS

1. For *in vitro* diagnostic use only.
2. Specimens may contain infectious agents and should be handled at Biosafety Level 2.
3. All reagents should be mixed gently before use.
4. Stored wash buffer may separate into layers. Shake well before use.
5. Do not interchange reagents from different kit lot numbers.
6. The substrate is sensitive to light; do not expose it to light.
7. Reagent vials should be held vertically at a suitable distance above the well to insure proper drop size and delivery.
8. Do not use kit components beyond the expiry date on the label.
9. Dispose of used wash buffer and all test materials in a manner appropriate for potentially biohazardous materials.
10. Avoid skin contact with Stop Solution; it contains 0.2 N sulfuric acid. Flush with water immediately if solution contacts skin or eyes.
11. Do not reuse microwells.
12. Exposing unused microwells to air for extended periods of time may compromise test results. It is important to protect strips from moisture during storage by replacing unused stripwells in the provided pouch.
13. Do not use a transfer pipette for more than one specimen.
14. When dispensing samples into a microwell avoid splashing by placing the transfer pipette tip about halfway into the well and trickling solution slowly down the side of well.
15. Stripwells should be washed precisely as directed in the assay procedure. Inadequate washing can elevate background readings and lead to false-positive results.
16. Any deviation from set incubation times can affect test performance. All parameters for this test have been optimized, and any deviation from test protocol could affect results.
17. Product contains material of animal origin and should be handled as a potential carrier and transmitter of disease.
18. Only faecal specimens without added preservatives may be used in the test.
19. Avoid scratching the microwells when handling, as scratches could affect absorbance readings.
20. It has been observed that strong positive samples may contaminate adjacent microwells leading to false positive test results. It is recommended that weakly positive samples be retested if they occur immediately adjacent to strongly positive samples.

REAGENT PREPARATION

1. Prepare 1X Wash Buffer from 20X Wash Buffer by mixing the supplied concentrate with 950 ml of distilled or deionized water.
2. Bring the entire kit, including plate pouch, to room temperature before use.

SPECIMEN STORAGE

Faecal specimens tested within 2 hours of collection do not require refrigeration. Specimens not tested within 2 hours of collection should be stored at 2-8°C and tested within 24 to 48 hours of collection, if possible. If specimens cannot be tested within 48 hours of collection, they should be stored frozen at

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20 Mural Street, Unit #4, Richmond Hill, ON, L4B 1K3

U.S.A. ☎ 800 522 7740 Fax 800 332 0450
21 Cypress Blvd., Suite 1070, Round Rock, Texas, 78665-1034

EC REP U.K. ☎ 0151 353 1613 Fax 0151 353 1614
3 Bassendale Road, Bromborough, Wirral, Merseyside CH62 3QL

≤-20°C (4). Avoid repeatedly freezing and thawing specimens, as this may lead to erroneous test results.

SAMPLE PREPARATION FOR MANUAL USE

1. Add 300 µl of Sample Diluent to a sample tube.
2. Transfer 100 µl of unformed faeces or approximately 20 µl of solid faeces (equivalent to a spherical mass with a diameter of approximately 4 mm) to the sample tube.
3. Vortex the sample tube for 10 seconds to thoroughly emulsify the specimen in Sample Diluent. Test sample immediately after preparation.
4. If the plate is to be washed with an automated plate washer, centrifuge the samples at ~5000 x g for 10 minutes (or until the particulate matter forms pellets) before adding the sample supernatant to the test wells. Large particulate matter in samples may interfere with automated plate washing.

TEST PROTOCOL FOR MANUAL USE

1. Cut the re-sealable foil pouch and carefully remove the assay plate from the pouch.
2. Remove the sealing tape from the stripwells. Return any extra wells to the pouch, re-seal the pouch and return it to storage at 2-8°C.
3. Add 1 drop (~50 µl) of the Immunoconjugate to the wells.
4. Use a transfer pipette to transfer 100 µl (equivalent to the first calibration point of the pipette) of diluted specimen to the wells, and add 100 µl of Positive Control, and 100 µl of Sample Diluent (negative control) to the appropriate wells.
5. Incubate the plate for 60 minutes at room temperature without shaking. Alternatively, incubate the plate for 20 minutes at room temperature with 1000 rpm shaking.
6. Discard the samples/controls from the strip(s) and wash the wells 5-7 times with 1X Wash Buffer.
   <u>Option 1</u>
   - Discard plate contents in an appropriate biohazard container
   - Strike the inverted plate firmly on a clean stack of paper towels
   - Completely fill all wells with 1X Wash Buffer using a wash bottle
   - Repeat washing cycle (discard, strike, and fill) 4-6 additional times
   - After the last refill, discard contents and strike the plates firmly on fresh paper towels to remove any excess wash buffer

   <u>Option 2</u>
   - Wash plate with an automated plate washer 5-7 times by filling the wells with 300 µl of 1X Wash Buffer
7. Add 100 µl of the Substrate Solution to each well, tap the plate holder gently and incubate for 10 minutes at room temperature.
8. Add 3 drops (~100 µl) of the Stop Solution to the wells and tap the plate holder gently to ensure that the contents are mixed properly.
9. Read the test results within 10 minutes after completion of Step 8. Ensure that bottom of wells are clean and dry. Use a lint-free towel to wipe the underside of wells when necessary.
10. Measure OD450/630 nm in a microplate reader.

SAMPLE PREPARATION FOR AUTOMATION USE

1. Add 600 µl Sample Diluent to a sample tube provided by an automated EIA system or equivalent tube.
2. Transfer 200 µl of unformed faeces or approximately 40 µl of solid faeces to the sample tube.
3. Cover the sample vials and vortex the sample tube for 10 seconds to thoroughly emulsify the specimen in the Sample Diluent.
4. Centrifuge the samples at least 5000 x g for 10 minutes at room temperature.

Note: If 5000 x g in a centrifuge for a specific sample vial is not available, a longer centrifugation time should be applied (e.g. 3000 x g for 20 minutes).

5. Do not disturb the sample tubes, and place the sample tube in an appropriate position in the automated EIA system.

TEST PROTOCOL FOR AUTOMATION USE

1. Cut the re-sealable foil pouch and carefully remove the assay plate from the pouch.
2. Remove the sealing tape from the stripwells. Return any extra wells to the pouch, re-seal the pouch and return it to storage at 2-8°C. Place the required strips with the holder in an appropriate position in the automated system.
3. Prepare adequate volume of 1X Wash Buffer by diluting 20X Wash Buffer in distilled or deionized water. Transfer the 1X Wash Buffer to an appropriate container in the automated system.

Carefully read the User Manual of the automated EIA system. Set up a program for running the Prolisa™ *C. difficile* GDH EIA in the automated EIA system according to the following policies (step 4-11). Contact Pro-Lab Diagnostics Technical Service for questions related to the set up of a program in an automated EIA system.

4. Transfer adequate volumes of the Immunoconjugate, the Substrate Solution and the Stop Solution to containers provided with the automated system and place them in the appropriate positions in the system.
5. Transfer 50 µl of the Immunoconjugate to each well.
6. Transfer 100 µl of Positive Control, and 100 µl of Sample Diluent (negative control) to the appropriate wells. Transfer 100 µl of diluted specimen to the wells.
7. Incubate the plate for 60 minutes at room temperature without shaking.
8. Aspirate the samples/controls from the strip(s) and wash the wells 5 times with 1X Wash Buffer.
9. Transfer 100 µl of the Substrate Solution to each well and incubate for 10 minutes at room temperature.
10. Transfer 100 µl of the Stop Solution to the wells and shake the plate briefly.
11. Measure OD450/630nm in the automated system within 10 minutes after Step 10.

Follow the maintenance and operation manual of the automated EIA system. Conduct a test of the Prolisa™ *C. difficile* GDH EIA kit in the automated EIA system and analyze the data in the system.

Note:
- *If a wavelengh 630 nm filter is not available in the automated EIA system, set up dual wavelength at 450 nm / 620 nm.*
- *It is recommended that the Positive Control and the Negative Control be added in well A1/B1 and C1/D1 in duplicate. The mean of OD readings of the Positive Control must be greater than 0.800 and the mean of OD readings of the Negative Control must be less than 0.100.*
- *It is recommended that "five (5) cycle washings with 300 µl 1X Wash Buffer in each well" be included in the running program for an automated EIA system. However, more washing cycles (> 5) may be required in different automated systems.*

QUALITY CONTROL PROCEDURES

The Positive and Negative Controls must be used with each assay run to assure quality of the reagents and test procedure.
1. The Positive Control should read > 0.800 at 450/630 nm.
2. The Negative Control should read < 0.100 but greater than 0.000 at 450/630 nm. If the Negative Control is <0.000, re-blank the plate reader to air and re-read the plate.
3. A well that is not visually positive (yellow) but has yielded a positive result should be wiped on the underside, repositioned, and reread.

INTERPRETATION OF THE RESULTS

Spectrophotometric Dual Wavelength (450/630nm)
- Negative = OD 450/630 nm < 0.100
- Positive = OD 450/630 nm ≥ 0.100

A positive result indicates the presence of GDH in the sample. A negative result indicates the absence of GDH in the sample, or a level of GDH below the level that can be detected by the test.
Samples containing high amounts of GDH can produce a visible black precipitate upon addition of Stop Solution. The presence of the precipitate will not affect interpretation of results.

LIMITATIONS OF THE PROCEDURE

1. The Prolisa™ *C. difficile* GDH EIA should not to be used alone to diagnose *C. difficile*-associated disease. Diagnosis should consider the test results, patient clinical history and the results of additional laboratory tests.
2. The Prolisa™ *C. difficile* GDH EIA does not distinguish between toxigenic and non-toxigenic *C. difficile*. Other tests are needed to confirm the presence of toxigenic *C. difficile*.
3. False positive test results can be obtained if plates are not washed adequately. Contact Pro-Lab Diagnostics Technical Service for assistance if false-positive test results are suspected.

PERFORMANCE CHARACTERISTICS

The clinical evaluation was performed at two external trial sites using faecal specimens submitted for routine testing for the presence of *C. difficile.* Samples were tested by the Prolisa™ *C. difficile* GDH EIA according to the instructions provided with the kit. Results from the test were compared to the results from *C. difficile* culture methods.

Table 1 summarizes the number of subjects and faecal *C. difficile* prevalence in the study. A total of 985 specimens were tested.

Table 1 – Distribution of Samples by Site

Study Site	Faecal Specimens		
	n	*C. difficile* Culture Positive	Prevalence
Site 1	483	67	13.9
Site 2	502	71	14.1
Sites Combined	985	138	14.0

Table 2 shows the sensitivity, specificity, and percent agreement values of the Prolisa™ *C. difficile* GDH EIA relative to recovery of *C. difficile* from faecal specimens by culture.

Table 2 – Performance of the Prolisa™ *C. difficile* GDH EIA Relative to Culture (Sites Combined)

Prolisa™ *C. difficile* GDH EIA Result	Culture Results			
		Positive	Negative	Totals
	Positive	128	74	202
	Negative	10	773	783
	Totals	138	847	985

Relative sensitivity: 92.8% [87.1 - 96.5%]*
Relative specificity: 91.3% [89.2 - 93.1%]
Positive percent agreement: 63.4% [56.3 - 70.0%]
Negative percent agreement: 98.7% [97.7 - 99.4%]
*95% confidence interval

Interfering Substances
Substances sometimes found in faeces of patients with diarrhoea, including common intestinal medications, barium sulfate, and blood, were not reactive and did not interfere with detection of GDH in the Prolisa™ *C. difficile* GDH EIA.

Assay Specificity
Forty-five different enteric micro-organisms, comprising 42 bacterial strains and 3 viruses, were tested both as pure samples and spiked into stool to determine

their reactivity with Prolisa. All bacteria were tested at >1 x 10^8 cfu/ml (viruses at 1 x 10^6 pfu/ml) in culture media, spiked into negative stool samples and spiked into negative stool samples containing GDH (contrived positive sample) to evaluate cross-reactivity and test interference. Table 3 lists the organisms that were tested and were non-reactive in the assay. Test reactivity was seen with *Clostridium sporogenes* which is not part of the normal intestinal flora. None of the remaining organisms were reactive in the GDH test nor did they interfere with test results. Prolisa was also tested for reactivity with 18 *C. difficile* strains. The test reacted with all of the tested strains including strains producing Toxin A and Toxin B, strains producing Toxin B only, and strains producing neither Toxin A or Toxin B.

Table 3 – Non-Reactive Microorganisms in Prolisa™ *C. difficile* GDH EIA

Adenovirus serotype 1	***Clostridium sordellii***	***Proteus mirabilis***
Aeromonas hydrophila	***Cocksackievirus***	***Proteus vulgaris***
Arcobacter butzleri	***Cytomegalovirus***	***Pseudomonas aeruginosa***
Bacillus cereus	***Enterobacter aerogenes***	***Salmonella typhimurium***
Bacillus subtilis	***Enterobacter cloacae***	***Serratia liquefaciens***
Campylobacter coli	***Enterococcus faecalis van B***	***Shigella boydii***
Campylobacter fetus	***Enterococcus faecium van A***	***Shigella dysenteriae***
Campylobacter jejuni	***Escherichia coli O157:H7***	***Shigella flexneri***
Citrobacter freundii	***Escherichia hermanii***	***Shigella sonnei***
Citrobacter braakii (freundii)	***Gardnerella vaginalis***	***Staphylococcus aureus***
Clostridium butyricum	***Klebsiella pneumonia***	***Staphylococcus epidermidis***
Clostridium histolyticum	***Listeria innocua***	***Streptococcus agalactiae***
Clostridium novyi	***Listeria monocytogenes***	***Vibrio parahaemolyticus***
Clostridium perfringens	***Peptostreptococcus anaerobius***	***Yersinia enterocolitica***
Clostridium septicum	***Prevotella melaninogenica***	

Analytical Sensitivity
The detection limit of the Prolisa™ *C. difficile* GDH EIA for recombinant *C. difficile* GDH is approximately 3.5 ng/ml in faecal sample.

Assay Precision
The interassay variability of the Prolisa™ *C. difficile* GDH EIA was evaluated with a panel of stool samples spiked with different amounts of GDH. The panel comprised negative, high negative, low positive and moderate positive samples. Testing was done by two operators at each of three sites on five different days. Between run variability of the positive samples ranged from 3.1%-12.2% and of negative samples ranged from 20.4%-50.6%. Intra-assay variability was 1.1%-29.5% on positive samples and 1.2%-39.4% on negative samples. Overall agreement between test result and expected sample result with negative samples was 100%. Overall agreement between test result and expected sample result with positive samples was 97%. The low positive sample was set at the limit of detection and thus it was anticipated that up to 5% could be negative due to assay variability.

REFERENCES

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