

INTENDED USE

The Prolisa™ EHEC EIA is a microwell assay for the qualitative detection of Shiga Toxins I (Stx1) and II (Stx2) in direct faecal samples, and from either selective broth or agar enrichment cultures of faecal samples. The Prolisa™ EHEC EIA is intended for use as an aid in the diagnosis of *Enterohaemorrhagic Escherichia coli* (EHEC) infections.

SUMMARY AND EXPLANATION
Mechanisms of Disease

EHEC are comprised of a group of organisms that produce Shiga toxins and are associated with the severe human diseases; haemorrhagic colitis and the haemolytic uraemic syndrome.^{7,9,11,16} *Escherichia coli* O157:H7 is the prototypical EHEC; however, numerous other organisms from serogroups such as O26, O111, and O145 are also reported to produce Stx and to cause disease in humans.^{2,4,8,9,10,12,13,17}

EHEC can be acquired from food, water, person-to-person contact or human-to-animal contact.⁶ The organisms are typically resistant to the acidic environment of the stomach and traverse the gastrointestinal tract to the colon where they attach to epithelial cells and produce potent cytotoxins called Stx.^{5,11} The toxins are absorbed by the systemic vasculature and are distributed to endothelial cells containing toxin receptors in sensitive tissues, primarily the kidney, colon and central nervous system. The toxins act locally to damage tissue by causing microvascular occlusions to form.¹⁵

Diagnosis of Disease

EHEC infections commonly affect the young and the elderly and are typically diagnosed by a combination of clinical and microbiological findings. Clinical findings may include bloody diarrhoea and kidney failure associated with haemolytic anemia. Microbiological findings include isolation of Stx-producing *E. coli* from stools, or detection of Stx directly in stools or in enrichment cultures of stools (either on selective agar or in broth culture). Toxins can be detected in stools using immunoassays.³ Polymerase chain reaction using toxin-specific primers may also be used to detect toxins indirectly.^{1,3,14,18}

Diagnosis of EHEC infections improves patient care, reduces person-to-person transmission and prevents spread of food-borne outbreaks of disease by facilitating early reporting and trace back activities.⁶

PRINCIPLE OF THE TEST

The Prolisa™ EHEC EIA test is performed by adding diluted samples to stripwells on which rabbit polyclonal anti-Stx1 and Stx2 are bound. The stripwells are incubated at room temperature. A wash is performed to remove unbound material. Monoclonal antibodies specifically recognizing Stx1 and Stx2 are added to the stripwells. After washing, enzyme-conjugated rabbit anti-mouse IgG polyclonal antibody is added and incubated at room temperature. A reactive antibody-enzyme complex is formed if toxin is present. After washing to remove unbound conjugate, substrate is added and incubated for 10 minutes at room temperature. Colour develops in the presence of bound enzyme. Stop solution is added and the results are interpreted spectrophotometrically.

MATERIALS PROVIDED

Component	Cat #	Amount / volume in each kit	Description of each component	Note
Coated and Stabilized Plate	PL.2109	1 plate / pouch	Rabbit polyclonal antibody to Stx1 and Stx2 coated in stripwells	Each pouch contains 1 plate with a sealer and 2 desiccants
EHEC EIA Positive Control	PL.2107	2.5 ml	Recombinant Stx1 (no toxicity) in a buffered protein solution with preservative	Dropper Bottle with Blue Cap
Negative Control	PL.2105	2.5 ml	A buffered protein solution with preservative	Dropper Bottle with White Cap
EHEC EIA Primary Antibody Solution	PL.2108	11 ml	Mouse monoclonal antibodies to Stx1 and Stx2 in a buffered protein solution with preservative	Dropper Bottle with Green Cap
EHEC EIA Secondary Antibody Solution	PL.2106	11 ml	Horse radish peroxidase-labelled rabbit anti-mouse IgG in a buffered protein solution with preservative	Dropper Bottle with Red Cap
20X Wash Buffer	PL.2101	25 ml X 4	Concentrated wash buffer containing detergent and preservative	White Bottle
Stop Solution	PL.2103	14 ml	0.2 N Sulfuric Acid	Dropper Bottle with Yellow Cap
Substrate Solution	PL.2104	14 ml	TMB (3,3',5,5'-tetramethylbenzidine) in a mildly acidic buffer	Amber Bottle
Sample Diluent	PL.2102	30 ml	A buffered protein solution with preservative	White Bottle
Plate Sealer	N/A	3		
Transfer pipette	N/A	100 in 4 bags		
Instruction for Use		1		

MATERIALS REQUIRED BUT NOT PROVIDED

1. Wooden applicator sticks or loop
2. Timer
3. Pipette capable of delivering 100 µl to 1000 µl
4. Pipette tips
5. Test tubes (12 X 75 mm or other suitable size) for dilution of sample
6. Distilled or deionized water
7. Wash bottle or a microplate washer
8. Graduated cylinder
9. EIA microplate reader with 450/630nm absorbance-reading capability

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 conditions after each use.

PRECAUTIONS

1. All reagents are for *in vitro* diagnostic use only.
2. Patient specimens may contain infectious agents. Specimens should be handled at Biosafety Level 2 as recommended in the CDC/NIH manual, "Biosafety in Microbiological and Biomedical Laboratories", (5th Edition -December 2009).
3. Mix all reagents gently before use.
4. Do not interchange reagents from different kit lot numbers.
5. Allow reagents to reach room temperature before use.
6. Reagent vials should be held vertically at a suitable distance above the microplate well to insure proper drop size and delivery.
7. Do not use kit components beyond labelled expiration date.
8. Dispose of used wash buffer and all test materials in an appropriate manner for potentially biohazardous materials.
9. Avoid skin contact with Stop Solution. If contact occurs flush with water immediately.
10. Stripwells are to be used only once.
11. Unused stripwells must be placed back inside the resealable pouch. It is important to protect stripwells from moisture.
12. Transfer pipettes provided are to be used for specimen preparation and transfer. Use one pipette per specimen.
13. Avoid splashing when dispensing diluted samples into microwell by placing transfer pipette tip about halfway into the microwell and dispensing slowly down the side of microwell.
14. Stripwell washing is to be performed precisely as directed in assay procedure. Inadequate washing may cause elevated background and false positive results.
15. All reagents are ready to use except the 20X Wash Buffer.
16. Any deviation above or below set incubation times may affect sensitivity and specificity.
17. Product contains material of animal origin. It should be handled as a potential carrier and transmitter of disease.

REAGENT PREPARATION

1. When the EHEC EIA kit is received, remove the 20X Wash Buffer (4 bottles) and store them at room temperature. Store the other components of the kit at 2-8°C.
2. Stripwells, Positive / Negative Control, Primary Antibody Solution, Secondary Antibody Solution, Stop Solution and Substrate Solution must be allowed to reach room temperature before use.
3. Prepare 1X Wash Buffer from 20X Wash Buffer as required.
For example: To make 500 ml 1X Wash Buffer add 25 ml of 20X Wash Buffer to 475 ml distilled or deionized water. 1X Wash Buffer is stable for three months at room temperature.

SPECIMEN SHIPPING AND HANDLING

On-Site Testing

All samples should be refrigerated at 2-8°C or frozen at -70°C upon collection. Repeated freezing and thawing is not recommended.

1. Stool Samples:

- Refrigerated specimens should be cultured within 1-2 hours. If culturing cannot be performed within 2 hours, place the specimen in Cary-Blair transport media or freeze at -70°C.
- Direct testing samples can be stored for up to 7 days at 2-8°C or frozen at -70°C.

2. Rectal Swabs: Place in Cary-Blair transport media immediately. Refrigerate at 2-8°C if culturing within 2-3 days. If culturing cannot be performed within this time period, freeze at -70°C.

3. Cary-Blair Transport Media – Store up to 3 days at 2-8°C or freeze at -70°C.

Off-Site Testing

Stool specimens intended for further testing and/or confirmation should be prepared and dispatched according to the process recommended by the selected testing centre.

SPECIMEN PREPARATION

Direct Stool

- Add 300 µl of Sample Diluent to a clean 12 X 75 mm test tube (or other suitable test tube).
- Mix stool as thoroughly as possible prior to pipetting.
 - Liquid, semi-solid stools or stools in transport medium: Using a transfer pipette, draw stool to the first calibration point (100 µl). Dispense the stool into the Sample Diluent. Using the same pipette, gently withdraw and expel the stool suspension several times. Leave the transfer pipette in tube for later use.
 - Solid stools: Using a wooden applicator stick or an inoculating stick/loop, transfer a small portion (~3-4 mm diameter, ~50 mg) of stool into 300 µl Sample Diluent and mix completely. Place a transfer pipette in the tube.

Enrichment Broth

- Add 50 µl of stool to 5 ml of MacConkey Broth or GN Broth.
- Incubate 16-24 hours at 37°C in a incubator.
- Add 100 µl of broth culture to 300 µl Sample Diluent in a clean 12 X 75 mm test tube. Leave the transfer pipette in the tube.

Note:

- Broths may be held up to seven days at 2-8°C before testing in the Prolisa™ EHEC EIA. If testing is not performed within this time period, the broth should be frozen at -70°C. Repeated freeze-thaw should be avoided.

Selective Enrichment Agar

- Add 20 µl of stool to a Sorbitol-MacConkey or MacConkey agar plate and spread the sample with a sterile loop.
- Incubate 16-24 hours at 37°C.
- Suspend individual colonies or colony sweeps in 200 µl of Sample Diluent in a clean 12 X 75 mm test tube.

TEST PROTOCOL

Note:

- The entire kit, including plate pouch, should be brought to room temperature before use.
- Three (3) free-falling drops of Positive Control, Negative Control, Primary Antibody Solution, Secondary Antibody Solution and Stop Solution from the dropper bottles in the kit are equivalent to 100 µl of each component.

- Cut the re-sealable foil pouch, and carefully remove the EIA plate from the pouch.
- Remove the sealing tape from the required number of stripwells. Unused stripwells must be resealed in the pouch immediately and stored at 2-8°C.
- Use a transfer pipette to transfer 100 µl (equivalent to the first calibration point of the pipette) diluted specimen to the wells, and add 100 µl of Positive Control and Negative Control to the appropriate wells. Incubate for 60 minutes at room temperature without shaking.
- Dilute the 20X Wash Buffer to 1X Wash Buffer with distilled or deionized water [50 ml 1X Wash Buffer is required for each strip (8 wells)].

- Discard the specimens/controls from the strip(s) and wash the wells 5 times with 1X Wash Buffer.

Option 1

- 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) four additional times.
- After the last fill, discard contents and strike the plate firmly on fresh paper towels to remove excess Wash Buffer.

Option 2

- Wash plate with an automated microplate washer 5 times by filling the wells with 300 µl of 1X Wash Buffer.
- Add 100 µl (3 drops) of Primary Antibody Solution to each well. Incubate for 30 minutes at room temperature without shaking.
 - Discard the Primary Antibody Solution and wash the wells 5 times with Wash Buffer (Repeat wash procedure from step 5).
 - Add 100 µl (3 drops) of Secondary Antibody Solution to each well. Incubate for 30 minutes at room temperature without shaking.
 - Discard the Secondary Antibody solution and wash the wells 5 times with Wash Buffer (Repeat wash procedure from step 5).
 - Add 100 µl of Substrate Solution to each well and incubate at room temperature for 10 minutes.
 - Add 100 µl (3 drops) of the Stop Solution to each well. Mix gently by lightly tapping the side of the Strip Holder.
 - Measure the OD450/630nm in a microplate reader immediately after completion of Step 11.

EXPECTED VALUES

The Prolisa™ EHEC EIA test detects the presence of Stx1 and/or Stx2. Expected values for a given population should be determined for each laboratory. The rate of positivity may vary depending on patient age, geographic location, method of specimen collection, handling and transportation, test employed and general health of the patient population under study.

QUALITY CONTROL PROCEDURES

- The Positive and Negative Controls are to be used with each assay run to provide quality assurance of the reagents.
- The Positive Control should read > 0.800 at 450/630nm.
- The Negative Control should read < 0.150 at 450/630nm but greater than 0.000. If the Negative Control is <0.000, re-blank the plate reader to air and re-read the plate.
- Any positive well without visible colour should be repositioned, wiped on the underside of the well and re-read.
- The kit components should be visually examined for obvious signs of microbial contamination, freezing or leakage at the time of each use.
- Results of each quality control check should be recorded in a log book to maintain high quality testing records.

INTERPRETATIONS OF RESULTS

Spectrophotometric Dual Wavelength (450/630nm)

Negative = OD450/630nm < 0.150

Positive = OD450/630nm ≥ 0.150

NOTE: A positive result indicates the presence of Stx1 and/or Stx2. A negative result indicates the absence of Shiga toxins, or that the level of toxin is below that which can be detected by the test.

LIMITATIONS OF THE PROCEDURE

- The Prolisa™ EHEC EIA test detects the presence of Stx1 and/or Stx2. The level of toxin has not been shown to be correlated with either the presence or severity of disease. As with all *in vitro* diagnostic procedures, test results should be interpreted by a physician in conjunction with other clinical information.
- A positive result does not preclude the presence of other infectious organisms.

PERFORMANCE CHARACTERISTICS

Sensitivity and Specificity

The Pro-Lab Prolisa™ EHEC EIA was evaluated at two hospitals and one reference laboratory in the United States and Canada. Samples were tested by both the Pro-Lab Prolisa EHEC EIA and a globally-marketed alternative EHEC EIA.

Samples were considered to be positive if the Verocell Cytotoxicity Assay (VCA) was positive. Samples were considered negative if the VCA was negative. The combined results of these studies are shown in the following table.

Performance	Direct Stool Assay		Enrichment Broth Assay	
	Prolisa™ EHEC EIA	Alternative EHEC EIA	Prolisa™ EHEC EIA	Alternative EHEC EIA
Sensitivity	56.7% (17/30)	56.7% (17/30)	86.5% (32/37)	70.3% (26/37)
Specificity	96.7% (502/519)	94.0% (488/519)	98.6% (493/500)	99.6% (498/500)
Overall	94.5% (519/549)	92.0% (505/549)	97.8% (525/537)	97.6% (524/537)

Assay Precision

Intra-assay Variability

Eight replicates of four different samples, spiked with differing amounts of Stx1 and Stx2 alone and in combination, were tested to determine intra-assay variability.

Sample	Mean OD 450/630 nm	SD	CV
Stx1 and Stx2	1.317	0.038	2.9%
Stx1	0.203	0.001	0.5%
Stx2	0.366	0.004	1.1%
No toxin	0.033	0.004	12.1%

Inter-assay Variability

Eight replicates of four different samples, spiked with differing amounts of Stx1 and Stx2 alone and in combination, were tested in a single assay on three different days to determine inter-assay variability.

Sample	Mean OD 450/630nm	SD	CV
STx1 and STx2	1.284	0.032	2.5%
STx1	0.204	0.004	1.8%
STx2	0.354	0.011	3.3%
No toxin	0.033	0.001	4.6%

Analytical Sensitivity

The Pro-Lab Prolisa™ EHEC EIA detects approximately 20-40 pg/ml of Stx1 and <20 pg/ml of Stx2.

Selective Enrichment Agar

Human isolates of twelve common serotypes producing Stx1 only (n=4), Stx2 or Stx2c only (n=6), or both Stx1 and Stx2 (n=2) and a non-toxicogenic *E. coli* reference strain were grown overnight at 37°C in Tryptic Soy Broth. A loopful of the broth culture was streaked for single colonies onto MacConkey agar and Sorbitol-MacConkey agar plates and the plates were incubated 16-24 hours at 37°C. Each of two single colonies from each agar was suspended individually in Sample Diluent and then tested in the EIA. All colonies of Stx-producing *E. coli* from both agars were positive in the test. All colonies from the non-toxicogenic *E. coli* reference strain from both agars were negative in the test.

Cross Reactivity

The following clinical isolates (CI) or reference strains were tested for cross reactivity and all were found to be negative.

Organism	ID
<i>Aeromonas hydrophila</i>	Clinical isolate (CI)
<i>Arcobacter butzleri</i>	CI
<i>Bacillus cereus</i>	ATCC 14179
<i>Bacillus subtilis</i>	ATCC 6051
<i>Campylobacter coli</i>	CI
<i>Campylobacter fetus</i>	CI
<i>Campylobacter jejuni</i>	ATCC 29428
<i>Citrobacter braakii (freundii)</i>	ATCC 43162
<i>Clostridium difficile</i>	CI
<i>Enterobacter aerogenes</i>	CI
<i>Enterobacter cloacae</i>	ATCC 13047
<i>Enterococcus faecalis</i>	ATCC 49149
<i>Escherichia coli non STEC</i>	ATCC 25922
<i>Escherichia coli O55:NM, EPEC</i>	ATCC 12014
<i>Escherichia coli O111:NM, ETEC/EPEC</i>	ATCC 43887
<i>Escherichia coli O124, EIEC</i>	ATCC 43893
<i>Escherichia hermannii</i>	ATCC 33650
<i>Klebsiella pneumoniae</i>	ATCC 27736
<i>Proteus vulgaris</i>	ATCC 33420
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Salmonella enteritidis</i>	CI
<i>Salmonella typhimurium</i>	CI
<i>Serratia marcescens</i>	CI
<i>Serratia liquefaciens</i>	ATCC 27592
<i>Shigella dysenteriae</i>	ATCC 49347
<i>Shigella flexneri</i>	ATCC 25929
<i>Shigella sonnei</i>	ATCC 25931
<i>Staphylococcus aureus</i>	ATCC 25923
<i>Vibrio parahaemolyticus</i>	CI
<i>Yersinia enterocolitica</i>	ATCC 23715

Interference Studies










Substances that might be expected to be present in stool samples were analysed to determine their effects on the Pro-Lab Prolisa™ EHEC EIA. The results of the study are presented in the following chart:



Interfering Substance	Buffer	Stool	Stool +Stx1	Stool +Stx2
Barium sulphate	negative	negative	positive	positive
Blood	negative	negative	positive	positive
Cary-Blair	negative	negative	positive	positive
Imodium	negative	negative	positive	positive
Kaopectate	negative	negative	positive	positive
PeptoBismol	negative	negative	positive	positive

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	= Use by
	= Lot number
	= Catalogue number
	= Manufacturer
	= Authorized Representative in the European Community
	= Contains sufficient for <n> tests
	= In vitro diagnostic medical device
	= Temperature limitation
	= Consult instructions for use

PL.2001 PL.2101	 Warning May cause an allergic skin reaction. Wear protective gloves. Avoid breathing vapor. Contaminated work clothing should not be allowed out of the workplace. IF ON SKIN: Wash with plenty of soap and water. Take off contaminated clothing and wash it before reuse. If skin irritation or rash occurs: Get medical attention. Dispose of contents and container in accordance with all local, regional, national and international regulations.
PL.2103	 Danger Causes severe skin burns and eye damage. Wear protective gloves. Wear protective clothing. Wear eye or face protection. Wash hands thoroughly after handling. IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER or physician. IF SWALLOWED: Immediately call a POISON CENTER or physician. Rinse mouth. Do NOT induce vomiting. IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water. Wash contaminated clothing before reuse. Immediately call a POISON CENTER or physician. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician. Store locked up. Dispose of contents and container in accordance with all local, regional, national and international regulations.