Department of Microbiology
Shiga Toxin Test Procedure (Immuno*Card* STAT! EHEC)

I. Purpose and Test Principle

Shiga toxin–producing Escherichia coli (STEC), also referred to as enterohemorrhagic *E. coli* (EHEC), are a leading cause of bacterial enteric infections. Prompt and accurate diagnosis of STEC infection is important because appropriate treatment early in the course of infection might decrease renal damage and improve patient outcome. Prompt laboratory identification of STEC strains is also essential for effective and timely outbreak responses and control measures. The Centers for Disease Control (CDC) recommends that all stools submitted for routine testing from patients with acute community-acquired diarrhea (regardless of patient age, season of the year, or presence or absence of blood in the stool) be simultaneously cultured for *E. coli* O157:H7 (O157 STEC) and tested with an assay that detects shiga toxins, in order to detect non-O157 STEC.

Immuno*Card* STAT! EHEC is an immunochromatographic rapid test for the qualitative detection of Shiga toxins 1 and 2 (ST1 and ST2) produced by *E. coli* in cultures derived from clinical stool specimens. The test utilizes monoclonal antibodies labeled with red-colored gold particles. The device has a circular sample port and an oval-shaped test and control window. The sample is applied to the chromatography paper via the circular sample port. The sample is absorbed through the pad to the reaction zone containing colloidal, gold-labeled antibodies specific to Shiga Toxins. Any Shiga toxin antigen present complexes with the gold-labeled antibody and migrates through the pad until it encounters the binding zones in the test (Toxin 1, Toxin 2) area. The binding zones contain another anti-ST1 or -ST2 antibody, which immobilizes any Shiga toxin-antibody complex present. Due to the gold labeling, a distinct red line is then formed. The remainder of the sample continues to migrate to another binding reagent zone within the control zone, and also forms a further distinct red line (positive control). Regardless of whether any Shiga toxin is present or not, a distinct red line should always be formed in the control zone and confirms that the test is working correctly.

II. Specimen Information

Collect stool sample in a clean, leak proof plastic container. If transportation time to the laboratory will exceed 2 h from time of collection, specimen should be refrigerated or placed in enteric transport medium (Modified Cary-Blair).

III. Reagents & Equipment

Kit components (store at 2 – 8° C when not in use)

- Immuno*Card* STAT! EHEC test devices are stable until the expiration date printed on the box when stored at 2 – 8° C. The test device should be used within 15 min after removal from the sealed foil pouch.
- Sample Diluent (Negative Control) Positive Control
- Disposable plastic transfer pipettes (150 µL)

Other Materials Needed

- Gram-Negative (GN) Broth
- Sterile swabs
- Aerobic incubator set at 35 ± 2° C
- Timer
- Personal protective equipment

IV. Procedure

A. Specimen Processing

1. Upon receipt, verify that the specimen was submitted in an appropriate transport device.
2. Accession the specimen using the appropriate workpar. The stool culture tests that require Shiga toxin testing include CSTLST, CSTLYS, and CECST.
3. Label the appropriate stool culture media and a GN broth tube. Write the current time on another accession label. This label should be placed on the cap of the GN broth tube after it is inoculated.
4. Using a sterile swab, mix the specimen thoroughly regardless of consistency. Ream off excess stool by rolling the swab against the wall of the collection container. Inoculate plated media and then the GN broth. DO NOT OVERINOCULATE the GN broth with excessive specimen.
5. Incubate inoculated broth <u>with caps loose</u> at 35 ± 2° C for 16 – 24 h.

B. Preparation for the Assay

Visually observe the broth tubes for growth. DO NOT PROCEED with testing if the broth tube does not exhibit growth after incubation as falsely negative results may occur.

- If the MacConkey agar plate in the stool culture set-up has growth, retrieve the original specimen and reset the GN broth.
- If the MacConkey agar and GN broth are both no growth, report the Shiga toxin results as negative. The lack of growth on the MacConkey agar plate indicates that there are no viable STEC present. Since the assay is not performed, credit the billing for the Shiga toxin test (SHIGCR).

Use only broth cultures that exhibit growth in the following steps.

1. Using the dropper vial, add five drops (150 µL) of Sample Diluent Buffer to a small test tube.
2. Mix broth culture thoroughly by tightening the loose cap and then gently inverting the tube.
3. Using the transfer pipette supplied with the kit, add 150 µL of sample (second mark from tip of pipette) to the tube containing Sample Diluent.

4. Gently mix the contents of the tube with the transfer pipette by squeezing the pipette bulb 3 times. Leave the transfer pipette in the tube for later use.
5. The diluted broth culture can be stored for up to 30 min at 20 – 25° C before testing.

C. Assay Procedure

1. Bring all test devices, reagents and samples to room temperature before testing.
2. Use one test device for each patient sample.
3. Remove the test device from its foil pouch. Label the device with the patient's identification.
4. Using the transfer pipette provided in the kit, add 150 µL of the diluted specimen (second mark from tip of pipette) to the sample port of the device.
5. Incubate the test at room temperature (20 – 25° C) for 20 min.
6. Read the results within 1 min after the end of incubation.

V. Interpretation, Reporting, Reference Testing

A. Negative test

1. Interpretation
   A negative test is indicated by a pink-red band at the Control line position with no other bands present.
2. Report
   Negative for Shiga Toxin 1 [ST1N]
   Negative for Shiga Toxin 2 [ST2N]

B. Positive test for Shiga toxin 1 and/or 2

1. Interpretation
   A pink-red band at the Control and Toxin 1 line and/or the Toxin 2 line positions indicates a positive test for ST1 and/or ST2. The appearance of a Toxin 1 or 2 test line, even if very weak, indicates the presence of ST1 or ST2. The intensity of the test line can be less than that of the Control line.
2. Report
   Positive for Shiga Toxin 1 [ST1P] and/or
   Positive for Shiga Toxin 2 [ST2P]
   The following comments should be attached to a positive Shiga toxin report:
   - **Antimicrobial therapy in patients infected with Shiga Toxin-producing *E. coli* is not recommended as it may increase risk of serious complications such as hemolytic-uremic syndrome. [STPS]**
   - **This is a REPORTABLE DISEASE. Please contact your County/State Health Department. [RPT2]**
   - **Sent to state public health laboratory for confirmatory testing. [STCONF]**

C. Positive test for Shiga toxin with no *E. coli* O157 isolated

1. Interpretation
   When the Shiga toxin assay is positive for ST1 or ST2 and the O157 culture is negative, it suggests that a non-O157 STEC strain may be present.
2. Preliminary Report – day 1
   Report Shiga toxin results as outlined above. DO NOT enter No enteric pathogens to date.
3. Preliminary Report – day 2
   If no other enteric pathogens are isolated, report **No Salmonella, Shigella, Campylobacter or E coli 0157 Isolated. [NSSCE]**
4. Final Report
   Do not finalize report until confirmation of STEC typing has returned from state lab.
5. Reference Testing
   If the Shiga toxin assay is positive but no O157 is isolated, send the GN broth to the appropriate state lab for confirmatory testing.

D. Invalid Test Results

If any result is difficult to interpret, the test should be repeated with the same sample to eliminate the potential for error. If possible, obtain a new sample, and retest when the original sample repeatedly produces uninterpretable results.

The test is not valid and results should not be reported if:

1. No band at the designated position for the Control line indicates that the test is invalid since the absence of a control band indicates the test procedure was performed improperly or that deterioration of reagents has occurred.
2. A pink-red band appearing at either the Toxin 1 or Toxin 2 test line position of the device after the defined incubation limit or a band of any color other than pink-red. Falsely positive results may occur if tests are incubated too long. Bands with colors other than pink-red may indicate reagent deterioration.

VI. Quality Control

At the time of each use, kit components should be visually examined for obvious signs of microbial contamination, freezing, or leakage. Do not use contaminated or suspect reagents.

A. **Internal controls**

Internal controls are contained within the test strip and therefore are evaluated with each test. A pink-red band appearing at the Control line serves as a procedural control and indicates the test has been performed correctly, that proper flow occurred and that the test reagents were active at the time of use. A clean background around the Control or Test lines also serves as a procedural control. Control or test lines that are obscured by heavy background color may invalidate the test and may

be an indication of reagent deterioration, use of an inappropriate sample or improper test performance.

B. **External controls**

New lots and/or shipments should be checked using the same lot of control material that was used to check the old lot. This is accomplished by saving a specific lot of control materials from a shipment of kits and then using that lot of controls for testing subsequent lots/shipments that are received. The control materials may be used until the manufacturer's expiration date printed on the bottle. Document the control material lot number used for QC on the Package Insert Verification log.

Quality control testing using external controls should be performed by following the procedure for testing patient samples. If external controls fail to produce the expected results, notify the supervisor and/or technical specialist. Lots and/or shipments that do not perform as expected cannot be used for patient testing. All results from external controls should be documented in LIS.

External Quality Control Procedure

1. Bring all test devices and reagents to room temperature (20-25° C) before testing.
2. Use one Immuno*Card* STAT! EHEC test device each for a positive and negative control.
3. Remove the Immuno*Card* STAT! EHEC test device from its foil pouch. Label the device with the control to be tested.
4. Add exactly 5 drops of the Positive Control reagent to the sample port of a device marked for the positive control.
5. Add exactly 5 drops of the Sample Diluent to the sample port of a device marked for the negative control.
6. Incubate the test at 20 – 25° C for 20 min.
7. Read the results within 1 min after the end of incubation.

Quality Control Results

The positive control should yield pink-red bands in both Toxin 1 and Toxin 2 test line positions. The negative control should not yield bands in either of the Toxin 1 or Toxin 2 test line positions. A pink-red band must be present at the internal control test line for the results to be valid.

VII. Limitations

A. The test is qualitative and no quantitative interpretation should be made with respect to the intensity of the positive line when reporting the result.
B. Test results are to be used in conjunction with information available from the patient clinical evaluation and other diagnostic procedures.
C. Failure to add 150 µL of broth culture to the 5 drops of Sample Diluent will lead to falsely negative results.

D. The addition of more than 5 drops of Sample Diluent can also lead to falsely negative test results.
E. Over incubation of tests may lead to false-positive test results. Incubating tests at reduced temperatures or times may lead to falsely negative results.
F. The performance of Immuno*Card* STAT! EHEC has not been evaluated with direct stool samples. The manufacturer has only evaluated its performance with SMAC plate culture or GN and MacConkey liquid cultures. The GN broth method is the only method validated for use in the PSHMC Microbiology lab.
G. Shiga toxin 1 produced by *E*. *coli* and the toxin produced by *Shigella dysenteriae* type 1 strains are nearly identical. Therefore, Immuno*Card* STAT! EHEC may give a positive result with toxins from *S. dysenteriae* type 1 strains.

VIII. Verification of Test Method

In this study, three different assays were evaluated for use. This included two EIA-based products, the Premier EHEC (Meridian Bioscience, Inc.) and ProSpecT Shiga Toxin (Remel). The EIA test results were interpreted visually. The third product is an immunochromatographic product, the ImmunoCard STAT! EHEC (Meridian Bioscience, Inc.).

In the first phase of the evaluation, 17 stool specimens that were submitted for routine culture were tested using all three Shiga Toxin assays. This included 7 fresh samples and 10 that were submitted in modified Cary-Blair (CB) transport medium. Each stool specimen was sampled using a sterile swab which was then used to inoculate a GN broth tube. The GN broths were incubated with loose caps overnight in an aerobic incubator at 35 ± 2°C. The GN broths were then used to perform the Shiga toxin assays. A total of 4 (24%) of the stool samples yielded *E. coli* O157 by culture with BD BBL™ CHROMagar™ O157. A total of 3 (18%) stool samples yielded positive results by all three toxin assays. One (6%) stool sample that was culture positive yielded false-negative results on the Shiga toxin assays. A MacConkey-Sorbitol culture of this stool specimen grew abundant fecal flora that completely obscured the clear colonies. The CHROMagar culture for this sample yielded only a few colonies of *E. coli* O157. Subcultures of the GN broth revealed that the *E. coli* O157 was still in proportionally low numbers with an abundance of enteric flora. To further evaluate this isolate, a colony was picked from the CHROMagar plate and inoculated into another GN broth. Testing of this broth yielded positive toxin results with all three assays. This proved that the isolate was a toxigenic strain. Presumably, it was not detected in the original GN broth cultures because the other enteric flora were able to outgrow the low numbers of the O157. The results from this phase of the study are summarized in Table 1 below.

Table 1: Evaluation of Clinical Specimens

	O157 Culture		Meridian Card		Meridian EIA		Remel EIA	
	n	%	n	%	n	%	n	%
Fresh Positive	3	18	2	12	2	12	2	12
CB Positive	1	6	1	6	1	6	1	6
Total Positive	**4**	**24**	**3**	**18**	**3**	**18**	**3**	**18**
Fresh Negative	6	35	7	41	7	41	7	41
CB Negative	7	41	7	41	7	41	7	41
Total Negative	**13**	**76**	**14**	**82**	**14**	**82**	**14**	**82**

Two of these O157 isolates were identified by the Washington State Public Health Laboratory as type H7, and the other two were identified as the non-motile strain, NM.

In the second phase of the study, 34 additional clinical STEC isolates were tested by seeding each isolate into a STEC-negative, pooled stool matrix. This included 14 *E. coli* O157 isolates from stock cultures and 20 non-O157 isolates that were obtained from the Washington State Public Health Laboratory. The O157 strains included one non-motile strain and 13 that were type H7. The non-O157 strains included a variety of serotypes, including three O26, two O111, one O103, and 14 undefined strains.

A 0.5 McFarland (approximately 10^8 CFU/mL) suspension was prepared from 24-h BAP growth of each test strain. Each suspension was then diluted 1:10 with saline to yield a concentration of approximately 10^7 CFU/mL. 100 μL of each suspension was then added to separate tubes containing 900 μL of the stool matrix to yield a final concentration of 10^6 CFU/mL of STEC. Each test suspension was vortexed and then sampled using a sterile swab which was used to inoculate individual GN broth tubes. The GN broth tubes were incubated with loose caps overnight in an aerobic incubator at 35 ± 2°C. All 14 (100%) of the *E. coli* O157 suspension cultures yielded positive results with the Shiga Toxin assays. A total of 19 (95%) of the non-O157 suspension cultures yielded positive Shiga Toxin results with the ImmunoCard STAT! EHEC assay. All 20 (100%) of the non-O157 suspension cultures yielded positive toxin results by both EIA products. However, 1 of these cultures was very weakly positive with both EIA tests. This was the same culture that yielded a negative result by the ImmunoCard assay. A new seeded specimen was prepared with this isolate and cultured in GN broth as before for repeat testing. The second cultured suspension yielded weak results by all three toxin assays. The results from this phase of the study are detailed in Table 2 below.

Table 2: Evaluation of STEC Seeded Stool Specimens (% Positive)

Serotype	# of isolates	Meridian Card	Meridian EIA	Remel EIA
O157	14	100	100	100
non-O157	20	100*	100	100

*1 non-O157 isolate yielded a negative initial result and a weak positive after repeat testing

For the ImmunoCard STAT! EHEC assay, 9 of the O157 strains were positive for both ST1 and ST2 and 5 strains were positive for ST2 only. A total of 18 of the non-O157 strains were positive for ST1 only, 1 strain was positive for ST2 only, and 1 strain was positive for both ST1 and ST2.

Table 3 below summarizes the data from the first two phases of the evaluation study. One of the clinical samples yielded a false-negative Shiga toxin result with all 3 assays. One of the STEC-seeded samples containing a non-O157 strain was initially negative by the ImmunoCard STAT! EHEC assay and weakly positive by both EIA assays. Repeat testing with the non-O157 strain yielded weak Shiga toxin results with all 3 assays.

Table 3: Data summary for clinical specimens and STEC-seeded stools.

	Negative Shiga Toxin	Positive Shiga Toxin	Total
Negative STEC Culture	13	1	14
Positive STEC Culture	0	37	37
Total	13	38	51

% Agreement = 98.0% (89.7 to 99.7%)
Positive Agreement = 97.4%
Negative Agreement = 100%

The third phase of the evaluation study examined specimen stability. Three specimens were available in pairs of fresh and modified Cary-Blair specimens. Initial colony counts were performed on the specimens. The culture plates were refrigerated to serve as reference comparisons later. The paired specimens were left at room temperature for 7 days after which colony count testing was repeated. The culture plates from the 7-day-old specimens were compared to the initial colony count plates. No difference in the number of colonies recovered was observed when comparing fresh vs. modified Cary-Blair specimens. All 3 fresh and 3 Cary-Blair specimens yielded positive toxin results when cultured in GN broth and when subsequently tested with the three Shiga Toxin assays.

As all 3 Shiga toxin assays performed essentially equally well, the assay selection decision was based on a balance of product and labor costs, along with the feasibility of incorporating the testing into the existing operational work flow in the Microbiology department. While the ImmunoCard STAT! EHEC was the most expensive product of the three, it also required the least amount of technical hands-on time. Each ImmunoCard STAT! EHEC test requires approximately 1 min of hands-on time to perform the test. The EIA products require several wash and incubation steps. The hands-on time is

about 30 – 45 min, but the actual time that a technologist is committed to the assay is about 2 h.

IX. References

A. Package insert: Meridian Immuno*Card* STAT! EHEC, Rev. 01/07.

B. CDC. Recommendations for Diagnosis of Shiga Toxin–Producing *Escherichia coli* Infections by Clinical Laboratories. MMWR 2009;58/RR–12.

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