Identification Guide

Isolation and Identification of Shiga Toxin-Producing *Escherichia coli* from Culture-independent Diagnostic Test Positive Specimens

APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES®

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Contents

Introduction

Purpose

A recommended workflow for the isolation and identification of Shiga toxin-producing *Escherichia coli* (STEC) from human fecal specimens identified as positive for STEC by a culture-independent diagnostic test (CIDT).

Background

The following is a workflow for the isolation and identification of STEC from human feces. STEC is any *E. coli* that contains either or both of the Shiga toxin genes (*stx*$_1$ and *stx*$_2$). STEC can cause severe or fatal infections. Sensitive and specific laboratory methods for the isolation, identification, serotyping and subtyping of STEC are key to monitoring and control efforts. Advances in clinical diagnostic testing have led to the use of CIDTs which are used to provide rapid and sensitive testing for enteric pathogens. While CIDTs are a benefit to patient care, they also come with a caveat that cultures for further characterization are not available for public health testing and surveillance. CIDTs thus have impacted the ability of public health laboratories to monitor the burden of STEC infections and identify and prevent foodborne illness outbreaks. In recent years, public health laboratories have received a drastic increase in primary specimens tested at the clinical laboratory using a CIDT platform for enteric pathogens. In many cases, the stool has tested positive for STEC at a clinical laboratory using a CIDT before submitting the stool specimen to a public health laboratory for culture and further characterization. The goal of this document is to provide public health laboratories with methods to isolate and identify STEC from stool specimens as efficiently as possible.

Fecal specimens are the preferred laboratory samples for diagnosis of infectious diarrhea. The recovery of enteric pathogens from feces is often complicated by multiple factors including prior antibiotic treatment, transport stress, intermittent shedding of pathogens in the feces, and a low abundance of STEC bacteria in relation to other enteric flora. These factors necessitate the use of culture algorithms that employ selective enrichment and the use of selective and differential media.

Selective enrichment suppresses fecal flora while allowing the target pathogen to grow. Selective media can also utilize various phenotypic characteristics to preliminarily differentiate potential pathogens from fecal flora.

The number of media and reagents used to identify STEC continues to expand. It is important to review literature and manufacturer product announcements. The manuscript by Parsons *et. al.* provides a robust review of the performance of many methods and is a valuable resource for STEC testing algorithm development and evaluation.[1]

Specimen Collection and Transport

The preferred specimen for STEC culture is fresh stool collected in transport media such as Cary-Blair (CB).[2] Stool in Gram Negative (GN) broth is commonly submitted and is acceptable. Unpreserved stool and fecal or rectal swabs submitted in non-transport media may also be acceptable; however, these samples should be considered for rejection if transit time exceeds limits stated by the receiving laboratory (often two hours after specimen collection[3]). If the unpreserved sample cannot reach the laboratory within the specified time, it is recommended that the raw stool cultures be transferred into a non-nutritive buffered transport medium (such as Cary-Blair) and stored at 4°C to preserve pathogen viability.[3] Rectal swabs are not a preferred sample and should only be utilized when the patient cannot produce a fecal sample. The rectal swab should be examined after collection; fecal matter should be visible on the swab. Suspect specimens for STEC culture collected in CB transport media, GN broth, or CB swabs can be shipped at room temperature.[3] However, if extremely high temperatures are anticipated, ice packs should be used. Ideally, specimens should be received as quickly as possible, not exceeding four days since collection, as isolate recovery may decline. At the public health laboratory, STEC testing should begin on the day of receipt or as soon as reasonably possible. Specimens should be held at 2–8°C until culture is completed.

Materials and Supplies

Media

- Selective/Differential Media (use at least one selective media): Sorbitol-MacConkey agar with cefixime-tellurite (CT-SMAC), STEC CHROMagar (Note: There may be multiple manufacturers of some media. Please refer to the instructions for use for each medium).
- Less-selective Media (use at least one non-selective media): Washed Sheep Blood with (WSBA) Agar, Washed Sheep Blood with Mytomycin C and $CaCl_2$ Agar (SHIBAM), Sorbitol-MacConkey Agar (SMAC), Chromogenic Agar (BCM O157 by Biosynth), MacConkey Agar (MAC), Blood Agar Plate (BAP).
- Enrichment broths: Gram-negative broth (GN), Trypticase Soy broth (TSB), MacConkey broth.
- Biochemicals for identification: Indole, Methyl Red, Voges-Proskauer, Citrate (For a full list of biochemicals, see **Appendix E: Typical Results of Biochemicals for *E. coli* on page 13**).
- Before using media, review the manufacturer's instructions to determine appropriate incubation times and organisms for media quality control. See **Serotyping Considerations (page 7)** for information about why specific media were included in the workflow.
- All STEC testing using commercial reagents should be performed according to the manufacturer's recommendations.

Other Supplies

- PCR reagents for detection of Shiga toxin genes by PCR (see **Appendix F** for information on detection of virulence genes by PCR)
- Inoculating loops
- Applicator swabs
- Supplies for MALDI-TOF (if performed)
- *E. coli* O157 Latex Test (multiple manufacturers), if performed

Culture and Identification Workflows

Standard Version

See "Appendix A: Standard Flow Diagram for Isolation and Identification of STEC" (page 9).

Day 0

Stool specimen collected in transport media is received at the public health laboratory.

1. Inoculate at least one of the following selective media: CT-SMAC, STEC CHROMagar (**Step 1** in Appendix A).
2. Inoculate one of the following less selective media: WSBA, SMAC, BCM, MAC (**Step 1** in Appendix A). Some STEC variants may grow better on less selective media. The use of an enrichment broth increases sensitivity and may be helpful where maximal sensitivity is required, such as an older specimen, a specimen of very high importance (e.g. outbreak investigation), or when a case has hemolytic uremic syndrome (HUS).[4] See workflow in Appendix B for a description of the steps involved in this workflow.

Day 1

Perform *stx*$_1$/*stx*$_2$ PCR on at least two suspicious colonies (see Appendix C, Appendix D and Appendix E for a description and images of suspicious colonies on the different media) as well as testing a loopful of bacteria from multiple quadrants (plate sweep) from all primary media used (**Step 2** in Appendix A).

1. If an individual colony is positive for *stx*$_1$, *stx*$_2$ or both *stx*$_1$ and *stx*$_2$, perform *E. coli* O157 latex agglutination (**Step 3** in Appendix A) and subculture to a BAP.
   - Testing for *E. coli* O157 can be performed on isolates from the primary media. It is important to read the package insert from the latex agglutination test kit to determine that the media is compatible with the assay. Subculture the isolated positive colony to a BAP (**Step 4** in Appendix A) for WGS and species identification (**Step 5** in Appendix A).
   - If the isolate is determined to be serotype O157, it can be reported according to the laboratory standard operating procedure. If the isolate is negative for O157, perform WGS on the isolate to determine the serotype.
2. If all *stx*$_1$/*stx*$_2$ PCRs are negative, report as negative (**Step 10** in Appendix A).
3. If the plate sweep tests positive for *stx*$_1$ or *stx*$_2$ but no colonies test positive for *stx*$_1$/*stx*$_2$ then:
   - Perform *stx*$_1$/*stx*$_2$ PCR on at least five additional isolated colonies from the plate that tested positive for *stx*$_1$ or *stx*$_2$ (**Step 6** in Appendix A). Note that some PHLs may opt to skip this step by performing *stx*$_1$/*stx*$_2$ PCR on additional colonies in **Step 6**.
   - If all five isolated colonies test negative, report as sweep PCR positive for *stx*$_1$ or *stx*$_2$, no isolate containing *stx*$_1$ or *stx*$_2$ was identified (**Step 11** in Appendix A).
   - One strategy to increase chances of identifying a *stx* positive isolate is to inoculate a BAP with growth from quadrant two and three of the plate that was sweep PCR positive. After overnight incubation, pick five isolated colonies of growth for PCR.
   - If a colony is positive for *stx*$_1$ or *stx*$_2$, perform *E. coli* O157 latex agglutination (**Step 7** in Appendix A) and subculture the isolated colony to a BAP (**Step 8** in Appendix A) for species identification and WGS (**Step 9** in Appendix A) the following day.
   - If the isolate is determined to be serotype O157, it can be reported according to the laboratory standard operating procedure. If the isolate is negative for O157, perform WGS on the isolate to determine the serotype.
   - Note: Depending on timing, some activities may occur the next day.

Day 2

If a colony tested positive for *stx*$_1$ or *stx*$_2$ (**Step 2** in Appendix A): From the sub-cultured BAP, perform species identification (**Step 9**) to confirm that it is an *E. coli* isolate (see Appendix F for a list of methods to identify *E. coli*) and perform WGS immediately (**Step 9** in Appendix A).

Optional Enrichment Version

See "Appendix B: Optional Enrichment Flow Diagram for Isolation and Identification of STEC" (page 10).

Day 0

Stool specimen collected in transport media is received at the public health laboratory.

1. Inoculate one of the following enrichment broths: TSB, GNB, MAC (**Step 1** in Appendix B).
   - Note: Enrichment broth may occasionally be overgrown with commensal bacteria. In such instances it may be necessary to plate directly from the stool (if this was not already done).

Day 1

1. Inoculate at least one of the following selective media: CT-SMAC, STEC CHROMagar (**Step 2** in Appendix B).
2. Inoculate one of the following less selective media: WSBA, SMAC, BCM, MAC (**Step 2** in Appendix B). Some STEC variants may grow better on less selective media.

Day 2

Perform *stx*$_1$/*stx*$_2$ PCR on at least two suspicious colonies (see Appendix C, Appendix D and Appendix E for a description and images of suspicious colonies on the different media) as well as testing a loopful of bacteria from multiple quadrants (plate sweep) from all primary media used (**Step 3** in Appendix B).

1. If an individual colony is positive for *stx*$_1$, *stx*$_2$ or both, perform *E. coli* O157 latex agglutination (**Step 4** in Appendix B) and subculture to a BAP.
   - Testing for *E. coli* O157 can be performed on isolates from the primary media. It is important to read the package insert from the latex agglutination test kit to determine that the media is compatible with the assay. Subculture the isolated positive colony to a BAP (**Step 5** in Appendix B) for WGS and species identification.
   - If the isolate is determined to be serotype O157, it can be reported according to the laboratory standard operating procedure. If the isolate is negative for O157, perform WGS on the isolate to determine the serotype.
2. If all *stx*$_1$/*stx*$_2$ PCRs are negative, report as negative (**Step 11** in Appendix B).
3. If the plate sweep tests positive for *stx*$_1$ or *stx*$_2$ but no colonies test positive for *stx*$_1$/*stx*$_2$ then:
   - Perform *stx*$_1$/*stx*$_2$ PCR on at least five isolated colonies from the plate that tested positive for *stx*$_1$ or *stx*$_2$ (**Step 7** in Appendix B). Note that some public health laboratories may opt to skip this step by performing *stx*$_1$/*stx*$_2$ PCR on additional colonies in **Step 3** in Appendix B.
   - If the five isolated colonies test negative, report as sweep PCR positive for *stx*$_1$ or *stx*$_2$, no isolate containing *stx*$_1$ or *stx*$_2$ was identified (**Step 12** in Appendix B).
   - One strategy to increase chances of identifying a *stx* positive isolate is to inoculate a BAP with growth from quadrant two and three of the plate that was sweep PCR positive. After overnight incubation, pick five isolated colonies of growth for PCR.
   - If a colony is positive for *stx*$_1$ or *stx*$_2$, perform *E. coli* O157 latex agglutination (**Step 8** in Appendix B) and subculture the isolated colony to a BAP (**Step 9**) for WGS and species identification (**Step 10**) the following day.
   - If the isolate is determined to be serotype O157, it can be reported according to the laboratory standard operating procedure. If the isolate is negative for O157, perform WGS on the isolate to determine the serotype.

Day 3

If a colony tested positive for *stx*$_1$ or *stx*$_2$ (**Step 2** in Appendix B):

1. From the sub-cultured BAP, perform species identification to confirm that it is an *E. coli* isolate (**Step 10** in Appendix B) (see Appendix F for a list of methods to identify *E. coli*).
2. Perform WGS (**Step 10**) on BAP from *stx*$_1$- or *stx*$_2$-positive isolate (**Step 6** in Appendix B).

Serotyping Considerations

- It is recommended that serotyping using antisera only be performed for *E. coli* O157. Determination of serotype is a routine analysis when WGS is performed. WGS is generally performed on every isolate, making additional serotyping redundant. Note that the serotype can only be reported to the submitter if the method has been validated for diagnostic testing.
- Flagellar typing (H typing) using antisera is costly to perform and has limited clinical value; however, it is useful for public health surveillance and can be determined for no additional cost from WGS data.
- Identifying isolates that are O157 and/or have a *stx*$_2$ gene should be a priority due to their increased virulence.[3,10] Such isolates should be reported to epidemiologists as soon as possible. The results can impact exclusions to work at or attend daycare or impact an employee working at an establishment that serves food. Note that some public health laboratories simplify their workflow and conserve resources by not performing O157 serotyping and instead focus on identifying *stx*$_2$ as the most important indicator of virulence. Public health laboratories should consult their epidemiologists to determine the best strategy for their jurisdiction.
- Immunomagnetic separation (IMS) for *E. coli* O157 can be performed to increase sensitivity for O157 detection in humans[12] and food.[5] The use of O157 IMS could be considered when maximum sensitivity is warranted such as in HUS cases or in outbreak settings for suspected *E. coli* O157 cases. IMS specific for other *E. coli* serotypes may increase their detection but is not warranted in most situations. IMS specific for *E. coli* O157 has shown to have cross-reactivity with other *E. coli* serotypes and it has proven to be useful to isolate rare and difficult to identify STEC serotypes.[6]
- Enzyme immunoassays (EIAs) are not included in the workflow as they have been largely replaced in public health laboratories by PCR for identification of Shiga toxins (or Shiga toxin genes) directly from stool. STEC EIAs have reduced sensitivity compared to that of PCR[1] and EIAs are more expensive than PCR. Some PHLs may consider using EIAs in situations where it is necessary to identify the production of Shiga toxins.
- Selective media that contain potassium tellurite (e.g., CT-SMAC, STEC CHROMagar) can facilitate the isolation of the most common serotypes, including *E. coli* O157. However, potassium tellurite can inhibit certain rare STEC serotypes, so it is important to include media that contains potassium tellurite and a less restrictive media into the workflow.
- Some public health laboratories may need to have a different workflow for diagnostic vs surveillance testing. These recommendations are based on the best information and data available for isolation and identification of STEC in public health laboratories. Additional studies to reduce the amount of media needed to detect STEC in public health laboratories would be greatly appreciated and may have additional cost reductions with minimal impact on the percentage of specimens that grow STEC. For questions or feedback, please contact entericreferencelab@cdc.gov or foodsafety@aphl.org.
- Examples of specific studies that could be helpful to better identify and isolate STEC include:
    - A study to determine the increased sensitivity of routine use of enrichment broth.
    - Studies to compare and evaluate the sensitivity and specificity of different media.
    - A study to determine the best quadrant for sweep PCR.

References

1. Parsons, B.D., et al., Detection, Characterization, and Typing of Shiga Toxin-Producing *Escherichia coli*. Front Microbiol, 2016. 7: p. 478.
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3. Humphries, R.M. and A.J. Linscott, Practical Guidance for Clinical Microbiology Laboratories: Diagnosis of Bacterial Gastroenteritis. Clin Microbiol Rev, 2015. 28(1): p. 3-31.
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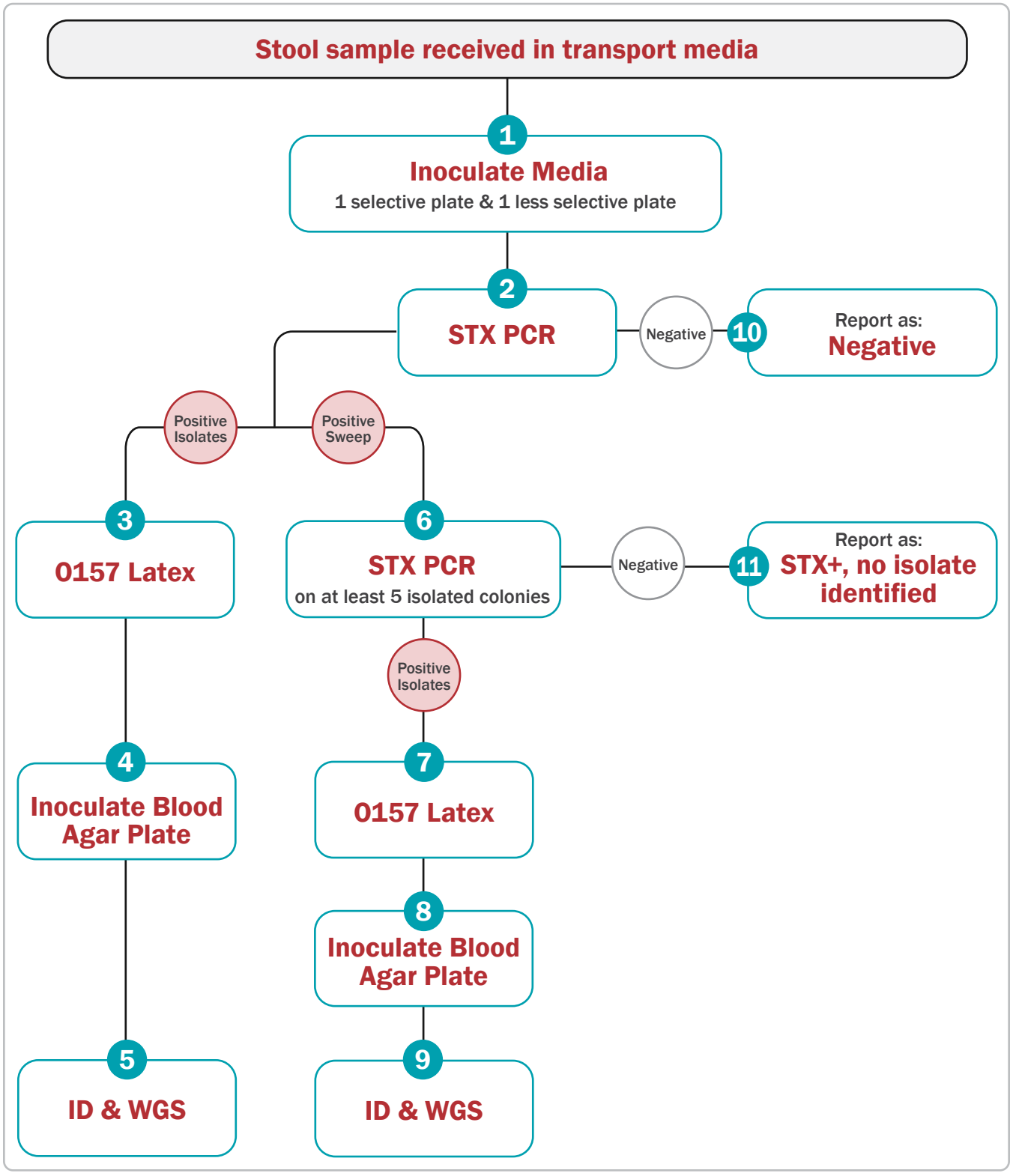
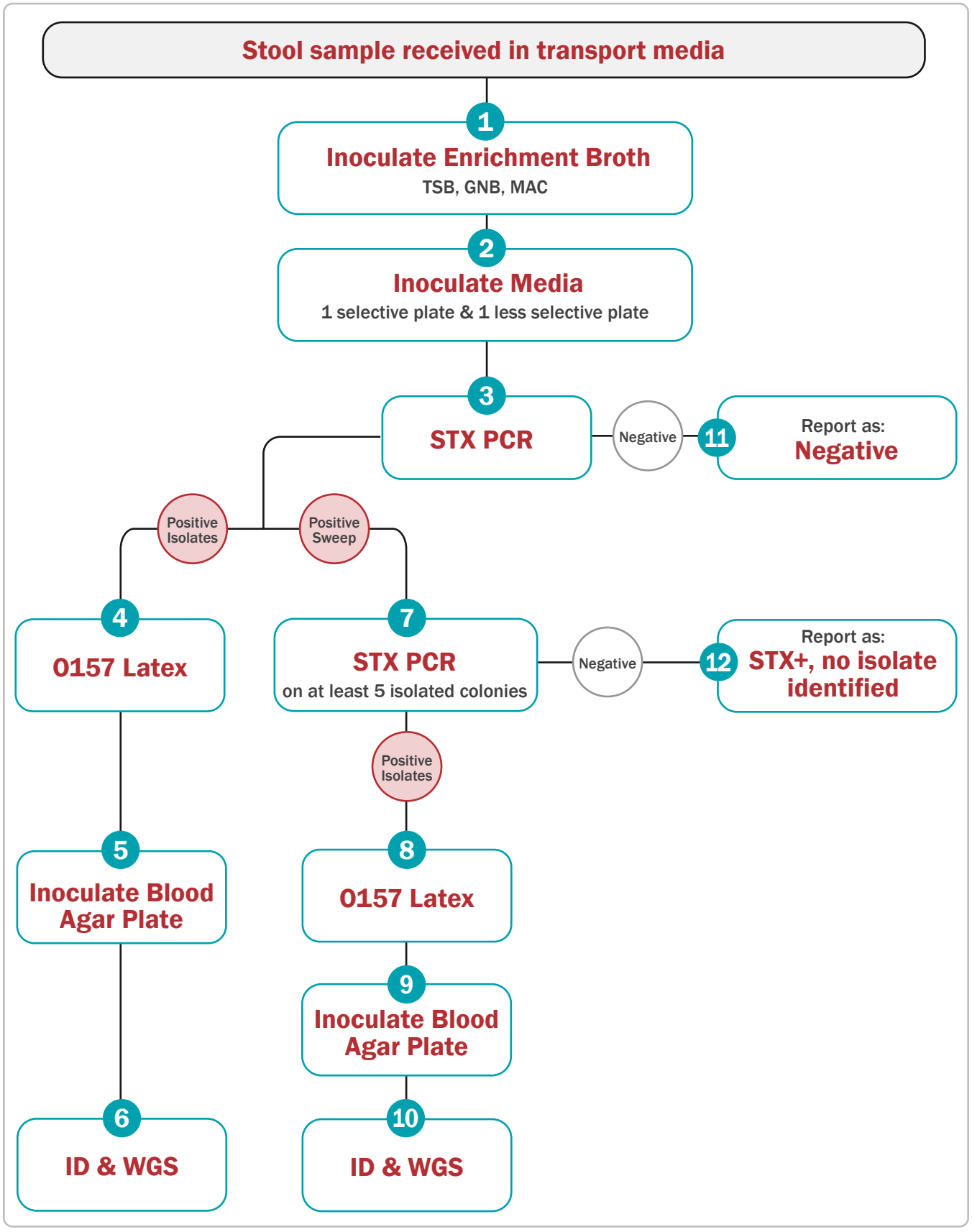
Appendix

Appendix A: Standard Flow Diagram for Isolation and Identification of STEC



Appendix B: Optional Enrichment Flow Diagram for Isolation and Identification of STEC



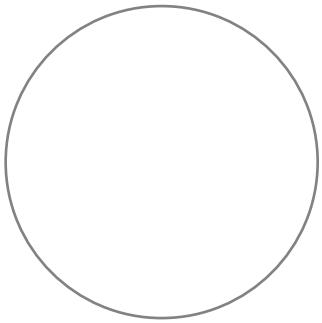
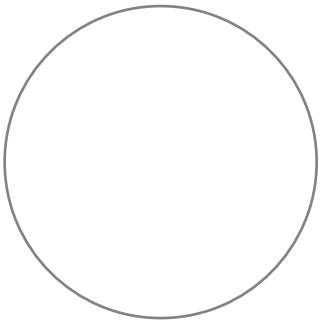
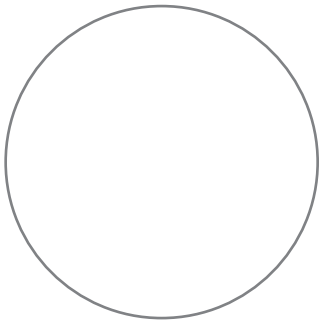
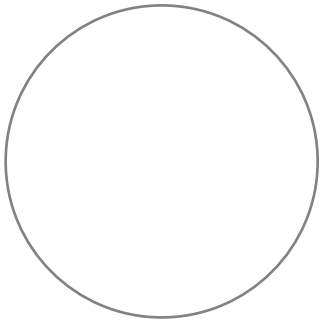
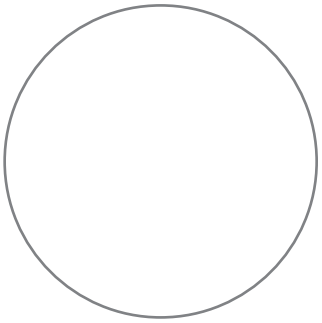
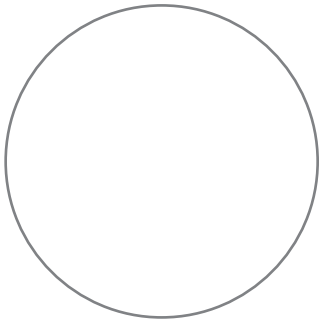
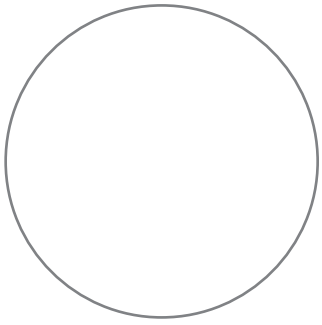
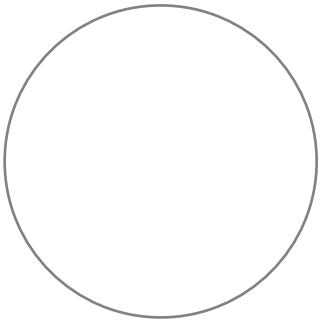
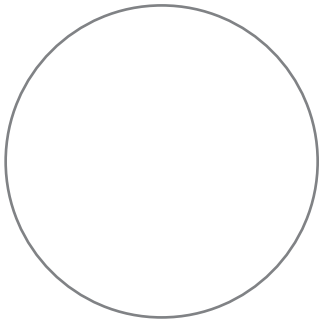
Appendix C: Typical Morphology Description of STEC on Selective Media

Media	O157 STEC	Non-O157 STEC	Commensal *E. coli*
CT-SMAC	Colorless colonies*	Pink colonies	Pink colonies
STEC CHROMagar	Mauve**	Mauve**	Colorless or blue**
Washed sheep blood	Colorless/non-hemolytic colonies	Colorless/non-hemolytic colonies	Colorless/non-hemolytic colonies
SHIBAM	Colorless/Beta-hemolytic colonies	Colorless/Beta-hemolytic colonies	Colorless/non-hemolytic colonies
SMAC	Colorless colonies*	Pink colonies	Pink colonies
BCM (*E. coli* O157 chromogenic agar)	Dark blue/black colonies with black precipitate	Dark green colonies with brownish center	Inhibited growth with clear to light green colonies
MAC	Colorless colonies (can appear pink)	Colorless colonies (can appear pink)	Pink colonies

* Some non-O157 strains may appear as colorless colonies on these two types of media (e.g. O111 serotype).

** There are multiple types of chromogenic agars that are used for STEC. Refer to the manufacturer's package insert before use.

Appendix D: Morphology of STEC on Selective and Differential Media

1. Non-O157 STEC on BCM Agar 	2. O157 STEC on BCM Agar 	3. STEC on MAC Agar 
4. Non-O157 STEC on SMAC Agar 	5. O157 STEC on SMAC Agar 	6. O157 STEC on CHROMagar (no fluorescence) 
7. Non-O157 STEC on CT-SMAC Agar 	8. O157 STEC on CT-SMAC Agar 	9. STEC on WSB Agar with beta-hemolysis (18-24 hours) 

Appendix E: Typical Results of Biochemicals for *E. coli*

Biochemicals*	Reaction**
TSI (slant)	K or A
TSI (butt)	A
TSI (H2S)	-
TSI (gas)	G
Indole at 37 °C	Usually produced (read at 24 hrs)
Methyl red reaction	+ (read at 48 hrs)
Voges-Proskauer reaction	-
Citrate utilization	- (read at 24 hrs)
Motility	+ (read at 24 hrs)

* For additional biochemical information, please refer to Biochemical Table 1 in APHL's Clinical STEC Guidance.

** K = alkaline slant, A = acidic slant, G = gas production

Appendix F: Methods for *E. coli* Identification

Techniques	Pros	Cons
Biochemicals	Common in PHLs to ID pathogens	Time-consuming and can be costly as *E. coli* requires multiple tests to ID
MALDI-TOF	Quick, cost effective and already performed in many PHLs, so would add minimal changes	Does not differentiate *E. coli* and *Shigella* spp. so additional methods are needed to ID
PCR	Quick, cost effective and already performed in many PHLs, so would add minimal changes	Laboratory diagnostic tests for *E. coli* specific target genes would require validation for diagnostic reporting.
WGS	All stx_1/stx_2 isolates are already sequenced in PHLs so would add minimal changes	Takes several days which can delay reporting results, and PulseNet cannot differentiate *E. coli* vs *Shigella* spp.

Biochemicals

The traditional method for identifying *E. coli* is through biochemicals. *E. coli* can be identified using biochemicals using a traditional method called IMViC (indole, methyl red, Voges-Proskauer, citrate) tests.[8] These four tests differentiate members of the order Enterobacterales. Additional biochemicals may be needed to identify *E. coli*; see **Appendix E** for an expanded list of biochemicals that may be used for *E. coli* identification. Note that some PHLs use API-20E as opposed to individual biochemicals for identification. API-20E is easy to use but does carry an increased cost.

MALDI-TOF

MALDI-TOF cannot routinely distinguish *Shigella* spp. and *E. coli*. The ability to identify *E. coli* can differ between manufacturers and libraries. To address this, some PHLs perform MALDI-TOF on only lactose-positive colonies while other PHLs utilize MALDI-TOF plus the indole spot test to discriminate *E. coli* and *Shigella* spp.

PCR

PCR can be used to identify *E. coli* and/or O157 directly from suspicious colonies reducing the need for identification by other methods. Contact entericreferencelan@cdc.gov for the most up to date information about stx1/stx2 variants.

- When detecting virulence genes by PCR, consider:
  - Routinely evaluating *stx*$_1$/*stx*$_2$ PCR assays to verify their ability to detect new and emerging *stx*$_1$ and *stx*$_2$ variants.
  - Intimin gene (*eae*), which is a virulence gene necessary for the attaching and effacing lesions on the intestinal epithelia. The presence of *eae* in combination with *stx*$_1$ or *stx*$_2$ increases the virulence of the STEC strain.
  - *E. coli* O157 specific target which can be used for rapid identification of O157 serotype to aid in investigations.
  - *E. coli* specific marker target, such as *lacY,* which can be used to confirm an isolate as *E. coli* to help mitigate the use of biochemicals or other methods for *E. coli* identification.
  - Hemolysin genes, such as *ehxA* and *hlyA*, which are potential virulence factors and are not frequently included in STEC PCR assays.
  - Targets for the "big six" STEC serotypes (O26, O45, O103, O111, O121, O145) which are known to cause severe illness in humans. These targets are not normally tested for at public health laboratories, although serotype determination is now standard practice with WGS minimizing the utility of testing specifically for these serotypes.
- When performing plate sweep PCR, testing a loopful of bacteria from quadrants one and two increases the possibility of detecting STEC.
- There are no definitive guidelines for the number of isolates to test from a STEC positive specimen. Usually, a STEC colony can be identified from a *stx*$_1$/*stx*$_2$ positive sweep by testing five colonies.[7] Testing additional colonies marginally increases the chance to identify STEC but also increases the cost of testing.

WGS

Current PulseNet protocols are not able to routinely distinguish *E. coli* and *Shigella* spp. New WGS analysis tools such as ECTyper[9] are able to distinguish *E. coli* and *Shigella* spp. and could be evaluated for use in PHLs to identify *E. coli*.

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