

EnT EnteroPluri Tube

Learning Objectives

The student will

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, and media types when making observations.
- Use the bright field light microscope to view microbes under oil immersion, make accurate observations and appropriate interpretations and store the microscope according to lab procedures.
- Properly prepare a bacterial smear for accurate staining.
- Accurately perform a Gram stain.
- Correctly perform various inoculation techniques including the T streak technique and describe each technique's purpose.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

Background/Theory

Identification of a microbial isolate is essential for the proper diagnosis and appropriate treatment of patients. Scientists have developed techniques that identify bacteria according to their biochemical characteristics. Typically, they either examine the use of specific carbon sources as substrates for fermentation or other metabolic reactions, or they identify fermentation products or specific enzymes present in reactions. In the past, microbiologists have used individual test tubes and plates to conduct biochemical testing as we have done. However, scientists, especially those in clinical laboratories, now more frequently use plastic, disposable, multitest panels that contain a number of miniature reaction tubes, each typically include a specific substrate and pH indicator. After inoculation of the test panel with a small sample of the microbe in question and incubation, scientists can compare the results to a database that includes the expected results for specific biochemical reactions for known microbes, thus enabling rapid identification of a sample microbe. These test panels have allowed scientists to reduce costs while improving efficiency and reproducibility by performing a larger number of tests simultaneously. (OpenStax CNX, 2018)

Many commercial, miniaturized biochemical test panels cover a number of clinically important groups of bacteria and yeasts. (OpenStax CNX, 2018) One such group is the family **Enterobacteriaceae**. Many members of this group are components of the natural intestinal flora but some members are pathogenic. More on pathogenic Enterobacteriaceae can be found at this link in [OpenStax Microbiology](#).

The Enterobacteriaceae are gamma proteobacteria that include familiar microbial genera like *Escherichia*, *Salmonella*, and *Yersinia*. Members of the Enterobacteriaceae are bacilli, and are typically 1-5 μm in length. Like other Proteobacteria they stain Gram negative and are facultative anaerobes. They ferment sugars (all can ferment glucose) to produce a mixture of solvents, organic acids, and gasses as end products (varying by strain). Most also reduce nitrate to nitrite via anaerobic respiration. Unlike similar bacteria, Enterobacteriaceae lack cytochrome c oxidase. Most are motile via many peritrichous (covering the entire surface) flagella, but a few genera are non-motile. They are non-spore forming. Most, but not all, members of the Enterobacteriaceae produce catalase. Some members of this family

are able to ferment lactose – these are referred to as **coliforms**. Coliforms are less commonly pathogenic, but, because they tend to inhabit mammalian digestive tracts, are used as indicators of water quality. Non-coliforms cannot ferment lactose and often act as human pathogens. (Franklund, 2018)

Because this group has clinical significance, several multitest systems have been developed to aid in their identification. In this course, you will use the EnteroPluri system (formerly Enterotube). This older system has been replaced by more complex and accurate systems in most clinical labs, but it will serve to expose you to the multitest concept. Keep in mind, the EnteroPluri system is only able to differentiate between members of this one family. Other methods must be used for organisms outside of the Enterobacteriaceae, Gram positives or other Gram negative rods.

The EnteroPluri tube is composed of several compartments containing differential media. Many of them are testing for the fermentation of a specific carbohydrate. Each of these chambers' media contains a sugar substrate and a pH indicator. Other chambers test for the metabolism of certain amino acids or other substances also utilizing pH indicators for the visualization of a positive result.

This and all biochemical tests require one to test a pure culture. If a mixed culture is tested, and a positive result is obtained, it is impossible to know which of the two organisms (or both) produced the result. If a multitest system is inoculated with a mixed culture, one erroneous positive result derived from even a small amount of the contaminant, can throw off the correct identification completely. To ensure a pure culture, the inoculum for an EnteroPluri tube, therefore, is taken from a single isolated colony. Using a MacConkey agar plate for the isolation streak provides evidence that one is testing a Gram negative organism.

The tube is constructed in a manner that may reveal an erroneous result. The chambers are inoculated by placing a mass of cells on the wire at the Citrate end of the tube. As the wire is drawn through the tube from the citrate compartment to the glucose compartment at the other end, cells are deposited in each chamber. The final chamber to receive cells is the glucose chamber. Because the ability to ferment glucose is common to all Enterobacteriaceae, a negative result in this chamber may indicate a problem with the test system. The inoculum may not have been heavy enough or viable. What other reason might there be for a negative in the glucose fermentation chamber?

Figure 1 shows an uninoculated EnteroPluri Tube. Each chamber is described in Table 1.



Figure 1 Uninoculated EnteroPluri Tube

Table 1 EnteroPluri tube chambers and results

Chamber	Test description	Negative result	Positive result	Interpretation of positive result
Glucose/Gas	Glucose fermentation. If glucose, present in the medium, is fermented, acid is produced lowering the pH. If this occurs, the pH indicator will change from red to yellow. Any sign	red	Yellow	Organism is capable of fermenting glucose.

Chamber	Test description	Negative result	Positive result	Interpretation of positive result
	of yellow is positive, orange is negative.			
	Gas production. A second byproduct of glucose fermentation may be a gas. If produced, a gas will cause the wax to separate from the medium.	No space between wax and medium	Wax lifting off the surface.	Gas is produced from glucose fermentation
Lysine	If the cells can remove the carboxyl group from the amino acid, lysine, they produce alkaline substances. This pH indicator starts out yellow and turns purple if the pH goes up.	Yellow	Purple	Organism is capable of lysine decarboxylation
Ornithine	This is also an amino acid. This test works the same way the lysine test works. The medium contains ornithine instead of lysine for the substrate.	Yellow	Purple	Organism is capable of ornithine decarboxylation
H ₂ S/Indole	The medium in this chamber is essentially the same as SIM. It tests for sulfur reduction and indole production.	Yellow	Black/ black around the inoculation line/diffuse brown	Organism capable of sulfur reduction
	Kovac's is added after incubation. A pink color indicates indole is produced	Yellow medium	Pink color develops anywhere even on the plastic	Tryptophanase is present and it converts tryptophan to pyruvate with indole as a byproduct.
Adonitol	This and the next three chambers test for the fermentation of a specific carbohydrate. These media work the same way the glucose test works. The sugar substrate in the medium is different. In all of them, if the sugar is fermented, acids are produced and the pH indicator changes color.	Red (orange)	Yellow (any degree of yellow)	Organism is capable of fermenting adonitol
Lactose	See above.	Red (orange)	Yellow (any degree of yellow)	Organism is capable of fermenting lactose
Arabinose	See above.	Red (orange)	Yellow (any degree of yellow)	Organism is capable of fermenting arabinose

Chamber	Test description	Negative result	Positive result	Interpretation of positive result
Sorbitol	See above.	Red (orange)	Yellow (any degree of yellow)	Organism is capable of fermenting sorbitol
VP	The Voges-Proskauer test indicates the presence of a certain metabolic pathway related to glucose fermentation.	No color after 20 min.	Red/pink develops within 20 min.	Acetoin is produced
Dulcitol/PA	Tests for the fermentation of dulcitol. Similar concept to Glu, Adon, Lac, Arab and Sorb compartments. The pH indicator is different. It is green, and then turns yellow when the pH goes down.	Green	Yellow/pale yellow/tan	Organism is capable of fermenting dulcitol
	Phenylalanine deaminase activity is detected by the formation of an iron salt black precipitate	Green	Black/smoky grey	PA-deaminase activity is present
Urea	Urea can be converted to ammonia (alkaline) through the action of urease. Rise in pH, changes indicator color to bright pink.	Beige/tan	pink	Organism has urease activity
Citrate	This medium is the same as Simmons Citrate media. Determines if citrate can be utilized from the medium.	green	Blue (any degree of blue)	Organism can utilize citrate

Experiment/Exercise

Materials per student pair

Lab 1

MacConkey agar plate
Gram staining reagents

Lab 2

Enteropluri tube

Lab 3

VP A reagent
VP B reagent
Indole reagent (Kovac's)
Results pad
Code book

Cultures

Fresh overnight broth cultures

Enteropluri unknowns labeled A-G; each student pair is assigned one to identify.

Mixed culture control for Gram Staining

Procedure Lab 1

1. Obtain your unknown and write the letter down in the data table.

2. Aseptically prepare two identical slides each with two smears. One smear will be your unknown and the other will be the mixed culture Gram Stain control. Allow the smears to air dry while you complete step 4 and other tasks. Heat fix both slides.
3. Perform a Gram stain on ONE of the slides. As usual, the instructor must sign off on the control under oil immersion. Observe the unknown under oil immersion and record the Gram stain result, the morphology and arrangement and draw a representative group of cells. (If your technique needs adjusting, you will have a second slide to use.)
4. Perform a T-streak on the MacConkey agar plate with the broth. Label it completely. For the organism name use "Unknown ___" adding the letter of your assigned unknown. Place the plate for incubation at 37°C for 24-30 hours. (Your TA will refrigerate the plate and then place it in the incubator 24-30 hours prior to your lab session next week.)

Procedure Lab 2

1. You will be using the MacConkey agar plate that you or your partner made from the "unknown" last week. Inspect plate. Make sure that you see only one colony type. Record observations (growth color and medium color) in the data table. Under interpretation, write down what these observations tell you about your organism. You may need to refer to the SDM1 exercise.
2. Watch the video clip demonstration for inoculation of the tube. These are expensive and each student pair is allowed only one.
3. Label the tube with your lab section, your names, date and unknown letter.
4. Locate the end of the tube with the Glucose/gas test. Remove the cap from this end. It should have a loop. This is the end that you can touch. One partner can hold the cap. See figure 2.
5. Remove the other cap. This is inoculating end. It should appear as a needle. Do NOT touch this end, it must remain sterile. Hand the cap to the partner. See figure 2.



Figure 2 EnteroPluri tube inoculation

6. Aseptically obtain a HEAVY inoculum of your unknown from a SINGLE colony on your MacConkey agar plate. There is no need to heat the needle tip because it is pre-sterilized.

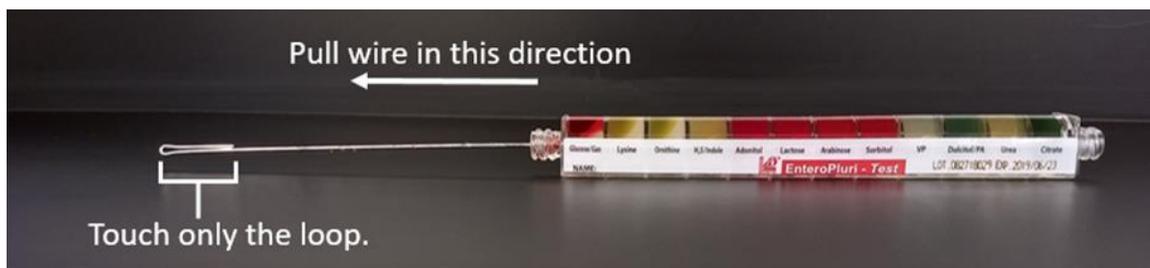


Figure 3

7. Carefully pull the wire through the tube, twisting as you go. Figure 3. The tip should reach the Glucose/gas chamber, but should NOT be completely removed from the tube. Figure 4.

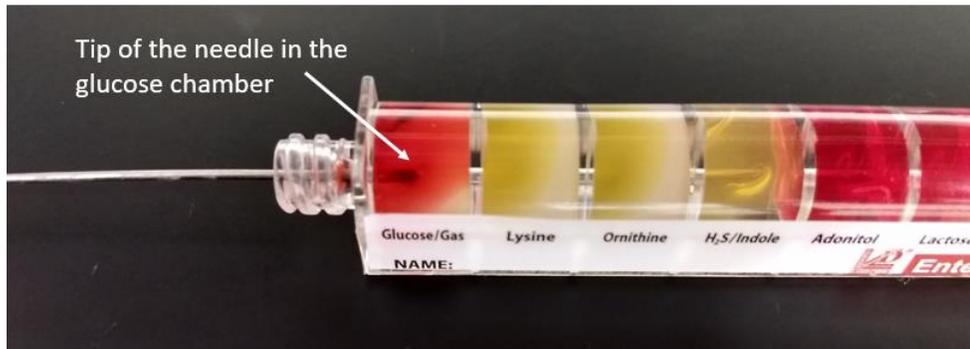


Figure 4

8. Then push the wire back into the tube until the tip reaches the citrate compartment. Figure 5.

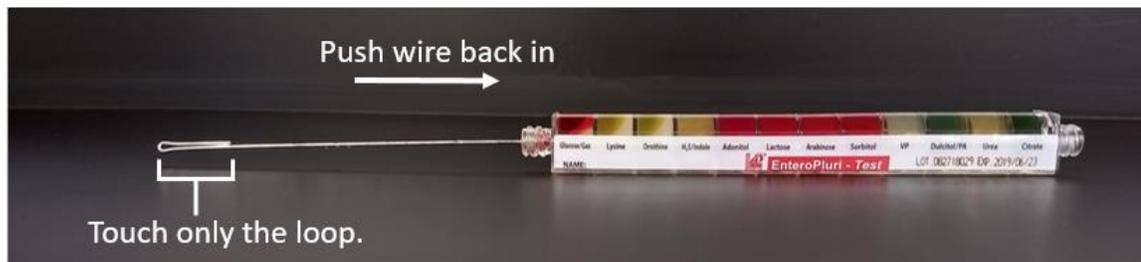


Figure 5

9. Notice the notch in the wire adjacent to the looped end that you are holding. Break the wire off at this point by bending it. Place the broken wire loop in the disposal container on the bench. See figures 6 and 7.



Figure 6

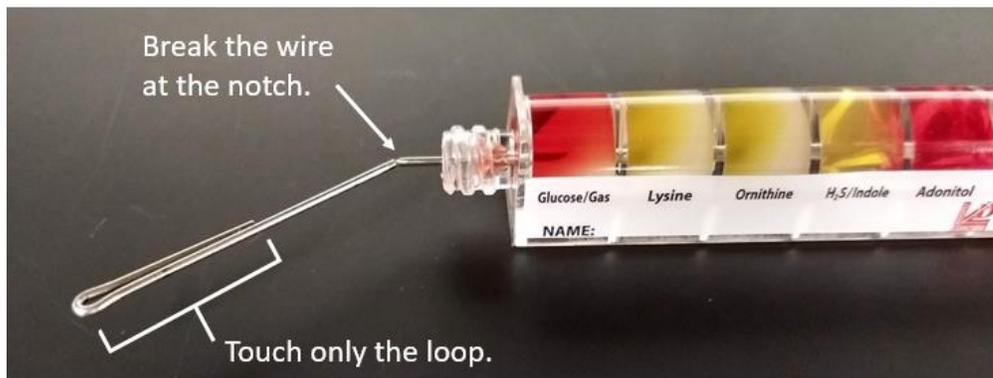


Figure 7

10. Replace the caps on the tube. Figure 8.



Figure 8

11. Locate the chambers that require access to oxygen. These are the last 8: Adon, Lac, Arab, Sor, VP, Dul/PS, Urea and Cit.
12. Incinerate the tip of an inoculating wire to sterilize it. Before it cools, use the hot tip to poke holes in each of those chambers at the notch.
13. Place the tube for incubation at 37°C for 24 hours.

Procedure Lab 3

1. Watch the video "Enterotube Results."
2. Compare the incubated EnteroPluri tube to the color cards provided or to figure 1. Determine which tests are positive. Observe all media colors first. For each positive test, circle the number corresponding to the test on the results sheet. Record all results except VP and Indole before going further. When a positive test is indicated by a color change in the medium, any degree of the positive color is considered a positive. In the sugar fermentation chambers, any yellow is positive, but orange is negative. Consult with the instructor if you are not sure of the result.
3. After the media colors have been observed, perform the two tests requiring the addition of reagents. See steps 4, 5 and 6. These reagents are hazardous! Please share an open vial with one or more other groups to minimize any extra reagent. After the vial is empty, it must be disposed of in the corresponding chemical waste container in the fume hood.
4. Perform the indole test. With a hot inoculating loop, melt a hole in the plastic covering the H₂S/Indole compartment. (Incinerate the loop before setting it down.) Add 1-2 drops of Indole reagent to the compartment. A positive test is indicated by a pink color developing in the medium or on the plastic film covering within 10-20 seconds.
5. For the VP test, create another hole in the film of the VP compartment as above. Add 3 drops of VP A and 2 drops of VP B to the compartment. Allow this to react for up to 20 minutes. Any pink forming in the solution, on the medium or on the plastic film is a positive result.
6. Please share the reagent vials with other groups at your bench. Do not place empty vials in the pipet containers. Instead, place them in special chemical hazard containers located in the chemical fume hood.
7. Add up the circled numbers to obtain the 5-digit code for your organism. Look up the code in the CCIS booklet and identify the organism. Check your identification with the instructor. Be sure to write down identification number on the data sheet as well.

EnT

Blank

Lab Report: Enteropluri Tube

Name _____

Lab Section _____

Data and Observations

MacConkey Agar

Organism	Growth on MacC +/-	Appearance on MacC	Interpretation
Unknown letter			

Gram Staining Results

Organism	color	Gram Rxn	Drawing	Morphology and Arrangement	instr initials
Mixed culture control			N/A	bacilli	
Mixed culture control			N/A	cocci	
Unknown letter:					N/A

Test Results

Glucose-Gas Lysine Ornithine H₂S-Indole Adonitol Lactose Arabinose Sorbitol VP Dulcitol-Phenylalanine Harnstoff-Urea/Uree Citrate

4 + 2 + 1 4 + 2 + 1 4 + 2 + 1 4 + 2 + 1 4 + 2 + 1

ID (Biocode)

Identification:

Post Lab Questions

1. What are the advantages of a multitest system? What are the disadvantages?
2. Which Enteropluri compartments contain media that you have already used?
3. List the characteristics common to all Enterobacteriaceae.
4. List characteristics that many Enterobacteriaceae have.
5. What is the purpose of Gram Staining the organism?
6. What is the purpose of streaking the culture onto a MacConkey agar plate?
7. Is your organism a coliform? How do you know?
8. If most coliforms are not pathogenic, why do we test for them in a water sample?

9. If the experimenter used a mixed (i.e. contaminated) culture in the inoculation of an EnteroPluri tube, how would the sensitivity or specificity be affected? Which is more likely, false positive or false negative results?

10. How are you minimizing the chances that you are using a mixed culture?

11. The following are identification numbers of 5 different isolates.

Isolate A 51743, Isolate B 00333, Isolate C 00001, Isolate D 75340, Isolate E 64261,

a. Which are not members of the family Enterobacteriaceae?

b. Which of these can ferment sorbitol?

c. Which of these can metabolize urea?

d. Which of these will you question the validity of? Explain why.

References

- Franklund, C. (2018). Microbiology Laboratory Manual, Observing and Recording the Microbial World. Git Hub, Inc. Retrieved 2018, from <https://github.com/WeeBeasties/microbiology-laboratory-manual>
- OpenStax CNX. (2018, Mar 19). OpenStax Microbiology. Retrieved from <http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24>