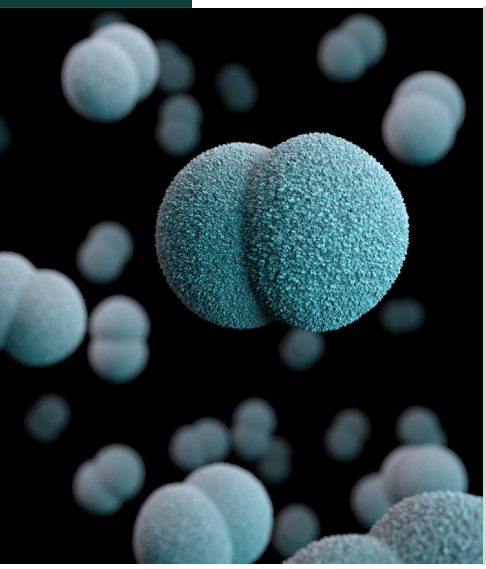
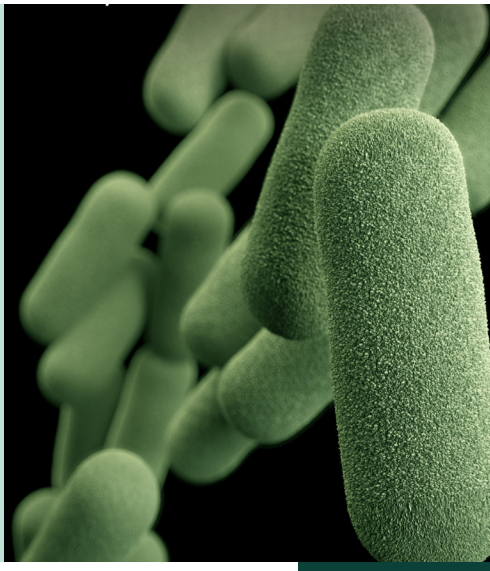
Standard Operating Procedures

for Identification and Characterization of *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*





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Preface/Summary

Bacterial meningitis, commonly caused by *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, is a public health threat worldwide. The highest burden of meningococcal meningitis is in a region of sub-Saharan Africa called the meningitis belt, which spans from Senegal to Ethiopia. Countries within the meningitis belt experience seasonal meningitis outbreaks punctuated by periodic epidemics (1). Prior to the introduction of the serogroup A meningococcal conjugate vaccine (MenAfriVac™) in 2010, 90% of meningococcal disease cases and epidemics were caused by serogroup A in the meningitis belt. MenAfriVac™ vaccination has eliminated meningitis epidemics caused by meningococcal serogroup A (2). However, non-NmA serogroups, including NmW, NmX, and NmC remain a threat and continue to cause outbreaks and disease in the region. Notably, a novel strain of NmC has emerged in recent years and has been responsible for multiple outbreaks and epidemics (3, 4, 5).

Rapid and accurate methods for the detection of these pathogens are critical for surveillance and outbreak response. This manual contains a set of standard operating procedures for the identification and characterization of bacterial meningitis pathogens from cerebrospinal fluid by culture. These procedures were utilized during two bacteriology workshops held in Atlanta, GA, USA: one for African francophone countries in September 2017 and another for African and South Asian anglophone countries in October 2018. Both workshops were co-sponsored by the Association of Public Health Laboratories (APHL), Georgia Public Health Laboratory, and Centers for Disease Control and Prevention (CDC). This manual will serve as a technical resource for laboratories at national and local levels to improve detection of bacterial meningitis and the quality of laboratory methods.

Citations

1. Lapeyssonnie L. [Cerebrospinal Meningitis in Africa]. Bull World Health Organ 1963; 28 Suppl:1-114.
2. Meyer SA, Novak RT. Effect of a vaccine to prevent serogroup A *N. meningitidis* epidemics in Africa. Lancet Infect Dis 2017; 17:789-90.
3. Sidikou F, Zaneidou M, Alkassoum I, et al. Emergence of epidemic *Neisseria meningitidis* serogroup C in Niger, 2015: an analysis of national surveillance data. Lancet Infect Dis 2016; 16:1288-94.
4. Nnadi C, Oladejo J, Yennan S, et al. Large outbreak of *Neisseria meningitidis* serogroup C - Nigeria, December 2016-June 2017. MMWR Morb Mortal Wkly Rep 2017; 66:1352-6.
5. Bozio CH, Vuong J, Dokubo EK, et al. Outbreak of *Neisseria meningitidis* serogroup C outside the meningitis belt-Liberia, 2017: an epidemiological and laboratory investigation. Lancet Infect Dis 2018; 18:1360-1367

Acknowledgments

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We specially thank APHL for technical and logistical support and the Georgia Public Health Laboratory for hosting the training based on this manual.

About the Editor

Dr. Seydou Diarra was born in Markala, Mali. He obtained his Bachelor and Master's degrees in Biology at Babes-Boyai University (Cluj-Napoca, Romania) in 1979. He then received a graduate diploma in food microbiology and hygiene and a certificate in hematology at the Institut Pasteur in Lille, France. Dr. Diarra's research interests include bacterial meningitis, bacterial and viral enteric diseases, antimicrobial resistance, and molecular diagnosis. Dr. Diarra has served as the Head of the Bacteriology and Virology Reference Laboratory at the Institut National de Recherche en Santé Publique (INRSP), Ministry of Health of the Republic of Mali from 2003 until his retirement in 2017. However, he continues to lead the Global Health Security Agenda program focused on antimicrobial resistance and laboratory system strengthening in Mali.

Dr. Diarra has published numerous scientific papers in national and international peer-reviewed journals and co-authored several laboratory manuals including Mali's National Quality Assurance Work Plan for Medical Laboratories, the National HIV/AIDS Laboratories Work Plan, and the Mali National Laboratory Policy and Laboratory Strategic Plan of 2013. He has also developed training modules on the use of laboratory diagnostic methods for diseases with epidemic potential (http://www.globe-network.org/fr/maladies-a-potentiel-epidemique).

Dr. Diarra served as a faculty instructor for a bacteriology workshop for African francophone countries that used the protocols published in this manual. The workshop was held in Atlanta, GA, USA (September 2017) and co-sponsored by the Association of Public Health Laboratories, Georgia Public Health Laboratory, and Centers for Disease Control and Prevention (CDC). Additionally, Dr. Diarra helped edit the French manual in collaboration with CDC subject matter experts during a sabbatical with the Bacterial Meningitis Laboratory at CDC in Atlanta in 2018.

1. CSF Cell Count (Quantitative Cytology)

1.0 Purpose

This standard operating procedure provides instructions for using a hemocytometer to obtain white blood cell (WBC) counts from cerebrospinal fluid (CSF) of patients suspected of bacterial meningitis..

2.0 Principle

The WBC (leukocyte) counts are conducted for suspected bacterial meningitis patients because an increased number of leukocytes can be indicative of infection. For this procedure, the leukocytes are counted using a hemocytometer (a specialized microscope slide that is designed to facilitate cell counting). The liquid chamber of a hemacytometer holds a very precise volume, permitting a small, representative number of cells to be counted and used to calculate the concentration of cells within a CSF. Furthermore, the hemocytometer chamber is etched with lines, to facilitate the counting process.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
CSF	Cerebrospinal fluid
PPE	Personal protective equipment
WBC	White blood cell or leukocyte

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. Microscope
3. Pipette (2-20 µL)
4. Pipettes (20-200 µL and 200-1000 µL) – only necessary if CSF dilutions are required
5. Hemocytometer
6. Hemocytometer cover slip (A hemocytometer cover slip is a specific size and weight).
    a. *Note*: Using a different type of coverslip could affect the chamber volume and the final counts, according to the manufacturer.
7. *Optional*: Handheld, mechanical tally counter

5.0 Reagents and Media

1. Distilled water
2. 70% Ethanol
3. 0.85% Saline (only used for turbid CSFs)
4. 1N Acetic acid (only used for bloody CSFs)
    a. Dilute to 0.5% prior to use (0.5 mL 1N acetic acid in 100 ml distilled water).

6.0 Supplies

1. Pipette tips (2-20 µL)

2. Pipette tips (20-200 µL and 200-1000 µL) – (only required if CSF dilutions are necessary)
3. 1.5 mL microfuge tube (only required if CSF dilutions are necessary)
4. Biohazard waste bag
5. Biohazards sharps container, if applicable.
6. *Optional*: Dacron polyester swab

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to acetic acid safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information/Processing

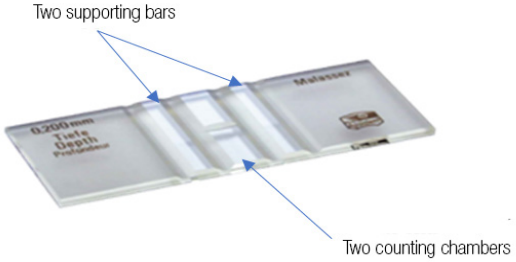
1. Freshly collected CSF (analyze within 30 minutes of collection).
2. The properties of the CSF (turbidity or the presence of red blood cells) will determine whether additional processing is required before counting.

9.0 Quality Control (QC)

Load only the reagent used as a diluent (saline solution or acetic acetic) onto the hemocytometer to confirm it is clear of artifacts or debris.

10.0 Test Procedure

1. Whenever possible, prepare, and load the CSF into the hemacytometer within a BSC.
2. Prepare the CSF for loading onto the hemacytometer.
    a. Clear CSF: No processing is required.
    b. Turbid CSF: Dilute CSF 1:10 with saline (example: 100 µL CSF into 900 µL saline).
    c. Bloody CSF: To lyse the red blood cells, dilute the CSF with 0.5% acetic acid using a dilution factor ranging from 1:2 to 1:20.
        i. If acetic acid is not available, the CSF can be diluted with saline. When selecting a dilution factor, remember that the WBCs will also be diluted.
3. Load the hemacytometer
    a. Place the hemocytometer chamber on a flat surface.
    b. *Optional*: Using distilled water, moisten the raised edges of the hemacytometer using a lightly damp swab to improve adhesion of the cover slip to the chamber.
        i. *Note*: Do not moisten the chamber itself and no excess water should be visible in the chamber.



c. Gently mix the CSF solution to achieve a homogenous solution prior to loading.

d. Using a pipette, load 10 µL CSF (or diluted CSF) into the hemacytometer chamber (dispensing the liquid at the junction of the hemocytometer and coverslip).

i. Note: Check for bubbles or an overflow from the chamber (liquid spillage into the troughs between the chamber and raised edges).

ii. If bubbles or an overflow is observed, clean the chamber with 70% ethanol and re-load the chamber.

4. Perform the cell counts using a microscope

a. Place the hemacytometer on the stage of the microscope and observe the cells using a 40X objective.

i. Note: It may be easier to initially find the hemacytometer gridlines using the 10X objective and then increasing the magnification to 40X.

b. General considerations for counting the cells:

i. WBCs should have crisp/defined cellular borders and diffract light as the focus is adjusted (almost appears like a glow/halo).

ii. Cellular debris or artifacts can be observed as spots that do not have clear cellular borders and do not diffract light. These should NOT be counted.

iii. Red blood cells will have a pigment. These should NOT be counted as a WBC; if a sample is very bloody, consider diluting as described above.

iv. A standardized method for how "clumped cells" will be counted should be established by the laboratory. For example:

1. Count each cell within the clump.

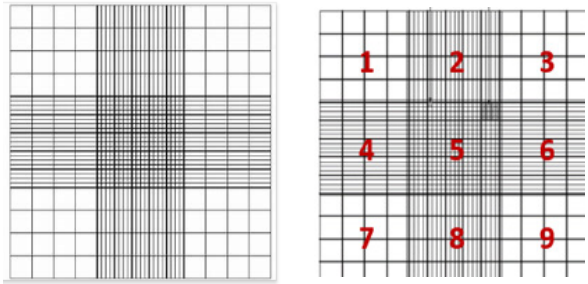
2. Note: If many clumps are present, consider mixing CSF gently and re-loading.

c. Count the cells present within the chamber

i. *Note: Exam*ples are provided below for counting with a Neubauer and Malassez hemacytometer but other types can also be used.

ii. Using a Neubauer hemacytometer.

1. Hemacytometer has 9 quadrants (labeled in red), which are oriented in a 3x3 layout and each quadrant holds a volume of 0.1 mm^3.



2. Count the cells within at least four quadrants of the counting chamber; if only a few cells are present, count all nine quadrants.

a. If cells are present on the outer borders of the quadrant: Include only cells touching the top and left border but exclude cells touching bottom and right outer borders

3. Calculate the WBC concentration using the following formula

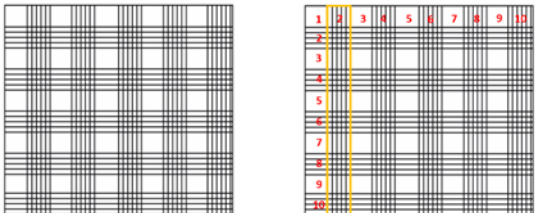
$$(\#\ cells\ counted \times dilution)/(\#\ quadrants\ used \times 0.1 mm^3) = cells/mm^3$$

Example: If you count 100 cells in 5 quadrants with no dilution used –

$$(100\ cells \times 1)/(5 \times 0.1\ mm^3) = 200\ cells/mm^3$$

iii. Using a Malassez hemacytometer.

1. Hemacytometer has 100 quadrants that are oriented in a 10x10 layout. 10 quadrants together form a band/strip within the hemacytometer (yellow box).
   a. The total grid volume is 1 µl (mm^3= µL), with each quadrant holding a volume of 0.01 mm^3. Each band contains 10 quadrants or a volume of 0.1 mm^3.



2. Count the cells within at least 50 quadrants (5 bands) of the counting chamber; if only a few cells are present, count all 100 quadrants (10 bands).
   a. If cells are present on the outer borders of the quadrant: Include only cells touching the top and left border but exclude cells touching bottom and right outer borders
3. Calculate the WBC concentration using the following formula
   a. If counting quadrants:

   $$(\#\ cells\ counted \times dilution)/(\#\ cells\ used \times 0.01\ mm^3) = cells/mm^3$$

   Example: If you count 100 cells in 50 quadrants with no dilution used –

   $$(100\ cells \times 1)/(50 \times 0.01\ mm^3) = 200\ cells/mm^3$$

   b. If counting bands:

   $$(\#\ cells\ counted \times dilution)/(\#\ bands\ used \times 0.1\ mm^3) = cells/mm^3$$

   Example: If you count 100 cells in 8 bands/strips with no dilution used –

   $$(100\ cells \times 1)/(8 \times 0.1\ mm^3) = 125\ cells/mm3$$

11.0 Interpretation of Results

1. Once the WBC counts have been obtained, refer to the appropriate guidance to determine if the patient's WBC counts are in the expected range. WHO guidance is provided in Chapter 6 of [3].
   a. *Note*: The expected WBC counts within CSF vary by patient age because infants often do not have a fully developed blood-brain barrier.

12.0 Test Limitations

1. If the test is not completed within 30 minutes of CSF collection, lysis of red or white blood cells may have already occurred, resulting in inaccurate interpretations.
2. Cellular debris or other artifacts may be inappropriately interpreted as cells, leading to inaccurate cell counts.
3. When few cells are present in the chamber, the test is more prone to sampling error and may lead to inaccurate cell counts.
4. Cell counts may vary between testing operators.

13.0 References

1. Rahimi J, Woehrer A. Overview of cerebrospinal fluid cytology. Handb Clin Neurol. 2017;145:563-71.

2. Deisenhammer F, Bartos A, Egg R, Gilhus NE, Giovannoni G, Rauer S, et al. Guidelines on routine cerebrospinal fluid analysis. Report from an EFNS task force. Eur J Neurol. 2006;13(9):913-22.

3. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

4. Deisenhammer F, Bartos A, Egg R, Gilhus NE, Giovannoni G, Rauer S, et al. Routine cerebrospinal fluid (CSF) analysis. In: Gilhus NE, Barnes P, Brainin M, editors. European Handbook of Neurological Management. 1. 2nd ed: Blackwell Publishing Ltd.; 2011. p. 5-17.

2. Differential CSF Cell Count – Qualitative cytology

1.0 Purpose

This standard operating procedure provides instructions for identifying and counting the different types of white blood cells (WBCs, leukocytes) within a cerebrospinal fluid (CSF) of patients suspected of bacterial meningitis.

2.0 Principle

The test determines the leukocyte formula (the percentage of different types of leukocytes in the blood smear or CSF). For this test, the cells from a CSF are put onto a microscope slide and stained with May-Grünwald and Giemsa reagents to differentiate the cellular nuclei from the cytoplasm. Using the cell shape and staining pattern, different types of leukocytes (eosinophils, lymphocytes, and neutrophils) can be identified and counted. The predominant type of leukocyte present in the CSF can indicate the type of infection present (viral or bacterial).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
CSF	Cerebrospinal fluid
PPE	Personal protective equipment
WBC	White blood cells

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. Microscope
3. Pipette (2-20 µL)
4. Microcentrifuge
5. *Optional*: Handheld, mechanical tally counter
6. *Optional*: Bunsen burner (only required if methanol is not available)

5.0 Reagents and Media

1. 0.85% saline
2. May-Grünwald-Giemsa Staining Kit
3. 70% Methanol (if unavailable, heat fixation can be used)

6.0 Supplies

1. Pipette tips (20-200 µL)
2. Glass slides
3. Biohazard sharps container
4. Biohazard waste bag

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to May-Grünwald-Giemsa Staining Kit product safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information/Processing

1. Freshly collected CSF (analyze within 30 minutes of collection).

9.0 Quality Control

Load only the reagent used as a diluent (saline solution or acetic acetic) onto the hemocytometer to confirm it is clear of artifacts or debris.

10.0 Test Procedure

1. Preparation of a CSF. Whenever possible, this step should be conducted within a BSC.
    a. Centrifuge 1 ml CSF at 3000 rpm/min for 5 minutes.
    b. Without touching the pellet, transfer the supernatant (the liquid portion) to another tube and save for latex testing, if applicable
    c. Resuspend the pellet in 200 µL saline solution
    d. Spread 100 µL of the pellet suspension onto a glass slide by using the tip of a pipette.
        i. Note: If the cell suspension is dense, spread it in a thin layer; if not dense, dispense the full volume onto a small portion of the slide.
    e. Allow to air dry
        i. Critical: The slides must be completely dry before proceeding
2. Fixation of slides. Whenever possible, methanol fixation should be conducted within a BSC.
    a. Methanol fixation (preferred method)
        i. Completely cover the dried cell spot (or flood the slide) with 95% methanol.
        ii. Let the dried cell spot incubate in methanol for two minutes.
        iii. Discard any excess methanol and allow to fully dry (do not touch smear surface).
        iv. Note: Methanol fixation is preferred because maintains specimen integrity better than heat fixation.
    b. Heat fixation (use this method only if methanol is not available)
        i. Quickly pass the slide through a flame three times.
        ii. Note: Do not over-heat the slide as overheating will cause significant distortion or destruction of the cells.
3. Stain the smear using the May-Grünwald-Giemsa according to the manufacturers' instructions.
    a. The exact incubation times and staining concentrations vary by manufacturer. Refer to the package insert to obtain the exact protocol that should be used.
    b. The general protocol includes:
        i. Incubate the smear with the May-Grünwald stain.

ii. Dilute the stain with a buffer.

iii. Incubate the smear with a Giemsa stain.

iv. Rinse the slide with an appropriate buffer.

v. Allow the slide to air dry.

4. Once dried, examine the stained smear using immersion oil under a microscope with a 100X objective.
   a. Note: It can be easier to first identify the proper focal plane for the smear using a 10X objective prior to adding immersion oil, and then to switch to a 100X objective after adding immersion oil.
5. Whenever possible, count at least 200 cells on the slide.
   a. Within a cell, the cytoplasm will appear pink and nucleus will appear blue.
   b. Considering the cell shape and staining pattern, count the number of polymorphonuclear lymphocytes (also called polymorphonuclear neutrophils or granulocytes), monocytes, and lymphocytes.
6. Decontaminate the glass slide in bleach solution after each use before disposal in a biohazard sharps container.

11.0 Interpretation of Results

1. To determine the different WBC types, refer to the manufacturers insert to obtain the expected staining patterns. Representative images are present in [1].
2. Once the WBC types have been identified, you can determine the leukocyte formula (the percentage of different types of leukocytes in the smear). Refer to the appropriate guidance to interpret the leukocyte formula. WHO guidance is provided in Chapter 6 of [3] and a general description is listed below:
   a. If the CSF predominantly contains polymorphonuclear lymphocytes (also called polymorphonuclear neutrophils or granulocytes), this can be indicative of a bacterial infection.
   b. If the CSF predominantly contains lymphocytes and monocytes, this can be indicative of a viral infection.
   c. If the ratio of lymphocytes and monocytes to the polymorphonuclear lymphocytes is similar, the results are inconclusive.

12.0 Test Limitations

1. If the test is not completed within 30 minutes of CSF collection, lysis of red or white blood cells may have already occurred, resulting in inaccurate interpretations.
2. When few cells are present in the CSF, the test is more prone to sampling error and may lead to inaccurate counts.
3. Cell counts may vary between testing operators.

13.0 References

1. Rahimi J, Woehrer A. Overview of cerebrospinal fluid cytology. Handb Clin Neurol. 2017;145:563-71.
2. Deisenhammer F, Bartos A, Egg R, Gilhus NE, Giovannoni G, Rauer S, et al. Guidelines on routine cerebrospinal fluid analysis. Report from an EFNS task force. Eur J Neurol. 2006;13(9):913-22.
3. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.
4. Deisenhammer F, Bartos A, Egg R, Gilhus NE, Giovannoni G, Rauer S, et al. Routine cerebrospinal fluid (CSF) analysis. In: Gilhus NE, Barnes P, Brainin M, editors. European Handbook of Neurological Management. 1. 2nd ed: Blackwell Publishing Ltd.; 2011. p. 5-17.

3. Gram Stain Procedure

1.0 Purpose

This standard operating procedure provides instructions for Gram staining bacterial isolates or directly on cerebrospinal fluid (CSF).

2.0 Principle

Gram stain is a method that differentiates bacterial species into two large groups (Gram-positive or Gram-negative) based on the structure of the cellular wall. Gram-positive organisms retain the color of the crystal violet dye because of the increased number of cross-linked teichoic acids present within the cell wall and decreased permeability to organic solvents due to their low lipid content. Although Gram-positive organisms also absorb the counterstain, it does not affect their color. Because Gram-negative organisms have a higher lipid content, they are permeable to organic solvents and lose the crystal violet dye during the decolorization step. Gram-negative bacteria absorb and retain the color of the counterstain dye, safranin.

The color of the stained bacteria (below) and the cellular shape can be used to differentiate among bacterial species.

- Gram-positive (+): Appear purple because they retain the primary stain (crystal violet and iodine complex).
- Gram-negative (-): Appear pink because the primary stain is removed by the decolorizer and the counterstain is absorbed (safranin).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
CSF	Cerebrospinal fluid
+	Positive
-	Negative
PPE	Personal protective equipment
QC	Quality control

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 packs are sufficient.
3. Timer
4. Microscope
5. Pipette (2-20 µL)
6. *Optional*: Bunsen burner (only required if methanol is not available)

5.0 Reagents and Media

1. Applicable agar media for growth
2. Gram stain reagent kit (store at room temperature)
   a. Crystal Violet
   b. Iodine

c. Decolorizer

d. Safranin

e. *Note*: If safranin is not available, fuchsin or other counterstains may be used. Dissolve the counterstain into the appropriate diluent (varies between products and manufacturers). Refer to the product insert to prepare a working solution.

3. 95% Methanol (if unavailable, heat fixation can be used)

4. Sterile water or 0.85% Sodium Chloride (NaCl, saline)

6.0 Supplies

1. Pipette tips (2 - 20 µL)
2. Inoculating loops
3. Biohazard sharps container
4. Glass Slides
5. Waterproof pen/wax pencil
6. Pencil (for labeling slides)
7. Immersion oil
8. Bibulous paper or paper towel
9. Biohazard waste bag

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. Glass slides should be disposed of in a puncture-resistant biohazard sharps container.
5. Refer to Gram stain product safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information/Processing

1. Gram stain can be performed using bacterial isolates or CSF.
2. When assessing bacterial isolates, Gram stain should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is conducted.
3. Label the slide with the sample identification number.

9.0 Quality Control

1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. .

a. Gram positive cocci (such as *Streptococcus pneumoniae* or *Staphylococcus aureus*)

b. Gram negative bacilli (such as *Haemophilus influenzae* or *Escherichia coli*)

2. Before use, test each new lot of reagent with a positive and negative control.

3. When performing the test, QC organisms must be included on each slide.

10.0 Test Procedure

1. Using a BSC whenever possible, streak the bacteria on an appropriate agar plate and incubate overnight at 35-37 ºC with 5% CO_2.

2. Preparation of smear. Whenever possible, this step should be conducted within a BSC.

   a. Preparation of a smear using a bacterial culture

      i. Draw a circle on the slide with a wax pencil or waterproof pen.

         1. Note: Smears for multiple isolates can be produced on a single slide (a maximum of 3 is recommended). However, this practice increases the chance of cross-contamination between samples and should only be used after an operator is fully proficient in the method.

      ii. Using a pipette, add approximately 10-20 µL of sterile water or saline to the circle.

      iii. Use an inoculating loop to pick up a single colony from the overnight agar plate.

      iv. Gently swirl the loop in the sterile water or saline to create a slightly turbid suspension.

      v. Allow to air dry.

      vi. Repeat steps i-v for additional isolates, as necessary.

   b. Preparation of a smear using a CSF

      i. Assess the volume of the CSF available and proceed to step ii or iii.

      ii. If < 1 mL is available, take few drops of the CSF for Gram staining (no centrifugation required) and the rest should be plated on agar for bacterial growth (incubate overnight at 37 °C with 5% CO_2).

         1. Draw a circle on slide with wax pencil or waterproof pen

         2. Using pipette, add a few drops of the CSF.

         3. Allow to air dry.

      iii. If > 1 mL is available, the CSF should be centrifuged prior to Gram stain

         1. Centrifuge the CSF for 10-15 minutes, 1000 x g

         2. Transfer the supernatant to another tube and save this supernatant for latex testing, if applicable.

         3. Vigorously mix the cellular pellet.

         4. Plate 1 drop (10-20 µL) of the CSF pellet onto agar for culture and incubate overnight at 37 °C with 5% CO_2.

         5. Prepare the Gram stain smear of CSF pellet

            a. Draw a circle on slide with wax pencil or waterproof pen

            b. Using pipette, add 1-2 drops of the CSF pellet on the slide.

         6. Allow to air dry.

      iv. Critical: The slides must be completely dry before proceeding

3. Fixation of slides. Whenever possible, methanol fixation should be conducted within a BSC.

   a. Methanol fixation (preferred method)

      i. Completely cover the dried cell spot (or flood the slide) with 95% methanol.

ii. Incubate for two minutes.

iii. Discard any excess methanol and allow to fully dry (do not touch smear surface).

iv. *Note*: Methanol fixation is preferred because it maintains the bacteria's structural integrity better than heat fixation.

b. Heat fixation (use this method only if methanol is not available)

i. Quickly pass the slide through a flame three times.

ii. Note: Do not over-heat the slide as overheating will cause significant distortion or destruction of the cells.

4. Staining slides

a. Note: Apply the reagents listed in the next few steps directly onto the fixed smear. Avoid touching the slide with tip of reagent bottle.

b. Flood the slide with crystal violet for 1 minute.

c. Rinse with distilled water then shake off excess water.

d. Flood the slide with Gram's iodine for 1-2 minutes (refer to manufacturer's instructions).

e. Rinse with distilled water then shake off excess water.

f. Rinse with decolorizer for 5-15 seconds (refer to manufacturer's instructions) until the solution becomes clear.

g. Rinse with distilled water then shake off excess water.

h. Flood the slide with safranin for approximately 1 minute to counterstain.

i. Rinse with distilled water then shake off excess water.

j. Gently blot/dab slides on bibulous paper/paper towel to remove excess water and let air dry.

k. Once dried, examine the stained smear under a microscope with 100X oil immersion objective.

i. Note: It can be easier to first identify the proper focal plane for the smear using a 10X objective, and then to switch to a 100X oil immersion objective.

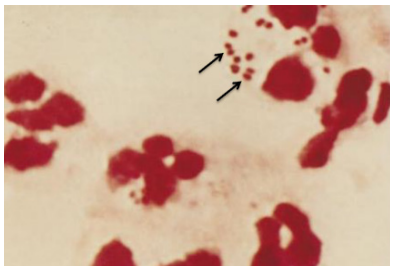
11.0 Interpretation of Results

1. Gram stain interpretation requires color and morphology to be recorded.

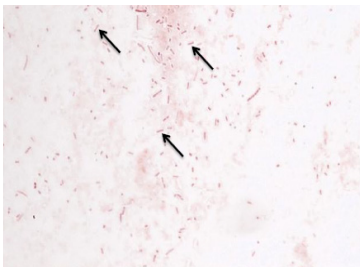
Organism	Gram	Morphology	Color (Safranin)
N. meningitidis	-	Diplococci or kidney bean-shaped in pairs	Pink to red
H. influenzae	-	Coccobacilli or rod-shaped	Pink to red
S. pneumoniae	+	Diplococci, cocci, lancet-shaped	Blue to purple

2. Representative Gram stain images (from [1]).

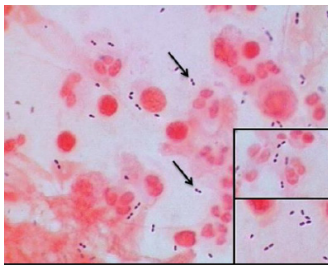
N. meningitidis



H. influenzae



S. pneumoniae



12.0 Test Limitations

1. It is recommended to use bacteria from an 18-24 hour culture grown on nonselective medium. Culture with growth >24 hrs will affect the gram stain results.
2. Insufficient or over-decolorization can affect interpretation.
   a. Gram (+) bacteria can appear Gram (-) by over-decolorization.
   b. Gram (–) can appear Gram (+) by insufficient decolorization.
3. When a smear is too thick, Gram (-) bacteria may not be fully decolorized during the decolorization steps and appear as Gram (+) bacteria.
4. Fixation with excessive heat can alter cell morphology.
5. Antibiotic treatments can affect the cell wall, resulting in misshapen or extremely long organisms and affect interpretation. For example, specimens with cocci shape may appear bacillus if the patient is on antimicrobial therapy.
6. Coccoid shapes or fungal elements, as well as other artifacts or background material, may appear on the slide as a result of precipitated crystal violet.

13.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.
2. Principles of Stains and Media. Manual of Clinical Microbiology, 9th Edition. 2007. ASM Press.

4. Catalase Test

1.0 Purpose

This standard operating procedure provides instructions for detecting expression of the catalase enzyme by an organism.

2.0 Principle

Some bacteria produce catalase, an enzyme that facilitates cellular detoxification. Catalase neutralizes the bactericidal effects of hydrogen peroxide (H_2O_2) by breaking down H_2O_2 into liquid water (H_2O) and oxygen gas (O_2). The bubbling observed during the reaction is due to the production and release of oxygen gas, which represent a positive catalase reaction.

$$2\ H_2O_2\ (\text{liquid}) + \text{Catalase} \rightarrow 2\ H_2O\ (\text{liquid}) + 1\ O_2\ (\text{gas})$$

The catalase test can be used to distinguish between Gram-positive cocci such as Staphylococci (catalase positive) and Streptococci/Enterococci (catalase negative).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.

5.0 Reagents and Media

1. Applicable agar media for growth
2. 3% H_2O_2 (light sensitive - store in a refrigerator in a dark bottle)
   a. If starting with 30% H_2O_2, dilute 1:10 in sterile water prior to use.

6.0 Supplies

1. Inoculating loops or Dacron polyester swabs
2. Transfer/Pasteur pipette
3. 5 mL test tubes or glass slides
4. Biohazard sharps container
5. Biohazard waste bag

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.

4. If applicable, sharps and glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to the H_2O_2 product safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information/Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is completed.
2. Label the tubes/slides with the sample identification number.

9.0 Quality Control

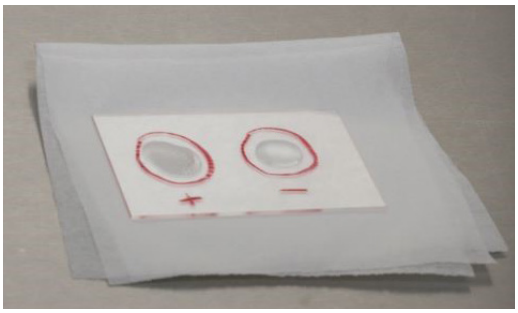
1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Staphylococcus aureus* (ATCC 6538)– positive (visible bubbling from O_2 production).
   b. *Streptococcus pneumoniae* (ATCC 6305)– negative (no bubbling from O_2 production).
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing the test, QC strains should be included each day the test is performed.

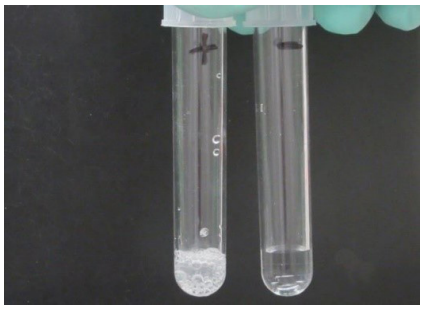
10.0 Test Procedure

1. Whenever possible, the following steps should be completed within a BSC.
2. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.
3. Tube Method (Strongly preferred for biosafety considerations. The catalase reaction produces aerosols, which are better contained within a tube than on a slide)
   a. Using an inoculating loop, collect a few colonies from a pure culture and smear the bacteria into the base of the test tube.
      i. Be careful to NOT transfer any of the blood agar (see test limitations)
   b. Add 4-5 drops of 3% H_2O_2 to each test tube.
   c. Quickly cap the tube.
   d. Immediately observe for bubbling.
4. Slide Method (only use this method if 5 mL tubes are not available)
   a. Using an inoculating loop, collect a few colonies from a pure culture and smear the bacteria onto a glass slide.
      i. Be careful NOT to transfer any of the blood agar (see test limitations).
   b. Add 1-2 drops of 3% H_2O_2 to the slide.
   c. Immediately observe for bubbling.

11.0 Interpretation of Results

1. Catalase Positive: Presence of bubbles immediately after the addition of H_2O_2.
2. Catalase Negative: Absence of bubbles 20 seconds after the addition of H_2O_2.





12.0 Test Limitations

1. Catalase is only expressed in viable cultures. Therefore, do not test cultures older than 24 hours, which can lead to false-negative results.
2. Red blood cells from the blood agar plates express the catalase enzyme. To avoid false-positive results, do not pick up any blood along with the bacteria.
3. Selecting colonies with a metal bacteriological loop can lead to false-positives.

13.0 References

1. Algorithm for Identification of Aerobic Gram-Positive Cocci. Manual of Clinical Microbiology, 9th Edition. 2007.
2. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

5. Oxidase Test

1.0 Purpose

This standard operating procedure provides instructions for performing the oxidase test on bacterial isolates.

2.0 Principle

The oxidase test detects the presence of the cytochrome oxidase enzyme and is useful for differentiating *Neisseria* and *Haemophilus* species from *Streptococcus* species. *Haemophilus* and *Neisseria* species express the cytochrome oxidase enzyme (oxidase positive), whereas *Streptococcus* species do not (oxidase negative). In this redox test, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) serves as an electron donor to cytochrome oxidase. If an organism expresses the cytochrome oxidase enzyme, TMPD is oxidized, resulting in an immediate color change from clear to blue (or dark violet).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.

5.0 Reagents and Media

1. Applicable agar media for growth
2. Oxidase strips (Hardy Diagnostics, Cat No. Z93)
   a. If oxidase strips are not available, you will need…
      i. Strips of filter paper (Whatman No. 2 or equivalent)
      ii. A clean petri dish or glass slide
      iii. Oxidase reagent (e.g., Kovac's reagent)
   b. Do not use expired or discolored reagents.

6.0 Supplies

1. Inoculating loops, plastic or platinum only
2. Biohazard waste bag
3. Paper towel

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.

4. Refer to safety data sheets when handling, working, or disposing of the oxidase reagent.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is conducted.

9.0 Quality Control

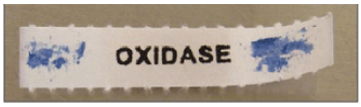
1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Pseudomonas aeruginosa* (ATCC 27853) – oxidase positive
   b. *Escherichia coli* (ATCC 51739) – oxidase negative
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing the test, QC strains should be included each day the test is performed.

10.0 Test Procedure

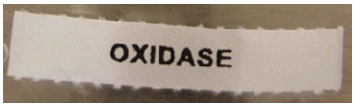
1. Prepare oxidase strips (skip to step 2 if you have commercially available oxidase strips)
   a. Place a strip of filter paper on a glass slide or in a Petri dish.
   b. Soak the filter paper with a few drops of the oxidase reagent.
   c. Let the filter paper air dry before use.
2. Whenever possible, the following steps should be completed within a BSC.
3. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.
4. To minimize the possible spread of contamination, place oxidase strip on a paper towel.
5. With an inoculating loop, collect an isolated colony from an overnight culture.
6. Apply the bacteria to the oxidase strip and observe for a change in color within 30 seconds.
   a. Note: Multiple isolates can be tested on a single oxidase strip – be careful to smear the bacteria onto distinct, non-overlapping portions of the strip.
7. After completion of the procedure, dispose the strip and paper towel in a biohazard waste bag.

11.0 Interpretation of Results

1. Oxidase positive: blue or dark violet color change within 30 seconds.
2. Oxidase negative: no color change within 30 seconds. A color change after 30 seconds is also interpreted as a negative result.
3. Note: If preparing oxidase strips as a part of this protocol, refer to the manufacturer's instructions for the specific oxidase reagent used to confirm the incubation time required for result interpretation.



Positive



Negative

Note: Images of oxidase positive and negative strips were obtained from [1].

Organism	**Oxidase**
N. meningitidis	+
H. influenzae	+
S. pneumoniae	-
P. aeruginosa	+
E. coli	-

12.0 Test Limitations

1. Do not use a nichrome or iron wire to select a colony because it can result in false-positives.
2. Timing is critical to accurate testing. Colonies older than 18-24 hours may produce weaker reactions.
3. Bacteria must be grown on an appropriate agar media before conducting the oxidase test.
   a. The oxidase test should not be performed on colonies grown on a medium containing a high concentration of glucose because the fermentation of glucose may inhibit oxidase activity.
   b. Use non-selective and non-differential media to ensure valid test results.

13.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.
2. Reagents, Stains, and Media: Bacteriology. Manual of Clinical Microbiology, 9th Edition. 2007.
3. Hardy Diagnostics. OxiStrips™ Oxidase Strips and OxiSticks™ Oxidase Swabs Available at: https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/OxiStripsOxisticks.htm. Accessed October 30, 2019.

6. *o*-Nitrophenyl β-D-Galactopyranoside (ONPG) Test

1.0 Purpose

This standard operating procedure provides instructions for detecting expression of the β-galactosidase enzyme, which ferments lactose.

2.0 Principle

o-Nitrophenyl β-D-galactopyranoside (ONPG) disks detect the expression of β-galactosidase and can be used to differentiate *Neisseria meningitidis* from other *Neisseria* species. The chemical structure of ONPG is similar to that of lactose and thus, can be cleaved by the β-galactosidase enzyme. Upon cleavage, ONPG produces galactose and o-nitrophenol, which has a yellow color at a physiological pH (7.5) or higher. *Neisseria lactamica* expresses the β-galactosidase enzyme (produces a yellow color change) but *Neisseria meningitidis* does not (no color change is observed).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
ONPG	*o*-nitrophenyl β-D-galactopyranoside
PPE	Personal protective equipment
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC)
2. 35–37°C Incubator (non-CO_2)
3. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.
4. Pipette (200-1000 µL)

5.0 Reagents and Media

1. Applicable agar media for growth
2. *o*-Nitrophenyl β-D-galactopyranoside (ONPG) Diatabs (Key Scientific, Rosco Diagnostica RO50311, 50 discs/tube).
3. 0.85% Saline solution

6.0 Supplies

1. Clean 5 mL test tube with cap
2. Test tube holder
3. Transfer/Pasteur pipettes
4. Pipette tips (200-1000 µL)
5. Inoculating loops or Dacron polyester swabs
6. Forceps
7. McFarland 4.0 standard

8. Biohazard waste bag
9. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to ONPG product safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37ºC with 5% CO_2) until a pure culture is available before testing is conducted.
2. Label the tubes with the sample identification number and “ONPG”.

9.0 Quality Control

1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer’s package insert for a complete list of recommended ATCC strains.
   a. *Neisseria lactamica* (ATCC 23970) – ONPG positive
   b. *Neisseria meningitidis* (ATCC 13077) – ONPG negative
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing testing, QC strains should be included every time a test is performed.
4. The saline solution must undergo QC testing for sterility prior to use in bacterial suspension preparations.
   a. Plate the saline solution onto the appropriate test media and incubate under normal growth conditions. Confirm no growth occurs before testing.

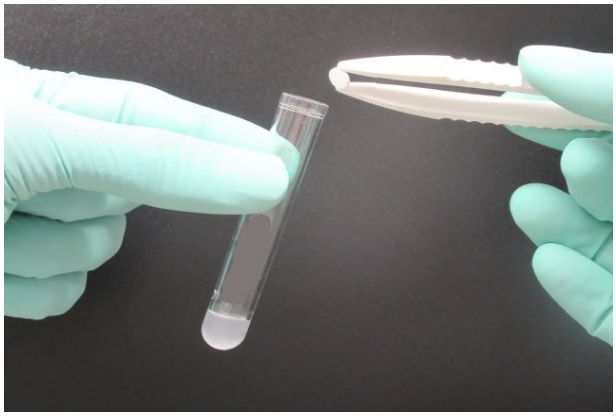
10.0 Test Procedure

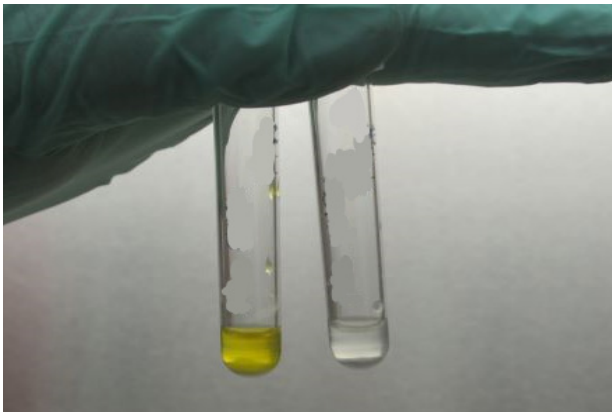
1. Whenever possible, the following steps should be completed within a BSC.
2. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37ºC with 5% CO_2.
3. Add 250 µL of saline to a 5 mL tube.
4. Using a swab or loop, prepare a heavy bacterial suspension in the tube equivalent to a McFarland 4.0 standard.
5. Carefully drop an ONPG Diatab into the tube using forceps.
6. Close the tube loosely with a cap.
7. Incubate at 35 – 37˚C (non-CO_2) for 4 hours.
8. Look for a color change and record the preliminary reading (see Interpretation of Results).

9. Incubate at 35 – 37˚C (non-CO_2) overnight.
10. Look for a color change the following morning and record the final reading.

11.0 Interpretation of Results

1. Color changes observed after either 4 hours or an overnight incubation are interpreted as positive results.
2. ONPG Positive: Yellow color is observed.
3. ONPG Negative: No color change is observed.





12.0 Test Limitations

1. If the bacterial culture alone appears to have a yellow color, the isolate should not be tested against ONPG. A bacterial suspension alone (with no tablet added) can be used as a control to confirm that any observed color change is not due to the culture itself.
2. The pH of the reaction is important for accurate interpretations.
   a. If the saline solution is buffered, results may be inaccurate.
   b. The reactions will not produce a change in color under acidic conditions.

13.0 References

1. Product insert with general instructions for use of Rosco Diagnostics Diatabs (Key Scientific). https://www.rosco.dk/gfx/pdf/Diatabs%20-%20Print%20Insert%20-%20Eng(3).pdf
2. User's Guide Diatabs: Diagnotic tablets for bacterial identification (Key Scientific, procedure on p. 62 and additional information beginning on p. 71). https://www.keyscientific.com/files/Other%20Manufacturers/Rosco/DiaTabs/Rosco%20DiaTabs.pdf

7. Gamma-Glutamyl-Beta-Naphthylamide (GGT) Test

1.0 Purpose

This standard operating procedure provides instructions for the gamma-glutamyl-β-naphthylamide (GGT) test on bacterial isolates.

2.0 Principle

The GGT test detects the expression of γ-glutamyl aminopeptidase and is useful for differentiating *Neisseria meningitidis* from other *Neisseria* species. The breakdown of GGT by γ-glutamyl aminopeptidase produces β-naphthylamine, which yields a red color in the presence of ρ-dimethyl-amino-cinnamaldehyde and acidic conditions (PEP reagent). *Neisseria meningitidis* expresses γ-glutamyl aminopeptidase (GGT positive) and therefore, will produce a red color in the presence of the GGT substrate.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
GGT	gamma-glutamyl-β-naphthylamide
PPE	Personal protective equipment
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC)
2. 35–37ºC Incubator (non-CO_2)
3. 35–37ºC Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37ºC incubator supplemented with a candle jar or CO_2 pack is sufficient.
4. Pipette (200-1000 µL)

5.0 Reagents and Media

1. Applicable agar media for growth
2. 0.85% Saline solution
3. Gamma-glutamyl-beta-naphthylamide (GGT) Diatabs (Key Scientific - Rosco Diagnostica RO46711, 50 discs/tube)
4. PEP reagent (ρ-dimethyl-amino-cinnamaldehyde in hydrochloric acid)
5. Note: May be supplied with the GGT discs. If not, use Key Scientific - Rosco Diagnostica RO92231, 3 mL/bottle)

6.0 Supplies

1. Clean 5 mL test tube with cap
2. Test tube holder
3. Transfer/Pasteur pipettes
4. Pipette tips (200-1000 µL)
5. Inoculating loops or Dacron polyester swabs

6. Forceps
7. McFarland 4.0 standard
8. Biohazard waste bag
9. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to the GGT and PEP reagent product safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is conducted.
2. Label the tubes with the sample identification number and "GGT."

9.0 Quality Control

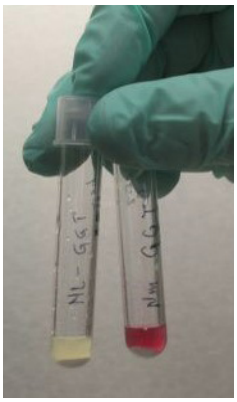
1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Neisseria meningitidis* (ATCC 23970) – GGT positive
   b. *Neisseria lactamica* (ATCC 13077) – GGT negative
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing testing, QC strains should be included each time a test is performed.
4. The saline solution must undergo QC testing for sterility prior to use in bacterial suspension preparations.
   a. Plate the saline solution onto the appropriate test media and incubate under normal growth conditions. Confirm no growth occurs before testing.

10.0 Test Procedure

1. Whenever possible, the following steps should be completed within a BSC.
2. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.
3. Add 250 µL of saline to the 5 mL tube.
4. Using a swab or loop, prepare a heavy bacterial suspension in the tube equivalent to a McFarland 4.0 standard.
5. Carefully drop one GGT Diatab into the tube using forceps.
6. Close the tube loosely with a cap.

7. Incubate the tube at 35-37˚C for 4 hours without CO_2.
8. After the 4 hour incubation, add three drops of PEP reagent.
9. Observe for a color change within five minutes of PEP reagent addition.

11.0 Interpretation of Results



1. Color changes observed after 4 hours and the addition of PEP reagent are interpreted as positive results.
2. GGT positive: Red color change within five minutes of PEP reagent addition.
3. GGT negative: No color change within five minutes of PEP reagent addition.

12.0 Test Limitations

1. If the bacterial culture alone appears to have a pink color, the isolate should not be tested against GGT. A bacterial suspension alone (with no tablet added) can be used as a control to confirm that any observed color change is not due to the culture itself.
2. The pH of the reaction is important for accurate interpretations.
   a. If the saline solution is buffered, results may be inaccurate.
   b. The reactions will not produce a change in color under basic pH conditions.

13.0 References

1. Product insert with general instructions for use of Rosco Diagnostics Diatabs (Key Scientific). https://www.rosco.dk/gfx/pdf/Diatabs%20-%20Print%20Insert%20-%20Eng(3).pdf
2. User's Guide Diatabs: Diagnotic tablets for bacterial identification (Key Scientific, procedure p. 13-15).https://www.keyscientific.com/files/Other%20Manufacturers/Rosco/DiaTabs/Rosco%20DiaTabs.pdf

8. Bile Solubility Test

1.0 Purpose

This standard operating procedure provides instructions for distinguishing *S. pneumoniae* from all other alpha-hemolytic streptococci using the bile solubility test.

2.0 Principle

The bile solubility test is a qualitative procedure based on the observation that *S. pneumoniae* lyse in the presence of bile salts under specific time and temperature conditions, but other alpha-hemolytic Streptococci do not. The working mechanism of the test is not clearly understood. It is thought that lysis depends on the presence of an intracellular autolytic enzyme, an amidase, which is produced by *S. pneumoniae*. Bile salts lower the surface tension between the bacterial cell membrane and the medium, thus accelerating the organism's natural autolytic process; bile salts also activate the autolytic enzyme which induces clearing of the culture. The bile solubility test may be performed using a bacterial suspension or by applying the Bile Spot Reagent directly onto a bacterial colony.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC) (recommended)
2. 35 – 37°C Incubator (non-CO_2)
3. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.
4. Equipment for the test tube method only
   a. Pipette (200-1000 µl)
   b. Vortex

5.0 Reagents and Media

1. Applicable agar media for growth
2. 0.85% Sterile saline
3. Bile salts (concentration required depends on the method used).
   a. Test tube method: 2% sodium deoxycholate (store 15-30°C, protected from light)
      i. If a 2% solution is not available, dissolve 0.2 g sodium deoxycholate in 10 mL of sterile water.
   b. Direct plate method: 10% sodium deoxycholate (store 15-30°C, protected from light).
      i. If the 10% solution is not available, this can be prepared from the powder. Dissolve 10 g sodium deoxycholate into 100 mL sterile water.
   c. If using a commercially available bile salt reagent, refer to the manufacturer's insert for concentration information and requirements (example: Bile Spot Reagent, Hardy Diagnostics, Cat #Z61).

6.0 Supplies

1. Biohazard waste bag
2. Transfer/Pasteur pipettes
3. Supplies specific for the test tube method
   a. Test tubes, 5 mL
   b. Polyester Dacron Swabs
   c. McFarland 0.5 or 1.0 standard
   d. Pipette tips (200-1000 µl)

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC.
4. Refer to the sodium deoxycholate or bile spot reagent safety data sheet for proper storage, handling, and disposal.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
2. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is conducted.
3. If using the test tube method, label two tubes with the sample identification number for each isolate and “Bile”.

9.0 Quality Control

1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer’s package insert for a complete list of recommended ATCC strains.
   a. *Streptococcus pneumoniae* (ATCC 49619) – Soluble in bile.
   b. *Enterococcus faecalis* (ATCC 29212) or *Streptococcus mitis* (ATCC 49456) – Insoluble in bile.
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing testing, QC strains should be included every time a test is performed.
4. The saline solution must undergo QC testing for sterility prior to use in bacterial suspension preparations.
   a. Plate the saline solution onto the appropriate test media and incubate under normal growth conditions. Confirm no growth occurs before testing.

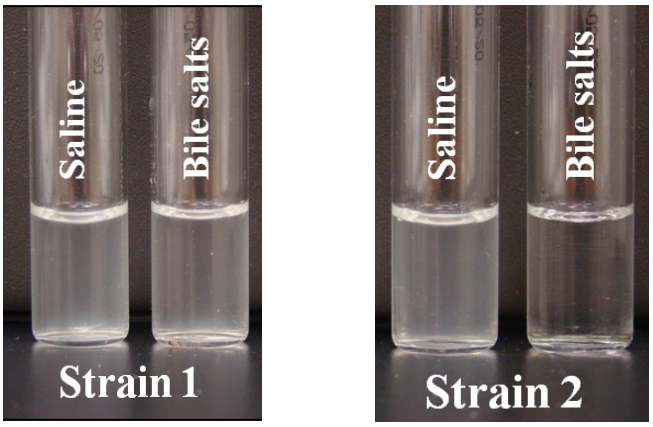
10.0 Test Procedure

1. Note: Storing the deoxycholate reagent at lower temperatures can cause it to thicken.
2. Warm the reagent bottle using a 37 °C incubator (non-CO_2 and not used for culturing pathogens) before use.
3. It is recommended that the following steps (for both the Test Tube method and the Direct Plate Method) be completed within a BSC, if possible.
4. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.

5. Test Tube Method (Preferred method)
    a. Pipette 1 mL of sterile 0.85% saline into one test tube.
    b. Using a swab, collect bacteria and resuspend in the saline tube to prepare a suspension equivalent to a 0.5 - 1.0 McFarland standard.
    c. Transfer half of the bacterial suspension (0.5 mL) to a second tube containing the specimen identification number.
    d. Label one tube “TEST” and the other “CONTROL”.
    e. Add 0.5 mL of 2% sodium deoxycholate (bile salt) into the tube labeled “TEST.”
    f. Add 0.5 mL of 0.85% saline into the tube labeled “CONTROL.”
    g. Gently mix each tube.
    h. Incubate the tubes at 35-37 °C (CO_2 is preferred but non-CO_2 can be used) for 10 minutes. Observe the “TEST” tube for any clearing of turbidity compared to the “CONTROL” tube.
    i. Continue to incubate the tubes for up to 2-3 hours if negative after 10 minutes. Observe the “TEST” tube for any clearing of turbidity compared to the “CONTROL” tube.
6. Direct Plate Method
    a. Note: The result interpretations by the direct plate method can be challenging, which is why the test tube method is preferred.
    b. Place a drop of Bile Spot Reagent (10% deoxycholate) near (or on) a suspected 18-24 hour old colony.
        i. Do not touch the agar surface with the tip of the dropper of bile reagent.
    c. Gently tilt the plate to roll the drop over several colonies.
        i. Be careful not to dislodge the colonies.
    d. Incubate the plate right-side up (agar on the bottom) at 35-37 ºC for 30 minutes (non-CO_2).
    e. When the reagent on the suspected colonies are dry - read, interpret, and record the results.

11.0 Interpretation of Results

1. Test Tube Method: Examine the “TEST” tube for clearing or loss of turbidity as compared to the “CONTROL” tube within two to three hours of incubation.
    a. Positive results: Bile solubility is demonstrated by the clearing or loss of turbidity in the “TEST” tube relative to the “CONTROL” tube.
        i. Note: Partial clearing (partial solubility) is not considered positive for *S. pneumoniae* identification. Partially soluble strains that have optochin zones of inhibition of less than 14 mm are not considered pneumococci.
    b. Negative results: Bile insolubility is demonstrated by little or no change in turbidity in the “TEST” tube relative to the “CONTROL” tube.



Bile Insoluble (-) Bile Soluble (+)

Figure 1: Tube method image from [1].

2. Direct Plate Method: Examine the plate for disintegration or flattening of the treated colonies and/or the appearance of α-hemolytic zone on the medium within 30 minutes of incubation.

   a. Positive results: Disintegration or flattening of the colonies and/or the appearance of α-hemolytic zone on the medium in the area treated with bile salts.

   b. Negative results: No change in the integrity of the colonies and/or a lack of a α-hemolytic zone on the medium in the area treated with bile salts.

   c. Images to aid in the interpretation of the Direct plate method are provided in [2].

3. Test Limitations [2] The test should not be performed on old cultures, as the active enzyme may be lost, resulting in a false-negative. Therefore, saline suspensions of fresh 18-24-hour cultures are used.

4. Normal autolysis of *S. pneumoniae* may be inhibited if too high of a concentration of bile salts is used. Evaporation may cause the reagent to become more concentrated, therefore affecting the test.

5. When performing the bile solubility tube test using saline or unbuffered broth, it is essential to adjust the pH to neutral before making the bacterial suspension and adding the reagent in order to avoid false negative reactions.

6. When using the plate method, be careful to not dislodge the colonies being tested, which may lead to false-positive results. If the direct plate is difficult to interpret, the test should be repeated using the test tube method.

12.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

2. Procedure and additional references are available in the product insert: https://catalog.hardydiagnostics.com/cp_prod/content/hugo/bilespotrgnt.htm

9. Cysteine Trypticase Agar (CTA) - Sugar Utilization Test

1.0 Purpose

This standard operating procedure provides instructions for differentiating fastidious organisms, such as *Neisseria* species, based on their ability to utilize carbohydrates.

2.0 Principle

The test uses five different carbohydrates or sugars and detects the ability of the organism to utilize each sugar independently: glucose (also called dextrose), maltose, lactose, fructose and sucrose. The carbohydrates are individually added to a cystine trypticase agar (CTA) base to obtain a final concentration of 1%. A phenol red indicator is also included in the medium, which develops a yellow color in the presence acid production (pH ≤ 6.8). If the organism is capable of utilizing the carbohydrate by oxidation (not fermentation), an acid will be produced, generating a color change (red to yellow) in the agar medium. Thus, this test can distinguish between multiple *Neisseria* species by identifying the unique combination of the 5 carbohydrates that can be utilized. For example, *N. meningitidis* oxidizes glucose and maltose, but not the lactose, fructose, or sucrose.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
CTA	Cysteine Trypticase Agar
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC)
2. 35-37°C Incubator (non-CO_2)
3. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.

5.0 Reagents and Media

1. Applicable agar media for growth
2. If commercial CTA are not available, each of the following agar bases should be prepared in a separate test tube
   a. Cysteine Trypticase Agar (base only)
   b. Cysteine Trypticase Agar with 1% Glucose (also called dextrose)
   c. Cysteine Trypticase Agar with 1% Maltose
   d. Cysteine Trypticase Agar with 1% Sucrose
   e. Cysteine Trypticase Agar with 1% Lactose
   f. Cysteine Trypticase Agar with 1% Fructose

6.0 Supplies

1. Inoculating loops
2. Test tube rack
3. Biohazard waste bag

4. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to the safety data sheet of the CTAs for information on proper storage, handling, and disposal.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before the testing is conducted.
2. Label each CTA sugar with the sample identification number.

9.0 Quality Control

1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Neisseria lactamica* (ATCC 23970) will utilize glucose, maltose and lactose but not sucrose and fructose.
   b. *Neisseria sicca* (ATCC 13077) will utilize glucose, sucrose, maltose, and fructose but not lactose.
2. Before use, test each new lot of reagent using two organisms for every sugar and include sterility controls.
   a. Sterility control: Loosen the caps for one complete set of CTA sugars (CTA base only and each sugar) and place into a non-CO_2 incubator for 72 hours to confirm sterility.
      i. Sterility tubes should all remain a reddish color and have no visible growth.
3. When performing testing, a control CTA base should be inoculated with every test. QC organisms are not required with each test.

10.0 Test Procedure

1. Whenever possible, the following steps should be completed within a BSC.
2. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.
3. Before starting the test, remove the sugar tubes from the refrigerator and allow them to reach room temperature.
4. Inoculate one set of tubes (1 base + 5 carbohydrates = 6 tubes in total) for each strain.
   a. Note: Be sure to change loops between each sugar to prevent carryover. If metal loops are being used, thoroughly clean and sterilize the loop between each sugar.
   b. Place the complete set of CTA tubes into a test tube rack.
   c. For each tube in the set:
      i. Use an inoculating loop to pick up a loopful of colonies from an overnight culture (18-24 hours).
      ii. Inoculate the media by stabbing into the upper third (10 mm) of the agar several times repeatedly (~8 times).

iii. Close the tubes loosely.

d. Incubate at 35 – 37 °C in an incubator (non-CO_2) for 24-72 hours.

5. Repeat step 4 for all isolates being tested that day.

6. Examine each tube daily (up to 72 hrs) for evidence of sugar utilization as indicated by a color change and bacterial growth.

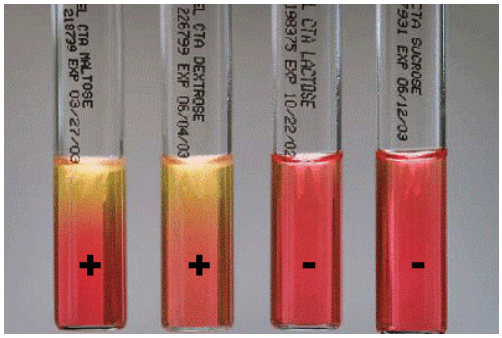
11.0 Interpretation of Results

1. When interpreting color change, it is helpful to consider the following:
   a. *Neisseria* species usually produce acid in the upper third of the media (around the site of inoculation), resulting in a color change.
   b. Any yellow color change observed throughout the media (whole tube and uninoculated sites) may indicate contamination.

2. The control CTA base has no carbohydrates in the media and therefore should not have a color change. If a color change is observed in this tube, the tube is contaminated.
   a. Consider repeat testing and discarding the results if contamination is suspected.
      i. To determine if the product is contaminated, incubate uninoculated CTA base for 72 hours and observe if a color change occurs.
      ii. If no color change is observed, proceed with retesting.

3. Positive: Yellow color on the surface of the medium (sugar was utilized, producing an acid that changed the color from red to yellow) and growth (visualized as turbidity) from the stab line of inoculation.

4. Negative: Orange/red color on the surface of the medium (no color change is observed). A yellow color change without visible growth is also considered a negative result.



Organism	Acid production from:				
	Glucose	Maltose	Lactose	Sucrose	Fructose
Neisseria meningitidis	+	+	-	-	-
Neisseria lactamica	+	+	+	-	-
Neisseria gonorrhoeae	+/-	-	-	-	-
Neisseria sicca	+	+	-	+	+
Moraxella catarrhalis	-	-	-	-	-

12.0 Test Limitations

1. Do not inoculate to the bottom of the tube; improper inoculation may lead to weak acid reactions, making interpretation challenging.

2. Aerobic incubation is necessary. Incubation with CO_2 may lead to erroneous results.

3. Lack of insufficient inoculum may lead to false negatives.

4. Although extremely rare, strains of *N. meningitidis* capable of utilizing glucose or maltose, but not both, have been reported.

13.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

10. Optochin Susceptibility Test

1.0 Purpose

This standard operating procedure provides instructions for differentiating *Streptococcus pneumoniae* from other α-hemolytic *Streptococcus* species based on their ability to grow in the presence of optochin.

2.0 Principle

The optochin susceptibility test is based on the differential sensitivity of *S. pneumoniae* and other α-haemolytic streptococci to optochin (hydrochloride ethylhydrocupreine). Organisms that are susceptible to optochin will form a region of no growth (called a zone of inhibition) around an optochin disc; organisms that are resistant will grow without inhibition. For this test, a filter paper disc with 5 µg of optochin is placed on the surface of a pure culture and the zone of inhibition is quantified. *S. pneumoniae* is susceptible to optochin (exhibits a zone of inhibition) but other α-haemolytic streptococci are resistant (no growth inhibition observed).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
BAP	Blood agar plate
QC	Quality control

4.0 Equipment

1. Biosafety cabinet Class IIA (BSC) (recommended)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.
3. Caliper or ruler (mm)

5.0 Reagents and Media

1. Applicable agar media for growth
2. 6mm Optochin disk (P-disk) (5 µg available from most vendors)
   a. If unavailable, a 1:4000 dilution of 100% ethylhydrocupreine hydrochloride (optochin) can be applied to sterile 6 mm filter paper disks.
3. Blood agar plate (BAP, 5% sheep blood)
   a. Note: Use of media other than 5% sheep blood is not recommended as it may yield a smaller zone of inhibition, resulting in incorrect interpretation.

6.0 Supplies

1. Inoculating loops
2. Sterile forceps
3. Biohazard waste bag

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.

2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC.
4. Refer to the safety data sheet of the product (s) that will be used during this test for information on proper storage, handling, and disposal.

8.0 Sample Information/Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is conducted.
2. Label each BAP plate with the sample identification number and "Optochin".

9.0 Quality Control

1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Streptococcus pneumoniae* (ATCC 49619) – Zone of inhibition ≥ 14 mm
   b. *Streptococcus mitis* (ATCC 49456) – No zone of inhibition
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing testing, QC strains should be included at least weekly. If the test is performed infrequently, QC strains should be included each day of testing.
   a. Confirm the disk integrity prior to each use. Do not use disks that appear damaged or failed initial QC.

10.0 Test Procedure

1. It is recommended that the following steps be completed within a BSC, if possible.
2. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.
3. Allow the BAP agar media to warm to room temperature.
4. Using an inoculating loop, select an isolated colony from an overnight culture of the α-hemolytic organism.
5. Streak the isolated colony onto a fresh BAP plate in at least two different directions to obtain confluent growth.
   a. Note: Two different isolates can be tested on the same plate, but you must ensure that the two streaking patterns do not overlap. Overlap will result in mixed cultures and invalid results.
6. Using forceps, place the optochin disk within the streaked area of the plate. Lightly push on the disk to ensure if adheres firmly to the agar plate.
   a. Note: Sterilize forceps between isolates if using non-disposable.
7. Incubate the BAP for 18-24 hours at 35 – 37 °C with 5% CO_2.
8. Observe the growth surrounding the disk and measure the diameter of the zone of inhibition using a millimeter ruler or caliper.
   a. The diameter can be measured by placing the ruler or caliper across the center of the zone of inhibition.
   b. Note: A culture that exhibits confluent growth around the disk will have a zone of inhibition equal to the diameter of the disk itself.

11.0 Interpretation of Results

1. The size of the zone of inhibition around a 6 mm disk can be interpreted using the following criteria:
   a. Positive results (Optochin susceptible): zone of inhibition is ≥14 mm
   b. Negative results (Optochin resistant): no zone of inhibition is observed
   c. Equivocal results: Any organism with a zone of inhibition 7-14 mm cannot be interpreted. A bile solubility test should be performed to determine if the bacteria is *S. pneumoniae*.
2. Note: For a 10 mm, 5 µg optochin disk, use as zone of inhibition diameter of ≥ 16 mm.
   a. Pneumococci will have a zone of inhibition ≥ 16 mm. Any organisms exhibiting growth inhibition < 16 mm must be tested by the bile solubility test.
3. A representative image is shown below (from [1]).

Resistant Susceptible

12.0 Test Limitations

1. A weak zone of inhibition (< 14 mm) indicates that the bile solubility test is necessary.
2. *S. pneumoniae* isolates should be incubated in a CO_2-enriched environment, as some isolates will grow poorly or not at all in the absence of CO_2. Poor growth conditions could affect interpretation of the zone of inhibition.

13.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

11. Growth Factor Disk Test

1.0 Purpose

This standard operating procedure provides instructions for differentiating between *Haemophilus* species, including Aggregatibacter aphrophilus, based on their requirements for growth factors X (hemin) and V (nicotinamide adenine dinucleotide, NAD).

2.0 Principle

Because different *Haemophilus* species have distinct requirements for X and V growth factors, this test can differentiate between *Haemophilus* species. The suspected *Haemophilus* isolates are grown on a limiting medium, which is not capable of sustaining *Haemophilus* growth on its own. The growth factors are impregnated in one of three types of disks (X only, V only, or X and V together) and placed on the surface of the agar. The growth factors diffuse into the medium from the disks, permitting growth around the disk itself. Using this test, you can determine if the *Haemophilus* species requires hemin, NAD, or both for growth.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
NAD	Nicotinamide adenine dinucleotide
Factor X	Hemin
Factor V	NAD
QC	Quality control

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.

5.0 Reagents and Media

1. Applicable agar media for growth
2. 0.85% Sterile saline
3. Growth factor-free growth medium
   a. Tryptic Soy Agar (TSA) (Cat No. G60, Hardy Diagnostics)
   b. Brain Heart Infusion Agar (BHI) (Cat No. W15)
   c. Mueller Hinton Agar (MHA) (Cat No. G45)
4. Growth factors disks (store -20°C to 8°C in the dark)
   a. X-Factor (Cat No. Z7031, Hardy Diagnostics)
   b. V-factor (Cat No. Z7041, Hardy Diagnostics)
   c. XV-Factor (Cat No. Z7051, Hardy Diagnostics)

6.0 Supplies

1. Clean 5 mL test tubes and test tube rack

2. Transfer/Pasteur Pipette
3. McFarland 0.5 standard
4. Dacron polyester swab
5. Sterile Forceps
6. Biohazard waste bag
7. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Refer to the safety data sheet of the product (s) that will be used during this test for information on proper storage, handling, and disposal.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before the test is conducted.
2. Label each agar plate with the sample identification number.

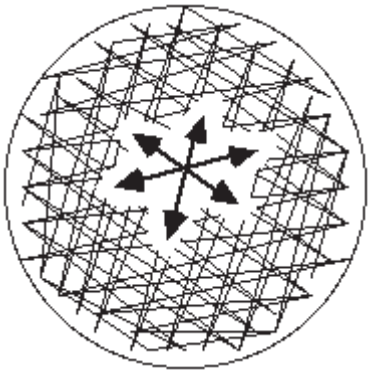
9.0 Quality Control

1. Use the following positive and negative control organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Haemophilus influenzae* (ATCC 10211): X- and V- factor: No Growth; XV-factor: Growth
   b. *Aggregatibacter aphrophilus* (ATCC 33389): X-, V-, XV-factor: Growth
2. Before use, test each new lot of reagent with a positive and negative control.
3. When performing testing, QC strains should be included each day that testing is completed.
4. The saline solution must undergo QC testing for sterility prior to use in bacterial suspension preparations.
   a. Plate the saline solution onto the appropriate test media and incubate under normal growth conditions. Confirm no growth occurs before testing.

10.0 Test Procedure

1. Note: This protocol is specific to Hardy Diagnostics products, although it may be applicable to additional manufacturers. Regardless of the brand, always consult the manufacturer's guidelines for the proper and updated procedure (Hardy Diagnostics, 2017).
2. Whenever possible, the following steps should be completed within a BSC.

3. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37ºC with 5% CO_2.
4. Make the bacterial suspension
   a. Dispense 1.0 mL of sterile saline into a 5 mL tube using a Pasteur pipette.
   b. Use a sterile swab to select several isolated colonies and prepare a suspension in the saline equivalent to a McFarland 0.5 standard.
5. Streak for confluent growth
   a. Dip a clean, fresh swab into the saline suspension.
   b. Remove excess liquid by rotating the swab along the inner walls of the tube above the solution.
   c. Swab the entire surface of the growth factor-free media by streaking in three different directions and rotating the plate in a 60-degree angle after each streaking (streaking for confluence).
   d. Avoid touching the sides of the plate to avoid aerosols. Finally, run swab around the edge of the agar to remove any excess moisture.
   e. Allow the media surface to dry for 3-5 minutes.



6. Apply the growth factor disks to the plate
   a. Using sterile forceps, apply disks around the plate (approximately 1-2 cm from the edge of the medium).
      i. Note: Sterilize forceps between isolates if using non-disposable.
   b. Apply disks in the following positions:
      i. Note: Space the disks as far apart as possible to ensure that the growth factors released from each disk do not overlap on the agar.

X-Factor Disk	12 o'clock
V-Factor Disk	4 o'clock
XV-Factor Disk	8 o'clock

7. Incubate the inoculated media 18-48 hours at 35–37 ºC in 5% CO_2.

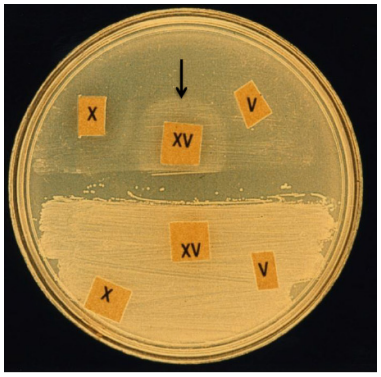
11.0 Interpretation of Results Procedure

1. Observe growth on the plate, specifically around the growth factor disks, up to 48 hours after inoculation.
2. Expected growth patterns:
   a. Organisms that require both X and V factor for growth
      i. Growth will only be observed around the XV disk.
      ii. *H. influenzae*, *H. aegyptius*, and *H. haemolyticus* will exhibit this type of growth.
   b. Organisms that only require X factor for growth
      i. Equivalent growth observed around both the X and XV disks.
      ii. *Haemophilus* species in this category will exhibit a pattern of growth consistent with only requiring one growth factor.
   c. Organisms that only require V factor for growth
      i. Equivalent growth observed around both V and XV disks.
      ii. *Haemophilus* species in this category will exhibit a pattern of growth consistent with only requiring one growth factor.

d. Organisms that do not require either X or V factor for growth

i. Growth will occur as a lawn across the inoculated medium including around all three disks.

3. A representative image is shown below (from [2]).



The top bacterial culture requires both X and V: Growth is only observed around the XV disk.

The bottom bacterial culture does not require either growth factor: Growth is observed throughout the inoculated agar including around each disk.

12.0 Test Limitations

1. Use care when inoculating the agar to prevent nutrient carryover.
2. The density of the culture and growth conditions are important for obtain accurate results (CO_2 is essential for *Haemophilus* growth).
3. If the disks are placed too close together, the growth factors can mix within the agar, leading to inaccurate results.
4. Because similarities exist in growth factor requirements of *Haemophilus* species, it is not recommended that this procedure be the sole criterion for species identification. Additional testing may be required.

13.0 References

1. Hardy Diagnostics. X- AND V-FACTOR DISKS Available at: https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/XVFactorDisks.htm. Accessed October 30, 2019.
2. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.
3. Haemophilus. Manual of Clinical Microbiology, 9th Edition. 2007. ASM Press.

12. Slide Agglutination Test

1.0 Purpose

This standard operating procedure provides instructions for performing slide agglutination on *Neisseria meningitidis* (Nm) isolates for serogrouping and on *Haemophilus influenzae* (Hi) isolates for serotyping.

2.0 Principle

Neisseria meningitidis and *Haemophilus influenzae* can express a polysaccharide capsule, which is often associated with virulence. The Nm serogroups include A, B, C, Z'/E, W, X, Y, and Z; the Hi serotypes are a, b, c, d, e, and f. In the slide agglutination test, a bacterial suspension is mixed with an antiserum (antibody). When the antibodies interact with the antigens on the bacterial cell surface, aggregated bacterial clumps will form; this formation is known as agglutination and is visible to the unaided eye. When determining the Nm serogroup or Hi serotype, an isolate is often assessed using multiple slide agglutination reactions for the serogroups/serotypes commonly associated with disease in that region.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
Nm	*Neisseria meningitidis*
Hi	*Haemophilus influenzae*
QC	Quality control

4.0 Equipment

1. Biosafety Class IIA (BSC IIA)
2. Chemical fume hood
3. 35–37ºC Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37ºC incubator supplemented with a candle jar or CO_2 pack is sufficient.
4. Pipettes (1-10 µL, 2-20 µL, 200-1000 µL)
5. Vortex
6. Timer
7. Electric pipette aid or a bulb for serological pipettes
8. Optional: Lamp for direct light

5.0 Reagents and Media

1. Applicable agar media for growth
2. The following antisera are commercially available for Nm and Hi:
   a. Nm: A, B, C, Z'/E, W, X, Y, Z
   b. Hi: a, b, c, d, e, f
   c. Antisera should be stored in a refrigerator (4 °C)
3. 0.85% Physiological Saline

4. 37% formaldehyde solution

6.0 Supplies

1. 2 inch x 3 inch glass slides
2. PAP pen
3. Pipette tips (1-10 µL, 2-20 µL, 200-1000 µL)
4. 1.5 mL or 2 mL microcentrifuge tubes
5. Dacron Polyester Swabs
6. McFarland 4.0 standard
7. Serological pipette (5 mL, 100 mL)
8. 200 mL beaker
9. Biohazard waste bag
10. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.
5. Antisera contain 0.1% sodium azide. Refer to the sodium azide product safety data sheet for proper storage, handling, and disposal.
6. Formaldehyde is hazardous substance. Refer to the formaldehyde product safety data sheet for proper storage, handling, and disposal.
    a. Even though the bacteria are suspended in a formaldehyde solution, the contact time during the course of the testing process is not sufficient to inactivate the bacteria. Thus, bacterial suspensions should be treated as infectious materials.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
    a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before testing is conducted.
2. Label each 1.5 or 2.0 mL microfuge tubes containing aliquots of formalized saline with the specimen ID.

9.0 Quality Control

1. Use at least one positive and negative control organism as well as a saline-only control when performing QC. Only well characterized strains such as ATCC strains should be used for quality control..
    a. If possible, a panel of strains representative of all serogroups/serotypes should be used. The saline-only negative control is used to test for auto-agglutination.

b. A *Neisseria meningitidis* strain for each serogroup

i. Confirm the antisera agglutinates with the target serogroup and that no agglutination is observed with non-targeted serogroups.

1. Exception: The NmE/Z' antiserum cross-reacts with NmZ.

ii. Note: A non-groupable strain (NmNG) is not expected to agglutinate with any of the above listed antisera.

c. A *Haemophilus influenzae* strain for each serotype

i. Confirm the antisera agglutinates with the target serotype and that no agglutination is observed with non-targeted serotypes.

ii. Note: A non-typeable strain (NTHi) is not expected to agglutinate with any of the above listed antisera.

2. Before use, test each new lot of reagent with positive and negative controls (as described above).

a. QC should be performed every six months to ensure it is performing as expected.

b. QC should be repeated as necessary, if a vial has been exposed to temperatures above 4°C or if there is reason to suspect that the vial has been contaminated.

3. When performing testing, a saline-only control should be included with every test.

a. If an unexpected or ambiguous agglutination pattern is observed, it may be helpful to repeat the test with QC strains to aid interpretation.

10.0 Test Procedure

1. Note: This procedure should only be completed on bacterial isolates that have been confirmed as *N. meningitidis* or *H. influenzae* by other assays.

2. Preparation of formalinized saline solution

a. Whenever possible, conduct steps 2b-2c in a chemical fume hood.

b. Combine 5 mL of the 37% formaldehyde solution with 95 mL of the 0.85% saline in a 200 mL beaker to make a 5% solution of formaldehyde (formalin or formalinized saline).

c. Mix by pipetting up and down with the serological pipette or gentle swirling.

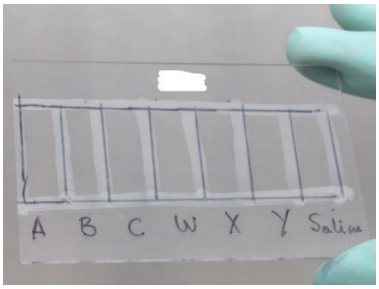
d. Within a sterile BSC (whenever possible), dispense 500 µL aliquots into 1.5 mL or 2 mL microcentrifuge tubes. Store at room temperature for up to 3 months.

3. Prepare the slides for agglutination reactions

a. Optional: Clean the glass slide with alcohol (only required if the slides are not pre-cleaned by the manufacturer)

b. Divide the slide into equal sections/chambers with a waterproof or PAP pen.

i. Note: Each isolate will require a separate section/chamber for each antiserum tested plus the one saline negative control.



4. Whenever possible, the following steps should be completed within a BSC.

5. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.

6. Prepare the bacterial suspension

a. Use a swab to collect bacterial colonies from an overnight culture.

b. Resuspend the bacteria in an aliquot of formalinized saline by gently swirling the swab and rubbing it against the side of the tube.

i. The bacterial suspension should have a density equivalent to a McFarland 4.0 standard or more.

c. Mix the suspension by either pipetting up and down or briefly vortexing.

d. Note: It is important to ensure no bacterial clumps are present in the suspension.

i. The suspension should be used within 15 minutes of preparation.

7. Slide agglutination procedure

a. Determine which antisera will be used for testing. Consider the following:

i. Only test antisera that are applicable to the organism.

ii. Consider first testing the prevalent serogroups/serotypes in the region.

iii. Remember: a saline negative control is always required.

b. Use a pipette to transfer 10 µl of the bacterial suspension to the lower portion of each chamber of the prepared slide.

c. In the upper portion of each chamber of the slide, add 10 µl of the appropriate antiserum or saline solution.

i. Note: Do not use the dropper provided with the antisera because it delivers larger than necessary quantities and can easily be contaminated.

ii. Note: If a micropipette and the tips are not available, a 10µL sterile, single-use loop can be used to transfer 10 µL of antisera. However, the loops often do not deliver the precise quantities.

d. Critical: Dispose of the tips/loops used to transfer antiserum after every use to avoid contamination of the antiserum. If the antiserum bottle becomes contaminated, a new vial should be used.

e. Gently rock the slide with a back and forth motion for 1 min (tilting it from front to back to efficiently mix the bacterial suspension with the antiserum).

i. Note: Do not make a circular motion. This can mix antisera between chambers, causing contamination and requiring the test to be repeated.

f. After 1 minute, examine the agglutination reactions under a bright light and against a black background.

g. Use the interpretation guide in Figure 1 to document the amount of agglutination observed.

h. Note: Return the antisera to the refrigerator as soon as testing is complete to avoid the loss of activity.

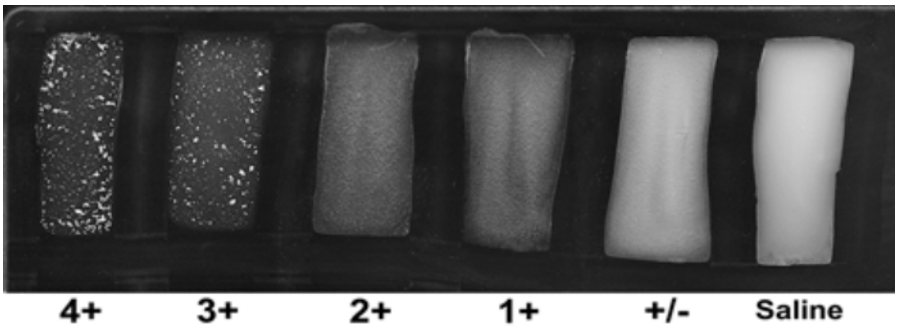
11.0 Interpretation of Results

1. Assess the intensity of the agglutination reactions

a. A positive result is designated by 3 + or 4 + (strong agglutination), except for serogroup B, which is considered to be positive with a rating of 2 + or more.

b. A negative result is designated by a 0, + /-, 1 + or 2 + (weak agglutination).

Figure 1: Assessment of the intensity of agglutination test (Image from [1]).



4+	All cells agglutinate and the cell suspension is clear
3+	75% of the cells agglutinate and the cell suspension is slightly turbid
2+	50% of the cells agglutinate
1+	25% of the cells agglutinate
+/-	Less than 25% of the cells agglutinate and fine granular material occurs
0	No visible agglutination; The suspension remains cloudy and smooth

2. Determination of the serogroup/serotype

   a. The serogroup is determined when a positive result occurs with one specific antiserum and not with the saline solution. Exception: Both meningococcal serogroups E/Z' and Z may agglutinate in antiserum E/Z'. To conclusively identify the serogroup, isolates suspected of being serogroup E/Z' or Z must be tested by both the Z and E/Z' specific antisera.

   b. The isolate is reported as Non-typeable (NT) for Hi or Non-groupable (NG) for Nm if one of the following observations are made (additional information in the table):

      i. Does not react with any of the serotype- or serogroup-specific antisera

      ii. Reacts with 2 or more antisera

      iii. Agglutinates with saline negative control

Slide Agglutination Result	**Agglutination Observations:**	
	Saline	**Antisera**
Serotype/Serogroup	Negative	Positive (one only)
NT/NG: Not Reactive	Negative	Negative
NT/NG: Auto-agglutination	Positive	Negative or Positive
NT/NG: Poly-agglutination/Cross-Reactive	Negative	Positive (2 or more)

3. Troubleshooting

   a. If the agglutination results are unclear, try the following steps.

      i. Repeat the test using the original suspension.

      ii. Make a new cell suspension and repeat the test.

      iii. Add 20 µL of antisera and saline directly to each chamber on a slide and then add a loopful of organism to each without diluting the isolate into the formalinized saline solution.

      iv. If results are still inconclusive. subculture a single colony and retest the next day.

         1. Note: If the original plate contains different size colonies, make a subculture for each type of colony and test each type the next day.

         2. It is also recommended to streak out QC strains to test alongside the isolates that had the ambiguous or unexpected agglutination.

12.0 Test Limitations

1. Cross-reactions have been observed with organisms of other species. It is important to identify the species by biochemical tests prior to conducting serological testing.

2. False negative results may be seen if the bacterial suspension is not of sufficient density.

3. Nm and Hi isolates are subject to variability (encapsulated versus non encapsulated small versus large colonies, growth slow versus fast growth, and strong agglutination versus weak agglutination) and agglutination can be ambiguous or difficult to interpret.

13.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

13. Kirby-Bauer Disk Diffusion

1.0 Purpose

This standard operating procedure provides instructions for the Kirby-Bauer disk diffusion method, which assesses antimicrobial susceptibility.

2.0 Principle

Antimicrobial susceptibility testing assesses the ability of an organism to grow in the presence of various antimicrobial agents. For the Kirby Bauer disk diffusion method, bacterial isolates are inoculated to confluence on an agar plate (observed as a lawn of bacteria). Prior to the growth incubation, commercially prepared filter paper disks impregnated with a specified single concentration of an antimicrobial agent are placed onto the agar, which diffuse into the growth medium immediately surrounding the disk. If growth of the organism is uninhibited, growth will be confluent around the disk. If the organism is sensitive to the antimicrobial agent, it will be unable to grow around the disk, creating a zone of inhibition. The diameter of the zone of inhibition can be measured and used to infer the susceptibility of the organism using interpretation tables, which define the thresholds for susceptible, intermediate, non-susceptible or resistant interpretations.

It is important to note that the zone of inhibition size is influenced by the rate of diffusion and other specific physical properties of each antibacterial agent. Thus, the disk diffusion interpretation will be distinct for every combination of antimicrobial agent and species, and can only be identified by referring to the published interpretation tables. Interpretation criteria and method requirements for antimicrobial susceptibility testing, including disk diffusion, are published by the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
CLSI	Clinical Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CFU	Colony forming units
QC	Quality control

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.
3. 35–37°C Incubator (non-CO_2) – required for growth of the *E. coli* QC organism
4. Caliper or ruler (mm)
5. Light source
6. Vortex
7. Test tube holder
8. Optional: Turbidity meter
9. Optional: Multi-disk dispensing apparatus

5.0 Reagents and Media

1. For each specific organism to be tested, the appropriate media need to be available.
   a. Growth media: agar that supports growth
   b. Dilution broth: used for inoculating the antimicrobial susceptibility test
   c. Testing media: used for growth in the presence of the antimicrobial agent
   d. A list of the CLSI specific media requirements is listed in the table below.
      i. Note: EUCAST methods may differ. Refer to the manual.

	Neisseria meningitidis	***Haemophilus influenzae***	***Streptococcus pneumoniae***	***Escherichia coli***
Growth Media	Chocolate Agar	Chocolate Agar	Blood Agar	Blood Agar
Dilution Broth	Mueller Hinton Broth	*Haemophilus* Test Medium Broth	Mueller Hinton Broth	Demineralized Sterile Water
Testing Media*	Mueller Hinton with 5% sheep blood Agar	*Haemophilus* Test Medium Agar	Mueller Hinton with 5% sheep blood Agar	Mueller Hinton Broth

2. Testing Media*: 100 or 150 mm agar plates can be used, depending on the number of antimicrobial agents that will be tested.
   a. Use a maximum of 4 disks on a 100 mm plate and 9 disks on a 150 mm plate.
3. Antimicrobial disks
   a. Refer to the CLSI/EUCAST document for the comprehensive list of antimicrobial disks that have interpretative criteria for the specific organism to be tested.
   b. To ensure optimal activity of antimicrobial disks, store at -20 °C or below in a frost-free freezer (or as directed by the manufacturer).

6.0 Supplies

1. McFarland 0.5 standard
2. Dacron polyester swabs (preferred) or inoculating loops
3. Sterile forceps
4. Biohazard waste bag
5. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
   a. Note: If plates are required to be opened when reading of the zone of inhibition, it is recommended that readings are completed within a BSC.
4. Refer to the safety data sheet of the product (s) that will be used during this test for information on proper storage, handling, and disposal.
5. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.

6. If a ruler or caliper contacts the agar or bacteria during measurement of the zone of inhibition, decontaminate it completely.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony until a pure culture is available before the testing is conducted. Note: If the isolate to be tested is being streaked from a frozen stock:
      i. Streak the isolate on the appropriate media and allow overnight growth.
      ii. Sub-culture a single colony to a fresh agar plate and allow overnight growth before inoculating a disk diffusion test, following the CLSI growth conditions listed in the procedure section below.
2. Label the agar plates and broth tubes used during testing with the appropriate specimen ID.

9.0 Quality Control

1. Use the recommended QC organisms from CLSI or EUCAST. Only ATCC strains should be used for QC. For each organism of interest, the routine QC strains suggested by CLSI are listed below:
   a. *N. meningitidis*: *S. pneumoniae* ATCC 49619 and *E. coli* ATCC 25922
   b. *H. influenzae*: *H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766, and *E. coli* ATCC 35218
   c. *S. pneumoniae*: *S. pneumoniae* ATCC 49619
   d. Note: EUCAST QC recommendations may differ. Consult the manual.
   e. Note: When designing a study for a specific set of antibiotics, confirm that the recommended QC strains have published acceptable ranges for the antibiotics of interest. If not, consider adding additional QC strains as necessary.
2. Before use, test each new lot of media for sterility and the ability to sustain growth.
   a. Complete a sterility test (incubate uninoculated media under normal growth conditions to confirm no growth occurs).
   b. Inoculate new media with a QC strain to ensure it can support normal growth.
3. When performing testing, QC strains should be included every week that testing is conducted to confirm the disk quality/performance and overall integrity of the testing process (see CLSI/EUCAST for additional recommendations).
   a. Test results from the QC organisms must be within the ranges provided in the CLSI/EUCAST manuals for the results obtained during that day to be considered valid.

10.0 Test Procedure

1. Allow agar plates and disks to equilibrate to room temperature before use.
   a. Agar plates can be placed into a 35–37 °C incubator (non-CO_2 and not used for culturing pathogens).
   b. The agar surface should be moist, but no water droplets should be visible on the agar or lid when inoculating.
   c. To avoid excessive condensation in the box of disks, do not open the cartridge until it reaches room temperature.
2. Whenever possible, the following steps should be completed within a BSC.
3. Streak the bacteria on an appropriate agar growth medium and incubate overnight.
   a. If starting from a frozen stock, see “8.0 Sample Information/Processing” of this procedure.
   b. The CLSI growth conditions are listed below:
      i. *H. influenzae*: 20-24 hrs, 35±2 °C with 5% CO_2
      ii. *N. meningitidis*: 20-24 hrs, 35±2 °C with 5% CO_2

iii. *S. pneumoniae*: 18-20 hrs, 35±2°C with 5% CO_2

iv. *E. coli*: 18-24 hrs, 35±2°C, ambient air

4. Inoculum preparation using the direct colony suspension method

   a. Using a swab, select several isolated colonies from an overnight culture and resuspend in the dilution broth appropriate for the organism being tested.

   b. Vortex until the suspension is uniform and has a concentration equivalent to a McFarland 0.5 standard (1x108 to 2x108 CFU/mL).

      i. If available, a turbidity meter can also be used to confirm suspension density.

   c. If necessary, adjust the bacterial suspension density.

      i. Add additional bacteria to increase turbidity

      ii. Dilute with sterile dilution broth to reduce turbidity.

5. Inoculation of the agar plate

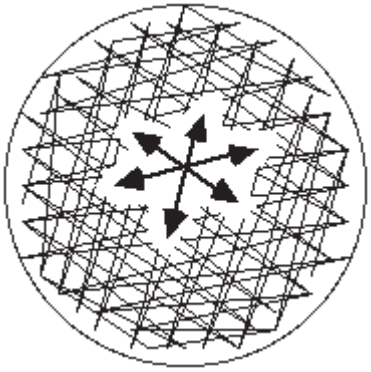
   a. Critical: Inoculate plate within 15 minutes of preparing the suspension.

   b. Dip a sterile polyester swab into the inoculum tube and rotate above the liquid and against the tube wall to remove excess liquid.

   c. Swab the entire surface of agar plate three times, rotating the plate approximately 60° between streaking to ensure even distribution (streaking for confluence/a bacterial lawn).

   d. Avoid touching the sides of the plate to avoid aerosols. Finally, run swab around the edge of the agar to remove any excess moisture.

   e. Allow inoculated plate to dry for 3 to 5 minutes before applying disks.



6. Application of disks

   a. Once the plate is dry, apply the disks to the agar surface with a dispenser or manually using sterile forceps.

      i. Use a maximum of 4 disks (100 mm plate) or 9 disks (150 mm plate).

      ii. Disks cannot be closer than 24 mm from center to center.

      iii. If the forceps touch the agar surface, decontaminate them before proceeding.

   b. Once a disk has made contact with the agar surface, do not move it. Movement will cause the zone of inhibition to be un-interpretable.

      i. If necessary, place a new disk in another location on the agar.

   c. Apply gentle pressure to each disk using the sterile forceps to ensure complete contact with agar (some dispensers do this automatically).

7. Incubation

   a. Invert plates and incubate within 15 minutes of disk application.

   b. Incubate agar plates in accordance with the CLSI/EUCAST recommendations. The CLSI incubation conditions are listed below:

      i. *H. influenzae*: 16-18 hrs, 35±2°C with 5% CO_2

      ii. *N. meningitidis*: 20-24 hrs, 35±2°C with 5% CO_2

      iii. *S. pneumoniae*: 20-24 hrs, 35±2°C with 5% CO_2

      iv. *E. coli*: 16-20 hrs, 35±2°C ambient air

8. Measuring the zone of inhibition

   a. Important considerations:

      i. If growth is intermittent, the inoculum was not sufficient and the procedure must be repeated. Only read plates that are at least nearly confluent (have a full bacterial lawn on the plate).

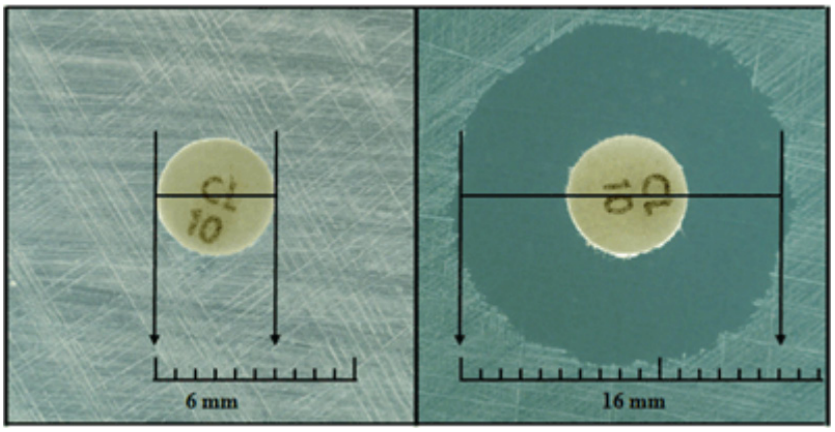
      ii. If plates are required to be opened when measuring the zone of inhibition, it is recommended to use a BSC.

      iii. When testing hemolytic organisms, measure the diameter of the zone of inhibition of growth and not the zone of inhibition of hemolysis.

      iv. If growth is confluent around the disk, the zone of inhibition will be the diameter of the disk itself.

   b. Use a caliper or ruler to measure and record the diameter of the zone of inhibition to the nearest whole millimeter. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye.

      i. Note: Using light from a lamp, with a black surface in the background may facilitate measurements.



      ii. Representative images from [1] are shown below.

   c. The media opacity will affect the way the plate is read.

      i. Translucent test media (ex: *Haemophilus* test medium)

         1. Measure the zone of inhibition can be measured from back of the plate while closed.

      ii. Opaque media (ex: Mueller Hinton with 5% sheep blood)

         1. Measure the zone of inhibition from the front of the plate. Do not touch the agar or bacterial growth with the ruler while taking measurements.
         2. Opaque plates can be illuminated from behind to facilitate reading.

11.0 Interpretation of Results

1. Once the zone of inhibition measurements has been obtained, refer to the CLSI or EUCAST manuals to interpret the susceptibility and obtain the categorical results (susceptible (S), intermediate (I), resistant (R), or non-susceptible (NS)).

2. Additional considerations

   a. Record the QC results first. If the QC strain are not within the expected ranges, the test is invalid and needs to be repeated.

   b. When measuring zones for sulfonamides (trimethoprim, or trimethoprim-sulfamethoxazole) disregard light growth (20% or less of lawn of growth). Measure the more obvious margin to determine the zone of inhibition.

12.0 Test Limitations

1. This assay depends on specific interpretation criteria. Strict adherence to the protocol and CLSI/EUCAST guidelines is necessary to ensure accurate results.

2. Factors that affect results, include inoculum sizes, rate of growth, formulation and pH of media, incubation environment, length of incubation, disk quality and drug diffusion rate.

13.0 References

1. European Committee on Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing - EUCAST disk diffusion method version 6.0: European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2017.

2. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

3. Clinical Laboratory Standards Institute M100: Performance Standards for Antimicrobial Susceptibility Testing, 28th Edition (M100S28E edition). January 2018.

4. Clinical Laboratory Standards Institute M7-A9: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard, 9th Edition (M7–A9 edition). January 2012

5. Murray et al. Manual of Clinical Microbiology, 9th Edition. ASM Press. 2007.

14. Epsilometer Test (E-Test)

1.0 Purpose

This standard operating procedure provides instructions for performing the Epsilometer Test (E-test) used to determine the minimum inhibitory concentration (MIC) for antimicrobial agents.

2.0 Principle

The Epsillometer Test (E-Test) assesses the ability of an organism to grow in the presence of an antimicrobial agents. Bacterial isolates are inoculated to confluence (a full bacterial lawn) on an agar plate. Before incubation, an E-test strip, which contains a concentration gradient of a single antibiotic (in 2-fold dilutions), is applied to the agar. The antimicrobial agent diffuses into the agar and provides a growth challenge to the organism. After overnight incubation, a growth inhibition ellipse will be observed around the E-test strip, which can be used to define the minimum inhibitory concentration (MIC) defined as the antimicrobial agent concentration where the growth inhibition ellipse intersects the strip.

Note: The E-test is not considered a reference method by either Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). However, according to the manufacturer, the MIC values obtained by E-test can be compared to the CLSI interpretation tables to determine the susceptibility interpretation (susceptible, intermediate, non-susceptible or resistant).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
CLSI	Clinical Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CFU	Colony forming units
MIC	Minimum inhibitory concentration
QC	Quality control

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C Incubator supplemented with a candle jar or CO_2 packs is sufficient.
3. Light source
4. Vortex
5. Test tube holder
6. Optional: Multi-strip dispensing apparatus
7. Optional: Mini Grip-ItTM
8. Optional: Turbidity meter

5.0 Reagents and Media

1. For each specific organism to be tested, the appropriate media must be available.
   a. Growth media: agar that supports growth

b. Dilution broth: used for inoculating the antimicrobial agent susceptibility test

c. Testing media: agar used for growth in the presence of the antimicrobial agent

d. A list of the specific media requirements is listed in the table below. Refer to the E-test package insert or CLSI documents to confirm the media requirements.

	Neisseria meningitidis	***Haemophilus influenzae***	***Streptococcus pneumoniae***	***Escherichia coli***
Growth Media	Chocolate Agar	Chocolate Agar	Blood Agar	Blood Agar
Dilution Broth	Mueller Hinton Broth	*Haemophilus* Test Medium Broth	Mueller Hinton Broth	Demineralized Sterile Water
Testing Media*	Mueller Hinton with 5% sheep blood Agar	*Haemophilus* Test Medium Agar	Mueller Hinton with 5% sheep blood Agar	Mueller Hinton Broth

2. Testing Media*: 100 or 150 mm agar plates can be used, depending on the number of antimicrobial agents that will be tested.

   a. Use a maximum of 2 strips on a 100 mm plate and 6 strips on a 150 mm plate.

3. E-test strips:

   a. To determine which strips to use, refer to the CLSI/EUCAST document for a comprehensive list of the antimicrobial agents that have MIC values with interpretations.

   b. To ensure optimal activity of E-test strips, store according to the temperature specified on the packaging, until the expiratoion date.

6.0 Supplies

1. McFarland 0.5 standard
2. Dacron polyester swabs (preferred) or inoculating loops
3. Sterile forceps
4. Biohazard waste bag
5. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.

   a. Note: If plates are required to be opened when reading of the MIC, use of a BSC is recommended during reading.

4. Refer to the safety data sheet of the product (s) that will be used during this test for information on proper storage, handling, and disposal.
5. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.

   a. If the culture is not pure, sub another single colony until a pure culture is available before testing is conducted.

b. Note: If the isolate to be tested is being streaked from a frozen stock:

i. Streak the isolate on the appropriate media and allow overnight growth.

ii. Sub-culture a single colony to a fresh agar plate and allow overnight growth before inoculating an E-test , following the CLSI growth conditions.

2. Label the agar plates and broth tubes used during testing with the appropriate specimen ID.

9.0 Quality Control

1. Use the recommended QC organisms from CLSI or EUCAST. Only ATCC strains should be used for QC. For each organism of interest, the routine QC strains suggested by CLSI are listed below:

a. *N. meningitidis*: *S. pneumoniae* ATCC 49619 and *E. coli* ATCC 25922

b. *H. influenzae*: *H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766, and *E. coli* ATCC 35218

c. *S. pneumoniae*: *S. pneumoniae* ATCC 49619

d. Note: EUCAST QC recommendations may differ. Consult the manual.

2. Before use, test each new lot of media for sterility and the ability to sustain growth.

a. Complete a sterility test (incubate uninoculated media under normal growth conditions to confirm no growth occurs).

b. Inoculate new media with a QC strain to ensure it can support normal growth.

3. When performing testing, QC strains should be included every week that testing is conducted to confirm the strip quality/performance and overall integrity of the testing process.

a. Test results from the QC organisms must be within the QC ranges provided in the CLSI/EUCAST manuals for the results obtained during that day to be considered valid.

10.0 Test Procedure

1. Allow agar plates and E-test strips to equilibrate to room temperature before use.

a. Equilibrate only enough E-test strips required for the day's testing (including a few extra) and immediately put the remaining E-test strips back at appropriate storage temperature.

b. Agar plates can be placed into a 35–37°C incubator (non-CO_2 and not used for culturing pathogens).

c. The agar surface should be moist, but no water droplets should be visible on the agar or lid when inoculating.

2. Whenever possible, the following steps should be completed within a BSC.

3. Streak the bacteria on an appropriate agar growth medium and incubate overnight.

a. If starting from a frozen stock, see "8.0 Sample Information/Processing".

b. Incubation conditions according to CLSI are listed below.

i. *H. influenzae*: 20-24 hrs, 35±2°C with 5% CO_2

ii. *N. meningitidis*: 20-24 hrs, 35±2°C with 5% CO_2

iii. *S. pneumoniae*: 18-20 hrs, 35±2°C with 5% CO_2

iv. *E. coli*: 18-24 hrs, 35±2°C, ambient air

4. Inoculum preparation using the direct colony suspension method

a. Using a swab, select several isolated colonies from an overnight culture and resuspend in the dilution broth appropriate for the organism being tested.

b. Vortex well.

c. Confirm the suspension is uniform and has a concentration equivalent to a McFarland 0.5 standard (1x108 to 2x108 CFU/ml).

i. If available, a turbidity meter can also be used to confirm suspension density.

d. If necessary, adjust the suspension density.

i. Add additional bacteria to increase turbidity

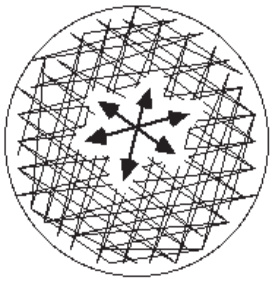
ii. Dilute with sterile dilution broth to reduce turbidity.

5. Inoculation of the agar plate

a. Critical: Inoculate plate within 15 minutes of preparing the suspension.

b. Dip a sterile polyester swab into the inoculum tube and rotate above the liquid and against the tube wall to remove excess liquid.

c. Swab the entire surface of agar plate three times, rotating the plate approximately 60° between streaking to ensure even distribution (streaking for confluence).



d. Avoid touching the sides of the plate to avoid aerosols. Finally, run swab around the edge of the agar to remove any excess moisture.

e. Allow inoculated plate to dry for 15-20 minutes before applying strips. The surface must be completely dry before applying the E-test gradient strips.

6. Application of strips

a. Note: Use a maximum of 2 strips (100 mm plate) or 6 strips (150 mm plate). Make sure that the printed MIC values are facing upward (ensures antibiotics contact the agar).

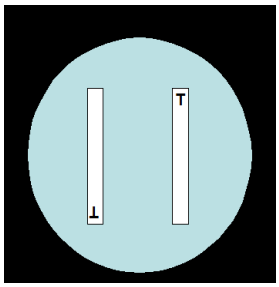
b. Once the plate is dry, apply strips to agar surface with a dispensing machine or manually using sterile forceps. Place the strips in the appropriate orientation to prevent overlap of the ellipses of inhibition.

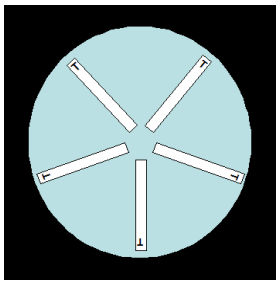
i. For a 100 mm plate, two E-strips are placed in opposite orientation. The “T” denotes the top of the strip.

ii. For a 150 mm plate, the lowest antibiotic concentration is oriented towards the center of the plate for 6 strips. The “T” denotes the top of the strip.

iii. Images are from [1].

iv. Once the plate is dry, apply strips to agar surface with a dispensing machine or manually using sterile forceps.If the forceps touch the agar surface, decontaminate them before proceeding.





c. Once a strip has made contact with the agar surface, do not move it. The antibiotic immediately begins diffusing in the agar and will be un-interpretable if moved.

i. If necessary, place a new strip in another location on the agar.

d. Apply gentle pressure to each strip using the sterile forceps to ensure complete contact with agar (some dispensers do this automatically).

7. Incubation

a. Invert plates and incubate within 15 minutes of applying the E-test strip.

b. Incubate agar plates in accordance with the CLSI/EUCAST recommendations. The CLSI incubation conditions are listed below:

i. *H. influenzae*: 20-24 hrs, 35±2°C with 5% CO_2

ii. *N. meningitidis*: 24 hrs, 35±2°C with 5% CO_2

iii. *S. pneumoniae*: 20-24 hrs, 35±2°C with 5% CO_2

iv. *E. coli*: 16-20 hrs, 35±2°C, ambient air

8. Determining the MIC

   a. Important considerations:

      i. If growth is intermittent, the inoculum was not sufficient and the procedure must be repeated. Only read the MIC from plates that are at least nearly confluent.

      ii. When testing hemolytic organisms, measure the diameter of the zone of inhibition of growth and not the zone of inhibition of hemolysis.

   b. Read the MIC as the value where complete inhibition of all growth intersects the strip.

      i. If no growth inhibition is observed, list the MIC is defined as > than the highest value on the strip.

      ii. If any questionable growth is observed, refer to the reading guide to help aid interpretation of the MIC (addresses organism specific effects, drug-related effects, resistance mechanism-related effects, etc.) It also provides guidance for general technical and handling issues such as inhibition intersecting the strip in between two MIC values or uneven growth on the two sides of the strip (http://www.ilexmedical.com/files/ETEST_RG.pdf)

      iii. Note: Using light from a lamp, with a black surface in the background may facilitate measurements. A magnifying glass may also be used.

11.0 Interpretation of Results

1. Once the MICs have been obtained, the E-test manufacturer recommends using criteria specified by CLSI to interpret the categorical result as susceptible (S), intermediate (I), resistant (R), or non-susceptible (NS) for each antimicrobial agent.

2. Additional considerations

   a. Record the QC results first. If the QC strain are not within the expected ranges, the test is invalid and needs to be repeated.

   b. Note any unusual observations such as a zone of incomplete killing (trailing endpoints) or single colonies growing within the ellipse.

12.0 Test Limitations

1. This assay depends on specific interpretation criteria. Strict adherence to protocol is necessary to ensure accurate results.

2. Factors that affect results include inoculum sizes, rate of growth, formulation and pH of media, incubation environment, length of incubation, strip quality, and drug diffusion rate.

13.0 References

1. World Health Organization & Centers for Disease Control and Prevention US. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*: WHO manual, 2nd ed. World Health Organization 2011.

2. Biomerieux. ETEST APPLICATION GUIDE. Available at: https://www.biomerieux-usa.com/sites/subsidiary_us/files/supplementary_inserts_-_16273_-_b_-_en_-_eag_-_etest_application_guide-3.pdf. Accessed October 30, 2019.

15. Sensititre Broth Microdilution Method

1.0 Purpose

This standard operating procedure provides instructions for the broth microdilution method for determining the minimum inhibitory concentration (MIC).

2.0 Principle

Antimicrobial susceptibility testing (AST) assesses the ability of an organism to grow in the presence of various antimicrobial agents. For the broth microdilution method, bacterial isolates are inoculated into a broth containing an antimicrobial agent. Broth microdilution assays are prepared in 96-well formats, with multiple concentrations (in 2-fold dilutions) present for each antimicrobial agent present on the panel. If growth of the organism is uninhibited, a bacterial pellet will be visible in the bottom of the well. If the organism is sensitive to the antimicrobial agent, the broth will be clear, as no growth will have occurred. The MIC is the lowest concentration of an antimicrobial agent that inhibits the macroscopic growth of a bacterium. Once the MICs have been obtained, the susceptibility of the organism can be inferred using interpretation tables, which define the thresholds for susceptible, intermediate, non-susceptible or resistant interpretations.

Interpretation criteria and method requirements for antimicrobial susceptibility testing, including broth microdilution, are published by the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST).

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
AST	Antimicrobial susceptibility test
CLSI	Clinical Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CFU	Colony forming units
MIC	Minimum inhibitory concentration
QC	Quality control
MHB	Mueller Hinton Broth
LHB	Lysed horse blood

4.0 Equipment

1. Biosafety Cabinet Class IIA (BSC)
2. 35–37°C Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37°C incubator supplemented with a candle jar or CO_2 pack is sufficient.
3. 35–37°C Incubator (non-CO_2)
4. Vortex
5. Sensititre AutoInoculator (AIM)
6. Sensititre Nephelometer™
7. Pipettes (2-20 µL and 20-200 µL)
8. Tube racks

9. Mirror Box

5.0 Reagents and Media

1. For each specific organism to be tested, the appropriate media need to be available.
   a. Growth media: agar that supports growth
   b. Dilution broth: used for inoculating the antimicrobial susceptibility test
   c. Testing media: broth used for growth in the presence of the antimicrobial agent
   d. A list of the CLSI media requirements is listed in the table below.
   e. Note: The EUCAST method may differ. Refer to the manual.

	Neisseria meningitidis	***Haemophilus influenzae***	***Streptococcus pneumoniae***	***Escherichia coli***
Growth Media	Chocolate Agar	Chocolate Agar	Blood Agar	Blood Agar
5 mL Dilution Broth	Mueller Hinton Broth (Remel; Catalog No. YT3462-05)	Mueller Hinton Broth (Remel; Catalog No. YT3462-05)	Mueller Hinton Broth (Remel; Catalog No. YT3462-05)	Demineralized Sterile water (Remel; Catalog No. YT3339)
11 mL Testing Media*	Mueller Hinton Broth with Lysed Horse Blood (Remel; Catalog No. YCP112-10)	Haemophilus Test Medium Broth (Remel; Catalog No. YT3470)	Mueller Hinton Broth with Lysed Horse Blood (Remel; Catalog No. YCP112-10)	Mueller Hinton Broth (Remel; Catalog No. YT3462)

2. Testing Media*: Broth tubes will need to have 11 mL for inoculation into the 96-well plate.
3. Antimicrobial agent panels can be obtained commercially or prepared using antimicrobial agent powders and media.
   a. Example of a commercially available panel: Sensititre HPB1 (*Haemophilus influenzae/Streptococcus pneumoniae*) Panel (Remel; Catalog No. YHPB1)
   b. When deciding which antimicrobial agents to test, refer to the CLSI/EUCAST manual for the comprehensive list of antimicrobial agents that have MICs with interpretation categories for the organism of interest.
   c. Panels can be stored at -20°C or according to the manufacturer's instructions.

6.0 Supplies

1. McFarland 0.5 standard (Remel; Catalog No. E1041)
2. Inoculating loops (1 µL and 10 µL)
3. Sensititre dosing heads (Remel; Catalog No. YT-E3010)
4. Dacron polyester swabs
5. Extra-long pipette tips (20-200 µL) (Rainin Catalog No. 17008887)
6. Pipette tips (2-20 µL)
7. Non-sticky, clear plastic seals (Remel Catalog No. YG523M)
8. Kim Wipes
9. Biohazard sharps container
10. Biohazard waste bag

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
   a. Note: If the plastic seals need to be removed during reading of the MIC, it is recommended that the reading is completed within a BSC.
4. Refer to the safety data sheet of the product (s) that will be used during this test for information on proper storage, handling, and disposal.
5. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony until a pure culture is available before the testing is conducted.
   b. Note: If the isolate to be tested is being streaked from a frozen stock, :
      i. Streak the isolate on the appropriate media and allow overnight growth.
      ii. Sub-culture a single colony to a fresh agar plate and allow overnight growth before inoculating a MIC panel following the CLSI growth conditions listed in the procedure.
2. Label the agar plates and broth tubes used during testing with the appropriate specimen ID.

9.0 Quality Control

1. Use the recommended QC organisms from CLSI or EUCAST. Only ATCC strains should be used for QC. For each organism of interest, the routine QC strains suggested by CLSI are listed below:
   a. *N. meningitidis*: *S. pneumoniae* ATCC 49619 and *E. coli* ATCC 25922
   b. *H. influenzae*: *H. influenzae* ATCC 49247 *H. influenzae* ATCC 49766, and *E. coli* ATCC 35218
   c. *S. pneumoniae*: *S. pneumoniae* ATCC 49619
   d. Note: EUCAST QC recommendations may differ. Consult the manual.
2. Before use, test each new lot of media for sterility and the ability to sustain growth.
   a. Complete a sterility test (incubate uninoculated media under normal growth conditions to confirm no growth occurs).
   b. Inoculate new media with a QC strain to ensure it can support normal growth.
3. When performing testing, QC strains should be included every week that testing is conducted to confirm the antibiotic panel quality/performance and overall integrity of the testing process (see CLSI/EUCAST for additional recommendations).
   a. On each antimicrobial susceptibility test (AST) panel, positive control wells (which lack antimicrobial agents) are inoculated to verify the broth media is still capable of sustaining growth.
   b. Test results from the QC organisms must be within the ranges provided in the CLSI/EUCAST manuals for the results obtained during that week to be considered valid.
4. To ensure the purity of inoculated bacteria, purity plates and colony counts are used.
   a. After inoculation of every AST panel, 1 µL from a positive control well is streaked onto the appropriate agar and incubated overnight using organism-appropriate conditions. Approximately ≥100 pure colonies should be detected.

b. On a periodic basis, an official colony count should be performed by taking 1 µL from a positive control well and diluting it into 50 µL of sterile water. Then, 1 µL of that dilution is streaked out onto the appropriate agar and incubated overnight using organism-appropriate conditions. After incubation, perform a colony count to confirm the inoculum is within range (2x105 – 7x105 CFU/mL).

10.0 Test Procedure

1. Whenever possible, the following steps should be completed within a BSC.
2. Streak the bacteria on an appropriate agar growth medium and incubate overnight.
   a. If starting from a frozen stock, see 8.0 Sample Information/Processing.
   b. The CLSI growth conditions are listed below:
      i. *H. influenzae*: 20-24 hrs, 35±2°C with 5% CO_2
      ii. *N. meningitidis*: 20-24 hrs, 35±2°C with 5% CO_2
      iii. *S. pneumoniae*: 18-20 hrs, 35±2°C with 5% CO_2
      iv. *E. coli* : 18-24 hrs, 35±2°C, ambient air
3. Allow agar, dilution media, and broth media to equilibrate to room temperature before use.
   a. Media can be placed into a 35–37°C incubator (non-CO_2 and not used for culturing pathogens).
4. Calibrate the Nephelometer™:
   a. Invert the McFarland 0.5 standard tube 5 to 6 times. Wipe the bottom of the tube with a KimWipe. (Note: Do NOT vortex the McFarland standard, which can result in inaccurate calibration of the nephelometer)
   b. Insert the tube into the Nephelometer.
   c. Press the “CAL” button on the panel.
   d. Wait until the lights on the panel turn green then stop flashing (this indicates that the device has been calibrated).
   e. If the lights are red or fail to turn green, wipe the bottom of the tube and repeat the previous steps (4a-d).
5. Prepare the bacterial suspension
   a. Loosen the cap of the 5 mL dilution broth tube.
   b. Use a sterile swab to transfer a few bacterial colonies from the growth agar into the appropriate 5 ml dilution broth.
      i. *H. influenzae*: Mueller Hinton broth
      ii. *N. meningitidis*: Mueller Hinton broth
      iii. *S. pneumoniae*: Mueller Hinton broth
      iv. *E. coli*: Sterile water
   c. Cap the lid tightly.
   d. Vortex the dilution broth tube for 10 seconds to create a homogeneous solution.
   e. Place the tube into the Nephelometer (turbidity meter) to confirm the concentration is equivalent to a McFarland 0.5 standard (1x108 to 2x108 CFU/mL).
      i. Note: When reading each tube in the Nephelometer, simply place the tube into the holder and do not press any buttons. The lights will change and display in green if the suspension is correct.
   f. If necessary, adjust the bacterial suspension density.
      i. Add additional bacteria to increase turbidity
      ii. Dilute with sterile dilution broth to reduce turbidity.

6. Inoculation of the antibiotic panel
    a. Note: Inoculation should be completed within 30 minutes of preparing the bacterial suspension to ensure an accurate inoculum is dispensed.
    b. Uncap the bacterial suspension and the 11 mL testing media broth.
    c. Using an extra-long tip, transfer the appropriate volume of the bacterial suspension into the 11 ml testing media tube. The testing media should give an inoculum of 1x105 CFU/ml for *E. coli* and 5x105 CFU/ml for *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*.
    d. Place a dosing head on the testing media tube and close it tightly. Invert the testing media tube 6-8 times to mix well.
        i. Note: Be careful not to touch the dosing head tip, which could potentially contaminate the culture.
    e. Insert the tube into the auto-inoculator machine (AIM) holder and secure the tube using the knob.
    f. Touch the screen twice to initiate the AIM and dispense the appropriate volume.
        i. The amount of volume that should be dispensed will depend on the specific MIC panel being used. For example, dispense 100µL per well when using the HPB1 panel from Remel.
        ii. Note: The volume and panel layout for dispensing by the AIM can be changed, so prior to starting the experiment, ensure that you have the appropriate panel layout and volume parameters set.
    g. Remove the test media broth tube from the AIM and discard the tube appropriately (use a sharps container if glass tubes are used).
        i. Note: It is important to remove the tube within 30 seconds of dispensing to avoid any leaks.
    h. Dip a 1 µl loop into a positive control well and streak for growth onto an organism-specific agar, which will serve as the purity plate for that specific bacterial isolate.
        i. Note: The colonies on this plate will be counted. Streak a line across the agar and then streak across the entire surface, in multiple directions to maximize colony spread.
        ii. If completing an official colony count, see “section 9.0 Quality Control”, #4.
    i. Depending on the incubation conditions (with or without CO_2), seal the panels accordingly.
        i. *S. pneumoniae*, *H. influenzae* and *E. coli* require non-CO_2 incubations. Seal the panels with a plastic film (non-sticky) and incubate for the appropriate time.
            1. Note: Make sure to press the clear film against the rim of the wells and ensure creases are not formed. This will prevent gas exchange.
        ii. *N. meningitidis* requires CO_2 during the incubation. Plates should be placed into nearly sealed bags to provide protection from aerosols but also permit gas exchange.
            1. Note: A site-specific risk assessment should be performed to ensure this can be completed safely.
    j. Incubate the antibiotic panels in accordance with the CLSI/EUCAST recommendations. The CLSI incubation conditions are listed below:
        i. *H. influenzae*: 20-24 hrs, 35±2°C, ambient air
        ii. *N. meningitidis*: 20-24 hrs, 35±2°C with 5% CO_2
        iii. *S. pneumoniae*: 20-24 hrs, 35±2°C, ambient air
        iv. *E. coli*: 16-20 hrs, 35±2°C ambient air
    k. Place the agar purity plates into an appropriate incubator (CO_2 or non-CO_2) for overnight growth.

7. Reading the antibiotic panels and confirming purity
    a. Place each antibiotic panel onto a mirror box to manually record the MIC.
        i. The MIC will be the lowest concentration of an antimicrobial agent that inhibits the growth an isolate within a well.

ii. Growth appears as turbidity or a deposit of cells at the bottom of the well.

iii. Note: Use of a dark background or a lamp may facilitate accurate reading of the MIC.

b. Check the agar purity plates for each isolate to confirm no contamination occurred during set up of the test.

i. The number of colonies present can be counted to infer the number of colonies in the testing broth used, but this is not the official colony count (see section "9.0 Quality Control", #4).

c. If applicable, complete an official colony count.

11.0 Interpretation of Results

1. To read the results, it may be necessary to remove the clear adhesive seal. If the seal must be removed, it is recommended that the MICs are read within a BSC.
2. Verify that the positive control wells on the panel have growth. If the positive control wells do not have growth, the tests are invalid and should be repeated.
3. Record the QC results first. If the QC strain are not within the expected ranges, the test is invalid and needs to be repeated.
4. The MIC can be recorded as <, =, or ≥ the value of the antibiotic in the well where no growth is observed.
   a. If no growth is observed within the well with the lowest concentration of an antibiotic, the MIC is < that value (ex: < 2, if the antibiotic range was 2-16).
   b. If no growth is observed within a well that is not the lowest concentration of an antibiotic, the MIC is = that value (ex: = 4 or = 16, if the antibiotic range was 2-16).
   c. If growth is observed in the well with the highest concentration of an antibiotic, the MIC is ≥ that value (ex: ≥ 16 if the antibiotic range was 2-16).
5. Use the criteria specified by CLSI or EUCAST to interpret the categorical result as susceptible (S), intermediate (I), or resistant (R) for each antimicrobial agent.

12.0 Test Limitations

1. Strict adherence to the protocol and CLSI/EUCAST guidelines is necessary to ensure accurate results.
2. Reading of the MIC may be challenging, particularly if growth occurs as a diffuse turbidity.

13.0 References

1. Manual Antimicrobial Susceptibility Testing | Thermo Fisher Scientific. https://www.thermofisher.com/.../antimicrobial-susceptibility-testing
2. CLSI M100 Performance standards for Antimicrobial Susceptibility Testing (M100S27E edition).
3. CLSI M7-A9 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically (M7–A9 edition).
4. European Committee on Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing - EUCAST disk diffusion method version 6.0: European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2017.

16. API NH Strips Test

1.0 Purpose

This standard operating procedure provides instructions for the rapid identification of *Neisseria* and *Haemophilus* species (and related genera), as well as Moraxella catarrhalis using API *Neisseria/Haemophilus* (NH) strips.

2.0 Principle

API NH test strips are a series of wells (cupules), which each contain dehydrated substrates for distinct biochemical tests. Each cupule detects an enzymatic activity or sugar fermentation by the inoculated organisms. After each biochemical reaction, a color change is produced spontaneously or after the addition of additional reagents to the cupule. The results from all the biochemical tests together are considered when determining the species identification.

3.0 Definitions

Term	Definition
BSC	Biological safety cabinet
PPE	Personal protective equipment
NH	*Neisseria*/Haemophilus
QC	Quality control

4.0 Equipment

1. Biosafety cabinet (BSC) Class IIA
2. Timer
3. Pipette (20-200 µL)
4. 35 – 37 °C incubator (non-CO_2)
5. 35–37ºC Incubator with 5% CO_2
   a. If a CO_2 incubator is not available, a 35–37ºC incubator supplemented with a candle jar or CO_2 pack is sufficient.

5.0 Reagents and Media

1. Applicable agar media for growth
2. Included in the API NH strip kit
   a. NH strip
   b. 2 mL 0.85% NaCl ampule
   c. Incubation trays and covers
   d. James reagent (expires a month after opening; must be protected from light)
   e. Zym B reagent (expires two weeks after opening)
   f. Instructions and result sheet

6.0 Supplies

1. Dacron polyester swabs
2. Pipette tips (20-200 µL)
3. Mineral oil

4. McFarland 4.0 standard
5. Biohazard waste bag
6. Biohazard sharps container

7.0 Safety Precautions

1. Wear proper PPE including lab coat and gloves.
2. All procedures must be carefully performed to minimize splashes and/or aerosols.
3. Ideally, all procedures that could result in aerosolization of a live organism should be completed within a BSC. If a BSC is not available, use of an N95 respirator is recommended.
4. Refer to the safety data sheet of the product (s) that will be used during this test for information on proper storage, handling, and disposal.
5. If applicable, sharps or glass must be disposed in a puncture-resistant biohazard sharps container.

8.0 Sample Information / Processing

1. Test should be performed using a pure single colony sub-culture, not more than 24 hours old.
   a. If the culture is not pure, sub another single colony (35–37°C with 5% CO_2) until a pure culture is available before the testing is conducted.
2. Label each NH strip with the specimen ID.

9.0 Quality Control

1. Use the following organisms when performing QC. Only well characterized strains such as ATCC strains should be used for quality control. Refer to the test manufacturer's package insert for a complete list of recommended ATCC strains.
   a. *Neisseria gonorrhoeae* (ATCC 31426): PEN/GLU/ProA (+), all others (-).
   b. *Haemophilus influenzae* (ATCC 10211): GLU/ODC/URE/PAL/IND (+), FRU variable, all others (-).
   c. *Haemophilus paraphrophilus* (ATCC 49917): GLU/FRU/MAL/SAC/PAL/BGAL/GGT (+), all others (-).
2. Before use, test each new lot of the NH strip kit with the QC organisms.

10.0 Test Procedure

1. Equilibrate 0.85% saline, NH strip, and reagents to room temperature.
2. Whenever possible, the following steps should be completed within a BSC.
3. Streak the bacteria on an appropriate agar plate and incubate overnight at 35–37°C with 5% CO_2.
4. Prepare the inoculum
   a. Open a 2 mL ampule of 0.85% NaCl using an ampule protector.
   b. Using a swab, collect several isolated colonies and resuspend in the saline ampule.
   c. Confirm the suspension is uniform and has a concentration equivalent to a McFarland 4.0 standard.
5. Inoculate each cupule with the bacterial suspension immediately after it is prepared.
   a. Note: Take care to avoid splashes and bubble formation during inoculation.
   b. Pipette 50 µL of bacterial suspension into the first 7 cupules (PEN to URE).
   c. Pipette 150 µL of the bacterial suspension into the last 3 cupules (LIP/ProA, PAL/GGT, and βGAL/IND).

d. Add 1-2 drops of mineral oil to each of the first 7 cupules (PEN to URE).

6. Close the incubation box with lid.

7. Incubate the NH strip covered tray for 2 hours +/- 15 minutes at 35–37 °C under aerobic conditions (non-CO_2 incubator).

8. After incubation, read the strip and record the results (see “Interpretation of Results”).

9. For the last 3 cupules (LIP/ProA, PAL/GGT, and βGAL/IND), two reactions are recorded.

   a. Record the results from the first reactions (LIP, PAL, βGAL).

   b. Add 1-2 drops of Zym B reagent into cupules 8 and 9 (labeled LIP/ProA and PAL/GGT)

      i. If the LIP reaction is blue, do not add Zym B reagent. Record the ProA reaction as negative.

   c. Add 1-2 drops of James reagent into cupule 10 (labeled βGAL/IND).

   d. Incubate for 3 minutes. Read the results, referring to the “Interpretation of Results”.

11.0 Interpretation of Results

1. Read and record results as (+) or (-) for all cupules on the worksheet provided in the kit.

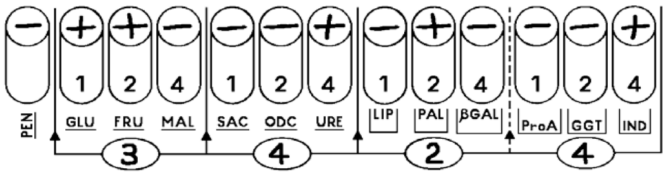
   a. Critical: The last three cupules contain two reactions that must be recorded (LIP/ProA, PAL/GGT, and βGAL/IND).

Detection of positive vs. negative results per cupule in the API NH strip	
Expected color after inoculation only	
Expected color after incubation if all cupules are negative	
Expected color after incubation if all cupules are positive	
Expected color after ZymB and James reagent incubation. Cupules 8-10 are positive for the second reaction	

2. Species identification is obtained through a numerical profile.

   a. To identify the numerical profile, each test/cupule is assigned a value of 1, 2 or 4, with the tests grouped into sets of three.

   b. Within each group, add the values corresponding to the positive reactions (as indicated in the table below) to generate the 4-digit numerical profile.

Recording on result worksheet	ID from worksheet
	3424

c. Compare the numeric profile to the “Identification Table” in the appendix of the API NH package insert.

   i. Note: The species identification and percentage can be obtained from the API NH Online Identification software. (https://apiweb.biomerieux.com/?action=prepareLogin)

12.0 Test Limitations

1. Reading enzymatic reactions beyond the allowable time limit can affect the results interpretation.

   a. If, after a 2-hour incubation period, several reactions (fermentation, penicillinase) are doubtful, re-incubate the strip for an additional 2 hours and read the reactions again.

   b. Critical: The enzymatic tests should not be re-read after the additional incubation.

2. False negative results may be seen if the bacterial suspension is not of sufficient density.

3. False negative or positive results may be observed when inaccurate dispensing of the bacterial suspension occurs.

4. From the manufacturer:

   a. The API NH system is intended uniquely for the identification of those species included in the Identification Table, i.e., those belonging to the genera *Neisseria* and *Haemophilus* (and related genera), and to the species *Moraxella catarrhalis* (*Branhamella catarrhalis*).

   b. API NH should not be used to identify any other microorganisms or to exclude their presence.

   c. Certain species of the genera *Moraxella*, *Oligella*, etc. may be wrongly identified as *Neisseria meningitidis* and *Neisseria gonorrhoeae* since their biochemical profiles on the API NH strip are very similar.

   d. *Neisseria meningitidis* identification can be confirmed by additional methods such as PCR.

   e. Only pure cultures of a single organism should be used in this assay. Mixed cultures could lead to inaccurate interpretations.

13.0 References

1. Biomerieux. API® NH. Available at: http://www.cantonhealth.org/pdf/400-001-06-13-A_API%20NH%20System%20BioMerieux%20Product%20Insert.pdf. Accessed October 30, 2019.

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