

ANNEX

Preparation of Media and Reagents

Quality control (QC) of media

Each batch of media prepared in the laboratory and each new manufacturer's lot number of media should be tested using appropriate QC reference strains for sterility, the ability to support growth of the target organism(s), and/or the ability to produce proper biochemical reactions. A QC record should be maintained for all media prepared in the laboratory and purchased commercially; including preparation or purchase date and QC test results. Any unusual characteristic of the medium, such as color or texture, or slow growth of reference strains should be noted.

I. Routine agar and broth media

All agar media should be aseptically prepared and dispensed into 15x100 mm Petri dishes (15-20 ml per dish). After pouring, the plates should be kept at room temperature (25°C) for several hours to prevent excess condensation from forming on the covers of the dishes. For optimal growth, the plates should be placed in a sterile plastic bag and stored in an inverted position at 4°C until use. All broth media should be stored in an appropriate container at 4°C until use.

A. Blood agar plate (BAP): trypticase soy agar (TSA) + 5% sheep blood

A BAP is used as a general blood agar medium. It is used for growth and testing of *N. meningitidis* and *S. pneumoniae*. The plate should appear a bright red color. If the plates appear dark red, they are either old or the blood was likely added when the agar was too hot. If so, the media should be discarded and a new batch should be prepared.

Media preparation

1. Prepare the volume of TSA needed in a flask according to the instructions given on the label of the dehydrated powder.
 - It is convenient to prepare 500 ml of molten agar in a 1-2 liter flask. If TSA broth powder is used, add 20 g agar into 500 ml of distilled water.
 - The media should be heated and fully dissolved with no powder on the walls of the vessel before autoclaving.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 60°C in a water bath.
4. Add 5% sterile, defibrinated sheep blood (5 ml sheep blood can be added to 100 ml of agar).

- If a different volume of basal medium is prepared, the amount of blood added must be adjusted accordingly to 5% (e.g., 50 ml of blood per liter of medium). Do NOT use human blood.
5. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
 6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *S. pneumoniae* or an *N. meningitidis* QC strain for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the BAP for specific colony morphology and hemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis).
- *N. meningitidis* should appear as large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- After 48 hours, the sterility test plate should remain clear.

B. Blood culture broth

Blood culture medium is used to grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*.

Media preparation

1. Follow the manufacturer's instructions on the label of each bottle of dehydrated trypticase soy broth (TSB).
2. Add 0.25 g sodium polyanetholsulfonate (SPS) per liter of medium.
 - SPS is especially important for recovery of *H. influenzae*.
3. Dispense in 20 ml (for a pediatric blood culture bottle) and 50 ml (for an adult blood culture bottle) amounts into suitable containers (tubes or bottles) with screw-caps with rubber diaphragms.
 - The amount of liquid in the containers should make up at least two-thirds of the total volume of the container.

4. Autoclave at 121°C for 15 minutes.
5. Allow to cool and store medium at room temperature (25°C).

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a BAP or CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Add 1-3 ml of sterile rabbit, horse, or human blood to 3 bottles of freshly prepared blood culture media.
3. Collect a loopful of overnight growth from each of the plates of bacteria and suspend it in 1-2 ml of blood culture broth (a different organism for each bottle).
4. Inoculate the bacterial suspensions into the 3 blood culture bottles.
5. Incubate the blood culture bottles at 35-37°C with ~5% CO₂ (or in a candle-jar) for up to 7 days and observe for growth.
6. Subculture bacteria onto appropriate media at 14 hours and 48 hours.
7. As a sterility test, incubate an uninoculated blood culture bottle for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- All three bacteria should be recovered on appropriate media after 24 and 48 hours.
- After 48 hours, the sterility test plate should remain clear.

C. Chocolate agar plate (CAP)

CAP is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO₂ atmosphere. CAP has a reduced concentration of agar, which increases the moisture content of the medium. It can be prepared with heat-lysed horse blood, which is a good source of both hemin and NAD, although sheep blood can also be used. Growth occurs on a CAP because NAD is released from the blood during the heating process of chocolate agar preparation (the heating process also inactivates growth inhibitors) and hemin is available from non-hemolyzed as well as hemolyzed blood cells.

Media preparation

1. Heat-lyse a volume of horse or sheep blood that is 5% of the total volume of media being prepared very slowly to 56°C in a water bath.
2. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
3. Place the plates in sterile plastic bags and store at 4°C until use.
4. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the CAP for specific colony morphology and hemolysis.

Passing result:

- *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.
- *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.
- After 48 hours, the sterility test plate should remain clear.

D. CAP with bacitracin

CAP with bacitracin is a selective medium used to improve the primary isolation of *H. influenzae* from specimens containing a mixed flora of bacteria and/or fungi.

Media preparation

1. Prepare double strength TSA (20 g into 250 ml distilled water) as the basal medium.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath.
 - Use a thermometer to verify the temperature in the water bath.
4. Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml of distilled water to form a smooth paste. Continue mixing as the rest of the water is added.

5. Autoclave at 121°C for 20 minutes.
6. Cool to 50°C in a water bath.
7. Add the hemoglobin solution to the double strength TSA and continue to hold at 50°C.
If a hemoglobin solution is unavailable, use the alternative method with defibrinated sheep blood described below:
 1. Add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood to 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water).
 - Do NOT use human blood.
 2. Heat to 56°C in a water bath then cool to 50°C.
8. After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50°C, add the growth supplement containing hemin and NAD to a final concentration of 1%. Mix the ingredients by gently swirling the flask in a figure 8 motion on the counter.
 - Avoid forming bubbles.
9. Prepare a stock solution of bacitracin by suspending 3 g bacitracin in 20 ml distilled water. Filter-sterilize, dispense into 1 ml amounts, and store at -20°C or -70°C.
10. While the medium is still at 50°C, add 1 ml stock solution of bacitracin (prepared in step 9) per 500 ml chocolate agar.
11. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
12. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow an *H. influenzae* QC strain for 18-24 hours on a CAP with bacitracin at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the CAP with bacitracin for specific colony morphology and hemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with bacitracin with no discoloration of the medium.

- After 48 hours, the sterility test plate should remain clear.

E. Chocolate agar with TSA and growth supplement

Chocolate agar with TSA and growth supplements is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO₂ atmosphere.

Media preparation

1. Prepare double strength TSA (20 g into 250 ml distilled water) as the basal medium.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath.
 - Use a thermometer to verify the temperature in the water bath.
4. Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml of distilled water to form a smooth paste. Continue mixing as the rest of the water is added.
5. Autoclave at 121°C for 20 minutes.
6. Cool to 50°C in a water bath.
7. Add the hemoglobin solution to the double strength TSA and continue to hold at 50°C.

If a hemoglobin solution is unavailable, use the alternative method with defibrinated sheep blood described below:

1. Add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood to 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water).
 - Do NOT use human blood.
2. Heat to 56°C in a water bath then cool to 50°C.
8. After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50°C, add the growth supplement containing hemin and NAD to a final concentration of 1%. Mix the ingredients by gently swirling the flask in a figure 8 motion on the counter.
 - Avoid forming bubbles.

9. Dispense 20 ml in each 15x100 mm Petri dish. Allow the media to solidify and condensation to dry.
10. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the chocolate agar with TSA and growth supplements for specific colony morphology and hemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.
- *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.
- After 48 hours, the sterility test plate should remain clear.

F. Chocolate agar with gonococcus medium (GC) base and growth supplement

Chocolate agar with GC base and growth supplement is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO₂ atmosphere.

Media preparation

1. Suspend 7.2 g of GC agar base in 100 ml distilled water in a flask. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.
2. Autoclave the flask at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Add 100 ml of warm distilled water to 2 g of soluble hemoglobin powder. Mix the powder with 5-10 ml of distilled water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water.
 - Alternatively, 100 ml ready-made 2% sterile hemoglobin solution, warmed to 50°C can be used.

5. Autoclave the solution at 121°C for 15 minutes. Cool to 50°C in a water bath.
6. Reconstitute lyophilized growth supplement containing NAD and hemin by aseptically transferring 10 ml of the accompanying diluent with a sterile needle and syringe. Shake to assure complete solution. After reconstitution, use immediately, or store at 4°C and use within 2 weeks.
7. Aseptically add 100 ml sterile hemoglobin solution and growth supplement to 100 ml of the GC agar base solution. Mix gently, but thoroughly, to avoid air bubbles in the agar.
8. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
9. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the chocolate agar with TSA and growth supplements for specific colony morphology and hemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.
- *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.
- After 48 hours, the sterility test plate should remain clear.

G. Chocolate agar slants

Chocolate agar slants for transport and short-term storage can be prepared in the same manner as described for agar plates with one difference: 4 ml of the medium should be dispensed into 16x125 mm screw-cap tubes and slanted before solidifying. Chocolate agar slants should look brown to brownish-red in color. Chocolate agar slants should be tested for QC using the same methods used for QC testing of CAP.

H. Cystine trypticase agar (CTA) with 1% carbohydrate (a semi-solid medium)

Media preparation

1. Follow the manufacturer's instructions for the amount of CTA medium to suspend in 900 ml of distilled water. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.
2. Autoclave the flask at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Prepare a 10% glucose (also called dextrose) solution by adding 10 g glucose to 100 ml distilled water. Filter-sterilize using a 0.22 micron filter.
5. Aseptically add 100 ml of the 10% glucose solution from step 4 to 900 ml of CTA medium to obtain a final concentration of 1% glucose.
6. Aseptically dispense 7 ml of the medium into 16x125 mm screw-cap glass tubes.
7. Repeat this procedure for the remaining 3 carbohydrates: maltose, lactose, and sucrose.
8. Store at 4°C and warm to room temperature (25°C) before use.

Quality control

1. Grow *N. meningitidis*, *N. lactamica*, and *N. sicca* QC strains to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Allow the 4 CTA sugars, glucose, maltose, lactose, and sucrose, to warm to room temperature (25°C) and label the tubes with the name of the QC strain.
3. Remove 3-5 colonies from overnight growth on the BAP using a 1 µl disposable loop.
4. Stab the CTA sugar several times into the upper 10 mm of medium. Approximately 8 stabs with the same loopful are sufficient.
 - Use a separate disposable loop for inoculating each carbohydrate to be tested.
5. Fasten the screw-cap of each tube loosely and place the tubes in a 35-37°C incubator without CO₂. Incubate the CTA sugars for at least 72 hours (and up to 5 days) before discarding as negative.
6. Observe the CTA sugars for development of visible turbidity and color change to yellow.

Passing result:

- Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth of bacteria and production of acid and is interpreted as a positive test.
- *N. meningitidis* should utilize glucose and maltose, but not lactose or sucrose.
- *N. lactamica* should utilize glucose, maltose, and lactose, but not sucrose.
- *N. sicca* should utilize glucose, maltose, and sucrose, but not lactose.

I. *Haemophilus* test medium (HTM) plates

HTM is used for antimicrobial susceptibility testing for *H. influenzae*.

Media preparation

The Mueller-Hinton agar used to make HTM should be thymidine free to obtain consistent results if susceptibility to cotrimoxazole is to be tested.

1. Prepare a fresh hemin stock solution by dissolving 50 mg of hemin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved.
2. Prepare a nicotinamide adenine dinucleotide (NAD) stock solution by dissolving 50 mg of NAD in 10 ml of distilled water; filter-sterilize.
3. Prepare Mueller-Hinton agar (MHA) from a commercially available dehydrated base according to the manufacturer's directions (or see protocol in section I.M.), adding 5 g of yeast extract and 30 ml of hemin stock solution to 1 L of MHA.
4. After autoclaving, cool the medium to 45°C to 50°C in a water bath.
5. Aseptically add 3 ml of the NAD stock solution.
6. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.
 - Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.
 - Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
 - Using more or less agar will affect the susceptibility results.
7. Allow the media to solidify and condensation to dry.
8. The pH should be 7.2-7.4.
 - Do not attempt to adjust the pH of the MHA test medium if it is outside the range.

9. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *H. influenzae* QC strain for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.
3. Inoculate a HTM plate with 10 µl of the cell suspension using a sterile loop and streak for isolation.
4. Observe HTM plate for specific colony morphology.
5. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the HTM plate with no discoloration of the medium.
- After 48 hours, the sterility test plate should remain clear.

J. Heart infusion agar (HIA) and trypticase soy agar (TSA)

HIA and TSA are general purpose media used with or without blood for isolating and cultivating a number of microorganisms. The media should appear straw colored (yellowish to gold coloring). HIA and TSA are also used for determining the hemin (X factor) and NAD (V factor) growth requirements of *H. influenzae*.

Media preparation

1. Prepare the volume of HIA or TSA needed in a flask, according to the instructions on the label of the dehydrated medium.
 - These media should be fully dissolved with no powder on the walls of the vessel, before autoclaving.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath and dispense 20 ml into each 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
4. Place the plates in plastic bags and store at 4°C until use.

Quality control

1. Grow a *H. influenzae* QC strain for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.
 - Do not transfer any of the chocolate agar media from the plate to the cell suspension as even the smallest amount of agar will affect the test and may lead to misidentification of the bacteria.
3. Inoculate one half of the HIA or TSA plate with 10 µl of the cell suspension using a sterile loop or swab and allow the suspension to dry.
4. Place paper disks or strips containing hemin, NAD, and hemin/NAD on the inoculated plate after the inoculum has dried.
5. Carefully invert the plates and incubate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
6. Observe growth on the HIA or TSA plate around the paper disks or strips.
7. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing Result:

- *H. influenzae* should grow only around the hemin/NAD disk.
- After 48 hours, the sterility test plate should remain clear.

K. Heart infusion rabbit blood agar plate (HIA - rabbit blood)

HIA-rabbit blood is used for determining the hemolytic reaction of *Haemophilus* species. This medium should appear bright red and look very similar to BAP. If the medium is dark red, it should be discarded and a new batch should be prepared. Horse blood may be substituted for rabbit blood in this medium.

Media preparation

1. Prepare the volume of HIA needed in a flask according to the instructions on the label of the dehydrated medium.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath.

4. Add 5% sterile, defibrinated rabbit blood (alternatively, 5 ml sheep blood can be added to 100 ml of agar).
5. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a hemolytic *H. haemolyticus* QC strain for 18-24 hours on a HIA-rabbit blood at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - After streaking the plate with the QC stain, stab the media with the inoculating loop.
2. Observe the HIA-rabbit blood for specific colony morphology and beta-hemolysis (clear).
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. haemolyticus* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies and be surrounded by a distinct zone of complete hemolysis which appears as a clear halo surrounding the colonies.
- After 48 hours, the sterility test plate should remain clear.

L. Horse blood agar (blood agar base)

This highly nutritive medium may be used for the primary isolation of *H. influenzae* and for the determination of the hemolysis with *H. haemolyticus* or other bacteria.

Media preparation

1. Prepare the volume of blood agar base needed in a flask according to the manufacturer's instructions on the label of the dehydrated medium.
2. Autoclave at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Add 5 ml horse blood per 100 ml of the medium.
5. Mix well and dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.

6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a hemolytic *H. haemolyticus* QC strain for 18-24 hours on a horse blood agar plate at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - After streaking the plate with the QC stain, stab the media with the inoculating loop.
2. Observe the horse blood agar for specific colony morphology and beta-hemolysis (clear).
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. haemolyticus* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies and be surrounded by a distinct zone of complete hemolysis which appears as a clear halo surrounding the colonies.
- After 48 hours, the sterility test plate should remain clear.

M. MacConkey (MAC) agar

MacConkey Agar is used for the isolation and differentiation of lactose-nonfermenting, gram-negative enteric bacteria from lactose-fermenting organisms. It is recommended that MAC medium be purchased commercially because preparing it with individual ingredients produces variability among lots.

Media preparation

1. Prepare MAC according to manufacturer's instructions.
2. Sterilize the medium by autoclaving at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
5. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow an *E. coli* QC strain for 18-24 hours on a MAC at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the MAC for specific colony morphology.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *E. coli* should appear as pink to red colonies.
- After 48 hours, the sterility test plate should remain clear.

N. Modified Thayer-Martin (MTM) agar medium

MTM is a selective medium used to improve the primary isolation of *N. meningitidis* from specimens containing a mixed flora of bacteria and/or fungi. MTM is a chocolate agar base containing vancomycin, colistin, nystatin, and trimethoprim lactate.

Media preparation

1. Suspend 7.2 g of GC agar base in 100 ml distilled water in a flask. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.
2. Autoclave the flask at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Add 100 ml of warm distilled water to 2 g of soluble hemoglobin powder. Mix the powder with 5-10 ml of distilled water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water.
 - Alternatively, 100 ml ready-made 2% sterile hemoglobin solution, warmed to 50°C can be used.
5. Autoclave the solution at 121°C for 15 minutes. Cool to 50°C in a water bath.
6. Reconstitute lyophilized growth supplement containing NAD and hemin by aseptically transferring 10 ml of the accompanying diluent with a sterile needle and syringe. Shake to assure complete solution. After reconstitution, use immediately or store at 4°C and use within 2 weeks.
7. Aseptically add 100 ml sterile hemoglobin solution and growth supplement to 100 ml of the GC agar base solution. Mix gently, but thoroughly, to avoid air bubbles in the agar.

8. To the agar base solution, add the following ingredients:
 - 3.0 µg/ml vancomycin
 - 7.5 µg/ml colistin
 - 12.5 units/ml nystatin
 - 5.0 µg/ml trimethoprim lactate
9. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
10. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *N. meningitidis* QC strain for 18-24 hours on a MTM at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the MTM for specific colony morphology.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *N. meningitidis* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the MTM with no discoloration of the medium.
- After 48 hours, the sterility test plate should remain clear.

O. Mueller-Hinton agar

Mueller-Hinton agar (MHA) is used for making the media required for susceptibility testing for *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. It is recommended that dehydrated Mueller-Hinton agar medium be purchased commercially because preparing it with individual ingredients can diminish the quality.

Media Preparation

1. Follow manufacturer's instructions to prepare MHA from a commercially available dehydrated base.
2. After autoclaving, cool the agar in a 45°C to 50°C water bath.
3. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.
 - Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.
 - Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

- Using more or less agar will affect the susceptibility results.
4. Allow the media to solidify and condensation to dry.
 5. The pH of MHA should be 7.2-7.4.
 - Do not attempt to adjust the pH of the MHA test medium if it is outside the range.
 6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow an *E. coli* QC strain for 18-24 hours on a MHA plate at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- Observe for *E. coli* growth.
- After 48 hours, the sterility test plate should remain clear.

P. Mueller-Hinton agar with 5% sheep or horse blood

Mueller-Hinton agar with 5% sheep or horse blood is used for susceptibility testing for *N. meningitidis* and *S. pneumoniae*. It is recommended that dehydrated MHA medium be purchased commercially because preparing it with individual ingredients can diminish the quality.

Media preparation

1. Prepare MHA as described above in Section I.M. through step 2.
2. Add 5% sterile defibrinated sheep or horse blood to the medium at 5% (i.e., 50 ml blood per liter of medium or 25 ml blood to 500 ml medium).
3. The pH of MHA after the addition of blood should be 7.2-7.4.
 - Do not attempt to adjust the pH if it is outside the range.
4. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.
 - Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.

- Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
 - Using more or less agar will affect the susceptibility results.
5. Allow the media to solidify and condensation to dry.
 6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *S. pneumoniae* or a *N. meningitidis* QC strain for 18-24 hours on Mueller-Hinton agar with 5% sheep or horse blood at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis).
- *N. meningitidis* should appear large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- After 48 hours, the sterility test plate should remain clear.

Q. Mueller-Hinton Broth

Mueller-Hinton broth is used to prepare the Mueller-Hinton broth (cation-adjusted).

Media Preparation

1. To 750 ml deionized H₂O add:
 - 3.0 g beef extract
 - 17.5 g acid hydrolysate of casein (casamino acids)
 - 1.5 g soluble starch
2. Adjust final volume to 1 liter.
3. Adjust pH to 7.3.
4. Autoclave the broth at 121°C for 20 minutes to sterilize.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

R. Mueller-Hinton broth (cation-adjusted)

Mueller-Hinton broth (cation-adjusted) is used to prepare dilutions equivalent McFarland standards with minimal loss of viability.

Media Preparation

1. Prepare a magnesium stock solution by dissolving 8.36 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 100 ml deionized H_2O to a final concentration of 10 mg/ml Mg^{2+} .
2. Prepare a calcium stock solution by dissolving 3.68 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml deionized H_2O to a final concentration of 10 mg/ml Ca^{2+} .
3. Filter-sterilize both stock solutions.
4. To Mueller-Hinton broth, add the magnesium stock solution to a final concentration of 10-12.5 $\mu\text{g/ml}$ Mg^{2+} .
5. Add the calcium stock solution to a final concentration of 20-25 $\mu\text{g/ml}$ Ca^{2+} .

Quality control

1. Streak 10 μl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO_2 (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

S. Trypticase soy broth (TSB)

TSB is used for making suspensions of *H. influenzae* prior to testing for hemin and NAD requirements. Heart infusion broth, sterile saline, or PBS may be substituted for TSB.

Media preparation

1. Prepare the volume of TSB needed in a flask according to the instructions on the label of the dehydrated medium.
2. Dispense 5 ml into 15x125 mm tubes.
3. Autoclave at 121°C for 20 minutes.
4. Cool and store at 4°C.

Quality control

1. Grow a *S. pneumoniae* QC strain for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - *H. influenzae* does not grow in TSB, but the medium should not be toxic to other bacteria. Therefore, *S. pneumoniae* should be used to QC for toxicity.
2. Inoculate a tube of TSB with a loop of overnight growth from the BAP and incubate overnight at 35°C.
3. The broth should be turbid the next day. Subculture the broth onto a BAP to test for proper growth characteristics of *S. pneumoniae*.
4. Observe the BAP for specific colony morphology and hemolysis.

Passing result: *S. pneumoniae* colonies are small and appear grey to grey-green surrounded by a distinct green halo (alpha-hemolysis).

II. Storage and transport media

A. Defibrinated sheep blood

Defibrinated sheep blood is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Mechanically shake 30 ml sheep blood with sterile glass beads or a wooden stick device in a 125-250 ml Erlenmeyer flask at approximately 90 rpm for 7-9 minutes.
 - Clotting factors will be visible in the flask as a translucent, fibrous web.
2. Remove the clotting factors using sterile forceps.
3. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

B. Dorset Transport medium

Dorset Transport medium is used for short term storage of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* isolates.

Media preparation

1. Combine sterile 0.85 % saline solution with beaten whole hen's eggs at a 1:3 ratio.
2. Inspissate (i.e., thicken) the mixture in an electric inspissator at 80°C for 60 minutes.
3. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

C. Greaves solution

Greaves solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. To 200.0 ml distilled water, add:
 - 10.0 g bovine albumin, fraction V
 - 10.0 g L-glutamic acid, sodium salt
 - 20.0 ml glycerol
2. Mix all ingredients listed below until they are completely dissolved.
3. Filter-sterilize the solution and transfer to a sterile flask.
4. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

D. Modified Trans-Isolate (MT-I) medium

Modified Trans-Isolate medium (MT-I) was designed and developed to be a simple and inexpensive medium for transport of CSF and growth of meningococci in large scale epidemics of meningococcal disease. It is less expensive and easier to produce than the standard T-I, and can be produced in most microbiology laboratories at approximately USD \$0.50/bottle, providing a relatively inexpensive, rapidly available (reducing the production and shipping time and cost) transport medium and diagnostic tool for use during a meningitis epidemic. The ingredients of the MT-I formulation are similar to those in T-I, with modifications to eliminate costly ingredients and save time.

Laboratory evaluation has demonstrated that growth/survival of isolates of meningococci was equal in T-I and MT-I under several environmental conditions. It has not been evaluated under field conditions with clinical specimens yet. It does not support the growth of *H. influenzae*; therefore, it is not used for routine surveillance of agents of bacterial meningitis.

0.5 oz, sterile round, clear glass screw-cap (with rubber liner) bottles should be used. MT-I bottles should be stored upright at 4°C when not in immediate use and warmed to room temperature (25-30°C) before use. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. MT-I media has a shelf life of 1 year with proper storage.

Media preparation

1. Weigh all ingredients listed below and place them into a 500mL Erlenmeyer flask.
 - 8 g 2.0% gelatin
 - 2 g 0.5% agar
 - 2.5 g Tris-HCl
 - 0.5 g Tris base
 - 12 g 3.0% Tryptic Soy Broth
2. Add 350 ml distilled, deionized water to the flask (pH should be 7.5 at 25°C).
3. Place a magnetic stirring bar in the flask and place the flask on hot plate stirrer.
4. Bring the solution to boiling (90-100°C) to melt and dissolve the gelatin until the medium is completely clear (about 30-45 minutes).
5. Remove the flask from the hot plate stirrer.
6. Mix 2.6 g 0.5% soluble starch with a small amount of cold water and dissolve completely.
7. Once the starch is evenly distributed throughout medium, add 1.6 g 0.4% activated charcoal and adjust water to 400ml.

8. Return the flask to the hot plate stirrer until all ingredients are thoroughly mixed (about 10 minutes).
 - The solution should be liquid, appear black in color, and should not have any clumps.
9. Turn down the heat to low so the flask can be handled comfortably while dispensing the medium.
 - Optional: At this step, the procedure can be stopped overnight if there is not enough time to dispense. Split the media into two flasks and place both flasks at 4°C overnight. The next day, apply heat until fully melted and aliquot (see step 10).
10. While the media is being stirred, use a sterile serological pipette to remove 7 ml of medium and dispense into each bottle.
11. Cap each bottle loosely with a screw cap.
12. Autoclave the bottles for 15 minutes at 121°C.
13. Tighten the caps as soon as possible after autoclaving.
14. Swirl the bottles to avoid the charcoal settling.
15. Slant the bottles overnight (or at least 4 hours) on wooden slanting stick (35 mm, 35 mm, 500 mm).
 - When slanted, the liquid should reach shoulder of the bottle.
16. Once the medium is firm, stand the bottles upright.
17. In approximately 1 hour, the weak slant should release some of the broth and the medium should appear biphasic.
18. Store at 4°C when not in use.

Quality control

1. Grow *N. meningitidis* and *S. pneumoniae* QC strains for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. For each organism, make a cell suspension equivalent to a 0.5 McFarland standard (~equivalent to 1.5x10⁸ CFU/ml) and serially dilute it to achieve an inoculum size of 10³ CFU/ml in brain heart infusion (BHI) broth.
3. Remove the screw cap from 3 MT-I bottles.

4. Inoculate the MT-I bottles with 100 μ l of the 10^3 CFU/ml suspensions within 15 minutes of preparation (inoculum size is 100 CFU) and replace the screw caps tightly.
 - After inoculation and replacement of the screw-caps, invert each MT-I bottle several times to mix.
5. Slightly loosen the screw-caps of the MT-I bottles to allow some air exchange.
6. Incubate the MT-I bottles for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
7. Close the screw-caps tightly and invert each MT-I bottle several times to mix.
8. Remove 10 μ l of broth from each MT-I bottle and inoculate a BAP for the MT-Is containing *N. meningitidis* and *S. pneumoniae* and a CAP for the MT-I containing *H. influenzae*.
9. Streak for isolation with a sterile loop and incubate plates for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to detect growth of the QC strains.

Passing results:

- *N. meningitidis* should appear large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis) on the BAP.

E. 10% skim milk and 15% glycerol solution

10% skim milk and 15% glycerol solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Place 10 g dehydrated skim milk and 85 ml distilled water into flask A. Swirl to mix.
2. Place 15 ml of glycerol into flask B.
3. Autoclave both flasks at 115°C for 10 min, and exhaust the pressure carefully.
4. While still hot, pour the contents of flask A into flask B in a safety cabinet.
5. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

F. Skim milk tryptone glucose glycerol (STGG) medium

STGG medium is used for transport and short term storage of nasopharyngeal swabs.

Media preparation

1. Add the following ingredients to 100 ml distilled water:
 - 2 g skim milk powder
 - 3 g TSB
 - 0.5 g glucose
 - 10 ml glycerol
2. Mix to completely dissolve all ingredients.
3. Dispense 1.0 ml amounts into 1.5 ml screw-cap vials.
4. Loosen the screw-caps and autoclave at 121°C for 10 minutes.
5. Tighten the caps after autoclaving and store at -20°C until use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

G. Trans-Isolate (T-I) medium

T-I is used to transport CSF and grow *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* from CSF. 10 cc tubing vials with rubber septum plugs and aluminum crimp seal caps should be used. T-I bottles should be stored upright at 4°C when not in immediate use. T-I media should be warmed to room temperature (25°C) before use. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. T-I media has a shelf life of 1 year with proper storage.

Media preparation

Diluent for solid and liquid phases: 3- (N-morpholino) propanesulfonic acid (MOPS) buffer; 0.1 M, pH 7.2

1. Dissolve 20.93g MOPS in 900ml distilled water.
2. Adjust to pH 7.2 with 1N NaOH.
3. Adjust volume to 1000 ml with distilled water.

Solid phase

Activated charcoal	2.0 g
Soluble starch	2.5 g
Agar	10.0 g

1. Suspend activated charcoal, soluble starch, and agar into 500 ml of MOPS buffer in a flask and add a magnetic bar to the flask.
2. Heat on a magnetic stirrer hot plate to dissolve the charcoal and starch and melt the agar.
3. While stirring to keep the charcoal in suspension, use a sterile serological pipette to remove 5.0 ml and dispense into each serum bottle.
4. Cap each bottle with a piece of aluminum foil and autoclave at 121°C for 20 minutes.
5. Remove from the autoclave and slant the bottles until they reach room temperature (25°C), so that the apex of the agar reaches the shoulder of each bottle.

Liquid phase

Tryptic soy broth (TSB)	30.0 g
Gelatin	10.0 g
MOPS buffer	500.0 ml

1. Heat the TSB, gelatin, and MOPS buffer to completely dissolve the gelatin and avoid coagulation.
2. Autoclave the media at 121°C for 15 minutes.

Optional additive for growth of *H. influenzae*

1. Once the bulk liquid phase medium has cooled to room temperature (25°C) after autoclaving, add 10 ml of a sterile liquid growth supplement containing NAD and hemin aseptically to help support growth of *H. influenzae*.
 - Alternatively, aseptically add 0.1 ml of the supplement to an individual T-I bottle (1% of the volume of both phases) or to a limited number of bottles, as needed.
 - If a commercial growth supplement is used (preferred method), it should be a sterile product and can be added directly to the T-I medium.
 - If the growth supplement is prepared in the laboratory, it should be filter-sterilized prior to being added to the T-I medium. To prepare the supplement in the laboratory:
 1. Dissolve the lyophilized supplement into an appropriate diluent (usually water).
 2. Filter-sterilize using a 0.45 micron pore size membrane.
 3. Use immediately.

T-I medium

1. Dispense 5 ml of the liquid phase aseptically into each of the bottles containing the solid phase slants.
2. Seal the bottles with sterile rubber septum plugs and aluminum caps. Use a hand crimping tool to fasten the aluminum caps if an automated system is not available.
3. Store at 4°C when not in use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a BAP or CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. For each organism, make a cell suspension equivalent to a 0.5 McFarland standard (~equivalent to 1.5X10⁸ CFU/ml) and serially dilute to achieve an inoculum size of 10³ CFU/ml in brain heart infusion (BHI) broth.
3. Using sterile forceps pull the aluminum cover of 3 T-I bottles away from the rubber stopper and wipe the stopper with 70% isopropanol or ethanol.
 - Do not use povidone-iodine.

4. Use a sterile syringe and needle to inoculate the T-I bottles with 100 μ l of the 10^3 CFU/ml suspensions within 15 minutes of preparation (inoculum size is 100 CFU).
 - Use a new needle and syringe for each T-I. After inoculation and removal of the syringe and needle from the rubber stopper, invert each T-I bottle several times to mix.
5. Insert a sterilized T-I venting needle through the rubber stopper of each of the inoculated T-I bottles.
 - Be sure that the venting needles do not touch the broth.
6. Incubate vented T-I bottles for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
7. Remove and discard venting needles. Invert each T-I bottle several times to mix.
8. Use a sterile syringe and needle to remove 100-200 μ l of broth from each T-I bottle and place the broth into a sterile, labeled 1.5 ml microcentrifuge tube. Inoculate a BAP for the T-Is containing *N. meningitidis* and *S. pneumoniae* and a CAP for the T-I containing *H. influenzae* with 10 μ l of this broth.
9. Streak for isolated colonies with a sterile loop and incubate plates for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to detect growth of the QC strains.

Passing results:

- *N. meningitidis* should appear as large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis) on the BAP.
- *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.

III. Miscellaneous reagents

A. Gram stain reagents

Ammonium oxalate-crystal violet

1. Dissolve 2.0 g certified crystal violet into 20.0 ml of 95% ethyl alcohol.
2. Dissolve 0.8 g ammonium oxalate into 80.0 ml distilled water.
3. Mix the two solutions together and allow them to stand overnight at room temperature (25°C).
4. Filter through coarse filter paper before use.

5. Store at room temperature (25°C).

Gram's iodine (protect solution from light)

1. Grind 1.0 g iodine (crystalline) and 2.0 g potassium iodide in a mortar. Small additions of distilled water may be helpful in preparing the solution.
2. Add to 300.0 ml distilled water.
3. Store at room temperature (25°C) in a foil-covered bottle.

Decolorizer is 95% ethyl alcohol

Counterstain (there are 2 options: safranin or carbol-fuchin)

Safranin

1. Add 2.5 g certified safranin-O to 100.0 ml 95% ethyl alcohol.
2. Add 10.0 ml safranin and ethyl alcohol solution made in step 1 to 90.0 ml distilled water.
3. Store at room temperature (25°C).

Ziehl-Nielsen carbol-fuchsin (may be a more effective counterstain than safranin)

1. Dissolve 0.3 g basic fuchsin in 10.0 ml 95% ethyl alcohol.
2. Add 5.0 ml melted phenol crystals to 95.0 ml distilled water.
3. Add the 5% phenol solution to the fuchsin solution and let stand overnight.
4. Filter through coarse filter paper.
5. Store at room temperature (25°C) in a foil-covered bottle for up to 1 year.

B. Iodine tincture

Iodine tincture is used as a skin antiseptic and should not be used to disinfect the rubber stoppers of T-I and blood culture bottles.

1. Add 1 g of iodine to 100 ml of 70% isopropyl alcohol.
2. Store at room temperature (25°C) in a foil-covered bottle for up to 1 year.

C. McFarland turbidity standards

1. Prepare a 1% solution of anhydrous BaCl₂ (barium chloride).
2. Prepare a 1% solution of H₂SO₄ (sulfuric acid).
3. Combine and completely mix the barium chloride and sulfuric acid solutions to form a turbid suspension of BaSO₄ in a specific proportion for each McFarland turbidity standard as shown in Table 1.
4. Place the resulting mixture in a foil-covered screw-cap tube.
5. Store the McFarland standard at room temperature (25°C) when not in use. Prepare a fresh standard solution every 6 months. McFarland standard density solution will precipitate and clump over time, and it needs vigorous vortexing before each use. Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred.

Table 1. McFarland turbidity standards

McFarland turbidity standard no.	0.5	1	2	3	4
1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1x10 ⁸ CFU/ml)	1.5	3.0	6.0	9.0	12.0

D. Phosphate buffered saline (PBS)

1. To 1000 ml distilled water, add the following ingredients for 0.1 M PBS:
 - 7 g sodium dihydrogen phosphate
 - 7 g disodium hydrogen phosphate
2. Mix to completely dissolve ingredients.
3. Adjust pH to 7.2 with 1 N acid or base.
4. Dispense buffer into a flask and autoclave at 121°C for 15 minutes.
5. Store at room temperature (25°C) for up to one year.

E. Physiological saline

1. Dissolve 8.5 g NaCl into 1 L distilled water.
2. Autoclave at 121°C for 15 minutes or sterilize using membrane filtration.
3. Store at room temperature (25°C) for up to 6 months.