APPENDIX 1

Standard Safety Practices in the Microbiology Laboratory

Laboratorians working with infectious agents are subject to laboratory-acquired infections as a result of accidents or unrecognized incidents. The degree of hazard depends upon the virulence of the biological agent concerned and host resistance. Laboratory-acquired infections occur when microorganisms are inadvertently ingested, inhaled, or introduced into the tissues. Laboratorians are relatively safe when working with *Haemophilus influenzae* and *Streptococcus pneumoniae;* however, persons who work with aerosolized *Neisseria meningitidis* are at increased risk of acquiring a meningococcal infection. The primary laboratory hazard associated with enteric pathogens such as *Shigella, Vibrio* or *Salmonella* is accidental ingestion. Biosafety Level 2 (BSL-2) practices are suitable for work involving these agents that present a moderate potential hazard to personnel and the environment. The following requirements have been established for laboratorians working in BSL-2 facilities.

- Laboratory personnel must receive specific training in handling pathogenic agents and be directed by competent scientists.
- Access to the laboratory must be limited when work is being conducted.
- Extreme precautions must be taken with contaminated sharp items.
- Certain procedures involving the creation of infectious aerosols or splashes must be conducted by personnel who are wearing protective clothing and equipment.

Standard microbiological safety practices

The following safety guidelines listed below apply to all microbiology laboratories, regardless of biosafety level.

Limiting access to laboratory

Sometimes, people who do not work in the laboratory attempt to enter the laboratory to look for test results they desire. Although this occurs more frequently in clinical laboratories, access to the laboratory should be limited, regardless of the setting.

Biohazard signs or stickers should be posted near all laboratory doors and on all equipment used for laboratory work (*e.g.*, incubators, hoods, refrigerators, and freezers). Children under 12 years of age and pets are not allowed in laboratory areas. All laboratories should be locked when not in use. In addition, all freezers and refrigerators located in corridors should be locked.

Handwashing

Each laboratory should contain a sink for handwashing. Hands should be washed for at least one minute. Frequent handwashing is one of the most effective procedures for avoiding laboratory-acquired infections. Hands should be washed with an appropriate germicidal soap before exiting the laboratory and after infectious materials are handled. (Laboratorians working with gram-positive organisms should use alcohol (70%) to cleanse their hands if germicidal soap is unavailable.)

Eating

Eating, drinking, and smoking are not permitted in laboratory work areas. Food must be stored and eaten outside of the work area in designated areas used for that purpose only. Personal articles (*e.g.*, handbags, eyeglasses, or wallets) should not be placed on the workstations.

Mouth pipetting

Mouth pipetting is **strictly prohibited** in the laboratory. Rubber bulbs or mechanical devices should be used.

Sharps

A high degree of precaution must always be taken with any contaminated sharp items, including needles and syringes, slides, pipettes, capillary tubes, and scalpels. Dispose of sharps in designated containers. To minimize finger sticks, used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal. Non-disposable sharps, including syringes, should be placed in a labeled discard pan for decontamination before cleaning. Broken glassware should not be handled directly by hand but should be removed by mechanical means (*e.g.*, a brush and dustpan, tongs, or forceps).

Aerosols

All procedures must be carefully performed to minimize splashes or aerosolization. Techniques that tend to produce aerosols should be avoided. Inoculating wires and

loops should be cooled by holding them still in the air for 5 – 10 seconds before they touch colonies or clinical material. Loops containing infectious material should be dried in the hot air above a burner before flaming. Vortexing and centrifugation should be done in closed containers. (If safety capped tubes are not available, sealed tubes should be used.) Gauze should be used to remove the tops on blood specimens and should be placed around the top of blood culture bottles to minimize aerosol production during removal of the needle. Needles should never be cut or removed from the syringe before autoclaving. All body fluids should be centrifuged in carriers with safety caps only.

When procedures with a high potential for creating infectious aerosols are conducted or when a procedure is used that can result in splashing or spraying of the face with infectious or other hazardous materials, laboratory work should be conducted in a safety cabinet or by laboratorians wearing the appropriate face-protection equipment (*e.g.*, goggles, mask, face shield, or other splatter guards). Procedures that pose a risk may include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of infectious materials whose internal pressures may be different from ambient pressures, inoculating animals intranasally, and harvesting infected tissues from animals or eggs. Face protection should also be used when working with high concentrations or large volumes of infectious agents.

Decontaminating bench tops and other surfaces

Bench tops should be wiped with a disinfectant (a phenolic disinfectant, 1% sodium hypochlorite [bleach], or 70% isopropyl alcohol) routinely after working with infectious agents or clinical specimens or after spills, splashes, or other contamination by infectious materials. Solutions of disinfectants should be maintained at the work station (see *Disinfectants*).

Disposal of contaminated materials

All discarded plates, tubes, clinical samples, and other contaminated materials should be placed in disposal containers at each bench. Special disposal boxes must be used for sharps (*e.g.*, syringes or broken glass) to minimize the risk of injury. Avoid overfilling such containers. Containers of contaminated material should be carefully transported to the autoclave room and autoclaved before disposal.

Autoclaving

An autoclave must be available for the BSL-2/3 laboratory and must be operated only by personnel who have been properly trained in its use. To verify that each autoclave is working properly, spore strips or other biological indicators designed to test for efficiency of sterilization should be included in autoclave loads on a

regular basis. Each autoclave load should be monitored with temperature-sensitive tape, thermograph, or by other means (*e.g.*, biological indicators).

General laboratory policies

All areas of the laboratory must be kept clean and orderly. Dirt, dust, crowding, or clutter is a safety hazard and is not consistent with acceptable biological research. Floors should be kept clean and free of unnecessary clutter. They should be washed with a germicidal solution on a regular basis and after any spill of infectious material has occurred.

Refrigerators and freezers

Refrigerators and freezers should be regularly inspected for the presence of broken vials or tubes containing infectious agents. When removing and discarding broken material, laboratorians should wear gloves and proper protective attire (*e.g.*, laboratory coat, goggles, or face-shield). Refrigerators and freezers should be regularly cleaned with a disinfectant and defrosted to prevent possible contamination and temperature failure.

Fire prevention

Burners should be used away from lamps and flammable materials. Bulk flammable material must be stored in the safety cabinet. Small amounts of these flammable materials (*e.g.*, ethyl acetate, ethyl alcohol, and methanol) can be stored in safety containers. Burners must be turned off when not in use. All laboratorians must know the location of fire extinguishers, fire blankets, and showers, and fire safety instructions and evacuation routes should be posted.

Special practices

Transport of biohazardous materials

Transport of biohazardous materials from one building to another increases the risk of breakage and spills. If transport is necessary, the primary infectious agent container (regardless of size) must be placed in an unbreakable second container that can be sealed (*e.g.*, using a screw-top tube or a plastic bag).

Disinfectants

Organisms may have different susceptibilities to various disinfectants. As a surface disinfectant, 70% alcohol is generally effective for the *Enterobacteriaceae,* but other organisms are more resistant. However, 70% isopropyl alcohol is not the

disinfectant of choice for decontaminating spills. Phenolic disinfectants, although expensive, are usually effective against many organisms. Always read disinfectant labels for manufacturers' recommendations for dilution and for exposure times for efficacy, especially before use on BSL-3 organisms (*e.g.*, *Mycobacterium tuberculosis*). **A effective general disinfectant is a 1:100 (1%) dilution of household bleach (sodium hypochlorite) in water; at this dilution, bleach can be used for wiping surfaces of benches, hoods and other equipment.** A 1:10 (10%) dilution of bleach is corrosive and will pit stainless steel and should not be used routinely; however, the 10% bleach solution may be used to clean up spills of cultured or concentrated infectious material where heavy contamination has occurred. If sodium hypochlorite is used as a disinfectant, the standard 1% dilutions should be made **daily** from a stock solution.

Decontamination of spills

The following procedure is recommended for decontaminating spills.

- Isolate the area to prevent anyone from entering.
- Wear gloves and protective clothing (*e.g.*, a gown or lab coat, shoes, and a mask [if the spill may contain a respiratory agent or if the agent is unknown]).
- Absorb or cover the spill with disposable towels.
- Saturate the towels with an appropriately diluted intermediate or high-level disinfectant (*e.g.*, a phenolic formulation or household bleach).
- Place disinfectant-soaked towels over the area and leave them in place for at least 15 minutes before removing and discarding them.
- Wipe area using clean disinfectant-soaked towels and allow area to air dry.
- Place all disposable materials used to decontaminate the spill into a biohazard container.
- Handle the material in the same manner as other infectious waste.

Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. When cuts or puncture wounds from potentially infected needles or glassware occur, the affected area should be promptly washed with disinfectant soap and water for 15 minutes. In the event of a centrifuge accident in which safety carriers have not been used, other personnel in the area should be warned immediately and the area isolated to prevent anyone from entering.

Protective clothing and equipment

Laboratory coats

Protective coats, gowns, smocks, or uniforms designated for laboratory use must be worn while working in the laboratory. Laboratory coats should fit properly and should cover arms to the wrist. This protective clothing should be removed and left in the laboratory before leaving for non-laboratory areas. All protective clothing is either disposed of in the laboratory or laundered by the institution; it should never be taken home by personnel.

Gloves

Regardless of the type of infectious material, gloves should be worn when performing potentially hazardous procedures (*e.g.*, slide agglutination) in which there is a risk of splashing or skin contamination or when the laboratory worker has cuts or broken skin on his or her hands. Gloves should always be worn when handling clinical specimens, body fluids, and tissues from humans and animals. These tissues should be assumed to be positive for hepatitis B virus, human immunodeficiency virus (HIV), other bloodborne pathogens, and *M. tuberculosis.* Gloves must be removed when contaminated by splashing or spills or when work with infectious materials is completed. Gloves should not be worn outside the laboratory. Personnel should not use the telephone or open doors with gloves that have been used in laboratory procedures. All used gloves should be disposed of by discarding them with other disposable materials and autoclaving. **Hands should be washed immediately after removing gloves.**

Barrier precautions

Clinical specimens, body fluids, and tissues from humans and animals should be assumed to be positive for hepatitis B virus, HIV, other bloodborne pathogens, and M. tuberculosis. These materials should be handled in a safety cabinet or using other barrier precautions (*e.g.*, goggles, mask, face shield, or other splatter guards) whenever a procedure is performed that can potentially create an aerosol.

Useful biosafety references for the laboratory

- Centers for Disease Control and Prevention, National Institutes of Health. *Biosafety in microbiological and biomedical laboratories.* Washington, DC: U.S. Government Printing Office; 1999: stock no. 017-040-00547-4.
- World Health Organization. *Laboratory Biosafety Manual,* 2nd edition. Geneva: WHO; 1993: ISBN 92 4 154450 3.

APPENDIX 2

Media, Reagents, and Quality Control

Each laboratory must ensure adequate control of the media and reagents it uses. Quality control (QC) includes the selection of satisfactory reagents, the preparation of media according to approved formulations or specific manufacturer's instructions, and the use of well-characterized reference strains to check prepared media. The World Health Organization (WHO) encourages central public health laboratories to participate in at least three external quality assessment surveys per year; for reference laboratories, this may involve an international testing scheme. National central laboratories (reference laboratories) should work to standardize procedures of regional and local laboratories to their own, so that observations can be interpreted in the same manner across sites.

Quality control of media

A summary of considerations for quality control of media, methods, and sources of quality control strains follows:

1) Considerations for quality control of media

Each batch of medium prepared from individual ingredients or each different manufacturer's lot number of dehydrated medium should be tested for sterility, the ability to support growth of the target organism(s), and/or the ability to produce appropriate biochemical reactions, as appropriate.

Sterility

- Incubate one tube or plate from each autoclaved or filter-sterilized batch of medium overnight at 35°–37°C and examine it for contaminants.

Ability to support growth of the target organism(s)

- Use at least one strain to test for ability of selective media to support growth of the target pathogen (*e.g.*, for MacConkey agar, a *Shigella* strain such as *S. flexneri*). Documentation should be made regarding whether this strain produces the appropriate biochemical reactions / color on the test medium.

(Discussions of specific biochemical reactions are included in the media section of this appendix.)

Ability to produce appropriate biochemical reactions

- **For selective media:** Use at least one organism that will grow on the medium and at least one organism that will not grow on the selective medium to test for the medium's ability to differentiate target organisms from competitors. If the medium is both selective and differential, it may be useful to include two organisms that will grow on the medium and produce different reactions (*e.g.*, for MacConkey agar: a lactose-nonfermenting organism such as *S. flexneri;* a lactose-fermenting organism such as *E. coli;* and, *S. aureus,* which should not grow).
- **For biochemical media:** Use at least one organism that will produce a positive reaction and at least one organism that will produce a negative reaction (*e.g.*, for urea medium, a urease-positive organism such as *Proteus* and a urease-negative organism such as *E. coli*).

2) Methods for quality control of media

When testing for ability of a medium to support growth, a small inoculum will give greater assurance that the medium is adequate for recovery of a small number of organisms from a clinical specimen; therefore, use a dilute suspension of control organisms to inoculate the medium for QC. An example of a protocol for quality control of media follows here:

a) Inoculate the control strain to nonselective broth (*e.g.*, a tryptone-based soy broth [TSB]) and incubate / grow overnight.

b) Prepare a standardized inoculum for testing the medium. The appropriate standard dilution differs for selective and nonselective media.

   - **If testing selective or inhibitory media:** To prepare a standardized inoculum for testing selective and inhibitory media, make a 1:10 dilution of the overnight nonselective broth culture.
   - **If testing nonselective media:** To prepare a standardized inoculum for testing nonselective media, make a 1:100 dilution of the nonselective broth culture.

c) Using a calibrated loop, if available, inoculate one tube or plate of each medium with a loopful of the standardized inoculum of the control strain(s). (If performing QC of plating medium, streak for isolation.) The same loop should be used for all QC of all media; it is more important to have the consistency of the same inoculating loop every time than it is to use a calibrated loop.

- **If testing selective or inhibitory media:** A nonselective plating medium (*e.g.*, heart infusion agar [HIA]) should be inoculated at the same time as the selective medium for comparison purposes.

3) Sources of quality control strains

Suitable QC strains may be obtained in the following ways.

- A laboratory may use strains isolated from clinical specimens or quality assurance specimens, provided the strains have been well characterized by all available methods (*e.g.*, biochemical, morphologic, serologic, and molecular).
- Many laboratories purchase QC strains from official culture collections (*e.g.*, the American Type Culture Collection [ATCC] and the National Collection of Type Cultures [NCTC]).[34] (Addresses for ATCC and NCTC are included in Appendix 13.)

Quality control strains appropriate for antimicrobial susceptibility testing as included in this manual

The following ATCC numbers can be used to identify the appropriate antimicrobial susceptibility testing QC organisms included in this laboratory manual.

Haemophilus influenzae	*H. influenzae* ATCC 49247 * (* *for testing of the antimicrobial agents included in this laboratory manual*)
Neisseria meningitidis	*S. pneumoniae* ATCC 49619
Streptococcus pneumoniae	*S. pneumoniae* ATCC 49619
Neisseria gonorrhoeae	*N. gonorrhoeae* ATCC 49226 *(other reference strains, available from CDC, may be used when testing antimicrobial agents not included in NCCLS criteria)*
Salmonella serotype Typhi	*E. coli* ATCC 25922
Shigella	*E. coli* ATCC 25922
Vibrio cholerae	*E. coli* ATCC 25922

[34] This manual presents the ATCC numbers for quality control organisms, but these ATCC strains may also be obtained from the NCTC.

Quality control of reagents

As with all other products used in testing, reagents (whether purchased or prepared in the laboratory) should be clearly marked to indicate the date on which they were first opened and the expiration date, if appropriate. Each reagent should be tested to make sure the expected reactions are obtained.

If the reagent is a rare, expensive, or difficult-to-obtain product (*e.g.*, diagnostic antiserum) it does not necessarily have to be discarded on the expiration date. If satisfactory sensitivity and specificity can still be verified by normal QC procedures, the laboratory may indicate on the vial label the date of verification of quality of the reagent. All reagents should be tested for quality at intervals established by each laboratory to ensure that no deterioration has occurred; if the quality of the reagent is being verified after the expiration date, testing should be performed more frequently.

Slide agglutination method for quality control of antisera

For QC of antiserum, two or more control strains (one positive and one negative) should be used to test the agglutination characteristics of the antiserum. The results of all reactions should be recorded. Following is an example of a typical QC procedure.

- Place a drop (about 0.05 ml, though as little as 10 µl can be used) of each antiserum on a slide or plate. Also, place a drop of 0.85% saline on each slide or plate to test each antigen for roughness or autoagglutination.
- Prepare a densely turbid suspension (2 or 3 McFarland turbidity standard, see Table 21) of each control isolate in 0.85% saline with growth aseptically harvested from an 18- to 24-hour culture from nonselective agar (*e.g.*, HIA or tryptone soy agar [TSA]).
- Add one drop of the antigen suspension to the antiserum and the saline. Mix thoroughly with an applicator stick, glass rod, or inoculating loop. Rock the slide back and forth for 1 minute.
- Read the agglutination reaction over a light box or an indirect light source with a dark background. The saline control must be negative for agglutination for the test to be valid.

The degree of agglutination should be read and recorded as follows:

Percentage of agglutination:	Record reaction as:
100%	4+
75%	3+
50%	2+
25%	1+
0%	negative

Advantages of centralized acquisition of media and reagents

Centralizing acquisition of media and reagents in the national reference laboratory or Ministry of Health can provide several benefits:

- Large amounts of a single lot of medium or reagent can be purchased and subsequently divided into smaller aliquots for distribution to provincial/district laboratories. This may be more cost effective because of, *e.g.*, discounts for larger orders, lower shipping costs, less waste because of product going past expiration date, etc.
- Quality control can be performed in the central laboratory, avoiding duplication of effort among provincial and district laboratories. An unsatisfactory medium or reagent can then be returned to the manufacturer before the lot is distributed to other laboratories.
- The standardization of methods among laboratories at all levels is facilitated by use of single lots of media.

Preparation of media and reagents

Each manufacturer's lot number of commercial dehydrated medium and each batch of medium prepared from individual ingredients should be quality controlled before use. Immediately after preparation, each medium should be tested, as appropriate, with a reference strain of *H. influenzae, N. meningitidis, S. pneumoniae, N. gonorrhoeae, S.* Typhi, *Shigella,* and/or *V. cholerae* O1/O139 for proper growth characteristics as described for each medium.

A record of all media preparation or purchase dates and quality control test results should be kept, and any unusual characteristic (*e.g.*, the color of the medium or slow growth of test bacteria) should be noted.

Many media call for the use of defibrinated blood. Defibrinated blood can be prepared mechanically by swirling 30 ml blood and (*e.g.*) sterile glass beads or a wooden-stick device in a 125–250 ml Erlenmeyer flask at approximately 90 rpm for 7–9 minutes. (Sterile paper clips in a flask can also serve to assist in defibrination.) Blood is defibrinated when clotting factors have been removed; they will be visible in the flask as a translucent, fibrous "web." (A useful reference for "low-technology" methods to defibrinate blood is in a publication by Kay *et al.* [1986], and is included in Appendix 15.)

Agar media should be dispensed into 15 x 100-mm or 15 x 150-mm Petri dishes to a uniform depth of 3–4 mm; approximately 20-ml of liquid agar medium will achieve this depth in a 15 x 100-mm plate. If agar is cooled to 50˚C prior to pouring, condensation is minimized. After pouring, the plates should be kept at

room temperature for several hours to prevent excess condensation from forming on the covers of the dishes. Another means by which condensation will be reduced is if plates are stacked so that they cool more slowly. Alternatively, if when preparing **selective** media (*e.g.*, MacConkey [MAC], xylose lysine desoxycholate [XLD], thiosulfate citrate bile salts [TCBS] agar, etc.), conditions are such that there is little chance that the cooling media will be contaminated, after the agar is poured into the plates, the lids can be placed on the dish so that a small opening is left to let the heat out, resulting in the formation of less condensation on the upper lid; the lid should remain slightly open like this for approximately 30 minutes, while the agar solidifies. If, however, it is likely that the agar will be contaminated if the lid is left partly open, the agar should be allowed to solidify with the lid closed.

> ***Note:*** Covering the agar while it is still hot will allow for the formation of a substantial amount of condensation on the upper lid. If the plates contain condensation, the plates should be covered at room temperature for 24 hours to allow the condensation to evaporate. After condensation has evaporated, the plates should be placed in an inverted position and stored in a plastic bag in an inverted position at 4°C.

Media for enrichment, identification, and antimicrobial susceptibility testing

Some of the culture media included in this manual are commonly referred to by their abbreviations rather than their full names. Therefore, when these media are mentioned in the methods for the preparation or quality control of other media in this section, they are referred to by their abbreviations.

Name of commonly abbreviated culture medium	Abbreviation
Alkaline peptone water	APW
Bismuth sulfite agar	BS
Cystine trypticase agar	CTA
Desoxycholate citrate agar	DCA
Gram-negative broth	GN
Hektoen enteric agar	HE
Heart infusion agar	HIA
Haemophilus test medium	HTM
Kligler iron agar	KIA
Lysine iron agar	LIA
MacConkey agar	MAC
Martin-Lewis agar	ML
Modified Thayer-Martin agar	MTM

Name of commonly abbreviated culture medium, *continued*	Abbreviation
Phosphate buffered saline	PBS
Selenite broth	SEL
Sulfide-indole-motility medium	SIM
Salmonella-Shigella agar	SS
Thiosulfate citrate bile salts sucrose agar	TCBS
Tryptone (Trypticase) soy agar	TSA
Tryptone (Trypticase) soy broth	TSB
Triple sugar iron agar	TSI
Xylose lysine desoxycholate agar	XLD

Methods for the preparation of media (from individual ingredients or commercially available preparations) follow, in alphabetical order.

Acidometric agar

This agar may be used to test *H. influenzae* for ß-lactamase if nitrocefin is not available.

Distilled water	100 ml
Agar	1.5 g
0.5% phenol red solution	0.2 ml
NaOH (1 Normal)	
Penicillin G powder (to prepare a 5000 unit/ml concentration)	

Combine distilled water, agar, and phenol red solution and boil until the agar dissolves. Adjust the pH of the solution with NaOH until the pH is in the range of 8.5 – 9.0. Dispense into 20-ml tubes and cool in a water bath to 50˚–55˚C. Add penicillin G powder (to yield a concentration of 5000 unit/ml) to the tube and vortex (or mix well). Re-check the pH for the proper range, pour the contents of the tube into a Petri plate and allow it to solidify.

Quality control:

- The acidometric agar surrounding ß-lactamase-positive colonies will be yellow in color after incubation for one hour at 35˚C.
- The acidometric agar surrounding ß-lactamase-negative colonies will exhibit no color change after incubation for one hour at 35˚C.

Alkaline peptone water (APW)

Alkaline peptone water (APW) can be used to enhance the recovery of *V. cholerae* when there are few organisms present. [***Note:*** There are several different published formulations for this medium.]

Peptone	10.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Add ingredients to the water and adjust to pH 8.5 with 3 M NaOH solution. Distribute into jars, bottles, or tubes, and autoclave at 121˚C for 15 minutes. Store at 4˚C for up to 6 months, making sure containers' caps are tightly closed to prevent a drop in pH or evaporation.

- ***Peptone water*** differs from APW in that it does not have added salt, nor is the pH adjusted to 8.5. Add 10 g of peptone to 1000 ml of distilled water; distribute, autoclave and store as previously described for APW.

Quality control: When inoculated into alkaline peptone water for quality control, *V. cholerae* O1 should show good growth at 6–8 hours.

Bismuth sulfite agar (BS)

Bismuth sulfite agar (BS), which is highly selective, is the preferred medium for isolation of *S.* Typhi. However, **bismuth sulfite agar should not be used for isolation of *S.* Typhi if it has been stored for more than 24–36 hours.** BS has been reported to inhibit *Salmonella* serotypes other than *S.* Typhi unless it is refrigerated at 4˚C for at least 24 hours before use. When culturing fecal specimens from suspected typhoid carriers, the use of a BS pour plate may enhance isolation.

Prepare according to manufacturer's instructions. [***Note:*** Several commercial brands of bismuth sulfite agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.] Heat to boiling to dissolve, but avoid overheating (*i.e.*, once dissolved, remove from heat). Do not autoclave.

For preparation of BS streak plates:

When cool enough to pour, dispense the BS medium into plates. Plates can be stored at 4˚C for up to 1 week if BS will not be used to isolate *S.* Typhi.

For preparation of BS pour plates:

For pour plates, the BS agar must be boiled and cooled to 50˚C in a water bath. A 5-ml quantity of fecal suspension is added to a Petri plate, after which approximately 20 ml of cooled BS is immediately poured into the plate. The plate is swirled to mix the fecal suspension and the BS agar, and the plate is left to harden.

Quality control: The following organisms should be adequate for quality control of BS agar:

- *S.* Typhi should produce excellent growth of black colonies with a metallic sheen;

- *E. coli* should grow poorly, if at all, and will appear as brown to green colonies.

Blood agar: TSA with 5% sheep blood

Sheep blood agar is used as a general blood agar medium, and consists of TSA plus 5% sheep's blood. The sheep blood agar plate should appear a bright red color. If the plates appear dark red, the blood has been added when the agar was too hot; if this happens, the medium should be discarded and a new batch prepared.

a) Prepare TSA according to the instructions given on the label of the dehydrated powder. For convenience, 500 ml of molten agar can be prepared in a l-liter flask. Add 20 g of agar into 500 ml of water. Heat to dissolve.

b) Autoclave at 121˚C for 20 minutes. Cool to 60˚C.

c) Add 5% sterile, defibrinated sheep blood (*i.e.*, add 25 ml sheep blood to 500 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).

d) Dispense 20 ml into 15 x 100-mm Petri dishes. Allow the medium to solidify and dry out, place in a plastic bag, and store at 4˚C.

Quality control: Test each new, freshly prepared or purchased batch of blood agar plates for growth and hemolytic reaction with a strain of *S. pneumoniae*. The colonies are small and should appear grey to grey-green surrounded by a distinct greenish halo in the agar (Figure 56).

Blood culture broth

Blood culture broth contains TSB and sodium polyanetholesulfonate (SPS).

a) Follow the instructions of the manufacturer on the label of each bottle of dehydrated trypticase soy broth.

b) Add 0.25 g SPS per liter of medium. SPS is especially important for recovery of *H. influenzae* since it prevents the inoculated blood from clotting.

c) Dispense in 20-ml (pediatric blood culture bottle) and 50-ml (adult blood culture bottle) amounts into suitable containers (*e.g.*, tubes or bottles) with screw-caps with rubber diaphragms. The amount of liquid in the containers should comprise at least two-thirds of the total volume of the container.

d) Sterilize by autoclaving at 121˚C for 15 minutes. Cool and store medium at room temperature.

Quality control: Each new batch of freshly prepared or purchased blood culture medium should be tested for supporting the growth of a variety of pathogens

including, *e.g.*, *H. influenzae, N. meningitidis, S. pneumoniae,* and *S.* Typhi. Add 1–3 ml of sterile rabbit, horse, or human blood to three bottles of freshly prepared blood culture medium. A fresh culture of each of the three bacteria should be inoculated into separate blood culture bottles. Make a dilute suspension of growth from an agar plate in broth by collecting a loopful of the growth and suspending it in 1–2 ml of broth. Inoculate this suspension into the blood culture broth to be tested. Incubate the broths at 35˚C for 7 days; observe for growth and subculture at 14 hours and 48 hours. All four bacteria mentioned above should be recovered on subculture after 14 and 48 hours.

Chocolate agar with TSA base and growth supplement

Chocolate agar with growth supplements is a medium that supports the special growth requirements needed for the isolation of fastidious organisms (when incubated in a 5% CO_2 atmosphere). Chocolate agar contains a reduced concentration of agar, which increases the moisture content of the medium. **Supplemented chocolate agar should support the growth of *H. influenzae.*** Chocolate agar slants for transport and short-term storage can be prepared in the same manner as that described for agar plates, except that the medium is dispensed in 16 x 125-mm screw-cap tubes and slanted before solidifying.

a) Use TSA as the basal medium. Prepare double strength (*i.e.*, 20 g in 250 ml distilled water). Autoclave, and then cool to 50˚C. Use the thermometer to verify the cooling temperature.

b) Prepare a solution of 2% hemoglobin (*i.e.*, 5 g in 250 ml distilled water). Mix the hemoglobin in 5–6 ml of the distilled water to form a smooth paste. Continue mixing as the rest of the water is added. Autoclave, and cool to 50˚C.

c) Add the hemoglobin solution to the double-strength TSA and continue to hold at 50˚C.
   - ***Alternative to steps a-c:*** If a hemoglobin solution is unavailable, an alternative is to add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (*i.e.*, 5 ml blood per 100-ml agar) to full-strength TSA (*i.e.*, 20 g in 500 ml distilled water). **DO NOT use human blood.** After the base medium has been autoclaved and cooled to 50˚C, add the blood and place in a hot water bath at no more than 80˚C for 15 minutes or until a chocolate color is achieved. Then cool to 50˚C.

d) After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50˚C, add growth supplement (*e.g.*, IsoVitaleX or Vitox) to a final concentration of 1%. Mix the ingredients by gently swirling the flask; avoid forming bubbles.

e) Dispense 15–20 ml in each 15 x 100-mm Petri dish.

Quality control: All freshly prepared or purchased chocolate agar media should be tested to determine the medium's capacity to support growth of the bacteria to be isolated, particularly *H. influenzae*. If the medium does not support the growth of one or all of the bacteria, the medium should be discarded, and a new batch of medium should be prepared or purchased.

- Chocolate agar should look brown to brownish-red in color. *N. meningitidis* and *H. influenzae* should appear as a greyish, almost translucent film on the slant's surface with no discoloring of the medium after 24 hours of incubation; *S. pneumoniae* should appear as small grey to grey-green colonies with a very distinct greenish discoloring of the medium (see Figures 61 and 62). **If *H. influenzae* does not grow, the growth supplement (IsoVitaleX or its equivalent) may have been inadvertently omitted.**

Chocolate agar with bacitracin

This medium is used for primary isolation of *H. influenzae* from respiratory sources. **Bacitracin-chocolate agar should not be used for subcultures.**

A stock solution of bacitracin can be made by suspending 3 g of bacitracin in 20 ml of distilled water. The solution should be sterilized by filtration. Dispense in l-ml amounts and store in a -20˚C to -70˚C freezer.

a) Prepare chocolate agar suspension according to the instructions for the preparation of chocolate agar outlined above (in steps a–d).

b) After adding the hemoglobin and growth supplement to the TSA base medium, cool the medium to 50˚C, and add 1.0 ml of stock solution of bacitracin per 500 ml of chocolate agar.

c) Dispense 15–20 ml in each 15 x 100-mm Petri dish (so that agar is a uniform 3–4 mm); this volume makes 25 to 30 bacitracin-chocolate agar plates.

d) After quality control testing, the plates should be placed in plastic bags and stored at 4˚C.

The final concentration of bacitracin in the chocolate agar is 300 µg/ml. The bacitracin does not change the color of the medium, and it should appear brown like standard supplemented chocolate agar (similar to the medium shown in Figure 58).

Quality control: For quality control of bacitracin-chocolate agar, a strain of *H. influenzae* (*e.g.*, ATCC 49247) should be tested for proper growth characteristics.

Cystine trypticase agar (CTA) with 1% carbohydrate

CTA medium is a semi-solid medium presented in this manual for the biochemical testing of *N. meningitidis* with glucose, maltose, lactose, and sucrose. It can be purchased as a ready-made medium or prepared from dehydrated media with the

addition of sugars. (Follow the manufacturer's instructions when using dehydrated medium.)

a) Suspend 28.0 g of cystine trypticase agar medium 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.

b) Autoclave the flask at 121˚C for 15 minutes. Cool to 50˚C.

c) Prepare a 10% glucose solution using 10 g of glucose in 100 ml of distilled water. Filter-sterilize the solution using a 0.22-micron filter.

d) Aseptically add this entire solution (100 ml of 10% glucose solution) to the 900 ml of the CTA medium to obtain a final 1% concentration of the glucose.

e) Dispense 7 ml into each 16 x 125-mm glass screw-capped tube.

f) Store at 4˚C.

g) Repeat this procedure for the remaining three carbohydrates (in place of glucose in step c): maltose, lactose, and sucrose.

Quality control: Testing of CTA agar with a reference strain of *N. meningitidis* will yield an acid reaction (indicated by a color change from red to yellow) in glucose and maltose (but not in lactose or sucrose). Reactions typical of other organisms are included in Table 3. It should be noted that because *N. gonorrhoeae* typically has a weak glucose reaction difficult to detect in CTA media, this laboratory manual suggests using a rapid acid-detection test (in place of CTA) for presumptive *N. gonorrhoeae.*

Desoxycholate citrate agar (DCA)

Desoxycholate citrate agar (DCA) is a differential selective plating medium for the isolation of enteric pathogens, particularly *Shigella* and *Salmonella.* Lactose-fermenting organisms produce pink colonies surrounded by a zone of bile precipitation. Colonies of lactose-nonfermenting strains are colorless. Several formulations of DCA, which may vary in selectivity, are available from different manufacturers.

Prepare according to manufacturer's instructions. [***Note:*** DCA may also be prepared from individual ingredients, but doing so can result in much greater lot-to-lot variation than when prepared from commercial dehydrated preparations.] DCA medium is very heat-sensitive, and overheating during boiling should be avoided; **do not autoclave DCA medium.** Plates can be stored at 4˚C for up to a week.

Quality control: For quality control of DCA, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *E. coli* may be somewhat inhibited, depending on the particular formulation used, but will produce pink colonies surrounded by a zone of precipitated bile; and,
- *S. flexneri* and *S. dysenteriae* 1 will produce fair-to-good growth of colorless colonies.

Formalinized physiological saline

(Refer to "Physiological saline," listed later in this Appendix.)

GC-Chocolate agar (gonococcus agar medium [GC] base plus hemoglobin and 1% defined growth supplement)

GC-chocolate agar is a nonselective medium used to grow pure cultures of *Neisseria gonorrhoeae*. Although chocolate agar is often made with fresh sheep blood, it is **not** recommended that fresh blood products be used in gonococcal medium because they vary in their ability to support the growth of *N. gonorrhoeae*. Hemoglobin powder must therefore be used to prepare a standardized GC-chocolate agar medium.

The following methods allow for the production of 100 ml of medium (five 15 x 100-mm diameter plates); adjust quantities proportionately for the production of larger volumes of media. It is suggested that laboratorians should prepare no more than 500 ml of media per individual container, because it is difficult to properly mix larger amounts of media.

a) Suspend 7.2 g of GC agar base in 100 ml of distilled water. Mix thoroughly, heat with frequent agitation, bring to a boil, and gently swirl to completely suspend the powder (approximately 1 minute). Autoclave the medium at 121˚ C for 15 minutes. Cool in a water bath set to 50˚ C.

b) Add 2 g of soluble hemoglobin powder to 5–10 ml of warm distilled water in a screw-cap bottle. Gradually add warm distilled water (to a total volume of 100 ml) and gently agitate the bottle until a smooth suspension is achieved. (This process is made easier if the solution can be stirred using a magnetic stirrer.) Autoclave the hemoglobin solution at 121˚C for 15 minutes. Cool in a water bath set to 50˚C.
   - If ready-made sterile hemoglobin solution is available, instead of following the methods described here in step b, use 100 ml of ready-made 2% sterile hemoglobin solution warmed in a water bath set at 50˚C.

c) Reconstitute the growth supplement (*e.g.*, IsoVitaleX).

d) Aseptically open the vial containing the lyophilized growth supplement.

e) Use a sterile needle and syringe to aseptically transfer 10 ml of the accompanying diluent to the vial.

f) Shake to ensure complete solution. After reconstitution, use growth supplement immediately, or store at 4˚C and use within 2 weeks.

g) Aseptically add 100 ml sterile hemoglobin solution and 2 ml of reconstituted growth supplement to 100 ml of GC agar base medium. Mix gently, but thoroughly, to avoid the formation of air bubbles in the agar (*i.e.*, foam). Holding the bottle in an upright position, bottle neck in the hand, and gently swirling it three times in one direction (*e.g.*, clockwise) and then three times in the other direction (*e.g.*, counter-clockwise) is a good, gentle mixing technique.

h) Dispense 15–20 ml volumes of medium into each sterile 15 x 100-mm Petri dish to achieve a uniform depth of 3–4 mm in the plate.

i) Replace the lid on the plate, and allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4˚C.

Quality control: Inoculated GC-chocolate agar should support the growth of *N. gonorrhoeae* (*e.g.*, the fastidious strain 14AHU [available from CDC, see Appendix 14] as well as ATCC 49226) after incubation at 35˚–36.5˚C in a 5% CO_2, humid atmosphere for 18–24 hours. QC strains may be inoculated onto either whole- or half-plates.

GC- susceptibility test medium (GC-agar base medium plus 1% defined growth supplement)

Mueller-Hinton agar does not support growth of *N. gonorrhoeae,* and therefore must not be used for antimicrobial susceptibility testing of this organism. **Antimicrobial susceptibility testing of *N. gonorrhoeae* is performed on a GC-susceptibility test medium,** a simple nonselective medium of GC agar base plus 1% defined supplement (*e.g.*,IsoVitaleX). GC-susceptibility test medium does not contain hemoglobin (*i.e.*, it is not a chocolate medium), unlike other GC test media.

GC-susceptibility test medium is similar to but simpler than the standard GC-chocolate medium; it is prepared in the same manner with the exception of the exclusion of hemoglobin from the GC-susceptibility testing medium. The methods described here allow for the production of 100 ml of medium (five 15 x 100-mm diameter plates); adjust quantities proportionately for the production of larger volumes of media. If a large number of plates is required, prepare no more than 500 ml of media per individual container (because it is difficult to mix larger quantities of the ingredients properly).

a) Suspend 7.2 g of GC agar base in 100 ml of distilled water. Mix thoroughly, heat with frequent agitation, bring to a boil, and gently swirl to completely suspend the powder (approximately 1 minute). Autoclave the medium at 121˚ C for 15 minutes. Cool in a water bath set to 50˚ C.

b) Reconstitute the growth supplement (*e.g.*, IsoVitaleX).

   1) Aseptically open the vial containing the lyophilized growth supplement.

   2) Use a sterile needle and syringe to aseptically transfer 10 ml of the accompanying diluent to the vial.
   3) Shake to assure complete solution. After reconstitution, use growth supplement immediately, or store at 4˚C and use within 2 weeks.

c) Aseptically add 2 ml of reconstituted growth supplement to 100 ml of GC agar base medium. Mix gently, but thoroughly, to avoid the formation of air bubbles in the agar (*i.e.*, foam). Holding the bottle in an upright position, bottle neck in the hand, and gently swirling it three times in one direction (*e.g.*, clockwise) and then three times in the other direction (*e.g.*, counter-clockwise) is a good, gentle mixing technique.

d) Dispense 15–20 ml volumes of medium into each sterile 15x100-mm Petri dish to achieve a uniform depth of 3–4 mm in the plate.

e) Replace the lid on the plate, and allow the medium to stay at room temperature for several hours. Place plates in a plastic bag and store at 4˚C.

Quality control: Each new lot of GC-susceptibility test medium should be quality controlled by testing both the NCCLS-recommended *N. gonorrhoeae* strain (ATCC 49226) and the other reference strains to be used when performing antimicrobial susceptibility testing of *N. gonorrhoeae*. Appropriate ranges of antimicrobial susceptibility test values for these QC strains are listed in Tables 9 and 10.

Gonococcal selective media

Gonococcal selective media support adequate growth of *N. gonorrhoeae* from clinical specimens while inhibiting commensal species and fungi. A variety of media share the same supplemented GC-chocolate base and antibacterial agents (vancomycin and colistin), and vary by antifungal agents (*e.g.*, anisomycin or nystatin) and additional antibacterial agents (*e.g.*, amphotericin B or trimethoprim lactate). *N. gonorrhoeae*, *N. meningitidis, N. lactamica,* and *K. denitrificans* are routinely able to grow on gonococcal selective media whereas strains of most commensal species cannot. Two commonly used selective gonococcal media mentioned in this manual are Modified Thayer-Martin and Martin-Lewis.

Modified Thayer-Martin (MTM) is GC-chocolate agar containing 3 µg/ml vancomycin, 7.5 µg/ml colistin, 12.5 units/ml nystatin and sometimes also 5 µg/ml trimethoprim lactate. Similarly, **Martin-Lewis (ML)** is GC-chocolate agar containing 4 µg/ml vancomycin, 7.5 µg/ml colistin, 20 µg/ml anisomycin and sometimes 5 µg/ml trimethoprim lactate. The antimicrobial/antifungal agents are commercially available as combined inhibitory supplements (*e.g.*, "VCA", "VCN", "VCNT", and GC-supplement).

Laboratories able to access inhibitory supplements can prepare their own gonococcal selective media by following the instructions included in this manual for the supplemented GC-chocolate base and following the manufacturer's instructions for the addition of the inhibitory supplement to the base medium. Alternatively, laboratories can purchase prepared gonococcal selective media from commercial suppliers.

Quality control: Gonococcal selective media should support the growth of *N. gonorrhoeae* (*e.g.*, ATCC 49226) after incubation at 35˚–36.5˚C in a 5% CO_2, humid atmosphere for 18–24 hours. *N. cinerea* is colistin-susceptible and should **not** grow on gonococcal selective media. Additional QC strains may be suggested on the manufacturer's package insert.

- ***Note:*** Because some gonococcal strains are vancomycin-sensitive, part of the quality assurance program should include comparing the rate of positive culture results with the rate of observation of gram-negative diplococci in urethral smears from the corresponding men with uncomplicated symptomatic gonorrhea. If a discrepancy is observed between these rates, laboratorians should culture the next (approximately) 50 specimens first on nonselective GC-chocolate agar and then on the selective medium. If cultures grow on the nonselective medium and exhibit poor growth or no growth on the selective medium, laboratorians should suspect that isolates are susceptible to vancomycin and consider sending strains to a reference lab for confirmation.

Gram-negative (GN) broth

Gram-negative (GN) broth is a selective / inhibitory medium for isolation of gram-negative organisms. It may be used for the enrichment of fecal specimens suspected to contain *Salmonella* serotype. Prepare GN broth according to manufacturer's instructions. [***Note:*** GN broth may also be prepared from individual ingredients, but doing so can result in much greater lot-to-lot variation than when prepared from commercial dehydrated preparations.]

Quality control: After overnight enrichment in GN broth, *S.* Typhi should produce good growth of colorless colonies on MacConkey agar.

Haemophilus test medium (HTM)

The unsupplemented Mueller-Hinton agar used for antimicrobial susceptibility testing of many bacteria included in this manual does not support growth of *H. influenzae* and therefore must not be used for the antimicrobial susceptibility testing of this organism. **Antimicrobial susceptibility testing of *H. influenzae* is performed on *Haemophilus* test medium (HTM).**

HTM can be prepared by supplementing thymidine-free Mueller-Hinton agar with 15 µg/ml ß-NAD (ß- nicotinamide adenine dinucleotide), 15 µg/ml bovine haematin and 5 mg/ml yeast extract. However, to decrease lot-to-lot variation, **it is suggested that laboratories prepare HTM from commercially available dehydrated preparations when possible** (or purchase ready-made plated media).

Prepare HTM according to manufacturer's instructions.

Quality control: Incubate HTM for 48 hours at 35°C, and then for 120 hours at room temperature to ensure purity in the closed medium container. Each new lot of HTM should also be quality controlled by testing the NCCLS-recommended *H. influenzae* strain (ATCC 49247). Appropriate ranges of antimicrobial susceptibility test values for this QC strain are listed in Table 2.

Heart infusion agar (HIA)

Heart infusion agar (HIA) is a general-purpose medium used with or without blood for isolating and cultivating a number of microorganisms. The medium should appear straw colored (a yellowish to gold coloring). HIA can also be used for determining the X- and V-factor requirements of *H. influenzae.*

a) Prepare the HIA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks. These media should be fully dissolved with no powder on the walls of the vessel before autoclaving; stirring over heat may help the powder dissolve more rapidly.

b) Autoclave at 121°C for 20 minutes.

c) Cool to 50°C and pour into 15 x 100-mm Petri dishes.

d) Allow medium to solidify and condensation to dry out before placing plates in plastic bags and storing at 4°C until used.

Quality control: Each freshly prepared or purchased batch of HIA should be quality control tested by determining the X and V requirements of *H. influenzae.* Inoculate a fresh plate of HIA with a control strain, such as *H. influenzae* ATCC 49247 (which should be readily available in laboratories performing antimicrobial susceptibility testing of *H. influenzae*); X, V, and XV disks should be placed on the inoculated plate identical to that shown in Figure 3. *H. influenzae* should grow only around the XV disk.

Heart infusion rabbit blood agar (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 blood agar plates. (Be sure to label the prepared medium carefully.) If the medium is dark red, discard and prepare a new batch. (Horse blood may be substituted for rabbit blood

in this medium; the preparation is exactly the same, with the exception of the blood source.)

a) Prepare the HIA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks and autoclave at 121˚C for 20 minutes. Cool to 50˚C in a water bath.

b) Add 5% sterile, defibrinated rabbit blood (5 ml/100 ml of medium) and dispense into 15x100-mm Petri dishes. Allow to solidify and dry for a few hours. Then, place in a plastic bag and store at 4˚C.

Quality control: A strain of *H. haemolyticus* should be used to quality control the proper growth and hemolytic reactions of the HIA-rabbit blood medium. *H. haemolyticus* should grow well and be surrounded by a distinct zone of complete hemolysis that appears as a clear halo surrounding the colonies.

Hektoen enteric agar (HE)

Hektoen enteric (HE) agar is a differential selective agar that is useful for isolation of *Salmonella* and *Shigella*. It has an H_2S-indicator system for selecting H_2S-producing *Salmonella,* which produce blue-green colonies with a black center. *Shigella* colonies are green wheras rapid lactose-fermenters (*e.g.*, *E. coli*) are pink to orange with a zone of bile precipitation.

Prepare HE according to manufacturer's instructions. [***Note:*** Several commercial brands of Hektoen enteric agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable when the medium is prepared in this manner than with a commercial dehydrated formulation.] Heat to boiling to dissolve, but avoid overheating (*i.e.*, remove from heat after the powder has dissolved); do not autoclave. When cool enough to pour, dispense the HE into plates. Plates can be stored at 4˚C for up to 1 week.

Quality control: For quality control of Hektoen enteric agar, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *E. coli* should produce colonies that are pink to orange surrounded by a bile precipitate; and,
- *S. flexneri* should produce fair to good growth of green colonies, but *S. dysenteriae* 1 colonies should be smaller.

Horse blood agar (blood agar base)

Horse blood agar is a highly nutritive medium that may be used for the primary isolation of *H. influenzae* and for the determination of the hemolysis with *H. haemolyticus* or other bacteria.

a) Prepare blood agar base according to the instructions on the label of the dehydrated medium. Oxoid number 2 base is best, but other blood agar bases may be substituted.

b) Autoclave at 121˚C for 15 minutes, and cool to 50˚C in a water bath.

c) Add horse blood (5 ml per 100 ml of medium).

d) Mix well, dispense in 15 x 100-mm Petri dishes. Allow to solidify and dry out excess moisture before placing in plastic bags and storing at 4˚C.

Quality control: The quality control testing of this medium is the same as that described for HIA-rabbit blood: a strain of *H. haemolyticus* should be used to quality control the proper growth and hemolytic reactions of horse blood agar medium. *H. haemolyticus* should grow well and be surrounded by a distinct zone of complete hemolysis that appears as a clear halo surrounding the colonies.

Kligler iron agar and triple sugar iron agar

Kligler iron agar (KIA) and triple sugar iron (TSI) agar are carbohydrate-containing screening media widely used for identification of enteric pathogens. Both media differentiate lactose fermenters from nonfermenters and have a hydrogen sulfide indicator. H_2S-producing organisms will cause blackening of the medium in both KIA and TSI.

KIA contains glucose and lactose. Organisms that ferment glucose cause the butt of the tube to become acid (yellow); some also produce gas. Lactose-fermenting organisms will produce an acid (yellow) slant; lactose-nonfermenting organisms will have an alkaline (red) slant.

TSI contains sucrose in addition to the ingredients in KIA. Organisms that ferment either lactose or sucrose will produce an acid (yellow) slant while organisms that ferment neither carbohydrate will have an alkaline (red) slant. As in KIA, in TSI glucose-fermenters produce an acid (yellow) reaction in the butt (sometimes with gas produced).

a) Prepare according to manufacturer's instructions. [***Note:*** There are several commercially available dehydrated formulations of KIA and TSI. These media can also be prepared from individual ingredients, but doing so may result in lot-to-lot variation.]

b) Dispense a quantity of medium in appropriate tubes with a sufficient volume to give a deep butt and a long slant (*e.g.*, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes).

c) Leave screw-caps loose, and autoclave the medium.

d) After autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is about 3.5-cm deep and the slant is about 2.5-cm long.

e) Tighten the screw-cap tops of the tubes and store at 4°C for up to 6 months.

Quality control: For quality control of KIA or TSI, the following organisms should be adequate for confirmation of biochemical response characteristics:

- *E. coli* should give an acid slant and butt, with the production of gas but no H_2S;
- *S. flexneri* should give an alkaline slant, acid butt, without production of gas or H_2S (as shown in Figure 38);
- an H_2S-producing *Salmonella* may be used to control this reaction, which would appear as blackening of the medium in a positive reaction.

Lysine iron agar (LIA)

Organisms that produce lysine decarboxylase in lysine iron agar (LIA) cause an alkaline reaction (purple color) in the butt of the medium and also on the slant (Figure 40). H_2S production is indicated by a blackening of the medium. Organisms lacking lysine decarboxylase (*e.g.*, *Shigella*) typically produce an alkaline slant (purple), an acid butt (yellow), no gas, and no H_2S (see Table 13). *Proteus* and *Providencia* species will often produce a red slant caused by deamination of the lysine. LIA must be prepared so that the volume of medium in the tube is sufficient to give a deep butt. LIA tubes must have a deep butt because the decarboxylation reaction occurs only in anaerobic conditions.

a) Prepare LIA medium according to manufacturer's instructions on the bottle. [***Note:*** There are several commercially available formulations of dehydrated LIA. LIA may also be prepared from individual ingredients, but doing so may result in lot-to-lot variation.]

b) Dispense a quantity of medium in appropriate containers such that the volume of medium is sufficient to give a deep butt and a long slant (*e.g.*, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes).

c) Leave the screw-caps loose), and autoclave the medium.

d) After autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is approximately 3.5-cm deep and the slant is approximately 2.5-cm long.

e) Tighten the screw-top caps and store at 4°C for up to 6 months.

Quality control: For quality control of LIA, the following organisms should be adequate to confirm the biochemical response properties of the medium:

- *S. flexneri* should produce an alkaline slant and an acid butt without production of H_2S;

- an H_2S-producing *Salmonella* strain may be used to control the H_2S reaction. *Salmonella* strains will most likely be lysine-positive and give an alkaline reaction in the butt of the tube.

MacConkey agar (MAC)

MacConkey agar (MAC) is a differential plating medium recommended for use in the isolation and differentiation of lactose-nonfermenting, gram-negative enteric bacteria from lactose-fermenting organisms. Colonies of *Shigella* on MAC appear as convex, colorless colonies about 2–3 mm in diameter. *S. dysenteriae* 1 colonies may be smaller. *S.* Typhi colonies are flat, colorless and usually 2–3 mm in diameter.

Several commercial brands of MAC are available. Most manufacturers prepare several formulations of MAC, which may vary in selectivity and thereby affect the isolation of *Shigella*. For example, some formulations of MAC do not contain crystal violet, a selective agent; these types are not as selective and should not be used for isolation of *Shigella*. Oxoid MacConkey Agar No. 3, Difco Bacto MacConkey Agar, and BBL MacConkey Agar are all suitable.

a) Prepare MAC according to manufacturer's instructions. [***Note:*** MAC can also be prepared from individual ingredients, but this produces more lot-to-lot variation than preparation of a commercially available dehydrated formulation.]

b) Sterilize the medium by autoclaving at 121˚C for 15 minutes.

c) Cool to 50˚C and pour into Petri plates (to a uniform depth of 3–4 mm).

d) Leave lids ajar for about 20 minutes so that the surface of the agar will dry. Close lids and store at 4˚C for up to 1 month. If plates are to be stored for more than a few days, put them in a sealed plastic bag to prevent drying.

Quality control: For quality control of MAC, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *E. coli* should produce pink to red colonies with good to excellent growth; and,
- *S. flexneri* should produce colorless colonies with fair to good growth, but *S. dysenteriae* 1 colonies may be smaller.

Martin-Lewis (ML) agar medium

(Refer to "Gonococcal Selective Media," listed earlier in this Appendix.)

Modified Thayer-Martin (MTM) agar medium

(Refer to "Gonococcal Selective Media," listed earlier in this Appendix.)

Motility medium

Because *Shigella* are always nonmotile, motility medium is a useful biochemical screening test. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 39). Nonmotile organisms do not grow out from the line of inoculation.

a) Follow manufacturer's instructions to weigh out and suspend the dehydrated medium. [***Note:*** Several commercial dehydrated formulations of motility agar are available. This medium can also be prepared from individual ingredients, but this results in more lot-to-lot variation than commercial preparations.]

b) Heat to boiling to make sure medium is completely dissolved.

c) Dispense into tubes with screw-caps (or other types of containers), leaving the caps loose, and sterilize at 121˚C for 15 minutes.

d) Allow the medium to solidify upright, forming a deep butt with no slant (*e.g.*, about 4–5 ml of medium per 13 x 100-mm screw-cap tube). When the medium is solidified and cooled, leave caps loose until the surface of the medium has dried.

e) Tighten caps and store at 4˚C for up to 6 months.

Quality control: For quality control of motility medium, the following organisms should be adequate:

- *E. coli* is motile;
- *Shigella* spp. are nonmotile.

The surface of the medium should be dry when used. If moisture has accumulated in the tube, carefully pour it out before the tube is inoculated. Moisture can cause a nonmotile organism to grow down the sides of the agar, creating a haze of growth and making it appear to be motile.

Mueller-Hinton agar

Mueller-Hinton agar is the NCCLS-recommended medium used for standardized antimicrobial susceptibility testing of certain bacteria; the organisms in this document for which it is appropriate to use this formulation of Mueller-Hinton medium (*i.e.*, unsupplemented Mueller-Hinton) are *S.* Typhi, *Shigella* spp., and *V. cholerae.*

[***Note:*** Several formulations of Mueller-Hinton agar are commercially available. This laboratory manual suggests that Mueller-Hinton agar medium should not be prepared from individual ingredients because this can diminish the quality. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

a) Follow manufacturer's instructions to prepare medium.

b) After autoclaving, cool medium to 50°C in a water bath.

c) Measure 60–70 ml of medium per plate into 15 x 150-mm plates, or measure 25–30 ml per plate into 15 x 100-mm plates. Agar should be poured into flat-bottom glass or plastic Petri dishes on a level pouring surface to a **uniform depth of 3–4 mm.** Using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.

d) Freshly prepared plates may be used the same day or stored in a refrigerator (at 2°–8°C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be placed in an incubator (35°–37°C) until the moisture evaporates (usually 10–30 min). **Do not leave lids ajar because the medium is easily contaminated.**

Quality control: Each new lot of Mueller-Hinton agar should be quality controlled before use by testing the *E. coli* ATCC 25922 standard strain for antimicrobial susceptibility testing. (This formulation of Mueller-Hinton agar can also be used for testing of gram-positive aerobes, in which case *S. aureus* ATCC 25923 can be used as a quality control strain.) **The pH of each new lot of Mueller-Hinton should be between 7.2 and 7.4; if the pH is outside this range,** the pH of the medium should not be adjusted by the addition of acid or base, *i.e.*, **the batch of Mueller-Hinton plates should be discarded and a new batch of plates prepared.** If the pH for every batch is too high or low, the entire lot of dehydrated medium may have to be returned to the manufacturer as unsatisfactory. Inhibition zone sizes / minimal inhibitory concentration (MIC) values for quality control are included in the antimicrobial susceptibility testing section of each pathogen-specific chapter.

Mueller-Hinton agar plus 5% sheep (or horse) blood

Mueller-Hinton agar plus 5% sheep (or horse) blood is the NCCLS-recommended medium used for standardized antimicrobial susceptibility testing of certain bacteria; the organisms in this document for which it is appropriate to use this formulation of Mueller-Hinton medium supplemented with sheep (or horse) blood are *S. pneumoniae* and *N. meningitidis.*

[***Note:*** Several commercial formulations of Mueller-Hinton agar are available. This medium should not be prepared from individual ingredients because this can diminish the quality and result in increased lot-to-lot variation. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

a) Follow manufacturer's instructions to prepare medium.

b) After autoclaving, cool medium to 50˚C in a water bath.

c) Add 5% sterile, defibrinated sheep (or horse) blood, *i.e.*, 50 ml of blood per liter of medium. (If a different volume of base medium is prepared, the amount of blood must be adjusted accordingly to 5%, *e.g.*, 25 ml of blood would be added to 500 ml of base medium.)

d) Measure 60–70 ml of medium per plate into 15 x 150-mm plates, or measure 25–30 ml per plate into 15 x 100-mm plates. Agar should be poured into flat-bottom glass or plastic Petri dishes on a level pouring surface to a uniform depth of 3–4 mm. Using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.

e) Freshly prepared plates may be used the same day or stored in a refrigerator (2˚–8˚C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be placed in an incubator (35˚–37˚C) until the moisture evaporates (usually 10–30 minutes). **Do not leave lids ajar because the medium is easily contaminated.**

Quality control: Each new lot of Mueller-Hinton plus sheep blood agar (or horse blood, if preparing Mueller-Hinton for antimicrobial susceptibility testing of *S. pneumoniae* with trimethoprim-sulfamethoxazole [cotrimoxazole]) should be quality controlled before use by testing the *S. pneumoniae* standard strain (for quality control of *S. pneumoniae* and *N. meningitidis*). **The pH of each new lot of Mueller-Hinton should be between 7.2 and 7.4; if outside this range,** the pH of the medium should not be adjusted by the addition of acid or base; **the batch of plates should be discarded and a new batch of plates prepared.** If the pH for every batch is too high or low, the entire lot of dehydrated medium may have to be returned to the manufacturer as unsatisfactory. Inhibition zone sizes and MIC values for quality control are included in the antimicrobial susceptibility testing section of each pathogen-chapter.

Phosphate buffered saline (PBS)

The formula for this medium is:

Sodium dihydrogen phosphate	7.0 g
Disodium hydrogen phosphate	7.0 g
Distilled water	1000.0 ml

To prepare 0.1 M PBS, pH 7.2: Dissolve ingredients in distilled water. Adjust pH to 7.2 with 1 N acid or base. Dispense buffer in 500-ml bottles, and autoclave at 121˚C for 15 minutes. Label bottles with the reagent name, date prepared, and the expiration date.

PBS has a shelf-life of one year if stored at room temperature (25˚C).

Physiological saline

(*0.85% saline, also referred to as "Physiologic" saline or "Normal" saline*)

Physiological saline is used in many different microbiological techniques. The formula for this saline is:

NaCl	8.5 g
Distilled water	1 liter

Dissolve the NaCl in water, heating if necessary. Physiological saline may be sterilized by autoclaving or membrane filtration. Store physiological saline at ambient temperature for up to 6 months with caps tightened to prevent evaporation.

Formalinized physiological saline is physiological saline with the addition of formalin (formaldehyde). Follow the instructions above for preparation of physiological saline, and **after autoclaving** add 5 ml of 36% – 38% formaldehyde solution. Do not autoclave after the addition of formaldehyde to the saline.

Polysaccharide medium

Polysaccharide medium is used to detect the production of polysaccharide from sucrose, and consists of TSA with 1% sucrose. The medium is included in this laboratory manual to assist in the identification of *N. gonorrhoeae,* which has a negative reaction. The formula for this medium is:

Tryptone soy agar (TSA)	40 grams
Distilled water (endotoxin-free; ETF)	1000.0 ml
Reagent-grade sucrose	(10% solution, preferably in distilled water)

If reagent-grade sucrose is not available, white table sugar may be an acceptable substitute, but brown sugar is not appropriate for preparation of this medium.

a) Suspend TSA in the distilled, ETF water.

b) Autoclave at 121˚C for 15 minutes. Cool to 50˚C.

c) Prepare a 10% sucrose solution using the reagent-grade sucrose, and filter-sterilize it using a 0.45-micron filter.

d) Aseptically add sucrose solution to agar to give a final concentration of 1% (wt/vol).

e) Dispense 20–25 ml volumes in 100-mm Petri dishes.

Store medium refrigerated (at 4˚–10˚C) until used. Pre-warm the medium to room temperature prior to inoculation.

Quality control: For quality control of polysaccharide medium, the following organisms may be used:

- *N. gonorrhoeae* and *N. meningitidis* are two examples of organisms that do not produce polysaccharide from sucrose, and therefore exhibit a negative reaction (no color change) with the addition of Gram's iodine to the incubated inoculated medium.
- *N. polysaccharea* and *N. mucosa* are two examples of polysaccharide-positive organisms, and will exhibit a color change to dark brown to blue-black with the addition of Gram's iodine to the incubated inoculated medium.

Salmonella-Shigella agar (SS agar)

SS agar is a highly selective medium for isolation of *Salmonella* and *Shigella*, although **it should not be used for isolation of *Shigella dysenteriae* type 1 because some strains are inhibited.** *S.* Typhi, which is lactose-negative, produces smooth, colorless, transparent or translucent colonies that may or may not have black centers indicating production of H_2S. Lactose-positive colonies are pink surrounded by a zone of bile precipitation.

Quality control: To quality control SS agar, *Salmonella* should produce good growth of colorless colonies that may have black centers, whereas *E. coli* should grow poorly and appear as pink colonies.

Selenite broth (SEL)

Selenite broth (SEL) is a frequently used as an enrichment broth for *Salmonella,* including *S.* Typhi. It may be advantageous for a laboratory to use SEL because it can also be used for enrichment for *Shigella.* SEL should only be incubated for 14–16 hours at 35˚–37˚C. After incubation, selenite broth should be streaked to selective agar (*e.g.*, HE or XLD).

Quality control: After overnight enrichment in SEL, *Salmonella* spp. typically produce good to excellent growth when streaked on MacConkey agar.

Sulfide-indole-motility medium (SIM)

Sulfide-indole-motility medium (SIM) is a commercially available combination medium that combines three tests in a single tube: hydrogen sulfide (H_2S) production, indole production, and motility. The indole reaction is not useful for screening suspected *Shigella* isolates because strains vary in their reactions in this test. SIM is inoculated in the same way as motility agar (*i.e.*, by using a needle to stab about 1–2 cm down into the medium, and is incubated overnight at 35˚–37˚C). The motility reaction in SIM is read the same as for motility medium. As in Kligler iron agar or triple sugar iron agar, H_2S production is indicated by blackening of the medium. Indole production can be tested by either the filter paper method or by adding Kovac's reagent to the tube.

a) Follow manufacturer's instructions to weigh out and suspend dehydrated SIM medium.

b) Heat to boiling to make sure the medium is completely dissolved.

c) Dispense into tubes and sterilize by autoclaving for 15 minutes at 121˚C.

Quality control: For quality control of SIM medium, the following organisms may be used:

- *E. coli* is indole positive, H_2S-negative, and motility positive;
- an H_2S-producing *Salmonella* strain may be used to control the H_2S reaction and will most likely be motile and indole negative;
- *Shigella* are motility negative and H_2S-negative but are variable for the indole reaction.

Thiosulfate citrate bile salts sucrose agar (TCBS)

TCBS is a selective medium used to isolate *V. cholerae* from fecal specimens.

a) Follow manufacturer's instructions to weigh out and suspend the dehydrated medium. [***Note:*** Several commercial brands of thiosulfate citrate bile salts sucrose agar (TCBS) agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.]

b) Heat with agitation, until the medium is completely dissolved.

c) Cool agar in a water-bath until cool enough to pour (50˚–55˚C).

d) Pour into Petri plates, leaving lids ajar about 20 minutes so that the surface of the agar will dry. Close lids and store at 4˚C for up to 1 week.

Quality control: Each new lot should be quality controlled before use because TCBS is subject to lot-to-lot and brand-to-brand variations in selectivity.

- *V. cholerae* O1 should show good growth of yellow colonies; and,
- *E. coli* should have none to poor growth of translucent colonies.

Todd-Hewitt broth

Todd-Hewitt broth is used (in the context of this laboratory manual) to incubate *S. pneumoniae* before re-testing when a Quellung reaction is not observed with growth in cell suspension from a blood agar plate. It is suggested that laboratories use a commercially available dehydrated formulation to prepare Todd-Hewitt broth, when possible.

a) Prepare the Todd-Hewitt broth according to the instructions on the label of the dehydrated medium.

b) Dispense 1 ml into 15 x 125-mm tubes, autoclave at 121˚C for 20 minutes, cool, and store at 4˚C.

Quality control: For quality control of Todd-Hewitt broth, inoculate a tube of medium with a loop of freshly growing strain of *S. pneumoniae;* incubate overnight at 35˚C; the broth should be turbid the next day. Subculture the broth onto a blood agar plate to test for proper growth characteristics of *S. pneumoniae.*

Triple sugar iron agar (TSI)

(Refer to "Kligler iron agar and triple sugar iron agar," listed earlier in this Appendix.)

Tryptone-based soy agar (TSA)

TSA is a general-purpose tryptone-based agar medium (also commonly referred to as Trypticase soy agar or Tryptic soy agar) used with or without blood for isolating and cultivating a number of microorganisms. The medium should appear straw colored (*i.e.*, a yellowish to gold coloring). TSA is also used for determining the X- and V-factor requirements of *H. influenzae* (as is HIA).

a) Prepare the TSA according to the instructions on the label of the dehydrated medium. Prepare the volume needed in flasks. The medium should be fully dissolved with no powder on the walls of the vessel before autoclaving; stirring over heat may help the powder dissolve more rapidly.

b) Autoclave at 121˚C for 20 minutes.

c) Cool to 50˚C and pour into 15x100-mm Petri dishes.

d) Allow medium to solidify and condensation to evaporate before placing plates in plastic bags and storing at 4˚C until they are used.

Quality control: Each freshly prepared or purchased batch of TSA should undergo quality control testing; follow instructions provided by the manufacturer.

- In general, *E. coli* is an organism that should show good growth on TSA.
- If a laboratory is using TSA to test suspect *H. influenzae* for growth factor requirements, it is suggested that the quality control include testing of a known *H. influenzae* isolate for X and V- factor requirements. To test the X and V requirements, inoculate a fresh plate of TSA with a control strain of *H. influenzae* (*e.g.*, ATCC 49247, which should be in stock for laboratories performing antimicrobial susceptibility testing): X, V, and XV disks should be placed on the inoculated plate identical to that shown in Figure 3. *H. influenzae* should grow only around the XV disk.

Tryptone soy broth (TSB)

TSB (also commonly referred to as Trypticase soy broth or Tryptic soy broth) is used for making suspensions of *H. influenzae* prior to testing for X- and V- factor requirements. (HIA, sterile saline or phosphate-buffered saline [PBS] may be substituted for TSB when making the *H. influenzae* suspension for X and V factor testing.)

a) Prepare the TSB according to the instructions on the label of the dehydrated medium.

b) Dispense 5 ml into 15 x 125-mm tubes, autoclave at 121˚ C for 20 minutes, cool, and store at 4˚ C.

Quality control: Inoculate a tube of medium with a loop of freshly growing strain of *S. pneumoniae;* incubate overnight at 35˚C; the broth should be turbid the next day. Use a blood agar plate to subculture the broth to test for proper growth characteristics of *S. pneumoniae.* [***Note:*** *H. influenzae* is not an appropriate organism for quality control of TSB because TSB lacks the X and V factors *H. influenzae* requires to grow.]

Tryptone soy sheep blood agar with gentamicin

Sheep blood agar with gentamicin is used for the primary isolation of *S. pneumoniae* from nasopharyngeal swabs.

Prepare a stock solution of gentamicin by adding 80 mg of gentamicin in 32 ml of distilled water; the stock solution contains 2.5 mg of gentamicin/ml. Sterilize by filtration and dispense in 1.0-ml amounts, store at -20˚C to -70˚C.

To prepare sheep blood agar with gentamicin:

a) Add 1 ml of the stock solution of gentamicin to 500 ml of molten agar (prepared according to manufacturer directions). Add the gentamicin at the same time the defibrinated sheep blood is added.

b) Place the plates in plastic bags and store them at 4˚C after they solidify. The agar should appear only slightly darker than the medium without gentamicin. If the agar is not bright red, discard and prepare a new batch.

Quality control: The quality control testing of freshly prepared or purchased gentamicin-blood medium is the same as for the (TSA-sheep) blood agar without gentamicin.

Urea medium

Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color (Figure 40). Urease-negative organisms do not change the color

of the medium, which is a pale yellowish-pink. *Shigella* are always urease-negative (Table 15).

a) Follow manufacturer's instructions for preparation of urea medium. [***Note:*** Several commercial brands of urea medium are available, some of which require the preparation of a sterile broth that is added to an autoclaved agar base. Some manufacturers have sterile prepared urea concentrate available for purchase.] Prepare urea agar base as directed on the bottle.

b) Sterilize at 121˚C for 15 min.

c) Cool to 50˚–55˚C, then add urea concentrate according to manufacturer's directions. (Before adding the urea to the agar base, make sure the agar base is cool since the urea is heat labile.)

d) Mix and distribute in sterile tubes. Slant the medium during cooling so that a deep butt is formed.

Quality control: For quality control of urea medium, the following organisms are adequate:

- *Proteus* species produce urease;
- *E. coli* is urease negative.

Xylose lysine desoxycholate agar (XLD)

Xylose lysine desoxycholate agar (XLD) is a selective differential medium suitable for isolation of *Shigella* and *Salmonella* from stool specimens. Differentiation of these two species from non-pathogenic bacteria is accomplished by xylose and lactose fermentation, lysine decarboxylation, and hydrogen sulfide production. *Shigella* colonies on XLD agar are transparent pink or red smooth colonies 1–2-mm in diameter (Figure 86). *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species (Figure 85). Coliforms appear yellow (Figure 87). *Salmonella* colonies are usually red with black centers but can also be yellow with black centers.

a) Prepare according to manufacturer's instructions. [***Note:*** Several commercial brands of XLD agar are available. This medium can also be prepared from individual ingredients, but results exhibit more lot-to-lot variation than a commercially available dehydrated formulation.]

b) Mix thoroughly.

c) Heat with agitation just until the medium boils. Do not overheat; overheating when boiling XLD or allowing the medium to cool too long may cause the medium to precipitate.

d) Cool flask under running water until just cool enough to pour; avoid cooling the medium too long.

e) Pour the XLD into Petri plates, leaving the lids ajar for about 20 minutes so that the surface of the agar will dry.

f) Plates can be stored at 4°C for up to a week.

Quality control: For quality control of XLD, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics:

- *S. flexneri* should produce fair to good growth of transparent pink or red smooth colonies that are 1–2 mm in diameter;
- *S. dysenteriae* 1 may produce very small transparent or red colonies;
- *E. coli* should produce poor to fair growth of yellow colonies.

Transport and storage media

Cary-Blair medium, Amies medium, and Stuarts medium

Prepare each of these transport media according to the manufacturer's instructions. [***Note:*** Several dehydrated formulations of Cary-Blair are commercially available; some require the addition of calcium chloride and some do not.] These media can also be prepared from individual ingredients; however, it is very difficult to make a well quality-controlled batch and so this manual recommends purchasing them from a manufacturer.

When the Cary-Blair medium is prepared, it should be dispensed into containers in sufficient volume so that swabs will be covered by at least 4 cm of medium. For example, 5- to 6-ml amounts may be dispensed into 13 x 100-mm screw cap tubes. With the caps loosened, sterilize the medium by steaming (**not** by autoclave) at 100°C for 15 minutes. Tighten the caps after sterilization, and store the medium at 15°–30°C.

These media are quite stable if stored in tightly sealed containers in a cool dark place so that the medium does not dry out. Each may be used for up to 1 year as long as no loss of volume, visible contamination (*e.g.,* foreign objects or bacterial growth), or color change is observed. Prepared Amies medium that has been stored for longer than 9 months, however, should be freshly steamed and the charcoal re-suspended before use.

Dorset egg medium

Dorset egg medium (DE) is a good choice for the long-term survival of *S. pneumoniae* isolates (up to 44 days), *H. influenzae* isolates (up to 21 days) and *N. meningitidis* isolates (up to 21 days) at room temperature. The formula for this medium includes physiologic (normal) saline and whole hen's eggs.

a) Combine sterile 0.85% (normal) saline solution with beaten whole hen's eggs in a 1:3 ratio.

b) Inspissate (*i.e.*, thicken) the mixture in an electric inspissator at 80°C for 60 minutes.

Greaves solution

Greaves solution can be used in the process of preparation of isolates for frozen storage, as described in Appendix XI of this manual. The formula for this medium is:

Albumin, bovine, fraction V	10.0 g
L-glutamic acid, sodium salt *(Fluka, Buchs, Switzerland, 49621)*	10.0 g
Glycerol	20.0 ml
Distilled water	200.0 ml

a) Mix all ingredients and let them dissolve for 2–3 hours.

b) Filter-sterilize the solution.

c) Transfer the solution to a sterile tube.

d) Incubate for 2 days at 35°–37°C (to control the sterility of the medium).

- If contamination is observed, discard the solution and prepare a new batch.

e) Store at 4°C.

JEMBEC ® Plates

Jembec® plates are commercially available kits containing a CO_2-generating system and a medium that will support the growth of gonococcus.

Skim-milk tryptone glucose glycerol (STGG) transport medium

STGG medium is used for transport (and sometime storage) of nasopharyngeal secretions on swabs. The formula for this medium is:

Skim milk powder (from grocery or, *e.g.*, Difco)	2 g
TSB (from, *e.g.*, Oxoid)	3 g
Glucose	0.5 g
Glycerol	10 ml
Distilled water	100 ml

a) Mix to dissolve all ingredients.

b) Dispense in 1.0 ml amounts in screw-capped 1.5-ml vials.

c) Loosen the screw-cap tops and autoclave for 10 minutes (at 15 pounds).

d) Tighten caps after autoclaving.

e) Store STGG frozen at -20°C or refrigerate until use.
   - Use STGG medium within 6 months of preparation.

Transgrow medium

Transgrow medium is a selective medium for the transport and isolation of *N. gonorrhoeae*. It should be prepared according to manufacturer's instructions. [***Note:*** Transgrow medium is a chocolate agar plus three antibiotics and may also be prepared from individual ingredients, but doing so can result in much greater lot-to-lot variation than when the medium prepared from commercially available dehydrated preparations.]

Trans-Isolate (T-I) Medium

T-I medium is a biphasic medium that is useful for the primary culture of *N. meningitidis*, *S. pneumoniae,* and *H. influenzae* from cerebrospinal fluid (CSF) samples. It can be used as a growth medium as well as a holding and transport medium.

When preparing T-I medium, use glass serum bottles with flange-type, slotted rubber plugs and aluminum crimp seals. Any size serum bottle that has at least a 20-ml capacity can be used, provided that the combined volume of solid and liquid phases equals approximately one-half the capacity of the bottle.

T-I medium includes solid and liquid phases; 0.1 M MOPS buffer (*i.e.*, 3-(N-morpholino) propanesulfonic acid buffer) with a pH of 7.2 is used in the preparation of both the solid and liquid phases of T-I medium. NaOH can be used to adjust the pH, and distilled water should be used to create the appropriate volume of 0.1 M solution (approximately 21 g MOPS: 1000 ml distilled water).

a) **Solid phase:**

Activated charcoal	2.0 g
Soluble starch	2.5 g
Agar agar (*e.g.*, *from Difco*)	10.0 g
0.1 M MOPS buffer, pH 7.2	500 ml

1. Suspend the activated charcoal, the soluble starch and the agar agar in 500 ml of MOPS buffer and add a magnetic bar to the flask.
2. Heat on a magnetic stirrer-heater to dissolve the starch and the agar.
3. With continued mechanical stirring to keep the charcoal in suspension, dispense 5.0 ml to each 20-ml serum bottle.
4. Cap each bottle with a piece of aluminum foil and autoclave in metal baskets at 121°C for 20 minutes.
5. Remove from the autoclave and slant the baskets until the bottles cool, so that the apex of the agar reaches the shoulder of each bottle.

b) **Liquid phase:**

TSB	30.0 g
Gelatin (*e.g.*, from Difco)	10.0 g
MOPS buffer [0.1 M, pH 7.2]	500.0 ml

1. Heat the medium to dissolve the gelatin and avoid coagulation.
2. Autoclave at 121˚C for 15 minutes.
3. *Optional:* the addition of growth supplement (*e.g.*, IsoVitaleX or Vitox) to the liquid phase of T-I medium can help support the growth of *H. influenzae.*
   - To add growth supplement to the entire batch of liquid phase medium, use aseptic technique to add a total of 10 ml of the growth supplement to the cooled liquid medium.
   - To add growth supplement to individual bottles, add 0.1 ml of the supplement to the liquid phase contained in an individual T-I bottle (1% of the volume of both phases) or to a limited number of bottles, as needed.

c) **Addition of the liquid phase to the solid phase**

1. Dispense 5 ml of the broth aseptically into each of the bottles containing the solid-phase slants.
2. Seal with sterile rubber stoppers and aluminum caps.
   - Use a hand-crimping tool to fasten the aluminum caps if an automated system is not available.

T-I bottles can be stored and used for at least 2 years if tightly capped and stored at 4˚C. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature.

Before use, perform quality control of the T-I medium: check several uninoculated bottles for sterility at 35˚C. Inoculate several bottles with *N. meningitidis* and check their ability to support meningococcal growth at 35˚C.

Before inoculation, the bottles should be pre-warmed in the incubator (at 35˚–37˚C) or allowed to reach room temperature (*i.e.*, 25˚–30˚C).

Miscellaneous reagents

Gram stain (Hucker Modification) reagents

The Gram stain (Hucker modification) requires the use of two stains (*e.g.*, crystal violet and safranin or carbol-fuchsin), Gram's iodine, and a decolorizing agent (*e.g.*, ethyl alcohol). Individual reagents and Gram stain kits are available commercially from several laboratory supply sources. Alternatively, follow the

methods for preparation of the individual reagents (as presented in steps a–d below).

a) **Ammonium oxalate-crystal violet** contains two solutions (solution a and solution b).

Solution a

Crystal violet (certified)	2.0 g
Dissolve in 95% ethyl alcohol	20.0 ml

Solution b

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

1. Mix solutions a and b.
2. Let stand overnight.
3. Filter through coarse filter paper before use.

b) **Gram's iodine** must be protected from light.

Iodine (crystalline)	1.0 g
Potassium	2.0 g
Distilled water	300.0 ml

1. Combine the crystalline iodine, potassium, and distilled water to prepare an iodine solution.
   - Grinding the dry chemicals in a mortar with small additions of distilled water may be helpful in preparing the iodine solution.
2. Store the Gram's iodine solution in a dark bottle and protected from light so it does not degrade.

c) **Decolorizer** is commonly ethyl alcohol. (Some kits use acetone or an acetone-alcohol combination.)

95% ethyl alcohol

d) **Counterstain** is commonly either safranin or carbol-fuchsin. Ziehl-Nielsen carbol-fuchsin is considered by many to be a more effective counter-stain than carbol-fuchsin.

1. **Safranin**

Stock solution:

Safranin-O (certified)	2.5 g
95% ethyl alcohol	100.0 ml

Working solution:

Safranin stock solution	10.0 ml
Distilled water	90.0 ml

a. Prepare the stock safranin solution by combining the Safranin-O with the 95% ethyl alcohol.

b. Combine 10-ml of the stock solution with 90-ml of distilled water.

OR

2. **Ziehl-Nielsen carbol-fuchsin**

Basic fuchsin	0.3 g
95% ethyl alcohol	10.0 ml
Phenol crystals, melted	5.0 ml
Distilled water	95.0 ml

a. Dissolve fuchsin in alcohol.

b. Add the 5% phenol solution.

c. Let stand overnight.

d. Filter through coarse filter paper.

- This solution can be used as described or diluted 1:10.

When limited by resource availability, counterstain can be prepared as a 0.3–0.5 aqueous solution of the basic fuchsin.

Loeffler's methylene blue stain

Loeffler's methylene blue provides a simple staining method for visualize the shape of bacterial cells; it does not determine whether bacteria are gram-positive or gram-negative. If determining whether an organism is gram-positive or gram-negative is essential, smears must be stained by Gram's method (*i.e.*, using reagents as described in "Gram stain", earlier in this Appendix). Because of the characteristic shape and arrangement of cells in *Neisseria* species, the methylene blue stain may provide an inexpensive, rapid method for detecting diplococci. (This laboratory manual recommends use of Loeffler's methylene blue stain in place of Gram stain for staining of suspect *N. gonorrhoeae* from specimens or cultures of non-sterile sites, but not for staining of *N. meningitidis* from sterile-site specimens.)

To prepare the Loeffler's methylene blue stain, add the components in the order presented in the following two steps to first prepare saturated ethanolic methylene blue and then the staining solution.

a) **Saturated ethanolic methylene blue**

Methylene blue powder	1.0 g
Ethanol (95%)	100 ml

b) **Staining solution**

KOH (1% aqueous solution)	1 ml
Distilled water	99 ml
Ethanolic methylene blue solution (*step 1*)	30 ml

Loeffler's methylene blue reagent must be 'ripened by oxidation,' and the ripened reagent is called polychrome methylene blue. Normally, oxidation takes several months, but it can be hastened by aerating the reagent: Place the reagent in bottles filled no more than half-full and shake the bottle frequently.

Loeffler's methylene blue stain improves with age, and the shelf-life of this reagent is 5–10 years; thus, the reagent can be prepared in batches large enough to last for this time period.

Nitrate reduction test reagents

These media and reagents are used to perform the nitrate reduction test for the confirmation of an isolate as *N. gonorrhoeae*. The test is performed in a nitrate broth composed of heart infusion broth containing 0.2% potassium nitrate.

The formula for the nitrate reduction test medium is:

Heart infusion broth	25.0 g
Potassium nitrate	2.0 g
Distilled water	1000.0 ml

Reagents for developing the nitrate reduction tests are as follows:

Nitrate Reagent A (Sulfanilic acid solution): 0.8% in 5 N acetic acid*

4-aminobenzene sulfonic acid	0.5 g
Acetic acid, glacial	20 ml
Distilled water	100 ml

1) Dissolve 0.5 g of 4-aminobenzene sulfonic acid in 30 ml of glacial acetic acid.

2) Add 100 ml of distilled water and filter.

Store Nitrate Reagent A at room temperature (15°–30°C) in the dark. Reagents may be stored in dark brown glass bottles or in clear bottles wrapped in aluminum foil to ensure darkness. **Nitrate Reagent A is stable for one month.**

Nitrate Reagent B (alpha-naphthylamine solution): 0.6% in 5 N acetic acid*

N,N-dimethyl-1 naphthylamine	0.1 g
Distilled water, boiling	100 ml
Acetic acid, glacial	30 ml

1) Dissolve 0.1 g of N,N-dimethyl-1 naphthylamine in 100 ml of boiling distilled water. Cool to room temperature.

2) Add 30 ml of glacial acetic acid.

3) Filter.

Store Nitrate Reagent B at room temperature (15°–30°C). Reagents may be stored in dark brown glass bottles or in clear bottles wrapped in aluminum foil to ensure darkness. **Nitrate Reagent B is stable for up to one week (7 days).**

Zinc powder: reagent grade. Store at room temperature.

* ***Warning:*** 5 Normal (glacial) acetic acid is corrosive. Contact with skin may cause blisters and burns. In case of contact, flush eyes and skin immediately with plenty of water for at least 15 minutes.

Nitrocefin reagent for ß-lactamase (penicillinase) test

The nitrocefin test is used to detect ß-lactamase. Reagents should be warmed to room temperature prior to use. There are two formulations of the liquid reagent for the nitrocefin test: one has a nitrocefin powder concentration of 500-µg/ml, and the other has a nitrocefin powder concentration of 25-µg/ml. The reagent used for the plate test contains 500 µg of nitrocefin powder/ml and is dropped directly onto colonies on culture medium; in contrast, the reagent used for the tube test (in which bacterial cells are suspended in the reagent) contains only 25 µg of nitrocefin/ml. Nitrocefin disks are also commercially available.

Because the nitrocefin reagent is expensive, this laboratory manual suggests that a commercially available nitrocefin disk be used because it is a more cost-effective means of performing the test than use of the liquid reagent (unless a laboratory is conducting surveillance for penicillin resistance in *N. gonorrhoeae* and will be performing the nitrocefin test on large numbers of isolates). If, however, a laboratory wants to prepare its own liquid nitrocefin reagent, instructions are below. (Methods for performing the nitrocefin test with liquid reagent are included in the *N. gonorrhoeae* chapter of this manual.) Because the reagent used for the tube method is more dilute than that used for the plate test, performing the nitrocefin test by the tube method using the liquid reagent is more cost-effective than the plate or disk method for testing large numbers of isolates.

Note that preparation of the nitrocefin solution requires dimethyl sulfoxide (DMSO; CH_2SO_4), and because of the hazardous nature of DMSO some suppliers may require a letter of justification for its purchase.

Materials for preparation of nitrocefin solution include:

Nitrocefin powder	(0.5 g for stock solution of 100 ml)
0.1 M phosphate buffer, pH 7.2	(100 ml for stock solution; 1:20 dilution for tube test)

DMSO (dimethyl sulfoxide)	
Graduated cylinder	(50 ml)
Screw-cap/snap-capped tubes	(5 ml capacity)
Pasteur pipets (sterile)	

Nitrocefin solution for plate test ("stock solution"; 500 µg/ml)

a) Weigh 0.5 g nitrocefin powder in a weigh boat or beaker. Transfer nitrocefin powder to graduated cylinder.

b) Using a sterile glass pipette, add a few drops of DMSO to the nitrocefin powder. Swirl until the powder dissolves.

c) Make volume up to 100 ml with 0.1 M phosphate buffer, pH 7.2.

d) Dispense nitrocefin reagent in 5 ml volume into screw-cap/snap-capped tubes.

e) Label tubes with the following information: Reagent name, date prepared, the expiration date when moved to storage at 4˚–10˚C, and hazard code for DMSO. (*This information should also be logged in the QC log.*)

Nitrocefin solution for tube test (25 µg/ml) *(Growth suspended in nitrocefin reagent contained in tubes / microtiter plate wells)*

a) Prepare the stock nitrocefin solution (500 µg nitrocefin/ml) as described in the "Plate test nitrocefin solution, stock solution" section above.

b) Dilute the stock solution 1:20 with 0.1 M phosphate buffer, pH 7.2.

c) Dispense 3-ml volumes of the diluted nitrocefin solution into screw-cap or snap-capped tubes.

d) Label tubes with the following information: reagent name, date prepared, the expiration date, and hazard code for DMSO. (*This information should also be logged in the work record.*)

Nitrocefin reagents may be prepared in bulk, dispensed in small aliquots (1–2 ml), and stored at -20˚C or -70˚C indefinitely if no color change (from colorless/yellow to pink) is observed. If a tube of the reagent is 'in-use,' the reagent may be stored for up to one year at 4˚–10˚C if no color change is observed.

Quality control: Perform quality control with each newly prepared batch of nitrocefin reagent or each newly purchased batch of nitrocefin disks.

- An example of a ß-lactamase negative control strain is *N. gonorrhoeae* ATCC 49226.
- Examples of ß-lactamase positive control strain are *H. influenzae* ATCC 49247 and *N. gonorrhoeae* P681E (available from the CDC's Gonorrhea Research Laboratory, see Appendix 14).

Oxidase reagent (Kovac's oxidase)

Kovac's oxidase reagent is used to test for the presence of cytochrome oxidase; *N. gonorrhoeae*, *N. meningitidis*, and *V. cholerae* are all oxidase-positive and exhibit a purple reaction when exposed to this reagent. The formula for Kovac's oxidase follows:

N,N,N',N' -Tetramethyl-ρ-phenylenediamine dihydrochloride	0.05 g
Distilled water	5.0 ml

Dissolve the reagent in purified water. (Do not heat to dissolve.)

Preparation of 1% Kovac's oxidase reagent from powder

To prevent deterioration of stock oxidase-reagent powder, store in a tightly closed bottle in a desiccator kept in a cool dark area. Prepare 10 ml of a 1.0% tetramethyl-ρ-phenylenediamine hydrochloride solution in distilled water. Dispense the reagent in 1-ml aliquots and store frozen at -20°C.

For use, thaw a 1-ml vial and either use the liquid reagent to moisten filter paper or a swab or prepare dried strips of filter paper.

- To prepare dried treated filter paper, immediately after the vial is thawed, wet as many strips of filter paper as possible on a nonporous surface (*i.e.*, Petri dish, glass plate). Let the strips dry in air or in the incubator. When the strips are completely dry, place them in a tightly capped tube/bottle and refrigerate at 4°C. The strips can then be used as needed.

Note: Oxidase reagent is intended only for *in vitro* diagnostic use; avoid contact with the eyes and skin because it can cause irritation. In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes.

Instead of Kovac's oxidase reagent (described above), some laboratories may use Gordon and McLeod's reagent. Gordon and McLeod's reagent is prepared to a 1% solution (as is Kovac's oxidase), but instead of the tetramethyl- reagent used for Kovac's reagent, Gordon and McLeod's reagent uses dimethyl-ρ-phenylenediame dihydrochloride. Gordon and McLeod's oxidase is a more stable reagent but oxidase reactions take up to 30 minutes to occur, instead of 5 minutes; it should also be noted that oxidase-positive reactions with Gordon and McLeod's reagent are blue (not purple). This laboratory manual suggests using Kovac's oxidase reagent if it is available.

Quality control: Positive and negative controls should be tested every time the reagent is prepared.

- *V. cholerae*, *N. meningitidis,* and *N. gonorrhoeae* are oxidase-positive
- *E. coli* and *S. pneumoniae* are oxidase negative.

Sodium desoxycholate reagent (0.5%) for string test

The string test is used to help identify *V. cholerae*. The formula for this reagent follows:

Sodium desoxycholate (also seen as "deoxycholate")	0.5 g
Sterile distilled water	100.0 ml

Add sterile distilled water to sodium desoxycholate and mix well. Store at room temperature for up to 6 months.

Quality control: Each new batch of sodium desoxycholate should be quality controlled before use.

- Use a *V. cholerae* O1 strain as a positive control.
- *E. coli* may be used as a negative control.

Preparation of turbidity standards

Turbidity standards (McFarland turbidity standards)

Commercially prepared 0.5 McFarland turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland turbidity standard may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_20$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid (H_2SO_4). The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland turbidity standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland turbidity standards may be stored for up to 6 months in the dark at room temperature (*i.e.*, 22°–25°C); discard after 6 months or sooner if any volume is lost. (Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred; if it has, a fresh turbidity standard should be prepared.) Before each use, shake the tube containing the turbidity standard well, so that the fine white precipitate of barium sulfate is mixed in the tube.

The composition of McFarland turbidity standards and the corresponding densities of bacteria (/ml) are presented in Table 23.

The accuracy of the density of a prepared McFarland turbidity standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland turbidity standard, the absorbance at a wavelength of 625 nm should be 0.08–0.1. Alternately, the accuracy of the McFarland turbidity standard may be verified by adjusting a suspension of a control strain (*e.g.*, *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts of colonies (Figure 50). The adjusted suspension should give a count of 108 colony forming units/ml. Figures 51 and 52 are helpful guides for how to read and compare the McFarland turbidity standard to a newly prepared cell suspension.

FIGURE 50: Flowchart procedure for preparation and quality control of the 0.5 McFarland turbidity standard

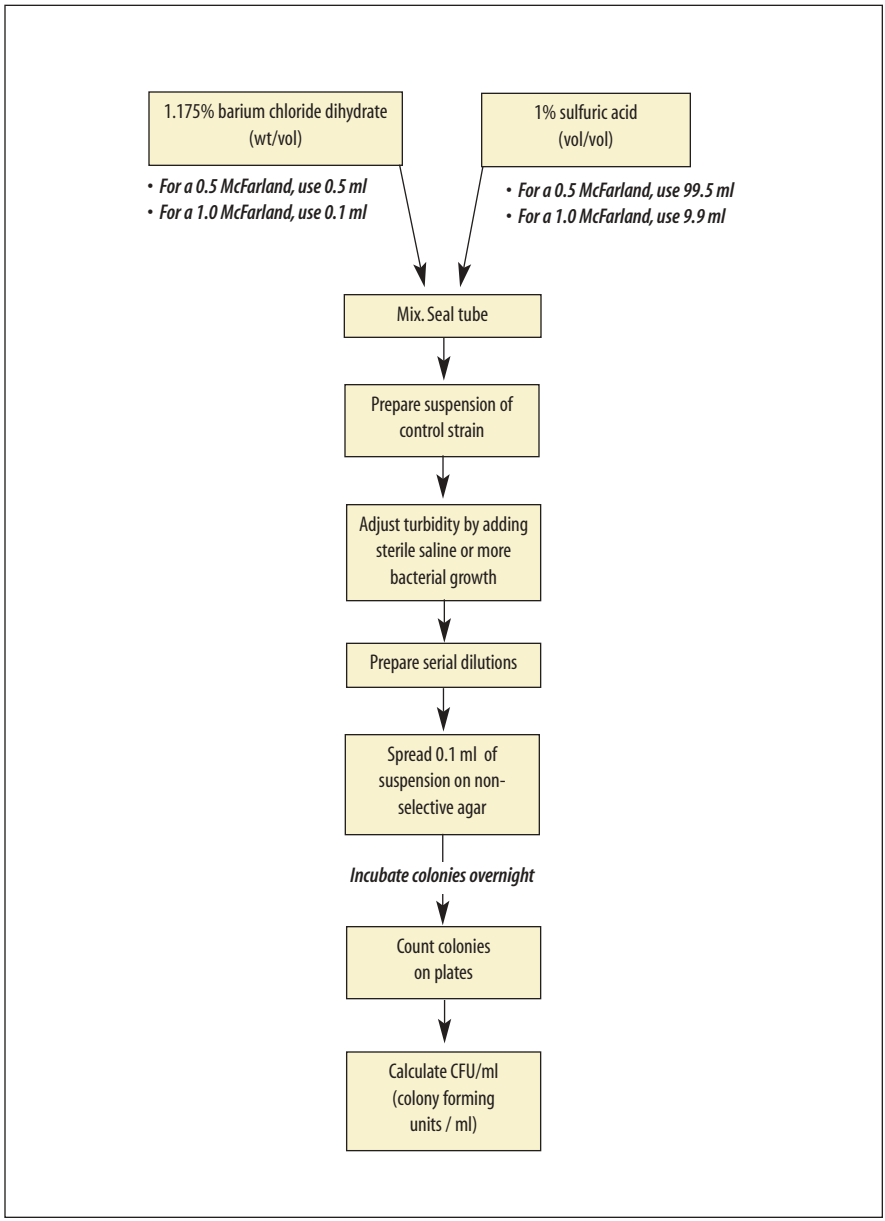

TABLE 23: Composition of McFarland turbidity standards

Turbidity standard number	Barium chloride dihydrate (1.175%)	Sulfuric acid (1%)	Corresponding approximate density of bacteria
0.5	0.5 ml	99.5 ml	1×10^8
1	0.1 ml	9.9 ml	3×10^8
2	0.2 ml	9.8 ml	6×10^8
3	0.3 ml	9.7 ml	9×10^8
4	0.4 ml	9.6 ml	12×10^8
5	0.5 ml	9.5 ml	15×10^8
6	0.6 ml	9.4 ml	18×10^8
7	0.7 ml	9.3 ml	21×10^8
8	0.8 ml	9.2 ml	24×10^8
9	0.9 ml	9.1 ml	27×10^8
10	1.0 ml	9.0 ml	30×10^8

FIGURE 51: Comparison of the 0.5 McFarland turbidity standard with inoculum suspension

FIGURE 52: Background lines for viewing turbidity of a suspension in comparison to a turbidity standard

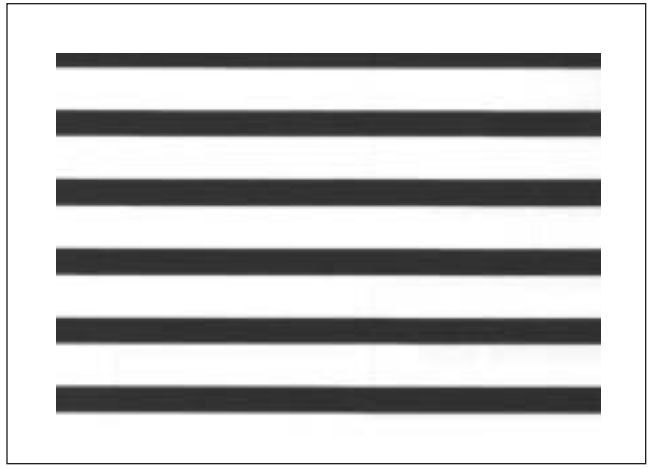

Plate count method for testing a 0.5 McFarland turbidity standard

The goal of this procedure is to determine the number of bacteria per ml of fluid. A bacterial suspension equivalent in turbidity to a 0.5 McFarland turbidity standard contains approximately 10^8 bacteria per ml.

1) Prepare 0.5 McFarland turbidity standard, as described above.

2) Prepare a suspension of a test organism (*e.g.*, *E. coli* ATCC 25922) to match the density of the McFarland turbidity standard.

3) Make serial, 10-fold dilutions of the bacterial suspension in a suitable broth medium. (Examples of suitable broth media include Mueller Hinton broth, TSB, or PBS.) The following steps *a* – *i* describe the procedure for making the serial dilutions.

   Materials necessary for testing of the 0.5 McFarland turbidity standard include: seven sterile screw-capped tubes, seven agar plates (with medium to support growth of the organism you're testing), and pipettes capable of measuring out 4.5 ml and 0.5 ml, respectively. In addition, a vortex machine is useful for vigorous mixing in tubes.

   a) Make sure you have seven screw-capped tubes, each capable of holding at least 10 ml of fluid. Prepare dilution tubes by adding 4.5 ml of sterile broth to each of the seven 10-ml tubes.

   b) Label the tubes from 1 to 7, indicating the dilution the tube will hold. Also label agar plates of the appropriate medium from 1 to 7.

   c) Add 0.5 ml of the bacterial suspension made up to 0.5 McFarland turbidity standard to the tube labeled 7 and mix vigorously.

d) **Using the same pipette as in step C,** draw up and release the suspension several times into the pipette and then transfer 0.5 ml from tube 7 to tube 6 and mix vigorously.

e) Continue this process of transferring 0.5 ml to each successive tube, **using the same pipette,** until you have completed the dilutions with tube 1. After vigorously mixing tube 1, use the pipette to draw up and release the suspension in the tube several times.

f) Using the same pipette, transfer 0.1 ml from tube 1 to the plate labeled 1.

g) Using the same pipette, transfer 0.1 ml from tube 2 to the plate labeled 2. Continue this process for tube 3 to plate 3, and tube 4 to plate 4. (If laboratorians unfamiliar with making bacterial suspensions to match a McFarland turbidity standard are responsible for the procedure, the process may be continued through tube 7. However, plating out the tubes with a higher concentrations of medium is not mandatory, because doing so would result in too many colonies to count when they grow.)

h) Using a bent rod and starting with plate 1, spread the fluid on each plate over the entire surface of the plate. A bent rod can be made by using heat to bend a 2–5 mm diameter glass rod to an approximately 60˚-angle, with the short end measuring approximately 5 cm. A bent, stainless steel metal rod of similar size can be used as an alternative to a glass rod. (The fluid can also be spread with a wire inoculating loop or needle bent to a 60˚-angle, but spreading the fluid evenly is more difficult using these methods.)

i) Incubate the plates overnight and count the number of colonies on each plate. It may be difficult to count the colonies on plates 4 through 7, and if there are more than 300 colonies per plate it should not be counted.

Interpretation of plate count results

A 0.5 McFarland turbidity standard is equivalent to approximately 10^8 bacteria per ml. The original bacterial suspension that resembles the 0.5 McFarland turbidity standard could have a range of 1.0 x 10^8 bacteria/ml to 9.0 x 10^8 bacteria/ml. Within this range, the 0.5 turbidity standard is accurate; the difference will be evident in the number of bacteria that grow out on the plates.

After 0.5 ml of the original bacterial suspension (*i.e.*, which is equivalent to the 0.5 McFarland turbidity standard) is added to the 4.5 ml of broth in tube 7, a suspension of bacteria is produced that contains approximately 10^7 bacteria per ml. Then 0.1 ml of this suspension has been transferred to the plate marked 7, which translates to approximately 10^6 (1,000,000–9,000,000) bacteria present on that plate. If the bacteria were diluted correctly: approximately 10^5 (or 100,000 –

[34] All lists are limited and incomplete. Note that inclusion of a company or specific product does not imply endorsement by CDC or WHO.

900,000) bacteria should be present on the plate labeled 6; approximately 10,000–90,000 bacteria on the plate labeled 5; approximately 1,000–9,000 bacteria present on plate 4; approximately 100 to 900 bacteria present on plate 3; approximately 10 to 90 bacteria on plate 2; and, approximately 1 to 9 bacterial colonies would be present on plate 1. Each plate should have one-tenth the bacteria as the plate with the next higher number. Generally, the plate labeled 3 will be the plate that is counted; however, if there are more than 300 colonies present on plate 3, then plate 2 should be counted.

Sources of prepared media and reagents

Although commercially prepared media and reagents are more expensive than media or reagents that can be prepared locally, commercially available items can be used (and may be preferable) in certain situations. Dehydrated media, for example, are often preferable to media prepared from individual components because of reduced lot-to-lot variation. It may also be desirable to purchase the supply of media and reagents to perform short-term studies rather than attempt formulation. The following media and reagents are available in most parts of the world from suppliers including, but **not limited to:** BBL (available from Becton, Dickinson and Company), bioMérieux, Difco (available from Becton, Dickinson and Company), Merck, Oxoid, and Quélab (Table 24); a partial listing of manufacturers, suppliers, and distributors with contact information is included in Appendix 13.[35] (The listing of supplies, media and reagents in this laboratory manual is not exhaustive, and availability of products from specific companies or suppliers may change. Inclusion of a company or product does not imply endorsement by CDC or WHO.) It is essential that each lot of materials has a satisfactory expiration date and that the date of expiration and lot number for commercial media are recorded in the laboratory.

In addition to media and reagents, laboratories must maintain their supplies (*e.g.*, glassware) and equipment; Developing Health Technology is a company that provides low-cost laboratory equipment for developing countries, nongovernmental organizations (NGOs) and aid agencies. Furthermore, as noted elsewhere in this document, the manufacturer of the Etest® (AB Biodisk) may make materials available at a reduced price to laboratories in developing country settings. Contact information for these companies is available in Appendix 13.

[35] All lists are limited and incomplete. Note that inclusion of a company or specific product does not imply endoresement by CDC or WHO.

TABLE 24: Partial listing of materials and suppliers / manufacturers

Description of item[a] [a] *This is not intended to be comprehensive catalog of materials and suppliers.*	Sample listing of manufacturers[b] [b] *Inclusion does not imply endorsement of commercial products or suppliers by CDC or WHO.*
Acetone-alcohol (decolorizing agent)	Remel; Difco (BD); BBL (BD)
Agar agar	Remel; Oxoid; BBL (BD)
Ampicillin disks	Remel; Oxoid; BBL (BD)
Azithromycin disks	Remel; Oxoid; BBL (BD)
Bile salts	Quélab; Remel; Oxoid; BBL (BD)
Bismuth sulfite (BS) agar	Quélab; Difco (BD); Oxoid; BBL (BD)
Blood culture medium - *prepared*	bioMérieux; Oxoid
Bovine hematin	Quélab; BBL (BD)
Brain-heart infusion (BHI)	Quélab; Difco (BD); Oxoid; BBL (BD); Remel
Cary-Blair medium	Quélab; Difco (BD); Oxoid; BBL (BD)
Cefixime disks	Remel; Oxoid; BBL (BD)
Ceftriaxone disks	Remel; Oxoid; BBL (BD)
Chloramphenicol disks	Remel; Oxoid; BBL (BD)
Chocolate blood agar - *prepared*	bioMérieux; Remel; BBL (BD)
Chocolate blood agar + bacitracin - *prepared*	Remel; BBL (BD)
Ciprofloxacin disks	Remel; Oxoid; BBL (BD)
CO_2-generating systems	Oxoid; Remel
Colistin disks	Quélab; Remel; Oxoid; BBL (BD)
Crystal violet	Quélab; Difco (BD); Remel; BBL (BD)
Cystine trypticase agar (CTA)	Quélab; Difco (BD); BBL (BD)
Defined growth supplement (*e.g.*, IsoVitaleX, Vitox, VX Supplement)	BBL (BD); Oxoid; Difco (BD)
Desoxycholate	Quélab; Remel
Desoxycholate citrate agar (DCA)	Quélab; Difco (BD); BBL (BD)
Etest® antimicrobial gradient strips	AB Biodisk; Remel; Fisher Scientific
Ethanol	Remel; Merck
Formalin (formaldehyde)	Remel; Merck
Furazolidone disks	Remel; Oxoid; BBL (BD)
Gonochek II® (enzyme substrate test)	TCS Microbiology
Gonococcus (GC) agar base medium	Quélab; Difco (BD); Oxoid; BBL (BD)
Gram stain kit	bioMérieux; Difco (BD); Remel; BBL (BD)
Gram-negative broth	Difco (BD); BBL (BD); Renek
Gram's iodine	Quélab; Difco (BD); Remel; BBL (BD)
H. influenzae serotyping antisera	Difco (BD); Remel

continued

TABLE 24: Partial listing of materials and suppliers / manufacturers, ***continued***

Description of item[a] *[a] This is not intended to be comprehensive catalog of materials and suppliers.*	Sample listing of manufacturers[b] *[b] Inclusion does not imply endorsement of commercial products or suppliers by CDC or WHO.*
Haemophilus test medium (HTM)	Oxoid; BBL (BD)
Heart infusion agar / broth	Quélab; Difco (BD); BBL (BD)
Hektoen enteric (HE) agar	Quélab; Difco (BD); Oxoid; BBL (BD)
Hemoglobin powder	Quélab; Difco (BD); Oxoid; BBL (BD)
Horse blood	Difco (BD); Oxoid
Jembec® plates	BBL (BD); Quélab
Kligler iron agar (KIA)	Quélab; Difco (BD); Oxoid; BBL (BD)
Kovac's oxidase reagent (*N,N,N',N'*-tetramethyl-ρ-phenylenediamine dihydrochloride)	Quélab; Merck
Lysine iron agar (LIA)	Quélab; Difco (BD); Oxoid; BBL (BD)
MacConkey agar (MAC)	Quélab; Difco (BD); Oxoid; BBL (BD)
Martin-Lewis medium - *prepared*	Quélab; BBL (BD)
Methylene blue	Quélab; Remel
Modified Thayer-Martin medium - *prepared*	Quélab; Remel; BBL (BD)
Motility medium	Quélab; Difco (BD); BBL (BD)
Mueller-Hinton agar - *prepared*	bioMérieux; Remel; BBL (BD)
Mueller-Hinton agar plus 5% sheep blood - *prepared*	bioMérieux; Remel; BBL (BD)
Mueller-Hinton agar/broth	Quélab; Difco (BD); Oxoid; Remel
N. meningitidis serogrouping antisera	Difco (BD); Remel
Nalidixic acid disks	Oxoid; BBL (BD)
Nicotinamide adenine dinucleotide (NAD; V factor)	Quélab; Merck
Nitrate broth	Quélab; Difco (BD)
Nitrate Reagents A and B	Remel; BBL (BD)
Nitrocefin (beta-lactamase)	Remel; Difco (BD); Oxoid; BBL (BD)
Optochin (p-disks)	Oxoid; BBL (BD)
Oxacillin disks	Oxoid; BBL (BD)
Penicillin disks	Oxoid; BBL (BD)
Peptone	Difco (BD); Oxoid
Permeable membrane screw-caps (for short-term storage of *N. meningitidis* at 4˚C)	(Biomedical Polymers, Inc., via) Fisher Scientific; VWR International
Phosphate buffered saline (PBS)	Quélab; Oxoid
Quad ID plate	Quélab; BBL (BD)
Quality control strains (type cultures)	American Type Culture Collection (ATCC); National Culture Type Collection (NCTC)
S. pneumoniae serotyping antisera	Omniserum; Remel; Difco (BD)

continued

TABLE 24: Partial listing of materials and suppliers / manufacturers, ***continued***

Description of item[a] *[a] This is not intended to be comprehensive catalog of materials and suppliers.*	Sample listing of manufacturers[b] *[b] Inclusion does not imply endorsement of commercial products or suppliers by CDC or WHO.*
Safranin	Quélab; Difco (BD); Remel; BBL (BD)
Salmonella (ser. Typhi) serotyping antisera	Remel; Difco (BD)
Salmonella-Shigella (SS) agar	Quélab; Difco (BD); Oxoid; BBL (BD)
Selenite broth (SEL)	Quélab; Difco (BD); Oxoid; BBL (BD)
Sheep blood	Remel; Quélab
Sheep blood agar (TSA + 5% sheep blood) - *prepared*	bioMérieux; BBL (BD)
Sheep blood agar + gentamicin - *prepared*	Quélab; BBL (BD)
Shigella antisera	Remel; Difco (BD)
Silica gel packets (*for transport and short-term storage of some pathogens*)	Scientific Device Laboratory, Inc.
Skim milk powder	Quélab; Difco (BD); BBL (BD)
Sodium polyanethol sulfonate (SPS)	Quélab; Oxoid
Spectinomycin disks	Oxoid; BBL (BD)
Sucrose (reagent-grade)	Quélab; BBL (BD)
Sulfide-indole-motility medium (SIM)	Remel; Oxoid; BBL (BD)
Superoxol reagent (30% H2O2)	Quélab; Merck
Tetracycline disks	Remel; Oxoid; BBL (BD)
Thiosulfate citrate bile salts sucrose agar (TCBS)	Quélab; Oxoid; BBL (BD)
Todd-Hewitt broth	Difco (BD); Oxoid; BBL (BD)
Trimethoprim-sulfamethoxazole (cotrimoxazole) disks	Remel; Oxoid; BBL (BD)
Triple sugar iron agar (TSI)	Quélab; Difco (BD); BBL (BD)
Tryptone (Trypticase) soy agar/broth	Quélab; Difco (BD); Oxoid; BBL (BD); bioMérieux
Tryptone (Trypticase) soy agar - *prepared*	BBL (BD); bioMérieux
Urea medium	Quélab; Difco (BD); Oxoid; BBL (BD)
V. cholerae antisera	Remel; Difco (BD)
VCA(T) supplement *(to prepare Martin-Lewis medium)*	Quélab; Oxoid; BBL (BD)
VCN(T) supplement *(to prepare Modified Thayer-Martin)*	Quélab; Oxoid; BBL (BD)
V-factor disks (NAD)	Remel; Oxoid
X-factor disks (haemin)	Remel; Oxoid; Quélab
XV-factor disks	Remel; Oxoid; Quélab
Xylose lysine desoxycholate agar (XLD)	Quélab; Difco (BD); Oxoid; BBL (BD); Remel
Zinc dust	Quélab; BBL (BD)