Chapter 9
Antimicrobial Susceptibility Testing (Agar Disk Diffusion Method)

The disk diffusion method presented in this chapter has been carefully standardized by the National Committee for Clinical Laboratory Standards (NCCLS) and if performed precisely according to the protocol below, will provide data that can reliably predict the in vivo effectiveness of the drug in question. However, any deviation from the method may invalidate the results. For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for susceptibility testing.

A. Considerations for Antimicrobial Susceptibility Testing

As antimicrobial resistance increases in many parts of the world, it becomes increasingly important to monitor the antimicrobial susceptibility of *Shigella* and *Vibrio cholerae* O1 and O139. However, because antimicrobial therapy for *Escherichia coli* O157:H7 infection has not been demonstrated to be efficacious or safe, except for cases of cystitis and pyelonephritis, determination of the antimicrobial susceptibility pattern is usually not meaningful.

See Chapters 3 and 5 for a discussion of appropriate antimicrobial agents for treatment of dysentery and cholera. Testing *Shigella*, *V. cholerae*, and *E. coli* O157:H7 against certain drugs may yield misleading results when in vitro results do not correlate with in vivo activity. *Shigella* spp., for instance, are usually susceptible to aminoglycosides (e.g., gentamicin, kanamycin) in the disk diffusion test, but treatment with these drugs is often not effective. Some special considerations for susceptibility testing of *V. cholerae* are discussed in section B below. Antimicrobial agents suggested for use in susceptibility testing of *Shigella* and *V. cholerae* are listed in Table 9-1.

B. Procedure for Agar Disk Diffusion

Figure 9-1 summarizes the disk diffusion method of susceptibility testing. Laboratory supplies required for *Shigella* and *V. cholerae* disk diffusion testing are listed in Annexes A and B.

1. Mueller-Hinton susceptibility test agar

Mueller-Hinton agar medium is the only susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar should always be used for disk diffusion susceptibility testing, according to NCCLS and international guidelines. Because the way Mueller-Hinton is prepared can affect disk diffusion test results, it is very important to refer to Section C below for instructions on preparation and quality control of this medium.

Table 9-1. Antimicrobial agents suggested for use in susceptibility testing of *Shigella* and *V. cholerae* O1 and O139

Agents for *Shigella*	Agents for *V. cholerae*
Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole
Chloramphenicol	Chloramphenicol
Ampicillin	Furazolidone
Nalidixic acid[a]	Tetracycline[b]

[a] If resistant to nalidixic acid, test with ciprofloxacin.
[b] The results from the tetracycline disk are used to predict susceptibility to doxycycline also.

2. McFarland turbidity standard

A McFarland 0.5 standard should be prepared and quality controlled prior to beginning susceptibility testing (see Section C). If tightly sealed to prevent evaporation and stored in the dark, the standard can be stored for up to 6 months. The McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility test.

3. Preparation of inoculum

Each culture to be tested should be streaked onto a noninhibitory agar medium (blood agar, brain heart infusion agar, or tryptone soy agar) to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline (see Section C) or nonselective broth (Mueller-Hinton broth, heart infusion broth, or tryptone soy broth) and vortex thoroughly. The bacterial suspension should then be compared to the 0.5 McFarland standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (see Figures 9-2 and 9-3). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the McFarland 0.5, the turbidity can be reduced by adding sterile saline or broth or increased by adding more bacterial growth.

Alternatively, the growth method may be used to prepare the inoculum. Four or five colonies are picked from overnight growth on agar and inoculated into broth (Mueller-Hinton broth, heart infusion broth, or tryptone soy broth). Incubate the broth at 35°C until turbid, and then adjust the turbidity to the proper density.

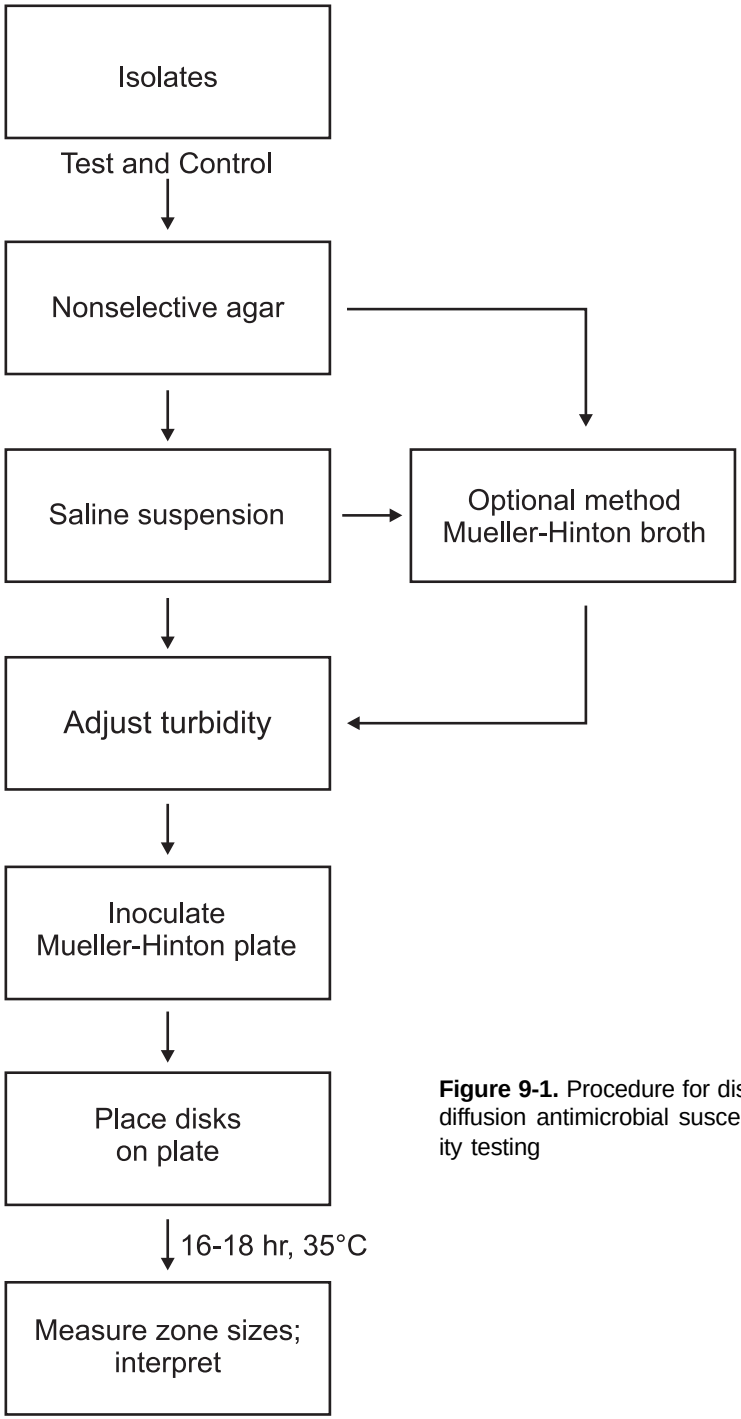

Figure 9-1. Procedure for disk diffusion antimicrobial susceptibility testing

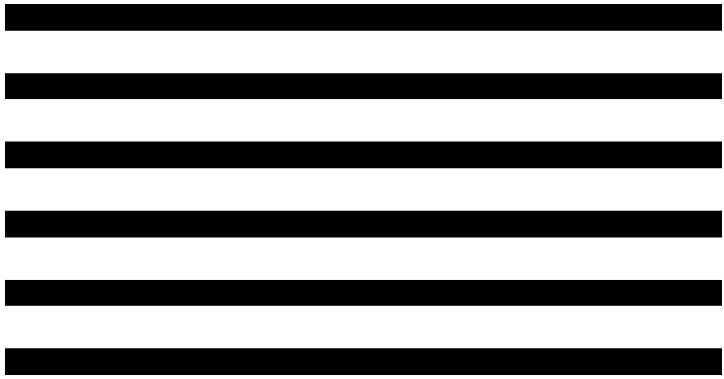

Figure 9-2. Background lines for viewing turbidity of inoculum

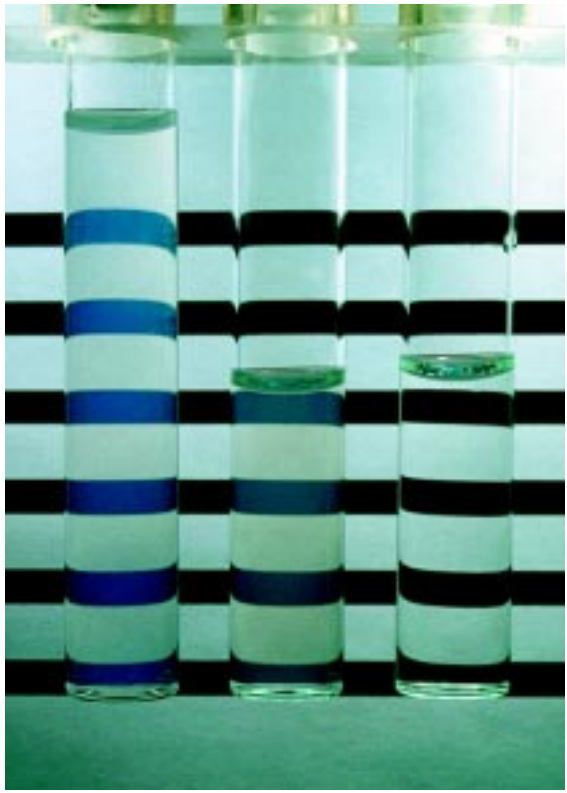

Figure 9-3. Comparison of McFarland 0.5 with inoculum suspension. From left to right, the tubes are the McFarland 0.5 standard, *E. coli* ATCC 25922 adjusted to the 0.5 McFarland turbidity, and uninoculated saline.

4. Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 9-4). Finally, swab all around the edge of the agar surface.

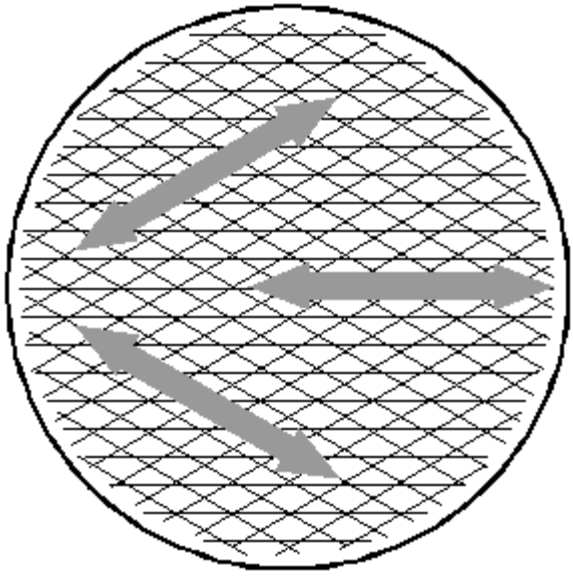

Figure 9-4. The Mueller-Hinton plate should be swabbed over the entire surface of the medium three times, rotating the plate 60 degrees after each application.

5. Antimicrobial disks

The working supply of antimicrobial disks should be stored in the refrigerator (4°C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before using.

Table 9-2. Zone size interpretative standards for *Enterobacteriaceae* for selected antimicrobial disks

Antimicrobial agent	Disk potency (µg)	Zone diameter (mm)			Zone diameter limits (mm) for *E. coli* ATCC 25922
		Resistant	Intermediate	Susceptible	
Chloramphenicol[a,b]	30	≤12	13-17	≥18	21-27
Ampicillin[a]	10	≤13	14-16	≥17	16-22
Furazolidone[c] for *V. cholerae*	100	<18	–	≥18	22-26
Trimethoprim-sulfamethoxazole[a]	1.25/ 23.75	≤10	11-15	≥16	24-32
Tetracycline[a]	30	≤14	15-18	≥19	18-25
Ciprofloxacin[a, d]	5	≤15	16-20	≥21	30-40
Nalidixic acid[a]	30	≤13	14-18	≥19	22-28
Nalidixic acid[c] for *V. cholerae*	30	<19	–	≥19	

[a]Source: National Committee on Clinical Laboratory Standards (NCCLS), 1998.
[b]Use these interpretive standards with caution as the disk diffusion test may misclassify many organisms (high minor error rate)
[c]Proposed interpretive criteria based on multi-laboratory studies. Criteria have not been established for *V. cholerae* by NCCLS.
[d]These zone sizes are valid for interpreting disk diffusion results for *Shigella* and *Enterobacteriaceae*. However, zone sizes for *V. cholerae* have not been established by NCCLS.

Apply the antimicrobial disks to the plates as soon as possible, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps or with a mechanical dispensing apparatus, and then gently press down onto the agar. In general, place no more than 12 disks on a 150-mm plate and no more than 4 disks on a 100-mm plate. This prevents overlapping of the zones of inhibition and possible error in measurement. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

6. Recording and interpreting results

After the disks are placed on the plate, invert the plate and incubate at 35°C for 16 to 18 hours. After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 9-5) and record it in millimeters (Figures 9-6, 9-7). The measurements can be made with a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (see Table 9-2), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. The distance from the colony(ies) closest to the disk to the center of the disk should be measured and then doubled to obtain a diameter. The diameter of the outer clear zone should be recorded as well and an interpretation recorded for each diameter. The colony(ies) inside the zone should be picked, re-isolated, re-identified, and retested in the disk diffusion test to confirm the previous results. The presence of colonies within a zone of inhibition may predict eventual resistance to that agent.

7. Quality control

To verify that susceptibility test results are accurate, it is important to include at least one control organism (ATCC 25922 is the *E. coli* control strain used when testing *Enterobacteriaceae* and *V. cholerae*) with each test. Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits (see Table 9-2 for diameters of the zones of inhibition for ATCC 25922). If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

Susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other factors. The medium used may be a source of error if it fails to conform to NCCLS recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs

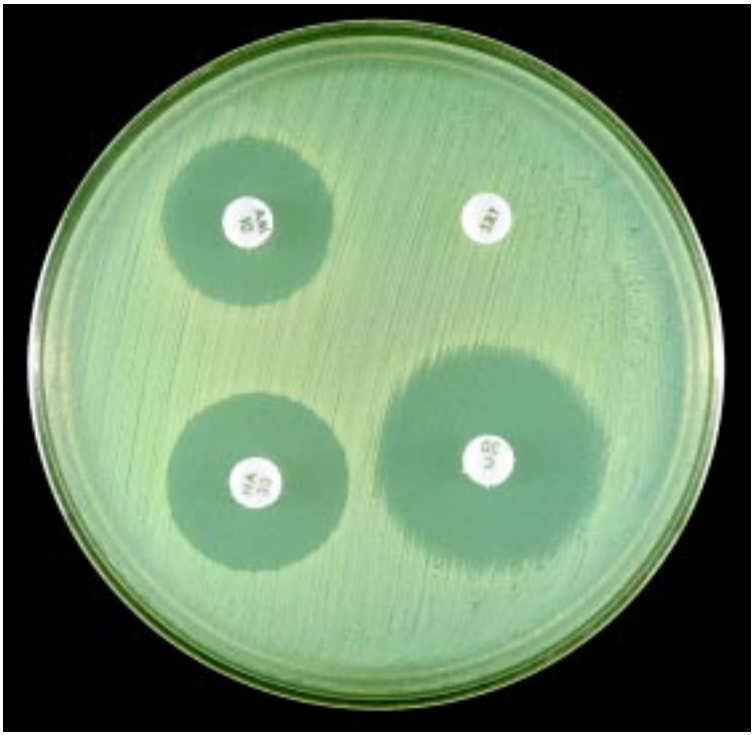

Figure 9-5. Results of the disk diffusion assay. This *Shigella* isolate is resistant to trimethoprim-sulfamethoxazole and is growing up to the disk (SXT), the zone of which is recorded as 6 mm.

when in fact they are not. If the depth of the agar in the plate is not 3 to 4 mm or the pH is not between 7.2 and 7.4, the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the McFarland standard, the susceptibility test results will be affected. For instance, a resistant organism could appear to be susceptible if the inoculum is too light. Also, if colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole disks even when testing susceptible isolates.

If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease; this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

Vibrio cholerae Antimicrobial Susceptibility Results

DATE TESTED:

SPECIMEN NUMBER	ORGANISM	TETRACYCLINE	FURAZOLIDONE	TRIMETHOPRIM/SULFA	CHLORAMPHENICOL
ATCC 25922					

Figure 9-6. Sample worksheet for recording disk diffusion susceptibility results for *V. cholerae* O1 or O139

***Shigella* Antimicrobial Susceptibility Results**

DATE TESTED:

SPECIMEN NUMBER	ORGANISM	TRIMETHOPRIM/SULFA	CHLORAMPHENICOL	AMPICILLIN	NALIDIXIC ACID	CIPROFLOXACIN (optional)
ATCC 25922						

Figure 9-7. Sample worksheet for recording disk diffusion susceptibility results for *Shigella* isolates

As mentioned above, testing some bacteria against certain antimicrobial agents may yield misleading results because these in vitro results do not necessarily correlate with in vivo activity. Examples include narrow- and expanded-spectrum cephalosporins and aminoglycosides (e.g., gentamicin) tested against *Shigella* spp. (see Chapter 3), and erythromycin tested against *V. cholerae* (see section C below).

C. Special Considerations for Susceptibility Testing of *V. cholerae*

Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, zone size interpretive criteria for *V. cholerae* O1 and O139 have been established only for ampicillin, chloramphenicol, sulfonamides, tetracycline and trimethoprim-sulfamethoxazole. It has been determined that disk diffusion results are not accurate for *V. cholerae* when testing erythromycin and doxycycline, and these agents should not be tested by this method. The results from the tetracycline disk should be used to predict susceptibility to doxycycline. If susceptible to tetracycline, the strain will be susceptible to doxycycline. At this time there is no in vitro method to accurately determine susceptibility to erythromycin.

The reliability of disk diffusion results for other antimicrobial agents, including ciprofloxacin, furazolidone and nalidixic acid, has not been validated. Until interpretive criteria have been established for *V. cholerae*, disk diffusion may be used to screen for resistance to ciprofloxacin, using interpretive criteria for the *Enterobacteriaceae* as tentative zone size standards. Tentative breakpoints have been proposed for testing furazolidone and nalidixic acid with *V. cholerae* (see Table 9-2). When screening with the disk diffusion method for these agents, results should be interpreted with caution. If zone sizes for these drugs fall within the intermediate range, the organism should be considered possibly resistant.

D. Preparation and Quality Control of Media and Reagents

1. Mueller-Hinton agar

[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. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

Follow manufacturer's instructions to prepare medium. After autoclaving, cool medium to 50°C. Measure 60 to 70 ml of medium per plate into 15 x 150-mm plates, or measure 25 to 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 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.

Freshly prepared plates may be used the same day or stored in a refrigerator (2° to 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° to 37°C) until the moisture evaporates (usually 10 to 30 min). Do not leave lids ajar because the medium is easily contaminated.

Each new lot should be quality controlled before use by testing the *E. coli* ATCC 25922 and/or *Staphylococcus aureus* ATCC 25923 standard strains. These standard strains are used with every test run for *Enterobacteriaceae* and gram-positive aerobes, respectively. The pH of each new lot of Mueller-Hinton should be between 7.2 to 7.4. If outside this range, the pH 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.

2. Turbidity standards (McFarland)

McFarland 0.5 turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland standards may be stored for up to 6 months in the dark at room temperature (22° to 25°C). Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube.

The accuracy of the density of a prepared McFarland standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm should be 0.08 to 0.1. Alternately, the accuracy of the McFarland 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 (see Figure 9-8). The adjusted suspension should give a count of 10^8 colony forming units/ml.

3. Physiological saline

NaCl	8.5 g
Distilled water	1 liter

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

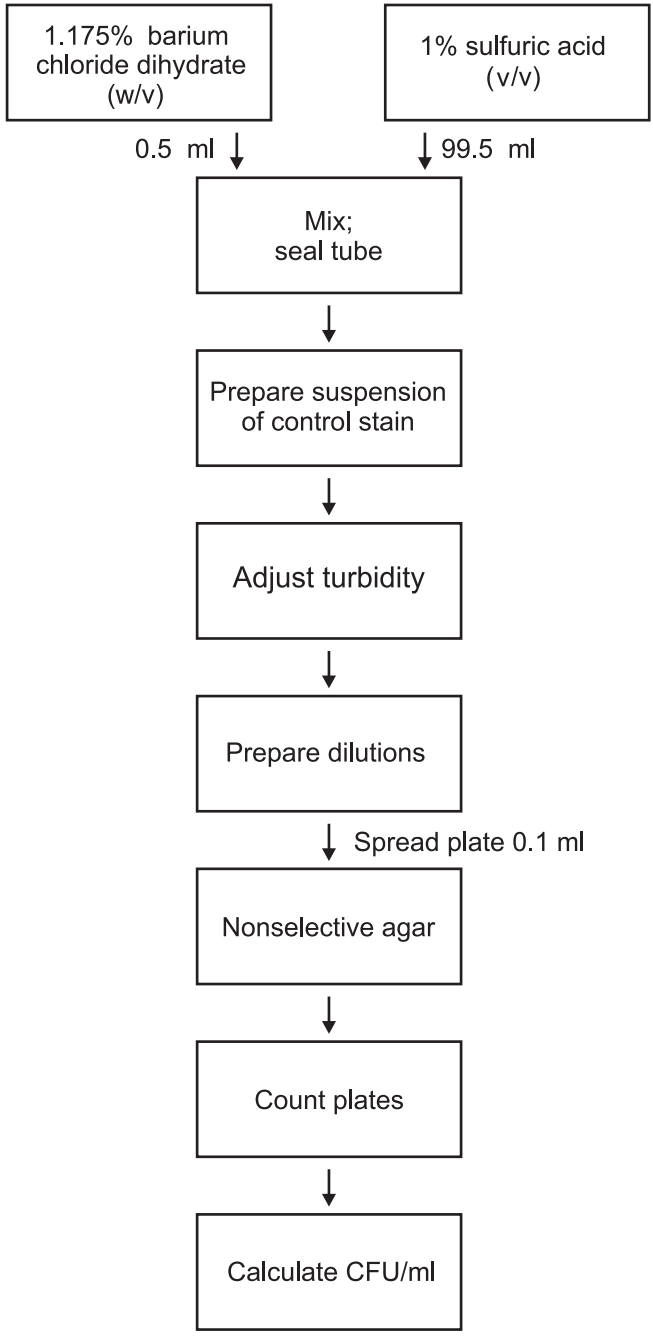

Figure 9-8. Procedure for preparation and quality control of the McFarland 0.5 standard

References

Jorgensen JH, Turnidge JD, Washington JA. Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of clinical microbiology, 7th ed.. Washington, DC: ASM Press; 1999:1526-1543.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; ninth informational supplement. Wayne, Pennsyslvania: NCCLS; 1999: document M100-S9, Vol. 19. No. 1, Table 2I.