

Antibiotic	Disc content	Individual tests		
		Zone diameter in millimeters		Permitted millimeter difference ATCC 25923- ATCC 25922
		With <i>S. aureus</i> ATCC 25923 ¹	With <i>E. coli</i> ATCC 25922 ¹	
Chloramphenicol	30 mcg	19-26	21-27	-4-1
Clindamycin	2 mcg	23-29		
Colistin	10 mcg		11-15	
Erythromycin	15 mcg	22-30	8-14	10-19
Gentamicin	10 mcg	19-27	19-26	-2-3
Kanamycin	30 mcg	19-26	17-25	-1-4
Methicillin	5 mcg	17-22		
Neomycin	30 mcg	18-26	17-23	0-3
Novobiocin	30 mcg	22-31		
Oleandomycin	15 mcg	19-28		
Penicillin G	10 units	26-37		
Polymyxin B	300 units	7-13	12-16	-7--2
Streptomycin	10 mcg	14-22	12-20	-1-5
Tobramycin	10 mcg	19-29	18-26	
Tetracycline	30 mcg	19-28	18-25	0-6
Vancomycin	30 mcg	15-19		

¹ Available from: American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852.

G. LIMITATIONS OF THE METHOD

The method of interpretation described in E above applies to rapidly growing pathogens and should not be applied to slowly growing organisms. The latter show larger zones of inhibition than those given in the table. Susceptibility of gonococci to penicillin, and of slow-growing strains, e.g., *Bacteroides* species and fastidious anaerobes to any antibiotic, should be determined by the broth-dilution or agar-dilution method unless specifically standardized diffusion tests are used.

(d) *Requests for certification; samples.*

(1) In addition to complying with the requirements of §431.1 of this chapter, a person who requests certification of a batch of antibiotic susceptibility discs shall submit with his request a statement showing the batch mark, the number of packages of each size in such batch, and, unless it was previously submitted, the date on which the latest assay of the antibiotic used in making such batch was completed, the potency of each disc, the quantity of each ingredient used in making the batch, the date on which the latest assay of the drug comprising such batch was completed, and a statement that each ingredient used in making the batch conforms to the requirements prescribed therefor by this section.

(2) Such person shall submit in connection with his request results of the tests and assays made by him on an accurately representative sample of the batch for potency.

(3) Such person shall submit in connection with his request an accurately representative sample of the batch consisting of one disc for each 5,000 discs in the batch, but in no case less than 36 discs collected by taking single discs at intervals throughout the entire time of packaging the batch so that the quantities packaged during the intervals are approximately equal.

[39 FR 19181, May 30, 1974, as amended at 41 FR 7093, Feb. 17, 1976; 41 FR 35061, Aug. 19, 1976; 44 FR 10376, Feb. 20, 1979; 44 FR 20666, Apr. 6, 1979]

§460.6 Tests and methods of assay for potency of antibiotic susceptibility discs.

(a) *Culture media.* Use ingredients that conform to the standards prescribed by the United States Pharmacopeia or The National Formulary. In lieu of preparing the media from the individual ingredients, they may be made from a dehydrated mixture which, when reconstituted with distilled water, has the same composition as such media. Minor modification of the specified individual ingredients is permissible if the resulting media possess growth-promoting properties at least equal to the media described.

(1) *Medium A:*

Peptone	6.0 gm.
Pancreatic digest of casein	4.0 gm.
Yeast extract	3.0 gm.

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Beef extract	1.5 gm.
Dextrose	1.0 gm.
Agar	15.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 6.5 to 6.6 after sterilization.	

(2) *Medium B.* Same as medium A, except that it also contains 300 milligrams of hydrated manganese sulfate per liter.

(3) *Medium C.* Same as medium A except that the final pH is adjusted from 7.9 to 8.1 after sterilization.

(4) *Medium D:*

Peptone	5.0 gm.
Yeast extract	1.5 gm.
Beef extract	1.5 gm.
Sodium chloride	3.5 gm.
Dextrose	1.0 gm.
Dipotassium phosphate	3.68 gm.
Potassium dihydrogen phosphate	1.32 gm.
Disilled water, q.s	1,000.0 ml.
pH 7.0 after sterilization	

(5) *Medium E:*

Peptone	6.0 gm.
Yeast extract	3.0 gm.
Beef extract	1.5 gm.
Agar	15.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 6.5 to 6.6 after sterilization.	

(6) *Medium F:*

Pancreatic digest of casein	17.0 gm.
Papaic digest of soybean	3.0 gm.
Sodium chloride	5.0 gm.
Dipotassium phosphate	2.5 gm.
Dextrose	2.5 gm.
Agar	20.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.3 after sterilization.	

(7) *Medium G.* Same as medium F except for the following:

Agar	12.0 gm.
Polysorbate 80 (Sterile)	10.0 gm.
Add polysorbate 80 after boiling.	

(8) *Medium H:*

Peptone	9.4 gm.
Yeast extract	4.7 gm.
Beef extract	2.4 gm.
Sodium chloride	10.0 gm.
Dextrose	10.0 gm.
Agar	23.5 gm.
Distilled water, q.s	1,000.0 ml.
pH 6.0 to 6.2 after sterilization.	

(9) *Medium I:*

Peptone	6.0 gm.
Yeast extract	3.0 gm.
Beef extract	1.5 gm.
Dextrose	1.0 gm.
Agar	15.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 6.6 after sterilization.	

(10) *Medium J:*

Pancreatic digest of casein	15.0 gm.
Papaic digest of soybean	5.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.3 after sterilization.	

(11) *Medium K:*

Pancreatic digest of casein	17.0 gm.
Papaic digest of soybean	3.0 gm.
Sodium chloride	5.0 gm.
Dipotassium phosphate	2.5 gm.
Dextrose	2.5 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.3 after sterilization.	

(12) *Medium L:*

Agar agar	15 gm.
Distilled water, q.s	1,000.0 ml.

(13) *Medium M:*

Beef, inclusion from	300 gm.
Acid hydrolysate of casein	17.5 gm.
Soluble starch	1.5 gm.
Agar	15 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.4 after sterilization.	

(14) *Medium N:*

Infusion from beef	300.0 gm.
Acid hydrolysate of casein	17.5 gm.
Starch	1.5 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.4 after sterilization.	

(15) *Medium O:*

Calf brains, infusion from	200.0 gm.
Beef heart, infusion from	250.0 gm.
Pancreatic digest of gelatin	10.0 gm.
Dextrose	2.0 gm.
Sodium chloride	5.0 gm.
Sodium phosphate dibasic (Na ₂ HPO ₄)	2.5 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.4 after sterilization.	

(16) *Medium P.* Same as medium J with 5 percent defibrinated sheep blood added.

(17) *Medium Q:*

Pancreatic digest of gelatin	17.0 gm.
Pancreatic digest of casein plus equal part of peptic digest of animal tissues	3.0 gm.
Lactose	10.0 gm.
Bile salts mixture	1.5 gm.
Sodium chloride	5.0 gm.
Agar	13.5 gm.
Neutral red	0.03 gm.
Crystal violet	0.001 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.1 after sterilization.	

(b) *Preparation of test organism suspensions—(1) Suspension 1. Staphylococcus aureus (ATCC 6538P)¹ is maintained and*

¹Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

grown on medium A. Wash the organisms from an agar slant, incubated for 24 hours at 32° C. to 35° C., with 3.0 milliliters of sterile sodium chloride solution onto the agar surface of a Roux bottle containing 300 milliliters of medium A. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate 24 hours at 32° C. to 35° C. Wash the resulting growth from the agar surface with about 50 milliliters of sterile sodium chloride solution. Standardize this stock suspension by determining the dilution that will permit 20 percent light transmission. Store the stock suspension in the refrigerator (1 week) and use the indicated dilution prepared daily.

(2) *Suspension 2.* Follow the procedure described for suspension 1, except standardize the bulk suspension so that a 1:10 dilution in saline solution gives 20 percent light transmission. In this case, the bulk suspension, and not the 1:10 dilution of it, is used for the inoculum.

(3) *Suspension 3.* The test organism is *Staphylococcus aureus* (ATCC 13150).¹ Follow the procedure described for suspension 1, but determine how much the bulk suspension should be diluted to obtain a suspension permitting 80 percent light transmission. Use the indicated dilution prepared daily for the inoculum for the plates.

(4) *Suspension 4. Sarcina lutea* (ATCC 9341)¹ is maintained on agar slants of medium A and transferred to fresh slants approximately every 2 weeks. This culture is incubated overnight at 26° C., and then stored in the refrigerator. Prepare an inoculum for the plates as follows: Streak an agar slant heavily with the test organism and incubate for 24 hours at 26° C. Wash the growth from the slant with 3 milliliters to 4 milliliters of medium D, and transfer to the surface of a Roux bottle containing 300 milliliters of medium A. Spread the suspension evenly over the entire surface with the aid of sterile glass beads. Incubate for 24 hours at 26° C. Wash the growth from the agar surface with 15 milliliters of medium D. If an aliquot of this bulk suspension when diluted 1:10 with medium D gives 10 percent light transmission, the bulk suspension is satisfactory for use. It

may be necessary to adjust the bulk suspension by dilution so that an aliquot of the adjusted suspension when diluted 1:10 will give the desired 10 percent light transmission. The adjusted bulk suspension only, and not the 1:10 dilution of it, is used in preparing the inoculum. Store the stock suspension in the refrigerator and use for 2 weeks.

(5) *Suspension 5. Bacillus subtilis* (ATCC 6633)¹ is maintained on agar medium A and transferred to a fresh slant every month. To prepare the spore suspension, inoculate a fresh slant of agar medium A with the test organism and incubate at 37° C. for 16 hours to 24 hours. Wash the culture from the slant with 3 milliliters of sterile sodium chloride solution onto the surface of a Roux bottle containing 300 milliliters of agar medium B. Incubate for 5 days at 37° C. Suspend the growth in 50 milliliters of sterile saline solution, centrifuge, and decant the supernatant liquid. Reconstitute the sediment and heat-shock the suspension by heating for 30 minutes at 70° C. Store the spore suspension in the refrigerator. It may be kept several months. Light transmission is not used for standardization.

(6) *Suspension 6. Staphylococcus epidermidis* (ATCC 12228)¹ is maintained on medium A and transferred to a fresh slant once a week. Inoculate a fresh slant of medium A with the test organism and incubate at 32° C. to 35° C. for 24 hours. Wash the culture from the slant with 3 milliliters of sterile sodium chloride solution onto the surface of a Roux bottle containing 300 milliliters of medium A. Incubate at 32° C. to 35° C. for 24 hours. Wash the resulting growth from the agar surface with about 50 milliliters of sterile sodium chloride solution. Standardize this stock suspension by determining the dilution that will give 80 percent light transmission. Store the stock suspension in the refrigerator (1 week) and use the indicated dilution prepared daily for the inoculum for the plates.

(7) *Suspension 7. Bordetella bronchiseptica* (ATCC 4617)¹ is maintained on medium F and transferred to a fresh slant every 2 weeks. To prepare a stock suspension inoculate a fresh slant of medium F and incubate at 37° C. for 16 hours to 24 hours. Wash the

culture from this slant with 3 milliliters of sterile distilled water onto the surface of a Roux bottle containing 300 milliliters of medium F, and incubate 24 hours at 37° C. Wash off the growth with 50 milliliters of sterile distilled water and standardize the resulting stock suspension by determining the dilution that will give 50 percent light transmission. Store the stock suspension in the refrigerator (2 weeks), and use the indicated dilution prepared daily for the inoculum for the plates.

(8) *Suspension 8. Saccharomyces cerevisiae* (ATCC 9763)¹ is maintained on slants of medium H and transferred once a week. After transfer, the culture is incubated at 37° C. for 24 hours and then kept refrigerated. Wash the organism from a freshly incubated agar slant with 3 milliliters of sterile saline solution onto the agar surface in a Roux bottle containing 300 milliliters of medium H. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate for 24 hours at 37° C. and then wash the resulting growth from the agar surface with about 25 milliliters of sterile saline solution. Store the suspension in the refrigerator and use for 1 month.

(9) *Suspension 9.* Follow the procedure described for suspension 1, except determine how much the bulk suspension should be diluted to obtain a suspension permitting 80 percent light transmission. Use the indicated dilution, prepared daily, for the inoculum for the plates.

(10) *Suspension 10: Klebsiella pneumoniae* (ATCC 10031),¹ noncapsulated, is maintained on medium A and transferred to a fresh slant once a week. Inoculate a fresh slant of medium A with the test organism and incubate overnight at 32° C.-35° C. Wash the culture from the slant with 3 milliliters of sterilized U.S.P. saline T.S. onto the surface of a Roux bottle containing 300 milliliters of medium A. Incubate at 32° C.-35° C. for 24 hours. Wash the resulting growth from the agar surface with about 50 milliliters of sterilized U.S.P. saline T.S. If an ali-

quot of this bulk suspension when diluted 1:9 with saline solution gives 40 percent light transmission, the bulk suspension is satisfactory for use. It may be necessary to adjust the bulk suspension by dilution so that an aliquot of the adjusted suspension when diluted 1:9 will give the desired 40 percent light transmission. The adjusted bulk suspension (not the 1:9 dilution) is used in preparing the inoculum. Store the suspension in the refrigerator and use for no more than 1 week.

(11) *Suspension 11 Streptococcus fecalis* (ATCC 14506)¹ is maintained on medium E and transferred to a fresh agar slant once a week. After transfer, the culture is incubated at 37° C. for 24 hours and then kept refrigerated. Transfer from a freshly incubated agar slant to a tube containing 10 milliliters of culture medium described in §147.3(b)(1). Incubate the broth culture for 16 to 18 hours at 37° C. and store in the refrigerator. This culture may be used for no more than 1 week.

The light transmission values referred to in this paragraph were determined with a Lumetron Model 400-A photoelectric colorimeter at a wavelength of 650 millimicrons. If other instruments are used, different light transmission readings will probably be obtained. The values given are to be used as guides in this paragraph.

(12) *Suspension 12. Pseudomonas aeruginosa* (ATCC 25619)¹ is maintained and grown on medium J and transferred to a fresh agar slant once a week. Inoculate a fresh slant of medium J with the test organism and incubate at 37° C. for 24 hours. Transfer the culture from this slant with sterile glass beads onto the agar surface of a Roux bottle containing 300 milliliters of medium J. Spread the organisms over the entire agar surface with the aid of the glass beads. Incubate 24 hours at 37° C. Wash the resulting growth from the agar surface with about 30 milliliters of medium K. Do not standardize the suspension. Store the stock suspension under refrigeration and use for 2 weeks.

(13) *Suspension 13. Escherichia coli* (ATCC 29214)¹ is maintained and grown on medium M. Wash the organisms from an agar slant, incubated for 24 hours at 37° C, with 3 milliliters of

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sterilized U.S.P. saline T.S. onto the surface of a Roux bottle containing 250 milliliters of medium M. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate for 24 hours at 37° C and then wash the resulting growth from the agar surface with 50 milliliters of sterilized U.S.P. saline T.S. Store the suspension in the refrigerator and use for 2 weeks.

(14) *Suspension 14.* The test organism is *Staphylococcus aureus* (ATCC 29213).

(i) *Stock culture.* Transfer a lyophilized culture into medium K in a sterile container and incubate at 37° C for 24 hours. Streak the culture onto the solidified agar surface of a plate containing medium P and incubate the plate at 37° C for 24 hours. Transfer 5 to 10 colonies into 3 milliliters of medium O in a sterile container and incubate at 37° C for 24 hours. Add 3 milliliters of sterile glycerol or 3 milliliters of sterile rabbit serum to the broth culture, mix well and pour the contents into a sterile flask containing a layer of sterile glass beads. Rotate the flask to coat the beads with the culture mixture and aseptically aspirate all the excess liquid from the flask. Store the flask containing the coated glass beads at –20° C to –70° C.

(ii) *Test suspension.* Aseptically add a coated glass bead to 0.5 milliliter of medium O and incubate at 37° C for 24 hours. Streak the culture onto the solidified agar surface of a plate containing medium P and incubate at 37° C for 24 hours. The streak plate may be used for 1 week if kept under refrigeration. On the day of test, transfer 4 to 10 colonies to a sterile tube containing 0.5 milliliter of medium O and incubate at 37° C for 4 to 6 hours. Pipet 0.05 milliliter of the test suspension into a screw-topped tube containing 25 milliliters of sterile distilled water and 0.005 milliliter of sterile polysorbate 80 and mix well (do not shake). Use this test culture suspension as the daily inoculum source.

(15) *Suspension 15.* The test organism is *Escherichia coli* (ATCC 25922).¹ Follow the procedure described for suspension 14 in paragraph (b)(14) of this section, except under paragraph (b)(14)(ii) of

this section use medium Q in place of medium P.¹

(16) *Suspension 16.* The test organism is *Streptococcus faecalis* (ATCC 29212)¹. Follow the procedure described for suspension 14 in paragraph (b)(14) of this section.

(17) *Suspension 17.* The test organism is *Pseudomonas aeruginosa* (ATCC 27853)¹. Follow the procedure described for suspension 14 in paragraph (b)(14) of this section, except under paragraph (b)(14)(ii) of this section use medium Q in place of medium P.

(18) *Suspension 18.* The test organism is *Staphylococcus aureus* (ATCC 29247)¹. Follow the procedure described for suspension 14 in paragraph (b)(14) of this section.

(19) *Suspension 19.* The test organism is *Enterobacter cloacae* (ATCC 29249)¹. Follow the procedure described for suspension 14 in paragraph (b)(14) of this section.

(20) *Suspension 20.* The test organism is *Pseudomonas aeruginosa* (ATCC 29248)¹. Follow the procedure described for suspension 14 in paragraph (b)(14) of this section, except under paragraph (b)(14)(ii) of this section use medium Q in place of medium P.

(c) *Preparation of plates—(1) Basalayer.* Depending on the particular antibiotic in the discs to be tested, add 42 milliliters of the appropriate medium prescribed in paragraph (c)(3) of this section to each Petri dish (20 millimeters x 150 millimeters) and allow to harden on a flat, level surface and dry slightly by raising the tops on one side.

(2) *Seed layer.* Add the appropriate amount of inoculum, as prescribed by paragraph (c)(3) of this section, to the seed agar which has been melted and cooled to 48° C. Swirl the flasks to obtain a homogeneous suspension. Add 8 milliliters of the appropriate seed agar, as specified in paragraph (c)(3) of this section, to each plate, spread evenly over the hardened base layer, and allow to harden and dry on a flat level surface. For accurate results, it is necessary to obtain uniform distribution of the agar over the surface of the plates.

¹Available from American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852.

(3) *Inoculum and media to be used.* Depending on the particular antibiotic in the disc to be tested, select from the following table the inoculum and media to be used:

Antibiotic	Volume of suspension added to each 100 ml. of seed agar used for test	Suspension number	Medium	
			Base layer	Seed layer
	<i>Ml.</i>			
Ampicillin	1.0	3	E	A
Bacitracin	1.0	3	E	A
Carbenicillin	3.0	12	F	G
Cefamandole (lithium)	1.0	10	E	A
Cefoxitin (sodium)	1.0	10	E	A
Cephaloglycin (dihydrate)	1.0	10	E	A
Cephaloridine	1.0	10	E	A
Cephalothin	1.0	10	E	A
Chloramphenicol	4.0	4	E	A
Clindamycin	2.0	2	A	I
Colistin (sulfate)	1.0	7	F	G
Erythromycin	2.0	11	C	C
Gentamicin (sulfate)	0.5	3	C	C
Kanamycin (sulfate)	1.0	9	E	A
Methicillin	1.0	3	E	A
Neomycin (sulfate)	2.5	6	C	C
Novobiocin (sodium)	4.0	5	E	A
Oleandomycin (phosphate)	2.0	3	E	A
Penicillin G	1.0	3	E	A
Polymyxin B (sulfate)	1.0	7	F	G
Rifampin	1.0	5	E	A
Rifampin discs for use in culture media	0.5	13	A	L
Streptomycin (sulfate)	3.0	1	C	C
Tetracycline (hydrochloride)	1.5	1	E	A
Tobramycin	0.5	3	C	C
Vancomycin	1.0	6	C	C

(d) *Preparation of control discs.* Use round, blank discs having a diameter of 1/4-inch made of clear-white paper weighing 30 milligrams ±4 milligrams per square centimeter, and which will absorb 2.5 to 3.0 times its own weight of distilled water. The paper shall contain no material that either enhances or inhibits the activity of any antibacterial agent impregnated thereon. In addition, the paper shall contain no materials which will affect the pH of any solvent placed on it or buffer any solution placed on it. The following methods shall be used to determine the suitability in this regard of any paper proposed for this use: Weigh 2 grams of paper or paper discs into a clean, glass-stoppered, 250-milliliter flask. Add 30 milliliters of freshly boiled and cooled distilled water (the pH of which has been determined). Stopper and shake vigorously for 1 hour on a shaking machine. Filter through a medium-porosity sintered glass filter. Determine the

pH of the filtrate. Take the two 10-milliliter aliquots. To one add 0.05 milliliter of 0.01N HCl. To the second aliquot add 0.05 milliliter of 0.01 N NaOH. Determine the pH of each solution. The paper shall be satisfactory for use, if (1) the pH of the paper filtrate was not more than ±0.3 pH units different from the pH of the distilled water used; (2) the pH of the acidified aliquot was lowered by at least 1.0 pH units; (3) the pH of the alkalinized aliquot was raised by at least 1.5 pH units. Place blank discs on aluminum or stainless steel wire mesh which is supported in a manner to allow circulation of air above and below the discs. Prepare the desired number of discs for each point on the standard curve by accurately adding 0.02-milliliter-increments of the appropriate standard stock solution to each disc, using a suitable pipette. Dry discs in circulating air or under vacuum. Discs may be stored for 2 weeks in a

desiccator under refrigeration. Depending on the antibiotic contained in the sample to be tested, prepare the stock solutions for the standard discs by dissolving an accurately weighed quantity

of the working standard in the solvent indicated to obtain stock solutions that will contain the following concentrations required for the standard discs:

Antibiotic	Solvent	Standard curve (antibiotic concentration per disc)
Ampicillin	Water	1.3, 2.4, 4.4, 8.1, 15.0µg.
Bacitracin	do	1.3, 2.4, 4.4, 8.1, 15.0 units.
Carbenicillin	Methyl alcohol	25.0, 35.5, 50.0, 70.7, 100µg.
Cefamandole (lithium)	50 percent methyl alcohol	5, 30, and 60 µgg.
Cefoxitin (sodium)	50 percent methyl alcohol	5, 30, 60 µg.
Cephalothin	50 percent methyl alcohol	15.0, 21.2, 30.3, 42.4, 60.0µg.
Chloramphenicol	do	3.3, 6.3, 12.2, 23.4, 45.0µg.
Clindamycin	do	1.0, 1.41, 2.0, 2.82, 4.0µg.
Colistin (sulfate)	Water	1.3, 2.4, 4.4, 8.1, 15.0µg.
Erythromycin	Methyl alcohol	1.3, 2.7, 5.4, 11.0, 2250µg.
Gentamicin (sulfate)	Water	5, 7.1, 10, 14.1, 20µg.
Kanamycin (sulfate)	do	3.3, 6.3, 12.2, 23.4, 45µg.
Methicillin	do	1.3, 2.4, 4.4, 8.1, 15.0µg.
Neomycin (sulfate)	do	3.3, 6.3, 12.2, 23.4, 45.0µg.
Novobiocin (sodium)	do	3.3, 6.3, 12.2, 23.4, 45.0µg.
Oleandomycin (phosphate)	do	1.3, 2.7, 5.4, 11.0, 22.5µg.
Penicillin G	do	1.3, 2.4, 4.4, 8.1, 15.0 units.
Polymyxin B (sulfate)	do	33, 63, 122, 234, 450 units.
Rifampin	Methyl alcohol	3.0, 6.0, 12.0, 24.0, 48.0µg.
Rifampin discs for use in culture media	do	12.5, 25, 50µg.
Streptomycin (sulfate)	Water	1.3, 2.4, 4.4, 8.1, 15.0µg.
Tetracycline (hydrochloride)	Methyl alcohol	3.3, 6.3, 12.2, 23.4, 45.0µg.
Tobramycin	Water	5, 10, and 20µg.
Vancomycin (hydrochloride)	do	3.3, 6.3, 12.2, 23.4, 45.0µg.

(e) Assay—(1) *Individual discs one-fourth inch in diameters*—(i) *Standard curves with five antibiotic concentrations.* On each of five plates prepared as directed in paragraph (c) of this section, place the five control discs for the standard curve and two discs from each batch to be tested. The control discs for the standard curve and the sample discs are placed on the plates in a random arrangement, with no discs being closer than 24 millimeters (on centers) to another disc. Discs are placed on the plates with the aid of forceps within as short a period of time as possible (not to exceed 3 minutes per plate) and tapped gently to ensure an even seal. Incubate the plates overnight at 32° C to 35° C, except if it is cephalothin, colistin, novobiocin, polymyxin, or viomycin, the incubation temperature is 37° C. After incubation, measure the diameter of each circle of inhibition, using calipers or a measuring device of comparable accuracy. Average the three zone sizes for each of the five standard-curve concentrations and plot the mean sizes on the arithmetic scale of semilogarithmic graph paper with the antibiotic concentrations on the

logarithmic scale. Use the following equation to calculate the best straight line:

$$L = (3a + 2b + c - e) / (5),$$

$$H = (3e + 2d + c - a) / (5),$$

where:

L = the calculated zone size of the low concentration;

H = the calculated zone size of the high concentration;

a, b, c, d, e = the observed average zone sizes for each respective concentration, *a* being that for the lowest concentration.

Plot the values obtained for *L* and *H* and connect these two points with a straight line. Average the six sample zone sizes and read the corresponding antibiotic concentration of this mean from the standard curve. This is the potency obtained for a single assay. Perform two or more replicate assays on each of 2 days. The average of all assays is the potency of the sample disc.

(ii) *Standard curves with three antibiotic concentrations.* On each of three plates prepared as directed in paragraph (c) of this section, place the three control discs for the standard

curve and two discs from each batch to be tested. The control discs for the standard curve and the sample discs are placed on the plates in a random arrangement, with no discs being closer than 24 millimeters (on centers) to any other discs. Discs are placed on the plates with the aid of forceps within as short a period of time as possible (not to exceed 3 minutes per plate) and tapped gently to ensure an even seal. Incubate the plates overnight at 32° C to 35° C, except if it is rifampin discs for use in culture media, the incubation temperature is 37° C. After incubation, measure the diameter of each circle of inhibition, using calipers or a measuring device of comparable accuracy. Average the three zone sizes for each of the three standard curve concentrations and plot the mean sizes on the arithmetic scale of semilogarithmic graph paper with the antibiotic concentrations on the logarithmic scale. Using the following equation to calculate the best straight line:

$$L = (5a + 2b - c) / (6),$$

$$H = (5c + 2b - a) / (6),$$

where:

- L* = calculated zone diameter of the lowest concentration of the standard curve;
- H* = calculated zone diameter of the highest concentration of the standard curve;
- a*, *b*, *c* = observed average zone sizes for each respective concentration, *a* being that for the lowest concentration.

Plot the values obtained for *L* and *H* and connect these two points with a straight line. Average the six sample zone sizes and read the corresponding antibiotic concentration of this mean from the standard curve. This is the potency obtained for a single assay. Perform two or more replicate assays on each of 2 days. The average of all assays is the potency of the sample disc.

(2) *Discs one-fourth inch in diameter attached to rings, spokes, or other devices.* Remove or cut the disc from the device, including a small portion of the device to which it is attached, before testing, and proceed as directed in paragraph (e)(1) of this section.

(3) *Individual discs with diameters larger than one-fourth inch but no larger than three-eighths inch for use in impregnating culture media.* Proceed as di-

rected in paragraph (e)(1) of this section, except instead of measuring the diameters of the zones of inhibition, measure the widths of the zones from any edge of the sample discs and the standard discs. The results obtained are multiplied by the factor 2 for determining whether the discs meet the requirements for uniformity prescribed by paragraph (f) of this section.

(f) The potency is satisfactory if the result obtained is not less than 67 percent and not more than 150 percent of that represented. The batch has a uniform potency if on the first or second test of six discs each, the diameter of the largest zone of inhibition is not more than 2.5 millimeters larger than the smallest zone, or if the number of zones that fall outside this range in three or more consecutive tests is not more than 10 percent of the total number of discs tested.

[39 FR 19181, May 30, 1974, as amended at 41 FR 7094, Feb. 17, 1976; 41 FR 53476, Dec. 7, 1976; 43 FR 9792, Mar. 10, 1978; 43 FR 12858, Mar. 28, 1978; 44 FR 10376, Feb. 20, 1979; 44 FR 20667, Apr. 6, 1979]

§ 460.11 Certification procedures for antibiotic elution susceptibility discs.

(a) *Requirements for certification—(1) Standards of identity, strength, quality, and purity.* Antibiotic elution susceptibility discs are round flat discs that have a diameter of 6.35 millimeters (¼ inch) and are made of absorbent paper containing antibiotic compounds. The identity of each disc is signified by means of an identifying sign. The absorbent paper and dye or ink used must not affect either bacterial growth or the antibiotic. Each disc shall have a potency that is equivalent to that contained in a standard disc prepared with the following quantities of antibiotic drugs:

- Ampicillin: 0.22 mcg.
- Ampicillin: 4.5 mcg.
- Bacitracin: 18.0 units.
- Carbenicillin: 120.0 mcg.
- Cephalothin: 15.0 mcg.
- Chloramphenicol: 4.0 mcg.
- Clindamycin: 2.0 mcg.
- Colistin: 13.0 mcg.
- Doxycycline: 0.5 mcg.
- Doxycycline: 1.6 mcg.
- Erythromycin: 2.5 mcg.
- Gentamicin: 9.0 mcg.