

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.

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§ 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.

Kanamycin: 22.0 mcg.
 Methicillin: 5.0 mcg.
 Neomycin: 24.0 mcg.
 Novobiocin: 2.5 mcg.
 Oleandomycin: 6.0 mcg.
 Penicillin: 0.2 unit.
 Streptomycin: 20.0 mcg.
 Tetracycline: 0.5 mcg.
 Tetracycline: 1.2 mcg.
 Tobramycin: 10.0 mcg.
 Vancomycin: 10.0 mcg.

The standard discs used to determine the potency shall be made of paper as described in §460.6(d). Each antibiotic compound used to impregnate such standard discs shall be equilibrated in terms of the working standard designated by the Commissioner for use in determining the potency or purity of such antibiotic.

(2) *Packaging.* The immediate container shall be a tight container as defined by the U.S.P. and shall be of such composition as will not cause any change in the strength, quality, or purity of the contents beyond any limit therefor in applicable standards, except that minor changes so caused that are normal and unavoidable in good packaging, storage, and distribution practice shall be disregarded. Each immediate container may contain a desiccant, and each may be packaged in combination with containers of suitable discs of drugs other than those described in paragraph (a)(1) of this section. Such other discs shall be suitable only if the manufacturer and packer have submitted to the Commissioner information of the kind described in §431.17 of this chapter, and such information has been accepted by the Commissioner.

(3) *Labeling.* Each package of discs shall bear on its label or labeling, as hereinafter indicated, the following:

(i) On the outside wrapper or container and the immediate container:

(a) The batch mark.

(b) The name and potency of each disc in the batch according to the following:

Name of disc and content of antibiotic in micrograms or units per disc

Ampicillin elution disc, 0.22 mcg.
 Ampicillin elution disc, 4.5 mcg.
 Bacitracin elution disc, 18.0 units.
 Carbenicillin elution disc, 120.0 mcg.
 Cephalothin elution disc, 15.0 mcg.
 Chloramphenicol elution disc, 4.0 mcg.

Clindamycin elution disc, 2.0 mcg.
 Colistin elution disc, 13.0 mcg.
 Doxycycline elution disc, 0.5 mcg.
 Doxycycline elution disc, 1.6 mcg.
 Erythromycin elution disc, 2.5 mcg.
 Gentamicin elution disc, 9.0 mcg.
 Kanamycin elution disc, 22.0 mcg.
 Neomycin elution disc, 24.0 mcg.
 Novobiocin elution disc, 2.5 mcg.
 Oleandomycin elution disc, 6.0 mcg.
 Penicillin elution disc, 0.2 unit.
 Methicillin elution disc, 5.0 mcg.
 Streptomycin elution disc, 20.0 mcg.
 Tetracycline elution disc, 0.5 mcg.
 Tetracycline elution disc, 1.2 mcg.
 Tobramycin elution disc, 10.0 mcg.
 Vancomycin elution disc, 10.0 mcg.

(c) The statement "Expiration date _____", the blank being filled in with the date that is 6 months after the month during which the batch was certified, except that the blank may be filled in with a date that is 12, 18, 24, 30, 36, 42, 48, 54, or 60 months after the month during which the batch was certified if the person who requests certification has submitted to the Commissioner results of tests and assays showing that such drugs as prepared by that person are stable for such longer period of time. If it is a packaged combination of discs of two or more drugs, its outside wrapper shall bear only one expiration date, and that date shall be the date that is required for the shortest dated discs contained in the package.

(d) The statement "FOR IN VITRO DIAGNOSTIC USE".

(ii) On the circular or other labeling within or attached to the package, adequate directions for the use of such discs.

(4) *Request for certification; samples.* (i) In addition to complying with the requirements of §431.1 of this chapter, a person who requests certification of a batch of antibiotic elution susceptibility discs shall submit with the request a statement showing the batch mark, the number of packages of each size in such batch, and the date on which the latest assay of the antibiotic used in making such batch was completed, the potency of each disc batch, the quantity of each ingredient used in making the batch, the date on which the latest assay of the drug constituting such batch was completed, and a statement that each ingredient used in

making the batch conforms to the requirements prescribed therefor by this section.

(ii) In connection with the request, such person shall submit results of the tests and assays made by him or her on an accurately representative sample of the batch for potency.

(iii) In connection with the request, such person shall submit an accurately representative sample of the batch, consisting of one disc for each 5,000 discs in the batch, but in no case collecting less than 100 discs. Single discs will be taken at regular intervals

throughout the entire time of packaging the batch.

(b) *Tests and methods of assay for potency of antibiotic elution susceptibility discs*—(1) *Preparation for assay.* Use culture media as directed in § 460.6(a).

(2) *Test organisms*—(i) *Culture of test organism suspensions.* For each test organism listed in the following table, select the appropriate medium (as listed in § 460.6(a)), incubation period of the Roux bottle, and suggested storage period under refrigeration for the particular test organism.

Test organism	Medium used for			
	Method used	Slants	Roux bottles	Storage ²
Suspension 1— <i>Staphylococcus aureus</i> (ATCC 29737) ¹	1	A	A	2 weeks.
Suspension 2— <i>Staphylococcus aureus</i> (ATCC 13150) ¹	1	A	A	1 week.
Suspension 3— <i>Pseudomonas aeruginosa</i> (ATCC 25619) ¹	6	J	J	1 week.
Suspension 4— <i>Klebsiella pneumoniae</i> (ATCC 10031) ¹	1	A	A	2 weeks.
Suspension 5— <i>Micrococcus luteus</i> (ATCC 9341) ¹	2	A	A	2 weeks.
Suspension 6— <i>Bordetella bronchiseptica</i> (ATCC 4617) ¹	1	A	A	2 weeks.
Suspension 7— <i>Streptococcus faecalis</i> (ATCC 14506) ¹	3	E	—	3 days.
Suspension 8— <i>Staphylococcus epidermidis</i> (ATCC 12228) ¹	4	A	—	3 days.
Suspension 9— <i>Bacillus subtilis</i> (ATCC 6633) ¹	5	A	B	1 year.

¹ Available from: American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852.
² Storage period under refrigeration.

(ii) *Methods of preparation of test organism suspensions*—(a) *Method 1.* Maintain organisms on agar slants containing 10 milliliters of the appropriate medium. Transfer organisms to fresh slants using an inoculating loop. Streak the fresh slants thoroughly. Incubate the slants for 24 hours at 37° C. Remove resulting growth from the agar slant with sterile glass beads. Transfer the cells onto a large agar surface, such as a Roux bottle, containing 250 milliliters of the appropriate medium. Spread the cells over the entire surface of the Roux bottle. Incubate the Roux bottle for 24 hours at 37° C. Wash the resulting growth from the agar surface with 50 milliliters of sterile U.S.P. saline test solution.

(b) *Method 2.* Proceed as directed in paragraph (b)(2)(ii)(a) of this section, except wash the growth from the surface of the Roux bottle with 20 milliliters sterile U.S.P. saline test solution.

(c) *Method 3.* Using an inoculation loop, transfer a portion of the growth on the slant to a culture tube contain-

ing 10 milliliters of sterile medium of the following composition:

- Calf brains, infusion from, 200.0 gm.
- Beef heart, infusion from, 250.0 gm.
- Proteose peptone, 10.0 gm.
- Dextrose, 2.0 gm.
- Sodium chloride, 5.0 gm.
- Sodium phosphate dibasic, 2.5 gm.
- Distilled water, q.s. pH 7.4 after sterilization, 1,000.0 ml.
- Incubate for 24 hours at 37° C.

(d) *Method 4.* Proceed as directed in paragraph (b)(2)(ii)(c) of this section, except transfer growth from the slant to a culture tube containing 50 milliliters of Medium D.

(e) *Method 5.* Proceed as directed in paragraph (b)(2)(ii)(a) of this section. Incubate the Roux bottle for 7 days at 37° C. Centrifuge the suspension at 3,500 RPM for 30 minutes. Decant the supernatant liquid. Resuspend the sediment in 50 milliliters sterile U.S.P. saline test solution. Heat-shock the suspension by placing in a 70° C. water bath for 30 minutes.

(f) *Method 6.* Proceed as directed in paragraph (b)(2)(ii)(b) of this section, except use 20 milliliters of Medium K.

(3)(i) *Preparation of plates.* Use volumes of appropriate media and plates as directed in § 460.6(c)(1) and (2).

(ii) *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	Medium		Suspension No.	Volume ³	Incub. temp.
	Base layer	Seed layer			
Ampicillin (.22 mcg)	E	A	1	1.5	32-35
Ampicillin (4.5 mcg)	A	L	2	¹ 1.0	32-35
B acitracin	A	L	2	¹ 1.0	32-35
Carbenicillin	A	L	3	5.0	37
Cephalothin	A	L	4	1.0	37
Chloramphenicol	A	L	5	1.0	32-35
Clindamycin	C	L	1	2.0	32-35
Colistin	G	L	6	0.3	37
Doxycycline	E	A	5	0.1	32-35
Erythromycin	C	L	7	0.2	37
Gentamicin	C	L	2	0.1	32-35
Kanamycin	A	L	1	¹ 1.0	32-35
Methicillin	A	L	2	0.1	32-35
Neomycin	C	L	8	6.0	32-35
Novobiocin	C	L	9	² 0.2	37
Oleandomycin	A	L	2	² 1.0	32-35
Penicillin	E	A	1	1.5	32-35
Streptomycin	C	L	1	0.1	32-35
Tetracycline	E	A	5	0.1	32-35
Tobramycin	C	C	2	0.1	32-35
Vancomycin	C	L	3	2.0	32-35

¹ Prepare a 1:100 dilution of the bulk suspension in sterile U.S.P. saline test solution. Use the indicated quantity of the diluted suspension to inoculate the seed medium.

² Prepare a 1:10 dilution of the bulk suspension in sterile U.S.P. saline test solution. Use the indicated quantity of the diluted suspension to inoculate the seed medium.

³ Suggested volume of suspension to be added to each 100 ml of seed agar.

(4) *Preparation of standard discs.* Depending on the concentration of antibiotic contained in the disc to be tested, prepare a stock solution for the standard disc by dissolving an accurately weighed quantity of the working standard in the solvent indicated to obtain an appropriate stock solution. Make further dilutions as required in the solvent indicated to obtain the following concentrations required on the standard discs:

Antibiotic	Solvent	Standard disc concentrations
Ampicillin (0.22 mcg.)	Water	0.1, 0.2, 0.4 mcg.
Ampicillin (4.5 mcg.)do	1, 5, 15 mcg.
Bacitracindo	5, 15, 30 units.
Carbenicillin	Methanol	24, 80, 240 mcg.
Cephalothin	50-percent methanol.	7.5, 20, 60 mcg.
Chloramphenicoldo	2, 4, 8 mcg.
Clindamycindo	1, 2, 4 mcg.
Colistin	Water	5, 12.5, 25 mcg.
Doxycycline	Methanol	0.25, 1, 3 mcg.
Erythromycindo	1.25, 5, 22.5 mcg.
Gentamicin	Water	5, 10, 20 mcg.
Kanamycindo	3, 15, 45 mcg.
Methicillindo	2.5, 5, 10 mcg.
Neomycindo	3, 15, 45 mcg.

Antibiotic	Solvent	Standard disc concentrations
Novobiocin	Methanol	1.25, 2.5, 5 mcg.
Oleandomycin	Water ¹	1.25, 5, 22.5 mcg.
Penicillindo	0.1, 0.2, 0.4 unit.
Streptomycindo	6.25, 25, 100 mcg.
Tetracycline	Methanol	0.25, 1, 3 mcg.
Tobramycin	Water	5, 10, 20 mcg.
Vancomycindo	3, 15, 45 mcg.

¹ If the chloroform adduct of oleandomycin is used as the standard, dissolve the weighing in absolute ethanol to a stock concentration of 10,000 micrograms per milliliter. Dilute this solution in water to achieve the working concentrations.

Use round, blank discs that conform to § 460.6(d). Place blank discs on aluminum or stainless steel wire mesh that is supported to allow circulation of air above and below the discs. Prepare the desired number of discs for each standard disc concentration by accurately adding 0.02-milliliter aliquots of the appropriate concentration of standard solution to each disc. Dry the discs in circulating air. Store standard discs under refrigeration in the presence of desiccant for a period not to exceed 2 weeks. Determine the stability of stored standard discs by assaying them

at daily and weekly intervals using freshly prepared standard disc for comparison.

(5) *Assay*—(i) *Individual discs*. On each of three plates prepared as directed in paragraph (b)(3) of this section, place standard disc and two or more discs from each batch to be tested. The standard disc and the sample discs are placed on the plates in a circular pattern with random arrangement, with no disc being closer than 24 millimeters (on centers) to any other disc. Discs are placed on the plates within as short a period of time as possible (not to exceed 3 minutes per plate) and tapped gently to ensure an even seal. After incubation as directed in paragraph (b)(3) of this section, measure the diameter of each circle of inhibition as accurately as possible. (In most cases, it is possible to estimate diameters to the nearest 0.1 millimeter).

(ii) *Estimation of potency*. Determine the logarithm of each dose of standard (x values) and the mean zone diameter for each dose of standard (y values). Using the three values of x and the three corresponding values of y, calculate Σx , Σx^2 , $(\Sigma x)^2$, Σy , and Σxy . Calculate the regression coefficient (slope, b) and the Y-intercept (a) of the standard response line by using the following equations:

$$b = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2}$$

$$a = \frac{\Sigma y - b\Sigma x}{n}$$

where n = the number of standard doses.

Determine the zone diameter (Y) for each sample disc being tested. Using the regression equation

$$X = \text{antilog} \frac{Y - a}{b}$$

calculate the concentration (X) for the mean response (Y) of the sample discs.

(6) *Potency*. The potency of the batch is satisfactory if the mean result obtained for the batch is not less than 85 percent and not more than 150 percent of that represented.

[45 FR 20668, Apr. 6, 1979]

§ 460.15 Streptomycin sulfate discs for use in culture media.

(a) *Requirements for certification*—(1) *Standards of identity, strength, quality, and purity*. Streptomycin sulfate discs for use in culture media are paper discs intended for impregnation of culture media in the sensitivity testing of mycobacteria. They conform to all requirements and to all procedures prescribed by § 460.1(a) for antibiotic sensitivity discs, except that each disc shall contain streptomycin sulfate equivalent to 10, 25, 50, or 500 micrograms of streptomycin.

(2) *Packaging*. It shall be packaged in accordance with the requirements of § 460.1(b).

(3) *Labeling*. In addition to complying with the requirements of § 460.1(c) of this chapter, the labeling shall also bear information indicating that the discs are for use in culture media for the sensitivity testing of mycobacteria and not for use in ordinary sensitivity disc plate tests.

(4) *Requests for certification; samples*. Requests for certification shall comply with § 460.1(d).

(b) *Tests and methods of assay; potency*. Proceed as directed in § 460.6 for the assay of streptomycin sulfate discs, except that:

(1) In the assay of streptomycin sulfate discs labeled to contain the equivalent of 10, 25, or 50 micrograms of streptomycin, the control discs shall be made to contain the equivalent of 6.25, 12.5, 25, 50, and 100 micrograms of streptomycin per disc.

(2) In the assay of streptomycin sulfate discs labeled to contain the equivalent of 500 micrograms of streptomycin:

(i) To each 100 milliliters of seed agar used for the test add 2.0 milliliters of suspension number 11.

(ii) The control discs shall be made to contain the equivalent of 50, 100, 200, 400, and 800 micrograms of streptomycin per disc.

§ 460.16 Rifampin discs for use in culture media.

(a) *Requirements for certification*—(1) *Standards of identity, strength, quality, and purity*. Rifampin discs for use in culture media are paper discs intended for impregnation of culture media in