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buffer wash. Extract the penicillin from the amyl acetate with a 10-milliliter aliquot of 1 percent potassium phosphate buffer, pH 6.0 (solution 1 as described in § 436.101). This is the assay solution.

(d) *Procedure for assay.* For the standard response line, use a total of 15 plates (three plates for each response line solution, except the reference concentration solution, which is included on each plate). On each set of three plates, fill three alternate cylinders with the reference concentration solution and the other three cylinders with the concentration of the response line under test. Thus, there will be 45 reference concentration zones of inhibition and nine zones of inhibition for each of the other concentrations of the response line. Treat a portion of the sample solution (2 to 5 milliliters) with 0.1 milliliter of penicillinase solution and incubate at 37° C. for 1 hour. For each sample tested, use three plates. On each plate fill two cylinders with the 0.050 unit of penicillin G per milliliter standard, two cylinders with the untreated sample, and two cylinders with the penicillinase-treated sample. Incubate all plates, including those of the standard response line, overnight at 30° C. A zone of inhibition with the untreated sample and no zone with the penicillinase-treated sample are a positive test for penicillin. If a positive test is obtained, measure the diameters of the zones of inhibition using an appropriate measuring device such as a millimeter rule, calipers, or an optical projector.

(e) *Estimation of penicillin G activity.* To prepare the standard response line, average the diameters of the standard reference concentration and average the diameters of the standard response line concentration tested for each set of three plates. Average also all 45 diameters of the reference concentration. The average of the 45 diameters of the reference concentration is the correction point of the response line. Correct the average diameter obtained for each concentration to the figure it would be if the average reference concentration diameter for that set of three plates were the same as the correction point. Thus, if in correcting the 0.025 penicillin G concentration, the average of the

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45 readings of the 0.050 unit of penicillin G-per-milliliter concentration is 18.5 millimeters and the average of the 0.050 unit of penicillin G-per milliliter concentration of this set of three plates is 18.3 millimeters, the correction is +0.2 millimeters. If the average reading of the 0.025 unit of penicillin G-per-milliliter concentration of these same three plates is 15.5 millimeters, the corrected value is 15.7 millimeters. Plot these corrected values, including the average of the 0.050 unit of penicillin G-per-milliliter concentration, on semilogarithmic graph paper using the penicillin concentration in units per milliliter on the logarithmic scale and the diameter of the zone of inhibition on the arithmetic scale. Draw the line of best fit through these points. To estimate the sample potency, average the zone diameters of the standard and the zone diameters of the sample on the three plates used. If the average zone diameter of the sample is lower than that of the standard, subtract the difference between them from the reference concentration diameter of the standard response line. From the response line, read the concentrations corresponding to these corrected values of zone diameters. Multiply the concentration by the dilution factor to obtain the units of penicillin G per sample size tested.

[39 FR 18944, May 30, 1974, as amended at 41 FR 34743, Apr. 17, 1976]

§ 436.105 Microbiological agar diffusion assay.

Using the sample solution prepared as described in the section for the particular antibiotic to be tested, proceed as described in paragraphs (a), (b), (c), and (d) of this section.

(a) *Preparation of inoculated plates.* For each antibiotic listed in the table in this paragraph, select the media (as listed by medium number in § 436.102(b)), the amount of media to be used in the base and seed layers, the test organism (as listed in § 436.103(a)), and the suggested inoculum and prepare the inoculated plates as follows: Prepare the base layer by adding the appropriate amount of melted agar to each Petri dish (nominal dimensions 20 by 100 millimeters). Distribute the agar

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evenly in each dish on a flat, level surface, placing a cover on each plate in turn; if a nonporous cover is used, leave it slightly ajar to prevent accumulation of condensed moisture from the hot agar base layer. After the agar hardens, seat the nonporous cover on each plate. To prepare the seed layer, add the suggested inoculum of the test organism suspension to a sufficient amount of agar, which has been melted and cooled to 48° C-50° C. Swirl the

flask to obtain a homogeneous suspension, and add the appropriate amount of the inoculated media to each of the plates containing the uninoculated base agar. Spread evenly over the agar surface, cover, and allow to harden on a flat, level surface. After the agar has hardened, place 6 cylinders described in § 436.100(a)(1) on the inoculated agar surface so that they are at approximately 60° intervals on a 2.8-centimeter radius.

Antibiotic	Media to be used (as listed by medium number in § 436.102(b))		Milliliters of media to be used in the base and seed layers		Test organism	Suggested volume of standardized inoculum to be added to each 100 milliliters of seed agar	Incubation Temperature for the plates
	Base layer	Seed layer	Base layer	Seed layer			
Amoxicillin	11	11	21	4	C	0.5	32-35
Amphotericin B	None	19	None	8	E	1.0	29-31
Ampicillin	11	11	21	4	C	0.5	32-35
Bacitracin	2	1	21	4	B	0.3	32-35
Bacitracin	2	1	21	4	L	0.3	32-35
Bleomycin	35	35	10	6	X	1.0	32-35
Carbenicillin	9	10	21	4	W	²0.5	36-37.5
Cefactor	2	1	21	5	A	0.05	36-37.5
Cefadroxil	2	1	21	4	A	0.05	36-37.5
Cefamandole	2	1	21	5	A	0.06	36-37.5
Cefazolin	2	1	21	4	A	0.05	32-35
Cefotaxime	2	1	21	5	A	0.1	36-37.5
Cefoxitin	2	1	21	5	A	0.1	36-37.5
Cephalexin	2	1	21	4	A	0.05	32-35
Cephaloglycin	2	1	21	4	A	0.2	32-35
Cephaloridine	2	1	21	4	A	0.1	32-35
Cephalothin	2	1	21	4	A	0.1	32-35
Cephapirin	2	1	21	4	A	0.08	32-35
Cephradine	2	1	21	4	A	0.05	32-35
Clindamycin	11	11	21	4	C	1.5	36-37.5
Cloxacillin	2	1	21	4	A	0.1	32-35
Colistimethate, sodium	9	10	21	4	F	0.1	36-37.5
Colistin	9	10	21	4	F	0.1	36-37.5
Cyclacillin	11	11	21	4	C	0.5	36-37.5
Dactinomycin	5	5	10	4	H	(¹)	36-37.5
Dicloxacillin	2	1	21	4	A	0.1	32-35
Dihydrostreptomycin	5	5	21	4	H	(¹)	36-37.5
Erythromycin	11	11	21	4	C	1.5	32-35
Gentamicin	11	11	21	4	D	0.03	36-37.5
Kanamycin B	5	5	21	4	H	(¹)	36-37.5
Methicillin	2	1	21	4	A	0.3	32-35
Mitomycin	8	8	10	4	H	0.5	36-37.5
Nafcillin	2	1	21	4	A	0.3	32-35
Natamycin	None	19	None	8	E	0.8	29-31
Neomycin	11	11	21	4	A	0.4	32-35
Neomycin	11	11	21	4	D	1.0	36-37.5
Netilmicin	11	11	20	5	D	0.25	36-37.5
Novobiocin	2	1	21	4	D	4.0	34-36
Nystatin	None	19	None	8	T	1.0	29-31
Oleandomycin	11	11	21	4	D	1.0	36-37.5
Oxacillin	2	1	21	4	A	0.3	32-35
Paromomycin	11	11	21	4	D	2.0	36-37.5
Penicillin G	2	1	21	4	A	1.0	32-35
Penicillin V	2	1	21	4	A	1.0	32-35
Plicamycin	8	8	10	4	A	0.1	32-35
Polymyxin B	9	10	21	4	F	0.1	36-37.5
Rifampin	2	2	21	4	H	0.1	29-31
Sisomicin	11	11	21	4	D	0.03	36-37.5
Streptomycin	5	5	21	4	H	(¹)	36-37.5
Ticarcillin	38	38	21	4	Y	1.5	36-37.5

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Antibiotic	Media to be used (as listed by medium number in § 436.102(b))		Milliliters of media to be used in the base and seed layers		Test organism	Suggested volume of standardized inoculum to be added to each 100 milliliters of seed agar	Incubation Temperature for the plates
	Base layer	Seed layer	Base layer	Seed layer			
Vancomycin	8	8	10	4	H	Milliliters (1)	Degrees C. 36-37.5

¹Determine the amount of the inoculum by the use of test plates.²Use dilution of the suspension that gives 25 percent light transmission in lieu of the stock suspension.

(b) *Preparation of working standard stock solutions and standard response line solutions.* For each antibiotic listed in the table in this paragraph, select the working standard drying conditions, solvent(s), concentrations, and storage time for the standard solutions and proceed as follows: If necessary, dry the working standard as described in § 436.200; dissolve and dilute an accurately weighed portion to the proper concentration to prepare the working

standard stock solution. Store the working standard stock solution under refrigeration and do not use longer than the recommended storage time. Further dilute an aliquot of the working standard stock solution to the proper concentrations to prepare the standard response line solutions. The reference concentration of the assay is the mid concentration of the response line.

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Antibiotic	Drying conditions (method number as listed in §436.200)	Working standard stock solutions				Storage time under refrigeration	Diluent	Standard response line concentrations
		Initial solvent	Diluent (solution number as listed in §436.101(a))	Final concentration units or milligrams per milliliter			
Amoxicillin	Not dried	Distilled water	1.0 mg	7 days	3 0.064, 0.080, 0.100, 0.125 and 0.156 µg. (Prepare the standard response line simultaneously with the sample solution.)
Amphotericin B	1	Dimethylsulfoxide	1 mg.	Use same day	10 0.64, 0.80, 1.00, 1.25, 1.56 µg. (Prepare the standard response line simultaneously with the sample solution.)
Ampicillin	Not dried	Distilled water	0.1	1 week	3 0.064, 0.080, 0.100, 0.125, 0.156 µg. (Prepare the standard response line simultaneously with the sample solution.)
Bacitracin zinc	1	0.01N HCl	100 units	Use same day	1 0.64, 0.80, 1.0, 1.25, 1.56 units.
Bleomycin	7	16	2 units	2 weeks	16 0.01, 0.02, 0.04, 0.08, 0.16 unit.
Carbenicillin	Not dried	1	1 mg	1 day	1 12.8, 16.0, 20.0, 25.0, 31.2 µg.
Cefaclor	Not dried	1	1 mg	Use same day	1 3.2, 4.0, 5.0, 6.25, 7.81 µg.
Cefadroxil	Not dried	3	1mg ^s	1 day	1 12.8, 16.0, 20.0, 25.0, and 31.2 µg.
Cefamandole	Not dried	10,000 µg per ml. in	1	1 mg	1 day	1 1.28, 1.60, 2.00, 2.50, 3.12 µg.
Cefazolin	Solution 6.	1	1 mg	5 days	1 0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cefotaxime	1	1	1 mg	Use same day	1 6.4, 8.0, 10, 12.5, 15.6 µg.
Cefoxitin	Not dried	1	1 mg	Use same day	1 12.8, 16.0, 20.0, 25, 31.2 µg.
Cephalexin	Not dried	Distilled water	100 µg	7 days	1 12.8, 16.0, 20.0, 25.0, 31.2 µg.
Cephalexolacin	1	1	1 mg	5 days	4 6.4, 8.0, 10, 12.5, 15.6 µg.
Cephaloridine	1	1	1 mg	5 days	1 6.4, 8.0, 10.0, 12.5, 15.6 µg.
Cephalothin	Not dried	1	1 mg	3 days	1 6.4, 8.0, 10.0, 12.5, 15.6 µg.
Cephradine	Not dried	1	1 mg	5 days	1 6.4, 8.0, 10.0, 12.5, 15.6 µg.
Clindamycin	Not dried	Distilled water	1 mg	1 month	3 6.4, 8.0, 10.0, 12.5, 15.6 µg.
Cloxacillin	Not dried	1	10,000 µg per ml. in	6	7 days	1 3.20, 4.00, 5.00, 6.25, 7.81 µg.
Colistimethate, sodium	1	10,000 µg per ml. in	6	Use same day	6 6.4, 8.0, 10.0, 12.5, 15.6 µg.
Colistin	1	distilled water	1 mg	2 weeks	6 0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cyclacillin	Not dried	Distilled water	1 mg	1 day	3 0.64, 0.80, 1.0, 1.25, 1.56 µg.
Dactinomycin	1	10,000 µg per ml. in	3	1 mg	3 months	(Prepare the standard response line simultaneously with the sample solution.)
Dicloxacillin	Not dried	1	1 mg	1 mg	7 days	3 0.50, 0.71, 1.00, 1.41, 2.00 µg.
Dihydrostreptomycin	5	3	1 mg	1 mg	30 days	1 3.20, 4.00, 5.00, 6.25, 7.81 µg.
								3 0.64, 0.80, 1.00, 1.25, 1.56 µg.

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Antibiotic	Drying conditions (method number as listed in §436.200)	Working standard stock solutions				Standard response line concentrations
		Initial solvent	Diluent (solution number as listed in §436.101(a))	Final concentration units or milligrams per milliliter	Storage time under refrigeration	
Erythromycin	1 3	10,000 µg. per ml. in methyl alcohol.	3 3	1 mg 1 mg	14 days 1 month	3 0.64, 0.80, 1.00, 1.25, 1.56 µg. 3 0.064, 0.080, 0.100, 0.125, 0.156 µg.
Gentamicin	Not dried	3	1 mg	1 month	3 0.64, 0.80, 1.00, 1.25, 1.56 µg.
Kanamycin B (use the kanamycin sulfate working standard).	Not dried	3	1 mg	4 days	1 6.4, 8.0, 10.0, 12.5, 15.6 µg.
Methicillin	Not dried	1	1 mg	14 days	1 0.50, 0.71, 1.0, 1.41, 2.0 µg.
Mitomycin	Not dried	1	1 mg	2 days	1 1.28, 1.60, 2.00, 2.50, 3.12 µg.
Nafcillin	Not dried	1 mg	Use same day	10 3.20, 4.00, 5.00, 6.25, 7.81 µg. (Prepare the standard response line solutions simultaneously with the sample solution to be tested using red low actinic glassware. Use solutions within 2 hours after preparation.)
Nalidixic acid	Not dried	3 0.64, 0.80, 1.00, 1.25, 1.56 µg. (If test organism D is used); 6.4, 8.0, 10.0, 12.5, 15.6 µg. (If test organism A is used), 0.064, 0.080, 0.100, 0.125, 0.156 µg.
Neomycin	1	3	1 mg	2 weeks	3 0.64, 0.80, 1.00, 1.25, 1.56 µg.
Neilmicin	Not dried ^a	3	1 mg	7 days	3 0.064, 0.080, 0.100, 0.125, 0.156 µg.
Novobiocin	5	10,000 µg. per ml. in absolute ethyl alcohol.	3	1 mg	5 days	6 0.320, 0.400, 0.500, 0.625, 0.781 µg.
Nystatin ¹⁰	4	Dimethylformamide	1,000 units ²	6 12.8, 16.0, 20.0, 25.0, 31.2 units. (Prepare the standard response line solutions simultaneously with the sample solution to be tested using red low actinic glassware.)
Oleandomycin	Not dried	10,000 µg. per ml. in ethyl alcohol.	3	1 mg	30 days	3 3.20, 4.00, 5.00, 6.25, 7.81 µg.
Oxacillin	Not dried	1	1 mg	3 days	1 3.20, 4.00, 5.00, 6.25, 7.81 µg. 3 0.64, 0.80, 1.00, 1.25, 1.56 µg.
Paromomycin	1	3	1000 units	3 weeks	1 0.64, 0.80, 1.00, 1.25, 1.56 units.
Penicillin G	Not dried	1	100 units	4 days	1 0.64, 0.80, 1.00, 1.25, 1.56 units.
Penicillin V Potassium	Not dried	1	0.1 mg	4 days	1 0.50, 0.7, 1.00, 1.41, 2.00 µg.
Plicamycin	7	Distilled water ³	6 10,000 units	2 weeks	6 6.4, 8.0, 10.0, 12.5, 15.6 units.
Polymyxin B	1	Distilled water ³	1 mg	1 day	1 3.20, 4.00, 5.00, 6.25, 7.81 µg.
Rifampin	Not dried	1	1 mg	30 days	3 0.64, 0.80, 1.00, 1.25, 1.56 µg.
Streptomycin	3

Sisomicin ⁶	Not dried ⁸	3	1 mg	14 days	3 0.064, 0.080, 0.100, 0.125,
Ticarcillin	Not dried	1	1 mg	1 day	1 0.156 µg.
Vancomycin	1	Distilled water	1 mg	1 week	4 3.20, 4.00, 5.00, 6.25, 7.81 µg. 4 6.4, 8.0, 10.0, 12.5, 15.6 µg.

¹ Further dilute aliquots of the working standard stock solution with dimethylsulfoxide to give concentrations of 12.8, 16, 20.0, 25, and 31.2 micrograms per milliliter.

² Further dilute aliquots of the working standard stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 units per milliliter.

³ Add 2 milliliters of distilled water for each 5 milligrams of weighed working standard material.

⁴ Further dilute aliquots of the working standard stock solution with dimethylformamide to give concentrations of 64, 80, 100, 125, and 156 micrograms per milliliter.

⁵ The final concentration of the working standard stock solution is allowed to hydrolyze in a 37° C. constant temperature water bath for 60 minutes.

⁶ Working standard should be stored below minus 20° C under an atmosphere of nitrogen. Sisomicin is hygroscopic and care should be exercised during weighing.

⁷ Further dilute aliquots of the working standard stock solution with dimethylsulfoxide to give concentrations 64.0, 80.0, 100, 125, and 156 micrograms per milliliter.

⁸ Weigh a separate portion of the working standard and determine the loss on drying by the method described in § 436.200(c) of this chapter. Use this value to determine the anhydrous content of the working standard.

⁹ Working standard should be stored below minus 10° C under an atmosphere of nitrogen. Netilmicin sulfate is hygroscopic and care should be exercised during weighing.

¹⁰ For assay of nystatin pastilles, use 80 percent aqueous dimethylformamide as the initial solvent and as diluent for all dilutions where dimethylformamide is required.

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(c) *Procedure for assay.* For the standard response line, use a total of 12 plates—three plates for each response line solution, except the reference concentration solution which is included on each plate. On each set of three plates, fill three alternate cylinders with the reference concentration solution and the other three cylinders with the concentration of the response line under test. Thus, there will be 36 reference concentration zones of inhibition and nine zones of inhibition for each of the four other concentrations of the response line. For each sample tested use three plates. Fill three alternate cylinders on each plate with the standard reference concentration solution and the other three cylinders with the sample reference concentration solution. After all the plates have incubated for 16 to 18 hours at the appropriate incubation temperature for each antibiotic listed in the table in paragraph (b) of this section, measure the diameters of the zones of inhibition using an appropriate measuring device such as a millimeter rule, calipers, or an optical projector.

(d) *Estimation of potency.* To prepare the standard response line, average the diameters of the standard reference concentration and average the diameters of the standard response line concentration tested for each set of three plates. Average also all 36 diameters of the reference concentration for all four sets of plates. The average of the 36 diameters of the reference concentration is the correction point of the response line. Correct the average diameter obtained for each concentration to the figure it would be if the average reference concentration diameter for that set of three plates were the same as the correction point. Thus, if in correcting the highest concentration of the response line, the average of the 36 diameters of the reference concentration is 16.5 millimeters and the average of the reference concentration of the set of three plates (the set containing the highest concentration of the response line) is 16.3 millimeters, the correction is +0.2 millimeter. If the average reading of the highest concentration of the response line of these same three plates is 16.9 millimeters, the corrected diameter is then 17.1 millimeters. Plot these

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corrected diameters, including the average of the 36 diameters of the reference concentration on 2-cycle semilog paper, using the concentration of the antibiotic in micrograms or units per milliliter as the ordinate (the logarithmic scale), and the diameter of the zone of inhibition as the abscissa. The response line is drawn either through these points by inspection or through points plotted for highest and lowest zone diameters obtained by means of the following equation:

$$L = \frac{3a + 2b + c - e}{5}$$

$$H = \frac{3e + 2d + c - a}{5}$$

where:

L=Calculated zone diameter for the lowest concentration of the standard response line;

H=Calculated zone diameter for the highest concentration of the standard response line;

c=Average zone diameter of 36 readings of the reference point standard solution;

a, b, d, e=Corrected average values for the other standard solutions, lowest to highest concentration, respectively.

To estimate the potency of the sample, average the zone diameters of the standard and the zone diameters of the sample on the three plates used. If the average zone diameter of the sample is larger than that of the standard, add the difference between them to the reference concentration diameter of the standard response line. If the average zone diameter of the sample is lower than that of the standard, subtract the difference between them from the reference concentration diameter of the standard response line. From the response line, read the concentrations corresponding to these corrected values of zone diameters. Multiply the concentration by the appropriate dilution factor to obtain the antibiotic content of the sample.

[39 FR 18944, May 30, 1974]

EDITORIAL NOTE: For FEDERAL REGISTER citations affecting § 436.105, see the List of CFR Sections Affected appearing in the Finding Aids section of this volume.