BRIEFING

⟨**81**⟩ **Antibiotics—Microbial Assays**, *USP 32* page 86. On the basis of comments received from the Monograph Development—Antibiotics Expert Committee and USP staff, this general chapter was revised by the General Chapter ⟨81⟩ Advisory Panel and endorsed by the Monograph Development—Antibiotics Expert Committee. The Advisory Panel consisted of representatives from the Food and Drug Administration as well as from pharmaceutical and related industries in the United States and overseas.

This proposal updates the entire chapter so that it reflects current industry practices. The major proposed revisions are as follows:

- The chapter was redesigned and the contents were reorganized for clarity.
- The tables were updated to delete references to 1) antibiotics for which the monograph specifies an HPLC procedure and 2) those for which there is no official *USP* monograph. The contents of all tables were sorted by antibiotic name for ease of use.
- The *Calculations* section was expanded to provide details about the interpretation of data. In an effort to make the chapter a more inclusive public standard, references to *Design and Analysis of Biological Assays* ⟨111⟩ were eliminated. Sample calculations were included in the chapter to provide guidance. In the future, USP intends to provide an online compendial tool that may be used to evaluate the data generated from microbial assay procedures.
- Because Federal Master Standards are no longer available, references to them were updated.
- A statement was added to indicate that the procedures should be performed under aseptic conditions.
- Additional information was provided about the maintenance of microorganisms for the assays.

Interested parties are invited to comment on the proposal.

NOTE—Because of the large number of crossed-off pages, the pages in the print version of *PF* 36(5) represent only the added proposed revision of ⟨81⟩. The complete text of this proposal is available on the USP website (www.usp.org) and in the online electronic version of *PF* 36(5).

(GCM: A. Wise; R. Tirumalai) RTS—C70647

⟨81⟩ ANTIBIOTICS—MICROBIAL ASSAYS

Change to read:

~~The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms. A reduction in antimicrobial activity also will reveal subtle changes not demonstrable by chemical methods. Accordingly, microbial or biological assays remain generally the standard for resolving doubt with respect to possible loss of activity. This chapter summarizes these procedures for the antibiotics recognized in this Pharmacopeia for which microbiological assay remains the definitive method.~~

~~Two general methods are employed, the cylinder-plate or "plate" assay and the turbidimetric or "tube" assay. The first depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a petri dish or plate to an extent such that growth of the added microorganism is prevented entirely in a circular area or "zone" around the cylinder containing a solution of the antibiotic. The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.~~

~~APPARATUS~~

~~All equipment is to be thoroughly cleaned before and after each use. Glassware for holding and transferring test organisms is sterilized by dry heat or by steam.~~

~~Temperature Control~~

~~Thermostatic control is required in several stages of a microbial assay, when culturing a microorganism and preparing its inoculum, and during incubation in plate and tube assays. Maintain the temperature of assay plates at ±0.5° of the temperature selected. Closer control of the temperature (±0.1° of the selected temperature) is imperative during incubation in a tube assay, and may be achieved in either circulated air or water, the greater heat capacity of water lending it some advantage over circulating air.~~

~~Spectrophotometer~~

~~Measuring transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength of the light source can be varied or restricted by the use of a 580-nm filter or a 530-nm filter for reading the absorbance in a tube assay. For the latter purpose, the instrument may be arranged to accept the tube in which incubation takes place (see *Turbidimetric Assay Receptacles*), to accept a modified cell fitted with a drain that facilitates rapid change of content, or preferably, fixed with a flow-through cell for a continuous flow-through analysis; set the instrument at zero absorbance with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test solution and formaldehyde as found in each sample.~~

~~NOTE—Either absorbance or transmittance measurement may be used for preparing inocula.~~

~~Cylinder Plate Assay Receptacles~~

~~For assay plates, use glass or plastic petri dishes (approximately 20 × 100 mm) having covers of suitable material. For assay cylinders, use stainless steel or porcelain cylinders with the following dimensions, each dimension having a tolerance of ±0.1 mm: outside diameter 8 mm; inside diameter 6 mm; and length 10 mm. Carefully clean cylinders to remove all residues. An occasional acid bath, e.g., with about 2 N nitric acid or with chromic acid (see *Cleaning Glass Apparatus* ⟨1051⟩) is needed.~~

~~Turbidimetric Assay Receptacles~~

~~For assay tubes, use glass or plastic test tubes, e.g., 16 × 125 mm or 18 × 150 mm that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. Tubes that are to be placed in the spectrophotometer are matched and are without scratches or blemishes. Cleanse thoroughly to remove all antibiotic residues and traces of cleaning solution, and sterilize tubes that have been used previously, before subsequent use.~~

MEDIA AND DILUENTS

Media

The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients, or reconstituted dehydrated media, may be substituted, provided the resulting media possess equal or better growth promoting properties and give a similar standard curve response.

Dissolve the ingredients in water to make 1 L, and adjust the solutions with either 1 N sodium hydroxide or 1 N hydrochloric acid as required, so that after steam sterilization the pH is as specified.

MEDIUM 1

Peptone	6.0 g
Pancreatic Digest of Casein	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 6.6 ± 0.1.

MEDIUM 2

Peptone	6.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 6.6 ± 0.1.

MEDIUM 3

Peptone	5.0 g
Yeast Extract	1.5 g
Beef Extract	1.5 g
Sodium Chloride	3.5 g
Dextrose	1.0 g
Dibasic Potassium Phosphate	3.68 g
Monobasic Potassium Phosphate	1.32 g
Water	1000 mL

pH after sterilization: 7.0 ± 0.05.

MEDIUM 4

Same as *Medium 2,* except for the additional ingredient 1.0 g of Dextrose.

MEDIUM 5

Same as *Medium 2,* except that the final pH after sterilization is 7.9 ± 0.1.

MEDIUM 8

Same as *Medium 2,* except that the final pH after sterilization is 5.9 ± 0.1.

MEDIUM 9

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose	2.5 g
Agar	20.0 g
Water	1000 mL

pH after sterilization: 7.2 ± 0.1.

MEDIUM 10

Same as *Medium 9,* except to use 12.0 g of Agar instead of 20.0 g, and to add 10 mL of Polysorbate 80 after boiling the medium to dissolve the agar.

pH after sterilization: 7.2 ± 0.1.

MEDIUM 11

Same as *Medium 1,* except that the final pH after sterilization is 8.3 ± 0.1.

MEDIUM 13

Dextrose	20.0 g
Peptone	10.0 g
Water	1000 mL

pH after sterilization: 5.6 ± 0.1.

MEDIUM 19

Peptone	9.4 g
Yeast Extract	4.7 g
Beef Extract	2.4 g
Sodium Chloride	10.0 g
Dextrose	10.0 g
Agar	23.5 g
Water	1000 mL

pH after sterilization: 6.1 ± 0.1.

MEDIUM 32

Same as *Medium 1,* except for the additional ingredient 0.3 g of Manganese Sulfate.

MEDIUM 34

Glycerol	10.0 g
Peptone	10.0 g
Beef Extract	10.0 g

Sodium Chloride	3.0 g
Water	1000 mL

pH after sterilization: 7.0 ± 0.1.

MEDIUM 35

Same as *Medium 34*, except for the additional ingredient 17.0 g of Agar.

MEDIUM 36

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.1.

MEDIUM 39

Same as *Medium 3*, except that the final pH after sterilization is 7.9 ± 0.1.

MEDIUM 40

Yeast Extract	20.0 g
Polypeptone	5.0 g
Dextrose	10.0 g
Monobasic Potassium Phosphate	2.0 g
Polysorbate 80	0.1 g
Agar	10.0 g
Water	1000 mL

pH after sterilization: 6.7 ± 0.2.

MEDIUM 41

Pancreatic Digest of Casein	9.0 g
Dextrose	20.0 g
Yeast Extract	5.0 g
Sodium Citrate	10.0 g
Monobasic Potassium Phosphate	1.0 g
Dibasic Potassium Phosphate	1.0 g
Water	1000 mL

pH after sterilization: 6.8 ± 0.1.

Phosphate Buffers and Other Solutions

Prepare as follows, or by other suitable means, the potassium phosphate buffers required for the antibiotic under assay. The buffers are sterilized after preparation, and the pH specified in each case is the pH after sterilization.

BUFFER NO. 1, 1 PERCENT, PH 6.0 —Dissolve 2.0 g of dibasic potassium phosphate and 8.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of 6.0 ± 0.05.

BUFFER NO. 3, 0.1 M, PH 8.0 —Dissolve 16.73 g of dibasic potassium phosphate and 0.523 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of 8.0 ± 0.1.

BUFFER NO. 4, 0.1 M, PH 4.5 —Dissolve 13.61 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of 4.5 ± 0.05.

BUFFER NO. 6, 10 PERCENT, PH 6.0 —Dissolve 20.0 g of dibasic potassium phosphate and 80.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of 6.0 ± 0.05.

BUFFER NO. 10, 0.2 M, PH 10.5 —Dissolve 35.0 g of dibasic potassium phosphate in 1000 mL of water, and add 2 mL of 10 N potassium hydroxide. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of 10.5 ± 0.1.

BUFFER NO. 16, 0.1 M, PH 7.0 —Dissolve 13.6 g of dibasic potassium phosphate and 4.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to a pH of 7.0 ± 0.2.

OTHER SOLUTIONS —Use the substances specified under *Reagents, Indicators, and Solutions*. For water, use *Purified Water*. For saline, use *Sodium Chloride Injection*. Dilute formaldehyde is *Formaldehyde Solution* diluted with water 1 : 3.

UNITS AND REFERENCE STANDARDS

The potency of antibiotics is designated in either "Units" or "μg" of activity. In each case the "Unit" or "μg" of antibiotic activity is established and defined by the designated federal master standard for that antibiotic. The corresponding USP Reference Standard is calibrated in terms of the master standard. USP Reference Standards for antibiotic substances are held and distributed by the U.S. Pharmacopeial Convention, Inc.

The concept of "μg" of activity originated from the situation where the antibiotic preparation selected as the reference standard was thought to consist entirely of a single chemical entity and was therefore assigned a potency of 1000 "μg" per mg. In several such instances, as a result of the development of manufacturing and purification methods for particular antibiotics, preparations became available that contained more than 1000 "μg" of activity per mg. It was then understood that such preparations had an activity equivalent to a given number of "μg" of the original reference standard. In most instances, however, the "μg" of activity is exactly equivalent numerically to the μg (weight) of the pure substance. Complications arise in some situations, e.g., where an antibiotic exists as the free base and in salt form, and the "μg" of activity has been defined in terms of one such form; where the antibiotic substance consists of a number of components having close chemical similarity but differing antibiotic activity; or where the potencies of a family of antibiotics are expressed in terms of a reference standard consisting of a single member which, however, might itself be heterogeneous. In such cases the "μg" of activity defined in terms of a "Master Standard" is tantamount to a "Unit." The "μg" of activity should therefore not be assumed necessarily to correspond to the μg (weight) of the antibiotic substance.

PREPARATION OF THE STANDARD

To prepare a stock solution, dissolve a quantity of the USP Reference Standard of a given antibiotic, accurately weighed, or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in that table, and then dilute to the required concentration as indicated. Store in a refrigerator, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1 : 1.25 for a cylinder-plate assay or smaller for a turbidimetric assay. Use the final diluent specified and a sequence such that the middle or median has the concentration designated.

PREPARATION OF THE SAMPLE

From the information available for the preparation to be assayed (the "Unknown"), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic but with the same final diluent as used for the USP Reference Standard. The assay with five levels of the Standard requires only one level of the Unknown at a concentration assumed equal to the median level of the Standard.

ORGANISMS AND INOCULUM

Test Organisms

The test organism for each antibiotic is listed in *Table 2*, together with its identification number in the American Type Culture Collection. The method of assay is given for each in *Table 1*. Maintain a culture on slants of the medium and under the incubation conditions specified in *Table 3*, and transfer weekly to fresh slants. For *K. pneumoniae* use a noncapsulated culture. For *Enterococcus hirae,* stab cultures may be used.

Table 1. Preparation of Stock Solutions and Test Dilutions of Reference Standards

	Stock Solution			Test Dilution	
Antibiotic and Type of Assay [Cylinder-plate (CP) or Turbidimetric (T)]	Initial Solvent (and initial concentration where specified); Further Diluent, if different	Final Stock Concentration per mL	Use Within	Final Diluent	Median Dose (µg of activity or Units per mL)
Amikacin (T)	Water	1 mg	14 days	Water	10 µg
Amphotericin B (CP)	Dimethyl sulfoxide	1 mg	Same day	*B.* 10	1.0 µg
Bacitracin Zinc (CP)	0.01 N hydrochloric acid	100 U	Same day	*B.* 1	1.0 U
Bleomycin (CP)	*B.* 16	2 U	14 days	*B.* 16	0.04 U
Candicidin (T)	Dimethyl sulfoxide	1 mg	Same day	Water	0.06 µg
Capreomycin (T)	Water	1 mg	7 days	Water	100 µg
Carbenicillin (CP)	*B.* 1	1 mg	14 days	*B.* 1	20 µg
Cephalothin (CP)	*B.* 1	1 mg	5 days	*B.* 1	1.0 µg
Cephapirin (CP)	*B.* 1	1 mg	3 days	*B.* 1	1.0 µg
Chloramphenicol (T)	Alcohol (10 mg/mL); [Water]	1 mg	30 days	Water	2.5 µg
Chlortetracycline (T)	0.01 N hydrochloric acid	1 mg	4 days	Water	0.06 µg
Cloxacillin (CP)	*B.* 1	1 mg	7 days	*B.* 1	5.0 µg
Colistimethate Sodium (CP)	Water (10 mg/mL); [*B.* 6]	1 mg	Same day	*B.* 6	1.0 µg
Colistin (CP)	Water (10 mg/mL); [*B.* 6]	1 mg	14 days	*B.* 6	1.0 µg
Cycloserine (T)	Water	1 mg	30 days	Water	50 µg
Demeclocycline (T)	0.1 N hydrochloric acid	1 mg	4 days	Water	0.1 µg
Dihydrostreptomycin (CP)	*B.* 3	1 mg	30 days	*B.* 3	1.0 µg
Dihydrostreptomycin (T)	Water	1 mg	30 days	Water	30 µg
Doxycycline (T)	0.1 N hydrochloric acid	1 mg	5 days	Water	0.1 µg
Erythromycin (CP)	Methanol (10 mg/mL); [*B.* 3]	1 mg	14 days	*B.* 3	1.0 µg
Gentamicin (CP)	*B.* 3	1 mg	30 days	*B.* 3	0.1 µg
Gramicidin (T)	Alcohol 95%	1 mg	30 days	Alcohol 95%	0.04 µg
Kanamycin (T)	Water	1 mg	30 days	Water	10 µg
Methacycline (T)	Water	1 mg	7 days	Water	0.06 µg
Nafcillin (CP)	*B.* 1	1 mg	2 days	*B.* 1	2.0 µg
Natamycin (CP)	Dimethyl sulfoxide	1 mg	Same day	*B.* 10	5.00 µg
Neomycin (CP)	*B.* 3	1 mg	14 days	*B.* 3	1.0 µg
Neomycin (T)	*B.* 3	100 µg	14 days	*B.* 3	1.0 µg
Netilmicin (CP)	*B.* 3	1 mg	7 days	*B.* 3	0.1 µg
Novobiocin (CP)	Alcohol (10 mg/mL); [*B.* 3]	1 mg	5 days	*B.* 6	0.5 µg
Nystatin (CP)	Dimethylformamide	1,000 U	Same day	*B.* 6	20 U
Oxytetracycline (T)	0.1 N hydrochloric acid	1 mg	4 days	Water	0.24 µg
Paromomycin (CP)	*B.* 3	1 mg	21 days	*B.* 3	1.0 µg
Penicillin G (CP)	*B.* 1	1,000 U	4 days	*B.* 1	1.0 U
Polymyxin B (CP)	Water; [*B.* 6]	10,000 U	14 days	*B.* 6	10 U
Rolitetracycline (T)	Water	1 mg	1 day	Water	0.24 µg
Sisomicin (CP)	*B.* 3	1 mg	14 days	*B.* 3	0.1 µg
Streptomycin (T)	Water	1 mg	30 days	Water	30 µg
Tetracycline (T)	0.1 N hydrochloric acid	1 mg	1 day	Water	0.24 µg
Thiostrepton (T)	Dimethyl sulfoxide	1 U	Same day	Dimethyl sulfoxide	0.80 U
Ticarcillin (CP)	*B.* 1	1 mg	1 day	*B.* 1	5.0 µg
Tobramycin (T)	Water	1 mg	14 days	Water	2.5 µg

Table 1. Preparation of Stock Solutions and Test Dilutions of Reference Standards *(Continued)*

Antibiotic and Type of Assay [Cylinder-plate (CP) or Turbidimetric (T)]	Stock Solution			Test Dilution	
	Initial Solvent (and initial concentration where specified); Further Diluent, if different	Final Stock Concentration per mL	Use Within	Final Diluent	Median Dose (µg of activity or Units per mL)
Troleandomycin (T)	Isopropyl alcohol–water (4 : 1)	1 mg	Same day	Water	25 µg
Tylosin (T)	Methanol (10 mg/mL); [*B.* 16]	1 mg	30 days	*B.*3: methanol (1 : 1)	4 µg
Vancomycin (CP)	Water	1 mg	7 days	*B.* 4	10 µg

NOTES—"*B*" denotes "buffer," and the number following refers to the potassium phosphate buffers defined in this chapter.

For amphotericin B, colistimethate sodium, and nystatin, prepare the USP Reference Standard solutions and the sample test solution simultaneously.

For amphotericin B, further dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8, 16, 20, 25, and 31.2 µg per mL prior to making the test dilutions. The *Test Dilution* of the sample should contain the same amount of dimethyl sulfoxide as the test dilutions of the USP Reference Standard.

For bacitracin zinc, each of the Standard test dilutions should contain the same amount of hydrochloric acid as the *Test Dilution* of the sample.

For neomycin turbidimetric assay, dilute the 100 µg per mL stock solution quantitatively with *Buffer No. 3* to obtain a solution having a concentration equivalent to 25.0 µg of neomycin per mL. To separate 50 mL volumetric flasks add 1.39, 1.67, 2.00, 2.40, and 2.88 mL of this solution, add 5.0 mL of 0.01 N hydrochloric acid to each flask, dilute with *Buffer No. 3* to volume, and mix to obtain solutions having concentrations of 0.69, 0.83, 1.0, 1.2, and 1.44 µg of neomycin per mL. Use these solutions to prepare the standard response line.

For nystatin, further dilute the stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 Units per mL prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilutions of the sample to be tested. The *Test Dilution* of the sample should contain the same amount of dimethylformamide as the test dilutions of the Standard. Use red low-actinic glassware.

For Polymyxin B, prepare the stock solution by adding 2 mL of water for each 5 mg of the weighed USP Reference Standard material.

Table 2. Test Organisms for Antibiotics Assayed by the Procedure Indicated in Table 1

Antibiotic	Test Organism	ATCC* Number
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Candicidin	*Saccharomyces cerevisiae*	9763
Capreomycin	*Klebsiella pneumoniae*	10031
Carbenicillin	*Pseudomonas aeruginosa*	25619
Cephalothin	*Staphylococcus aureus*	29737
Cephapirin	*Staphylococcus aureus*	29737
Chloramphenicol	*Escherichia coli*	10536
Chlortetracycline	*Staphylococcus aureus*	29737
Cloxacillin	*Staphylococcus aureus*	29737
Colistimethate Sodium	*Bordetella bronchiseptica*	4617
Colistin	*Bordetella bronchiseptica*	4617
Cycloserine	*Staphylococcus aureus*	29737
Demeclocycline	*Staphylococcus aureus*	29737
Dihydrostreptomycin (CP)	*Bacillus subtilis*	6633
Dihydrostreptomycin (T)	*Klebsiella pneumoniae*	10031
Doxycycline	*Staphylococcus aureus*	29737
Erythromycin	*Micrococcus luteus*	9341
Gentamicin	*Staphylococcus epidermidis*	12228
Gramicidin	*Enterococcus hirae*	10541
Kanamycin	*Staphylococcus aureus*	29737

Table 2. Test Organisms for Antibiotics Assayed by the Procedure Indicated in Table 1 *(Continued)*

Antibiotic	Test Organism	ATCC* Number
Methacycline	*Staphylococcus aureus*	29737
Nafcillin	*Staphylococcus aureus*	29737
Neomycin (CP)	*Staphylococcus epidermidis*	12228
Neomycin (T)	*Klebsiella pneumoniae*	10031
Netilmicin	*Staphylococcus epidermidis*	12228
Novobiocin	*Staphylococcus epidermidis*	12228
Nystatin	*Saccharomyces cerevisiae*	2601
Oxytetracycline	*Staphylococcus aureus*	29737
Paromomycin	*Staphylococcus epidermidis*	12228
Penicillin G	*Staphylococcus aureus*	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Rolitetracycline	*Staphylococcus aureus*	29737
Sisomicin	*Staphylococcus epidermidis*	12228
Spectinomycin	*Escherichia coli*	10536
Streptomycin (T)	*Klebsiella pneumoniae*	10031
Tetracycline	*Staphylococcus aureus*	29737
Thiostrepton (T)	*Enterococcus hirae*	10541
Tobramycin	*Staphylococcus aureus*	29737
Troleandomycin	*Klebsiella pneumoniae*	10031
Tylosin	*Staphylococcus aureus*	9144
Vancomycin	*Bacillus subtilis*	6633

* American Type Culture Collection, 10801 University Boulevard, Manassas VA 20110-2209. (http://www.atcc.org).

Table 3. Preparation of Inoculum

Test Organism & (ATCC No.)	Incubation Conditions: Medium	Incubation Conditions: Temp. (°)	Incubation Conditions: Time	Suggested Inoculum Composition: Medium	Suggested Inoculum Composition: Amount (mL per 100 mL)	Antibiotics Assayed
Bacillus subtilis (6633)	32	32 to 35	5 days	5	As required	Dihydrostreptomycin
				8	As required	Vancomycin
Bordetella bronchiseptica (4617)	1	32 to 35	24 hr.	10	0.1	Colistimethate Sodium, Colistin, Polymyxin B
Escherichia coli (10536)	1	32 to 35	24 hr.	3	0.7	Chloramphenicol
Klebsiella pneumoniae (10031)	1	36 to 37.5	16 to 24 hr.	3	0.05	Capreomycin
					0.1	Streptomycin, Troleandomycin, Dihydrostreptomycin
				39	2	Neomycin
Micrococcus luteus (9341)	1	32 to 35	24 hr.	11	1.5	Erythromycin
Micrococcus luteus (10240)	1	32 to 35	24 hr.	1	0.3	Bacitracin
Mycobacterium smegmatis (607)	36	36 to 37.5	48 hr.	35	1.0	Bleomycin
Pseudomonas aeruginosa (25619)	1	36 to 37.5	24 hr.	10	0.5	Carbenicillin
Saccharomyces cerevisiae (9763)	19	29 to 31	48 hr.	13	0.2	Candicidin
				19	1.0	Amphotericin B
Saccharomyces cerevisiae (2601)	19	29 to 31	48 hr.	19	1.0	Nystatin
Staphylococcus aureus (9144)	3	35 to 39	16 to 18 hr.	39	2 3	Tylosin
Staphylococcus aureus (29737)	1	32 to 35	24 hr.	1	0.1	Cephalothin, Cephapirin, Cloxacillin
				1	0.3	Nafcillin
				1	1.0	Penicillin G
				3	0.1	Amikacin, Chlortetracycline, Demeclocycline, Doxycycline, Methacycline, Oxytetracycline, Rolitetracycline, Tetracycline
				3	0.2	Kanamycin
				3	0.4	Cycloserine
				3	0.15	Tobramycin
Staphylococcus epidermidis (12228)	1	32 to 35	24 hr.	11	0.25	Netilmicin
				1	4.0	Novobiocin
				11	0.03	Gentamicin, Sisomicin
				11	0.4	Neomycin
				11	2.0	Paromomycin
Enterococcus hirae (10541)	3	36 to 37.5	16 to 18 hr.	3	1.0	Gramicidin
	40	36 to 37.5	18 to 24 hr.	41	0.2	Thiostrepton

NOTE—For *Pseudomonas aeruginosa* (ATCC 25619) in the assay of Carbenicillin, use 0.5 mL of a 1 : 25 dilution of the stock suspension per 100 mL of *Medium 10*.

Preparation of Inoculum

Preparatory to an assay, remove the growth from a recently grown slant or culture of the organism, with 3 mL of sterile saline TS and sterile glass beads. Inoculate the surface of 250 mL of the agar medium specified for that organism in *Table 3* and contained on the flat side of a Roux bottle except in the case of *Enterococcus hirae* and *Staphylococcus aureus* (ATCC 9144), which are grown in a liquid medium. Spread the suspension evenly over the surface of the agar with the aid of sterile glass beads, and incubate at the temperature shown for approximately the indicated length of time. At the end of this period, prepare the stock suspension by collecting the surface growth in 50 mL of sterile saline TS, except for Bleomycin (use 50 mL of *Medium 34*).

Determine by trial the quantity of stock suspension to be used as the *Inoculum*, starting with the volume suggested in *Table 3*. The trial tests should be incubated for the times indicated in the section *Turbidimetric Method* for *Procedure*. Adjust the quantity of *Inoculum* on a daily basis, if necessary, to obtain the optimum dose-response relationship from the amount of growth of the test organism in the assay tubes and the length of the time of incubation. At the completion of the incubation periods described in the section *Turbidimetric Method* for *Procedure*, tubes containing the median dose of the Standard should have absorbances of at least 0.3 absorbance unit, except for Amikacin, Chlortetracycline, Gramicidin, and Tetracycline (0.35 absorbance unit), and Capreomycin, Methacycline, and Tobramycin (0.4 absorbance unit).

For the cylinder-plate assay, determine by trial the proportions of stock suspension to be incorporated in the *Inoculum*, starting with the volumes indicated in *Table 3*, that result in satisfactory demarcation of the zones of inhibition of about 14 to 16 mm in diameter and giving a reproducible dose relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45° to 50°, and swirling to attain a homogeneous suspension.

PROCEDURE

Assay Designs

Microbial assays gain markedly in precision by the segregation of relatively large sources of potential error and bias through suitable experimental designs. In a cylinder-plate assay, the essential comparisons are restricted to relationships between zone-diameter measurements within plates, exclusive of the variation between plates in their preparation and subsequent handling. To conduct a turbidimetric assay so that the differences in observed turbidity will reflect the differences in the antibiotic concentration requires both greater uniformity in the environment created for the tubes through closer thermostatic control of the incubator and the avoidance of systematic bias by use of a random placement of replicate tubes in separate tube racks, each rack containing one complete set of treatments. The essential comparisons are then restricted to relationships between the observed turbidities within racks.

NOTE—For some purposes, the practice is to design the assay so that a set of treatments consists of not fewer than three tubes for each sample and standard concentration, and each set is placed in a single rack.

Within these restrictions, the assay design recommended is a 1-level assay with a standard curve. For this assay with a standard curve, prepare solutions of 5, 6, or more test dilutions, provided they include one corresponding to the reference concentration (S_3), of the Standard and a solution of a single median test level of the Unknown as described under *Preparation of Standard* and *Preparation of the Sample*. Consider an assay as preliminary if its computed potency with either design is less than 80% or more than 125% of that assumed in preparing the stock solution of the Unknown. In such a case, adjust its assumed potency accordingly and repeat the assay.

Microbial determinations of potency are subject to inter-assay as well as intra-assay variables, so that two or more independent assays are required for a reliable estimate of the potency of a given assay preparation or Unknown. Starting with separately prepared stock solutions and test dilutions of both the Standard and the Unknown, repeat the assay of a given Unknown on a different day. If the estimated potency of the second assay differs significantly, as indicated by the calculated standard error, from that of the first, conduct one or more additional assays. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes.

Cylinder-Plate Method

To prepare assay plates using Petri dishes, place 21 mL of *Medium 2* in each of the required number of plates, and allow it to harden into a smooth base layer of uniform depth, except for Amphotericin B and Nystatin, where no separate base layer is used. For Erythromycin, Gentamicin, Neomycin B, Paromomycin, and Sisomicin, use *Medium 11*. For Bleomycin, use 10 mL of *Medium 35*. For Dihydrostreptomycin use *Medium 5*. For Vancomycin, use 10 mL of *Medium 8*. For Carbenicillin, Colistimethate Sodium, Colistin, and Polymyxin B, use *Medium 9*. For Netilmicin, use 20 mL of *Medium 11*. Add 4 mL of seed layer inoculum (see *Preparation of Inoculum* and *Table 3*), prepared as directed for the given antibiotic, except for Bleomycin (use 6 mL), for Netilmicin (use 5 mL), and for Nystatin and Amphotericin B (use 8 mL), tilting the plate back and forth to spread the inoculum evenly over the surface, and allow it to harden. Drop six assay cylinders on the inoculated surface from a height of 12 mm, using a mechanical guide or other device to insure even spacing on a radius of 2.8 cm, and cover the plates to avoid contamination. After filling the six cylinders on each plate with dilutions of antibiotic containing the test levels specified below, incubate the plates at 32° to 35°, or at the temperature specified below for the individual case, for 16 to 18 hours, remove the cylinders, and measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm. Incubate the plates at 29° to 31° for Amphotericin B and Nystatin. Incubate at 34° to 36° for Novobiocin. Incubate at 36° to 37.5° for Carbenicillin, Colistimethate Sodium, Colistin, Dihydrostreptomycin, Gentamicin, Neomycin, Netilmicin, Paromomycin, Polymyxin B, Sisomicin, and Vancomycin.

For the 1-level assay with a standard curve, prepare dilutions representing five test levels of the Standard (S_1 to S_5) and a single test level of the Unknown U_3 corresponding to S_3 of the standard curve, as defined under *Preparation of the Standard* and *Preparation of the Sample*. For deriving the standard curve, fill alternate cylinders on each of three plates with the median test dilution (S_3) of the Standard and each of the remaining nine cylinders with one of the other four dilutions of the Standard. Repeat the process for the three dilutions of the Standard. For each Unknown, fill alternate cylinders on each of three plates with the median test dilution of the Standard (S_3), and the remaining nine cylinders with the corresponding test dilution (U_3) of the Unknown.

Turbidimetric Method

On the day of the assay, prepare the necessary doses by dilution of stock solutions of the Standard and of each Unknown as defined under *Preparation of the Standard* and *Preparation of the Sample*. Add 1.0 mL of each dose, except for Gramicidin, Thiostrepton, and Tylosin (use 0.10 mL) to each of 3 prepared test tubes, and place the 3 replicate tubes in a position, selected at random, in a test tube rack or other carrier. Include similarly in each rack 1 or 2 control tubes containing 1 mL of the test diluent (see *Table 1*) but no antibiotic. Upon completion of the rack of test solutions (with Candicidin, within 30 minutes of the time when water is added to the dimethyl sulfoxide stock solution), add 9.0 mL of inoculum to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at 36° to 37.5°, except for Candicidin (incubate at 27° to 29°). Incubate the tubes for 4 to 5 hours, except for Capreomycin, Chloramphenicol, Cycloserine, Dihydrostreptomycin, Spectinomycin, Streptomycin, and Troleandomycin (incubate these for 3 to 4 hours), Tylosin (incubate for 3 to 5 hours), and Candicidin (incubate for 16 to 18 hours). After incubation add 0.5 mL of dilute formaldehyde to each tube, except for Tylosin (heat the rack in a water bath at 80° to 90° for 2 to 6 minutes or in a steam bath for 5 to 10 minutes, and bring to room temperature), taking one rack at a time, and read its transmittance or absorbance in a suitable spectrophotometer fitted with a 530-nm or 580-nm filter (see *Spectrophotometer* under *Apparatus*).

For the 1-level assay with a standard curve, prepare dilutions representing 5 test levels of the Standard (S_1 to S_5) and a single test level (U_3) of each of up to 20 Unknowns corresponding to S_3 of the Standard. Prepare also an extra S_3 as a test of growth. Add 1 mL of each test dilution, except for Gramicidin, Thiostrepton, and Tylosin (use 0.10 mL) to 3 tubes and 1 mL of antibiotic-free diluent to 6 tubes as controls. Distribute one complete set, including 2 tubes of controls, to a tube rack, intermingling them at random. Add 9.0 mL of inoculum, except for Thiostrepton (use 10.0 mL of inoculum), incubate, add 0.5 mL of dilute formaldehyde, and complete the assay as directed above. Determine the exact duration of incubation by observation of growth in the reference concentration (median dose) of the dilutions of the Standard (S_3).

CALCULATION

To calculate the potency from the data obtained either by the cylinder-plate or by the turbidimetric method, proceed in each case as directed under *Potencies: Interpolated from a Standard Curve* (see *Design and Analysis of Biological Assays* ⟨111⟩), using a log transformation, straight-line method with a least-squares fitting procedure, and a test for linearity. Where a number of assays of the same material are made with the same standard curve, calculate the coefficient of variation of results of all of the assays of the material. Where more than one assay is made of the same material with different standard curves, average the two or more values of the potency.

Change to read:

▪INTRODUCTION AND GENERAL INFORMATION

The activity (potency) of antibiotics can be demonstrated by their inhibitory effect on microorganisms under suitable conditions. A reduction in antimicrobial activity may not be adequately demonstrated by chemical methods. This chapter summarizes procedures for the antibiotics recognized in the *United States Pharmacopeia* (*USP*) for which the microbiological assay is the standard analytical method.

Two general techniques are employed, the *cylinder-plate* (or *plate*) assay and the *turbidimetric* (or *tube*) assay. *Table 1* lists all the antibiotics that contain microbial assays and specifies the type of assay (cylinder-plate or turbidimetric).

Table 1

Antibiotic	Type of Assay
Amphotericin B	Cylinder-plate
Bacitracin	Cylinder-plate
Bleomycin	Cylinder-plate
Capreomycin	Turbidimetric
Carbenicillin	Cylinder-plate
Chloramphenicol	Turbidimetric
Chlortetracycline	Turbidimetric
Cloxacillin	Cylinder-plate
Colistemethate	Cylinder-plate
Colistin	Cylinder-plate
Dihydrostreptomycin	Cylinder-plate
	Turbidimetric
Erythromycin	Cylinder-plate
Gentamicin	Cylinder-plate
Gramicidin	Turbidimetric

Table 1 *(Continued)*

Antibiotic	Type of Assay
Nafcillin	Cylinder-plate
Natamycin	Cylinder-plate
Neomycin	Cylinder-plate
	Turbidimetric
Novobiocin	Cylinder-plate
Nystatin	Cylinder-plate
Oxytetracycline	Turbidimetric
Paromomycin	Cylinder-plate
Penicillin G	Cylinder-plate
Polymyxin B	Cylinder-plate
Sisomicin	Cylinder-plate
Tetracycline	Turbidimetric
Thiostrepton	Turbidimetric
Troleandomycin	Turbidimetric
Tylosin	Turbidimetric
Vancomycin	Cylinder-plate

[NOTE—Perform all monograph procedures aseptically. Take adequate safety precautions while performing these assays because of possible allergies to drugs and because live cultures of organisms are used in the procedures.]

Cylinder-plate assay: The cylinder-plate assay depends on diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate. The growth of the specific microorganisms inoculated into the agar is prevented in a circular area or *zone* around the cylinder containing the solution of the antibiotic.

Turbidimetric assay: The turbidimetric assay depends on the inhibition of growth of a microorganism in a uniform solution of the antibiotic in a fluid medium that is favorable to the growth of the microorganism in the absence of the antibiotic.

Units and Reference Standards: The potency of antibiotics is designated in either units (U) or μg of activity. In each case the unit or μg of antibiotic activity was originally established against a United States Federal Master Standard for that antibiotic. The corresponding USP Reference Standard is calibrated in terms of the master standard.

Originally, an antibiotic selected as a reference standard was thought to consist entirely of a single chemical entity and was therefore assigned a potency of 1000 μg/mg. In several such instances, as the manufacturing and purification methods for particular antibiotics became more advanced, antibiotics containing more than 1000 μg of activity/mg became possible. Such antibiotics had an activity equivalent to a given number of μg of the original reference standard. In most instances, however, the μg of activity is exactly equivalent numerically to the μg (weight) of the pure substance. In some cases, such as those listed below, the μg of activity defined in terms of the original master standard is equal to a unit:

1. Where an antibiotic exists as the free base and in salt form and the μg of activity has been defined in terms of one of these forms
2. Where the antibiotic substance consists of a number of components that are chemically similar but differ in antibiotic activity
3. Where the potencies of a family of antibiotics are expressed in terms of a reference standard consisting of a single member which, however, might itself be heterogeneous

Do not assume that the μg of activity corresponds to the μg (weight) of the antibiotic substance.

Apparatus: Labware used for the storage and transfer of test dilutions and microorganisms must be sterile and free of residues that may affect the assay (see *Cleaning Glass Apparatus* ⟨1051⟩). Use a validated sterilization method, such as dry heat, steam, or radiation; or use sterile, disposable labware.

Temperature control: Thermostatic control is required in several stages of a microbial assay: when culturing a microorganism and preparing its inoculum, and during incubation in plate and tube assays. Refer to specific temperature requirements below for each type of assay.

Test organisms: The test organism for each antibiotic is listed in *Table 3* for the cylinder-plate assay and *Table 8* for the turbidimetric assay. The test organisms are specified by the American Type Culture Collection (ATCC) number.

In order to ensure acceptable performance of test organisms, store and maintain them properly. Establish the specific storage conditions during method validation or verification. Discard cultures if a change in the organism's characteristics is observed.

Prolonged storage: For prolonged storage, maintain test organisms in a suitable storage solution such as 50% fetal calf serum in broth, 10%–15% glycerol in tryptic soy broth, defribinated sheep blood, or skim milk. Prolonged-storage cultures are best stored in the freeze-dried state; temperatures of –60° or below are preferred; temperatures below –20° are acceptable.

Primary cultures: Prepare primary cultures by transferring test organisms from prolonged-storage vials onto appropriate media, and incubate under appropriate growth conditions. Store primary cultures at the appropriate temperature, usually 2°–8°, and discard after three weeks. A single primary culture can be used to prepare working cultures only for as many as seven days.

Working cultures: Prepare working cultures by transferring the primary culture onto appropriate solid media to obtain isolated colonies. Incubate working cul-

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tures under appropriate conditions to obtain satisfactory growth for preparation of test inocula. Prepare fresh working cultures for each test day.

Uncharacteristic growth or performance of a test organism: Use new stock cultures, primary cultures, or working cultures when a test organism shows uncharacteristic growth or performance.

Assay designs: Suitable experimental designs are key to increasing precision and minimizing bias. Control of the incubation parameters, temperature distribution and time, is critical for minimizing bias; it can be accomplished by staging the plates and racks as described for each assay.

Cylinder-plate assay: The comparisons are restricted to relationships between zone diameter measurements within plates, excluding the variation between plates. Individual plate responses are normalized on the basis of the relative zone size of the standard compared to the mean zone size of the standard across all plates.

Turbidimetric assay: To avoid systematic bias, place replicate tubes randomly in separate racks so that each rack contains one complete set of treatments. The purpose of this configuration is to minimize the influence of temperature distribution on the replicate samples. The turbidimetric assay, because of the configuration of the samples in test tube racks, is sensitive to slight variations in temperature. The influence of temperature variation can also be decreased by ensuring proper airflow or heat convection during incubation. At least three tubes for each sample and standard concentration (one complete set of samples) should be placed in a single rack. The comparisons are restricted to relationships between the observed turbidities within racks.

Potency considerations: Within the restrictions listed above, the recommended assay design employs a five-concentration standard curve and a single concentration of each sample preparation.

For the *cylinder-plate assay*, each plate includes only two treatments, the reference treatment (median level standard, i.e., S_3) and one of the other four concentrations of the standard (S_1, S_2, S_4, and S_5) or the sample (U_3). The concentration of the sample is an estimate based on the target concentration. The sample should be diluted to give a nominal concentration that is estimated to be equivalent to the median reference concentration (S_3) of the standard. The purpose of diluting to the median reference concentration is to ensure that the sample result will fall within the linear portion of the standard curve. The test determines the relative potency of U_3 against the standard curve. The sample (U_3) should have a relative potency of about 100%. The final potency of the sample is obtained by multiplying the U_3 result by the dilution factor.

An assay should be considered preliminary if the computed potency value of the sample is less than 80% or more than 125%. In this case, the results suggest that the sample concentration assumed during preparation of the sample stock solution was not correct. In such a case, one can adjust the assumed potency of the sample on the basis of the preliminary potency value and repeat the assay. Otherwise, the potency will be derived from a portion of the curve where the standard and sample responses will likely not be parallel.

Microbial determinations of potency are subject to inter-assay as well as intra-assay variables; therefore two or more independent assays are required for a reliable estimate of the potency of a given sample. Starting with separately prepared stock solutions and test dilutions of both the standard and the sample, perform additional assays of a given sample on a different day. The mean potency should include the results from all the valid independent assays. The number of assays required in order to achieve a reliable estimate of potency depends on the variability of the assay and the required maximum uncertainty for the potency estimate. The latter is assessed by

the width of the confidence interval (refer to *Calculations, Confidence limits and combinations of assay calculations*). The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than one from a single large assay with the same total number of plates or tubes. Note that additional assays or lower variability allows the product to meet tighter specification ranges. Reducing assay variability achieves the required confidence limit with fewer assays.

CYLINDER-PLATE METHOD

Temperature control: Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified in *Table 3*.

Apparatus

Plates: Glass or disposable plastic Petri dishes (approximately 20 × 100 mm) with lids

Cylinders: Stainless steel or porcelain cylinders; 8 mm ± 0.1 mm o.d.; 6 mm ± 0.1 mm i.d.; 10 mm ±0.1 mm high. [NOTE—Carefully clean cylinders to remove all residues; occasional cleaning in an acid bath, e.g., with about 2 N nitric acid or with chromic acid (see *Cleaning Glass Apparatus* ⟨1051⟩) is required.]

Standard solutions: To prepare a stock solution, dissolve a suitable quantity of the USP Reference Standard of a given antibiotic, or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in *Table 2*; and dilute to the specified concentration. Store at 2°–8° and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, in which the successive solutions increase stepwise in concentration, usually in the ratio of 1 : 1.25. Use the final diluent specified such that the median has the concentration suggested in *Table 2*.

Sample solutions: Assign an assumed potency per unit weight or volume to the sample. On the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard (*Table 2*). Dilute the sample stock solution in the specified final diluent to obtain a nominal concentration equal to the median concentration of the standard (S_3).

Table 2

	Stock Solution					Test Dilution	
Antibiotic	Initial Solvent	Initial Concentration	Further Diluent	Final Concentration	Use Within	Final Diluent	Median Concentration (S_3)[a,b]
Amphotericin B[c,d]	dimethyl sulfoxide	—	—	1 mg/mL	same day	B.10[e]	1 µg/mL
Bacitracin[f]	0.01 N hydrochloric acid	—	—	100 U/mL	same day	B.1[e]	1 U/mL
Bleomycin	B.16[e]	—	—	2 U/mL	14 days	B.16[e]	0.04 U/mL
Carbenicillin	B.1[e]	—	—	1 mg/mL	14 days	B.1[e]	20 µg/mL
Cloxacillin	B.1[e]	—	—	1 mg/mL	7 days	B.1[e]	5 µg/mL

Table 2 *(Continued)*

Antibiotic	Stock Solution					Test Dilution	
	Initial Solvent	Initial Concentration	Further Diluent	Final Concentration	Use Within	Final Diluent	Median Concentration (S_3)[a,b]
Colistemethate[c]	water	10 mg/mL	B.6[e]	1 mg/mL	same day	B.6[e]	1 μg/mL
Colistin	water	10 mg/mL	B.6[e]	1 mg/mL	14 days	B.6[e]	1 μg/mL
Dihydrostreptomycin[g]	B.3[e]	—	—	1 mg/mL	30 days	B.3[e]	1 μg/mL
Erythromycin	methanol	10 mg/mL	B.3[e]	1 mg/mL	14 days	B.3[e]	1 μg/mL
Gentamicin	B.3[e]	—	—	1 mg/mL	30 days	B.3[e]	0.1 μg/mL
Nafcillin	B.1[e]	—	—	1 mg/mL	2 days	B.1[e]	2 μg/mL
Natamycin	dimethyl sulfoxide	—	—	1 mg/mL	same day	B.10[e]	5 μg/mL
Neomycin[g]	B.3[e]	—	—	1 mg/mL	14 days	B.3[e]	1 μg/mL
Novobiocin	alcohol	10 mg/mL	B.3[e]	1 mg/mL	5 days	B.6[e]	0.5 μg/mL
Nystatin[c, h]	dimethylformamide	—	—	1000 U/mL	same day	B.6[e]	20 U/mL
Paromomycin	B.3[e]	—	—	1 mg/mL	21 days	B.3[e]	1 μg/mL
Penicillin G	B.1[e]	—	—	1000 U/mL	4 days	B.1[e]	1 U/mL
Polymyxin B[i]	water	—	B.6[e]	10,000 U/mL	14 days	B.6[e]	10 U/mL
Sisomicin	B.3[e]	—	—	1 mg/mL	14 days	B.3[e]	0.1 μg/mL
Vancomycin	water	—	—	1 mg/mL	7 days	B.4[e]	10 μg/mL

[a] It is acceptable to adjust the median concentration to optimize zone sizes if the data remain in the linear range.
[b] μg in this column refers to μg of activity.
[c] Prepare the USP Reference Standard and sample test dilutions simultaneously.
[d] Further dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8 μg/mL, 16 μg/mL, 20 μg/mL, 25 μg/mL, and 31.2 μg/mL before making the test dilutions.The test dilution of the sample should contain the same amount of dimethyl sulfoxide as the test dilutions of the USP Reference Standard.
[e] The letter B refers to buffer. See *Media and Solutions, Buffers* for a description of each buffer listed in this table.
[f] Each of the standard test dilutions should contain the same amount of hydrochloric acid as the test dilution of the sample.
[g] The turbidimetric assay can be used as an alternative procedure.
[h] Further dilute the stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 U/mL before making the test dilutions. Prepare the standard test dilutions simultaneously with test dilutions of the sample to be tested. The test dilution of the sample should contain the same amount of dimethylformamide as the test dilutions of the standard. Use low-actinic glassware.
[i] Prepare the stock solution by adding 2 mL of water for each 5 mg of the USP Reference Standard.

Inocula: Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium (See *Table 3*).

Incubate for the specified time and at the temperature as specified in *Table 3*, or until growth is apparent.

After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS (except use *Medium 34* for bleomycin; see the section *Media and Solutions*), using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass bottle. This is the harvest suspension.

Dilute an appropriate amount of the harvest suspension with sterile saline TS. Using the UV-visible spectrophotometer, measure % transmittance at 580 nm. The target value is approximately 25% transmittance at 580 nm. This value is used to standardize the harvest suspension volume added to the seed layer agar.

Starting with the suggested volumes indicated in *Table 3*, determine during method verification the proportions of stock suspension to be added to the inoculum medium that result in satisfactory zones of inhibition of approximately 14–16 mm in diameter for the median concentration of the standard (S_3). [NOTE—Zone sizes that are outside the 11–19-mm range are not desirable, because these contribute to assay variability.] If the dilution percentage transmittance is above 25%, a ratio may be used to normalize the addition of organism to the seed layer. The normalization factor can be determined by dividing the percentage transmittance obtained from the dilution by 25. This ratio can then be multiplied by the suggested inoculum amount to obtain the volume (mL) of harvest suspension that needs to be added to the seed layer. Adjust the quantity of inoculum on a daily basis, if necessary, to obtain an optimum concentration–response relationship.

Alternatively, determine during method verification the proportion of harvest suspension to be incorporated into the inoculum, starting with the volumes indicated in *Table 3*, that result in satisfactory demarcation of the zones of inhibition of about 14–16 mm in diameter for the median concentration of the standard (S_3) and giving a reproducible concentration–response relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45°–50°. Swirl the mixture without creating bubbles in order to obtain a homogeneous suspension.

Table 3

Antibiotic	Test Organism	ATCC[a] Number	Incubation Conditions			Suggested Inoculum Composition	
			Medium[b]	Temperature (°)	Time	Medium[b]	Amount (mL/100 mL)
Amphotericin B	*Saccharomyces cerevisiae*	9763	19	29–31	48 h	19	1.0
Bacitracin	*Micrococcus luteus*	10240	1	32–35	24 h	1	0.3
Bleomycin	*Mycobacterium smegmatis*	607	36	36–37.5	48 h	35	1.0
Carbenicillin[c]	*Pseudomonas aeruginosa*	25619	1	36–37.5	24 h	10	0.5
Cloxacillin	*Staphylococcus aureus*	29737	1	32–35	24 h	1	0.1
Colistimethate	*Bordetella bronchiseptica*	4617	1	32–35	24 h	10	0.1
Colistin	*Bordetella bronchiseptica*	4617	1	32–35	24 h	10	0.1
Dihydrostreptomycin	*Bacillus subtilis*	6633	32	32–35	5 days	5	as required
Erythromycin	*Micrococcus luteus*	9341	1	32–35	24 h	11	1.5
Gentamicin	*Staphylococcus epidermidis*	12228	1	32–35	24 h	11	0.03
Nafcillin	*Staphylococcus aureus*	29737	1	32–35	24 h	1	0.3
Neomycin	*Staphylococcus epidermidis*	12228	1	32–35	24 h	11	0.4
Novobiocin	*Staphylococcus epidermidis*	12228	1	32–35	24 h	1	4.0
Nystatin	*Saccharomyces cerevisiae*	2601	19	29–31	48 h	19	1.0

Table 3 *(Continued)*

Antibiotic	Test Organism	ATCC[a] Number	Incubation Conditions			Suggested Inoculum Composition	
			Medium[b]	Temperature (°)	Time	Medium[b]	Amount (mL/100 mL)
Paromomycin	*Staphylococcus epidermidis*	12228	1	32–35	24 h	11	2.0
Penicillin G	*Staphylococcus aureus*	29737	1	32–35	24 h	1	1.0
Polymyxin B	*Bordetella bronchiseptica*	4617	1	32–35	24 h	10	0.1
Sisomicin	*Staphylococcus epidermidis*	12228	1	32–35	24 h	11	0.03
Vancomycin	*Bacillus subtilis*	6633	32	32–35	5 days	8	as required

[a] American Type Culture Collection, 10801 University Boulevard, Manassas VA 20110-2209 (http://www.atcc.org)
[b] See *Media and Solutions, Media.*
[c] Use 0.5 mL of a 1 : 25 dilution of the stock suspension/100 mL of *Medium 10*.

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Analysis: Prepare the base layer for the required number of assay Petri plates, using the medium and volume shown in *Table 4*. Allow it to harden into a smooth base layer of uniform depth. Prepare the appropriate amount of seed layer inoculum (*Table 5*) as directed for the given antibiotic (*Table 3*) with any adjustments made based on the preparatory trial analysis. Tilt the plate back and forth to spread the inoculum evenly over the base layer surface, and allow it to harden.

Table 4 (base layer)

Antibiotic	Medium[a]	Target Volume (mL)
Amphotericin B[b]	—	—
Bleomycin	35	10
Carbenicillin	9	21
Colistimethate	9	21
Colistin	9	21
Dihydrostreptomycin	5	21
Erythromycin	11	21
Gentamicin	11	21
Neomycin	11	21
Nystatin[a]	—	—
Paromomycin	11	21
Polymyxin B	9	21
Sisomicin	11	21
Vancomycin	8	10
All others	2	21

[a] See *Media and Solutions, Media*.
[b] No base layer is used.

[NOTE—The base layer may be warmed to facilitate a uniform seed layer.]

Drop six assay cylinders on the inoculated surface from a height of 12 mm, using a mechanical guide or other device to ensure even spacing on a radius of 2.8 cm, and cover the plates to avoid contamination. Fill the six cylinders on each plate with dilutions of antibiotic containing the test levels (S_1–S_5 and U_3) specified in the following paragraph. Incubate the plates as specified in *Table 6* for 16–18 h, and remove the cylinders. Measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm.

Table 5 (seed layer)

Antibiotic	Medium[a]	Target Volume (mL)
Amphotericin B	Refer to *Table 3*	8
Bleomycin		6
Nystatin		8
All others		4

[a] See *Media and Solutions, Media*.

Table 6

Antibiotic	Incubation Temperature (°)
Amphotericin B	29–31
Carbenicillin	36–37.5
Colistimethate	36–37.5
Colistin	36–37.5
Dihydrostreptomycin	36–37.5
Gentamicin	36–37.5
Neomycin	36–37.5
Novobiocin	34–36
Nystatin	29–31
Paromomycin	36–37.5
Polymyxin B	36–37.5
Sisomicin	36–37.5
Vancomycin	36–37.5
All others	32–35

The standards (S_1–S_5) and a single test level of the sample (U_3) corresponding to S_3 of the standard curve, as defined under *Standard solutions* and *Sample solutions* will be used in the assay. For deriving the standard curve, fill alternate cylinders on each of three plates with the median test dilution (S_3) of the standard and each of the remaining nine cylinders with one of the other four test dilutions of the standard. Repeat the process for the three test dilutions of the standard. For the sample, fill alternate cylinders on each of three plates with the median test dilution of the standard (S_3), and fill the remaining nine cylinders with the corresponding test dilution (U_3) of the sample.

TURBIDIMETRIC METHOD

Temperature control: Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified in *Table 8*. [NOTE—Temperature control can be achieved using either circulating air or water. The greater heat capacity of water lends it some advantage over circulating air.]

Spectrophotometer: Measuring absorbance or transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength can be varied or restricted by use of 580-nm or 530-nm filters. Alternatively, a variable-wavelength spectrophotometer can be used and set to a wavelength of 580 nm or 530 nm.

The instrument may be modified as follows:

1. To accept the tube in which incubation takes place (see *Apparatus* below)
2. To accept a modified cell fitted with a drain that facilitates rapid change of contents
3. To contain a flow cell for a continuous flowthrough analysis

Autozero the instrument with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test dilution (including formaldehyde if specified) as found in each sample.

Either absorbance or transmittance can be measured while preparing inocula.

Apparatus: Glass or plastic test tubes, e.g., 16 × 125 mm or 18 × 150 mm. [NOTE—Use tubes that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. In the spectrophotometer, use matched tubes that are free from scratches or blemishes. Clean tubes thoroughly to remove all antibiotic residues and traces of cleaning solution. Sterilize tubes before use.]

Standard solutions: To prepare a stock solution, dissolve a quantity of the USP Reference Standard of a given antibiotic or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in *Table 7*, and dilute to the required concentration. Store at 2°–8°, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1 : 1.25. [NOTE—It may be necessary to use smaller ratios for the successive dilutions from the stock solution for the turbidimetric assay.] Use the final diluent specified such that the median level of the standard (S_3) has the concentration suggested in *Table 7*.

Sample solutions: Assign an assumed potency per unit weight or volume to the unknown, and on the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard (*Table 7*). Dilute the sample stock solution in the specified final diluent at a nominal concentration equal to the median concentration of the standard (S_3) as specified in *Table 7*.

Table 7

Antibiotic	Stock Solution					Test Dilution	
	Initial Solvent	Initial Concentration	Further Diluent	Final Stock Concentration	Use Within	Final Diluent	Median Concentration (S_3)
Capreomycin	water	—	—	1 mg/mL	7 days	water	100 μg/mL
Chloramphenicol	alcohol	10 mg/mL	water	1 mg/mL	30 days	water	2.5 μg/mL
Chlortetracycline	0.01 N hydrochloric acid	—	—	1 mg/mL	4 days	water	0.06 μg/mL
Dihydrostreptomycin[b]	water	—	—	1 mg/mL	30 days	water	30 μg/mL
Gramicidin	alcohol	—	—	1 mg/mL	30 days	alcohol	0.04 μg/mL
Neomycin[b,d]	B.3[c]	—	—	100 μg/mL	14 days	B.3[c]	1.0 μg/mL
Oxytetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	4 days	water	0.24 μg/mL
Tetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	1 day	water	0.24 μg/mL
Thiostrepton	dimethyl sulfoxide	—	—	1 U/mL	same day	dimethyl sulfoxide	0.80 U/mL
Troleandomycin	isopropyl alcohol and water (4 : 1)	—	—	1 mg/mL	same day	water	25 μg/mL

Table 7 *(Continued)*

	Stock Solution					Test Dilution	
Antibiotic	**Initial Solvent**	**Initial Concentration**	**Further Diluent**	**Final Stock Concentration**	**Use Within**	**Final Diluent**	**Median Concentration (S_3)**
Tylosin	methanol	10 mg/mL	B.16[c]	1 mg/mL	30 days	methanol and B.3[c] (1 : 1)	4 μg/mL

[a] μg in this column refers to μg of activity.
[b] The cylinder-plate assay can be used as an alternative procedure.
[c] The letter B refers to buffer. See *Media and Solutions, Buffers* for a description of each buffer listed in this table.
[d] Dilute the 100 μg/mL stock solution with *Buffer B.3* to obtain a solution having a concentration equivalent to 25 μg/mL of neomycin.To separate 50-mL volumetric flasks add 1.39, 1.67, 2.00, 2.40, and 2.88 mL of this solution. Add 5.0 mL of 0.01 N hydrochloric acid to each flask, dilute with *Buffer B.3* to volume, and mix to obtain solutions having concentrations of 0.69, 0.83, 1.0, 1.2, and 1.44 μg/mL of neomycin. Use these solutions to prepare the standard response line.

Inocula: Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. *Enterococcus hirae* (ATCC 10541) and *Staphylococcus aureus* (ATCC 9144) are grown in a liquid medium, not on agar. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium (see *Table 8*). Incubate at the time and temperature specified in *Table 8*, or until growth is apparent.

After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS, using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass bottle. This is the harvest suspension.

Determine during method verification the quantity of harvest suspension that will be used as the inoculum, starting with the volume suggested in *Table 8*. Prepare also an extra S_3 as a test of growth. Incubate the trial tests for the times indicated in *Table 11*. Adjust the quantity of inoculum daily, if necessary, to obtain the optimum concentration–response relationship from the amount of growth of the test organism in the assay tubes. At the completion of the specified incubation periods, tubes containing the median concentration of the standard should have absorbance values as specified in *Table 9*. Determine the exact duration of incubation by observing the growth in the reference concentration (median concentration) of the standard (S_3).

Table 8

Antibiotic	Test Organism	ATCC[a] Number	Incubation Conditions			Suggested Inoculum Composition	
			Medium[b]	Temperature (°)	Time	Medium[b]	Amount (mL/100 mL)
Capreomycin	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24 h	3	0.05
Chloramphenicol	*Escherichia coli*	10536	1	32–35	24 h	3	0.7
Chlortetracycline	*Staphylococcus aureus*	29737	1	32–35	24 h	3	0.1
Dihydrostreptomycin[d]	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24 h	3	0.1
Gramicidin	*Enterococcus hirae*	10541	3	36–37.5	16–18 h	3	1.0
Neomycin	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24 h	39	2
Oxytetracycline	*Staphylococcus aureus*	29737	1	32–35	24 h	3	0.1
Tetracycline	*Staphylococcus aureus*	29737	1	32–35	24 h	3	0.1
Thiostrepton	*Enterococcus hirae*	10541	40	36–37.5	18–24 h	41	0.2
Troleandomycin	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24 h	3	0.1
Tylosin	*Staphylococcus aureus*	9144	3	35–39	16–18 h	39	2–3

[a] American Type Culture Collection, 10801 University Boulevard, Manassas VA 20110-2209 (http://www.atcc.org)
[b] See *Media and Solutions, Media*.

Table 9

Antibiotic	Absorbance, NLT (a.u.)
Capreomycin	0.4
Chlortetracycline	0.35
Gramicidin	0.35
Tetracycline	0.35
All others	0.3

Analysis: On the day of the assay, prepare the necessary concentration of antibiotic by dilution of stock solutions of the standard and of each sample as specified under *Standard solutions* and *Sample solutions*. Prepare five test levels, each in triplicate, of the standard (S_1–S_5) and a single test level (U_3), also in triplicate, of up to 20 samples corresponding to S_3 (median concentration) of the standard.

Table 10

Antibiotic	Volume of Test Dilution (mL)	Volume of Inoculum (mL)
Gramicidin	0.10	9.0
Thiostrepton	0.10	10.0
Tylosin	0.10	9.0
All others	1.0	9.0

Place the tubes in test tube racks or other carriers. Include in each rack 1–2 control tubes containing 1 mL of the inoculum medium (see *Table 8*) but no antibiotic. Add the volumes of the standard and sample test dilutions as indicated in *Table 10*. Randomly distribute one complete set, including the controls, in a tube rack. Add the volume of inoculum specified in *Table 10* to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at the temperature specified in *Table 8* and for the time specified in *Table 11*.

Table 11

Antibiotic	Incubation Time (h)
Capreomycin	3–4
Chloramphenicol	3–4
Cycloserine	3–4
Dihydrostreptomycin	3–4
Streptomycin	3–4
Troleandomycin	3–4
Tylosin	3–5
All others	4–5

After incubation, immediately inhibit the growth of the organism by adding 0.5 mL of dilute formaldehyde to each tube, except for tylosin. For tylosin, heat the rack in a water bath at 80°–90° for 2–6 min or in a steam bath for 5–10 min, and bring to room temperature. Read absorbance or transmittance at 530 or 580 nm, analyzing one rack at a time.

MEDIA AND SOLUTIONS

The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients are acceptable; and reconstituted dehydrated media can be substituted, provided that the resulting media possess equal or better growth-promoting properties and give a similar standard curve response.

Media: Dissolve the ingredients in water to make 1 L, and adjust the solutions with either 1 N sodium hydroxide or 1 N hydrochloric acid as required, so that after steam sterilization the pH is as specified.

Medium 1

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	6.6 ± 0.1

Medium 2

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	6.6 ± 0.1

Medium 3

Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
Sodium chloride	3.5 g
Dextrose	1.0 g
Dibasic potassium phosphate	3.68 g
Monobasic potassium phosphate	1.32 g
Water	1000 mL
pH after Sterilization	7.0 ± 0.05

Medium 4

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	6.6 ± 0.1

Medium 5

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	7.9 ± 0.1

Medium 8

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	5.9 ± 0.1

Medium 9

Pancreatic digest of casein	17.0 g
Papaic digest of soybean	3.0 g
Sodium chloride	5.0 g

Medium 9 *(Continued)*

Dibasic potassium phosphate	2.5 g
Dextrose	2.5 g
Agar	20.0 g
Water	1000 mL
pH after Sterilization	7.2 ± 0.1

Medium 10

Pancreatic digest of casein	17.0 g
Papaic digest of soybean	3.0 g
Sodium chloride	5.0 g
Dibasic potassium phosphate	2.5 g
Dextrose	2.5 g
Agar	12.0 g
Water	1000 mL
Polysorbate 80 (added after boiling the medium to dissolve the agar)	10 mL
pH after Sterilization	7.2 ± 0.1

Medium 11

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	8.3 ± 0.1

Medium 13

Peptone	10.0 g
Dextrose	20.0 g
Water	1000 mL
pH after Sterilization	5.6 ± 0.1

Medium 19

Peptone	9.4 g
Yeast extract	4.7 g
Beef extract	2.4 g
Sodium chloride	10.0 g
Dextrose	10.0 g
Agar	23.5 g
Water	1000 mL
pH after Sterilization	6.1 ± 0.1

Medium 32

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Manganese sulfate	0.3 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	6.6 ± 0.1

Medium 34

Glycerol	10.0 g
Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	3.0 g
Water	1000 mL
pH after Sterilization	7.0 ± 0.1

Medium 35

Glycerol	10.0 g
Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	3.0 g
Agar	17.0 g
Water	1000 mL
pH after Sterilization	7.0 ± 0.1

Medium 36

Pancreatic digest of casein	15.0 g
Papaic digest of soybean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 mL
pH after Sterilization	7.3 ± 0.1

Medium 39

Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
Sodium chloride	3.5 g
Dextrose	1.0 g
Dibasic potassium phosphate	3.68 g
Monobasic potassium phosphate	1.32 g
Water	1000 mL
pH after Sterilization	7.9 ± 0.1

Medium 40

Yeast extract	20.0 g
Polypeptone	5.0 g
Dextrose	10.0 g
Monobasic potassium phosphate	2.0 g
Polysorbate 80	0.1 g
Agar	10.0 g
Water	1000 mL
pH after Sterilization	6.7 ± 0.2

Medium 41

Pancreatic digest of casein	9.0 g
Dextrose	20.0 g
Yeast extract	5.0 g
Sodium citrate	10.0 g
Monobasic potassium phosphate	1.0 g
Dibasic potassium phosphate	1.0 g
Water	1000 mL
pH after Sterilization	6.8 ± 0.1

Solutions

Buffers: Prepare as in *Table 12*, or by other suitable means. The buffers are sterilized after preparation; the pH specified in each case is the pH after sterilization.

Table 12. Buffers

Buffer	Concentration of Dibasic Potassium Phosphate (g/L)	Concentration of Monobasic Potassium Phosphate (g/L)	Volume of 10 N Potassium Hydroxide (mL)	pH After Sterilization[a]
Buffer B.1 (1%, pH 6.0)	2	8	—	6.0 ± 0.05
Buffer B.3 (0.1 M, pH 8.0)	16.73	0.523	—	8.0 ± 0.1
Buffer B.4 (0.1 M, pH 4.5)	13.61	—	—	4.5 ± 0.05
Buffer B.6 (10%, pH 6.0)	20	80	—	6.0 ± 0.05
Buffer B.10 (0.2 M, pH 10.5)	35	—	2	10.5 ± 0.1
Buffer B.16 (0.1 M, pH 7.0)	13.6	4	—	7.0 ± 0.2

[a] Adjust the pH with 18 N phosphoric acid or 10 N potassium hydroxide.

Other solutions: See *Reagents, Indicators, and Solutions.*

Water: Use *Purified Water.*

Saline: Use saline TS.

Dilute formaldehyde: Formaldehyde solution and water (1 : 3)

CALCULATIONS

Introduction: Antibiotic potency is calculated by interpolation from a standard curve using a log-transformed straight-line method with a least-squares fitting procedure (see below for calculation details). The analyst must consider three essential concepts in interpreting antibiotic potency results:

1. Biological concentration–response relationships generally are not linear. The antibiotic potency method allows fitting the data to a straight line by evaluating a narrow concentration range where the results approach linearity. The assay results can be considered valid only if the computed potency is 80%–125% of that assumed in preparing the sample stock solution. When the calculated potency value falls outside 80%–125%, the result for the sample may fall outside the narrow concentration range where linearity has been established. In such a case, adjust the assumed potency of the sample accordingly, and repeat the assay to obtain a valid result.
2. The most effective means of reducing the variability of the reportable value (the geometric mean potency across runs and replicates) is through independent runs of the assay procedure. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes. Three or more independent assays are required for antibiotic potency determinations.
3. The number of assays needed in order to obtain a reliable estimate of antibiotic potency depends on the required specification range and the assay variability. The confidence limit calculation described below is determined from several estimated log potencies that are approximately equal in precision. If the value calculated for the width of the confidence interval, W, is too wide, no useful decision can be made about whether the potency meets its specification.

The laboratory should predetermine in its standard operating procedures a maximum acceptable value for the confidence interval width. This maximum value should be determined during development and confirmed during validation or verification. If the calculated confidence interval width exceeds this limit, the analyst must perform additional independent potency determinations to meet the limit requirement. Note that the decision to perform additional determinations does not depend on the estimated potency but only on the uncertainty in that estimate as determined by the confidence interval width. Assay variability has a greater impact on the calculated confidence limit than does the number of independent potency determinations. As a result, the analyst should first consider decreasing variability to the extent possible before conducting potency determinations.

The following sections describe the calculations for determining antibiotic potency as well as for performing the confidence limit calculation. Methods for calculating standard error are also shown in order to allow estimates of assay variance. Where logarithms are used, any base log is acceptable. *Appendix 1* provides formulas for hand calculations applicable when the concentrations are equally spaced in the log scale. Alternative statistical methods may be used if appropriately validated.

Cylinder-plate assay: This section details analysis of the sample data and determination of the potency of an unknown, using the cylinder-plate assay.

Sample data: *Table 13* shows the data from one assay that will be used as an example throughout this section. For each of the 12 plates, zones 1, 3, and 5 are the reference concentration and the other three zones are for one of the other four concentrations, as shown. Other columns are needed for calculations and are explained below.

Step 1: Perform initial calculations and variability suitability check.

For each set of three plates, average the nine reference values and average the nine standard values.

Example (see *Table 13*)

$$15.867 = \bar{X}(16.1, 15.6, \ldots, 15.8)$$

$$14.167 = \bar{X}(14.6, 14.1, \ldots, 14.8)$$

For each set of three plates determine the standard deviation of the nine reference values and the standard deviation of the nine standard values. For each standard deviation, determine the corresponding relative standard deviation.

Example (see *Table 13*)

$$0.200 = \sigma(16.1, \ldots, 15.8)$$

$$1.3\% = (0.200/15.867) \times 100$$

$$0.324 = \sigma(14.6, \ldots, 14.1)$$

$$2.3\% = (0.324/14.167) \times 100$$

For a variability suitability criterion, each laboratory should determine a maximum acceptable value for the relative standard deviation. If any of the eight relative standard deviations (four for the reference and four for the standard) exceed this predetermined maximum, the assay data are not suitable and should be discarded. [NOTE—The suggested limit for relative standard deviation is NMT 10%.]

Step 2: Perform a plate-to-plate variation correction.

This correction is applied to convert the average zone measurement obtained for each concentration to the value it would be if the average reference concentration measurement for that set of three replicate plates were the same as the value of the correction point:

$$\bar{X}_C = \bar{X}_S - (\bar{X}_R - P)$$

$\bar{X}_C$ = corrected standard mean

$\bar{X}_S$ = original standard mean

$\bar{X}_R$ = reference mean

P = correction point

Example: For the first set of three plates in *Table 13* (S_1), the correction is:

$$14.022 = 14.167 - (15.867 - 15.722) = 14.167 - 0.145$$

Step 3: Determine the standard curve line.

Generate the standard curve line by plotting the corrected zone measurements versus the log of the standard concentration values. Calculate the equation of the standard curve line by performing a standard unweighted linear regression on these values, using appropriate software or the manual calculations of *Appendix 1*. [NOTE—Use either the natural log or the base 10 log to plot the standard curve and determine the regression equation;

both provide the same final test result.] Each laboratory should determine a minimum value of the coefficient of determination (%R^2) for an acceptable regression. The regression is acceptable only if the obtained %R^2 exceeds this predetermined value. [NOTE—The suggested limit for the percentage coefficient of determination is NLT 95%.]

Table 13: Sample Data (Cylinder-Plate Assay)

Standard	Concentration (U/ml)	Plate Replicate	Reference (S_3)						Sample						Corrected mean (mm)
			Zone 1 (mm)	Zone 3 (mm)	Zone 5 (mm)	Mean	SD	%RSD	Zone 2 (mm)	Zone 4 (mm)	Zone 6 (mm)	Mean	SD	%RSD	
S_1	3.20	1	16.1	15.6	15.8	15.867	0.200	1.3	14.6	14.1	13.5	14.167	0.324	2.3	14.022
		2	16.0	15.9	16.2				14.5	14.1	14.4				
		3	15.7	15.7	15.8				14.0	14.2	14.1				
S_2	4.00	1	15.8	15.6	15.5	15.567	0.158	1.0	14.7	15.1	14.8	14.833	0.265	1.8	14.989
		2	15.7	15.5	15.6				14.7	14.9	15.2				
		3	15.7	15.4	15.3				14.8	15.0	14.3				
S_4	6.25	1	15.6	15.8	16.0	15.789	0.169	1.1	16.6	16.8	16.3	16.578	0.233	1.4	16.511
		2	15.8	15.6	15.7				16.6	16.5	16.2				
		3	16.1	15.7	15.8				16.9	16.5	16.8				
S_5	7.8125	1	15.6	15.6	15.5	15.667	0.141	0.9	17.3	17.0	17.0	17.167	0.224	1.3	17.222
		2	15.6	15.7	15.5				17.3	17.4	17.2				
		3	15.9	15.8	15.8				17.3	17.3	16.7				
						15.722[a]									
U_3	Unknown	1	15.7	15.8	15.7	15.678	0.179	1.1	15.3	15.8	15.7	15.478	0.307	2.0	15.522
		2	15.9	15.7	15.7				15.8	15.8	15.5				
		3	15.5	15.8	15.3				15.2	15.1	15.1				

[a] This is the value of the overall reference mean, referred to as the 'correction point'.

Example: *Table 14* summarizes the portion of *Table 13* needed for this part of the calculation.

Table 14

Standard Set	Corrected zone Measurements (mm)	Concentration (U/mL)
S_1	14.022	3.2
S_2	14.989	4.0
Reference (S_3)	15.722	5
S_4	16.511	6.25
S_5	17.222	7.8125

Linear regression results

Standard curve line:

$$Z = [3.551 \times \ln(C)] + 9.978$$

Z = corrected zone measurement

C = concentration

$\%R^2 = 99.7$

Sample potency determination: To estimate the potency of the unknown sample, average the zone measurements of the standard and the zone measurements of the sample on the three plates used. Correct for plate-to-plate variation using the correction point determined above to obtain a corrected average for the unknown, $\bar{U}$. [NOTE—An acceptable alternative to using the correction point is to correct using the value on the estimated regression line corresponding to the log concentration of S_3.] Use the corrected average zone measurement in the equation of the standard curve line to determine the log concentration of the sample, L_U, by:

$$L_U = (\bar{U} - a)/b$$

a = intercept of the regression line

b = slope of the regression line

To obtain the potency of the unknown, take the antilog of L_U and multiply the result by any applicable dilution factor. This value can also be expressed as a percentage of the reference concentration value.

Example: Corrected sample zone measurement (*Table 13*) = 15.522

Natural log of sample concentration:

$$L_U = (15.522 - 9.978) / 3.553 = 1.561$$

Sample concentration:

$$C_U = e^{1.561} = 4.765$$

Percentage of reference concentration:

$$\text{Result} = (4.765/5.000) \times 100 = 95.3\%$$

Turbidimetric assay: This section details analysis of the sample data and determination of the potency of an unknown using the turbidimetric assay. The method assumes that the tubes are randomly distributed within the heat block or other temperature control device. If the device has a temperature profile that is not uniform, a randomized blocks design is preferred. In such a design, the rack is divided into areas (*blocks*) of relatively uniform temperature and at least one tube of each standard concentration and of each unknown is placed in each area. The data analysis of a randomized block design is different from the following.

Sample data: *Table 15* shows the data from one assay that will be used for an example throughout this section. Other columns are needed for calculations and are explained below.

Table 15: Sample Data (Turbidimetric Assay)

Standard	Concentration (μg/mL)	Replicate	Absorbance (a.u.)	Average (a.u.)	Standard Deviation
S_1	64	1	0.8545	0.8487	0.0062
		2	0.8422		
		3	0.8495		
S_2	80	1	0.8142	0.8269	0.0125
		2	0.8273		
		3	0.8392		
S_3	100	1	0.6284	0.6931	0.0640
		2	0.6947		
		3	0.7563		
S_4	125	1	0.6933	0.6827	0.0119
		2	0.6850		
		3	0.6699		
S_5	156	1	0.5299	0.5465	0.0272
		2	0.5779		
		3	0.5316		
U_3	unknown	1	15.7	0.7430	0.0460
		2	15.9		
		3	15.5		

Step 1: Perform initial calculations and variability suitability check.

For each concentration (including the sample), average the three absorbance values.

Example: See S_1 in *Table 15*.

$$0.8487 = \bar{X}(0.8545, 0.8422, 0.8495)$$

For each concentration, determine the standard deviation of the three readings and a combined standard deviation for all the concentrations.

Example: See S_1 in *Table 15*.

$$0.0125 = SD(0.8545, 0.8422, 0.8495)$$

The combined value is calculated by taking the square root of the average of the five variances:

$$0.0325 = \{[(0.0062)^2 + (0.0125)^2 + (0.0640)^2 + (0.0119)^2 + (0.0272)^2]/5\}^{1/2}$$

For a variability suitability criterion, each laboratory should determine a maximum acceptable combined standard deviation. If the combined standard deviation exceeds this predetermined maximum, the assay data are not suitable and should be discarded. [NOTE—The suggested limit for the combined standard deviation is NMT 10% of the average absorbance value across the five concentrations.] If the number of replicates per concentration is at least five, then a relative standard deviation can be computed for each concentration after checking for outliers and compared to a maximum acceptable relative standard deviation. [NOTE—The suggested limit for the relative standard deviation is NMT 10%.]

Step 2: Determine the standard curve line.

Generate the standard curve line by plotting the average absorbance values versus the log of the standard concentration values. Calculate the equation of the standard curve line by performing an unweighted linear regression on these values using appropriate software or the manual calculations of *Appendix 1*. [NOTE—Use either the natural log or the base 10 log to plot the standard curve and determine the regression equation; both provide the same final test result.] Each laboratory should determine a minimum value of the percentage coefficient of determination (%R^2) for an acceptable regression. The regression is acceptable only if the %R^2 value obtained exceeds this predetermined value. [NOTE—The suggested limit for the percentage coefficient of determination is NLT 90%.]

Example: *Table 16* summarizes the portion of *Table 15* needed for this part of the calculation.

Table 16

Set of Standards	Average Absorbance Values (a.u.)	Concentration (μg/mL)
S_1	0.8487	64
S_2	0.8269	80
S_3	0.6931	100
S_4	0.6827	125
S_5	0.5465	156

Linear regression results

Standard curve line:

$$\text{Absorbance} = 2.2665 - [0.7735 \times \log_{10}(\text{concentration})]$$

$$\%R^2 = 93.0\%$$

Sample potency determination: To estimate the potency of the unknown sample, average the three absorbance measurements to obtain an average for the un-

known, $\overline{U}$. Use this average measurement in the equation of the standard curve line to determine the log concentration of the unknown sample, L_U, by:

$$L_U = (\overline{U} - a)/b$$

a = intercept of the regression line

b = slope of the regression line

To obtain the potency of the unknown, take the antilog of L_U and multiply the result by any applicable dilution factor. This value can also be expressed as a percentage of the reference concentration value.

Example: Average sample absorbance *Table 15* = 0.7430.

$$\log_{10}(C_U) = (0.7430 - 2.2665)/(-0.7735) = 1.9696$$

$$C_U = 10^{1.9696} = 93.2$$

$$\text{Percentage of reference concentration} = (93.2/100.0) \times 100 = 93.2\%$$

C_U = concentration of sample

Confidence limits and combination of assays calculations: Because of interassay variability, three or more independent determinations are required for a reliable estimate of the sample potency. For each independent determination, start with separately prepared stock solutions and test dilutions of both the standard and the sample, and repeat the assay of a given sample on a different day.

Given a set of at least three determinations of the unknown potency, use the method of *Appendix 2* to check for any outlier values. This determination should be done in the log scale.

To obtain a combined estimate of the unknown potency, calculate the average, M, and the standard deviation of the accepted log potencies. [NOTE—Use either the natural log or the base 10 log.] Determine the confidence interval for the potency as follows:

$$\text{antilog}[M - t(0.05, N - 1) \times SD/\sqrt{N}], \text{antilog}[M + t(0.5, N - 1) \times SD/\sqrt{N}]$$

M = average

SD = standard deviation

N = number of assays

t(0.5, N–1) = the two-sided 5% point of a Student's t-distribution with N–1 degrees of freedom

NOTE—The t value is available in spreadsheets, statistics texts, and statistics software.

$$W = \text{antilog}\{M - [t(0.05, N - 1) \times SD/\sqrt{N}]\}$$

W = half-width of the confidence interval

Compare the half-width of the confidence interval to a predetermined maximum acceptable value. If the half-width is larger than the acceptance limit, continue with additional assays.

Example: Suppose the sample is assayed four times, with potency results in the natural log scale of 1.561, 1.444, 1.517, and 1.535. Then:

$$N = 4$$

$$M = \overline{X}(1.561, 1.444, 1.517, 1.535) = 1.514$$

$$SD = \sigma(1.561, 1.444, 1.517, 1.535) = 0.050$$

$$t = 3.182$$

The confidence interval in the log scale is

$$1.514 \pm (3.182 \times 0.050/\sqrt{4}) = (1.434, 1.594)$$

Taking antilogs, the estimated potency is

$$e^{1.514} = 4.546$$

with a 95% confidence interval for the potency of $e^{1.434}$, $e^{1.594} = (4.197, 4.924)$.

The confidence interval half-width to compare to an acceptance value is the ratio 4.924/4.546 = 1.083.

APPENDIX 1. FORMULAS FOR MANUAL CALCULATIONS OF REGRESSION AND SAMPLE CONCENTRATION

If the concentrations are equally spaced in the logarithmic scale, the calculations can be performed using the following formula. Let:

$\bar{S}_k$ = mean corrected zone measurement (cylinder-plate assay) or average absorbance value (turbidimetric assay) for standard set k

$k = S_1, S_2, S_3, S_4, S_5$

$\bar{S}$ = mean of the five $\bar{S}_k$ values

L_k = logarithm of the kth concentration. [NOTE—Use either the natural log or the base 10 log. Slope of the regression line is calculated by:]

$$b = (Y_{high} - Y_{low}) / (X_{high} - X_{low})$$

$$Y_{high} = \tfrac{1}{5}(3S_5 + 2S_4 + S_3 - S_1)$$

$$Y_{low} = \tfrac{1}{5}(3S_1 + 2S_2 + S_3 - S_5)$$

$X_{high} = L_5$

$X_{low} = L_1$

Combine and simplify to:

$$b = (4\bar{S}_5 + 2\bar{S}_4 - 2\bar{S}_2 - 4\bar{S}_1) / [5(L_5 - L_1)]$$

The log of the concentration of the sample is found using:

$$L_U = L_{reference} + [(\bar{U} - \bar{S})/b]$$

For example, using the data for the cylinder-plate assay in *Table 13* and natural logarithms:

$$b = [(4 \times 17.222) + (2 \times 16.511) - (2 \times 14.989) - (4 \times 14.020)] / \{5[\ln(7.81)] - \ln(3.2)\} = 3.553$$

$$\bar{S} = (14.020 + 14.989 + 15.722 + 16.511 + 17.222)/5 = 15.693$$

$$\text{Natural log of sample concentration} = \ln(5) + [(15.522 - 15.693)/3.553] = 1.561$$

$$\text{Sample concentration} = e^{1.561} = 4.765$$

APPENDIX 2. PROCEDURE FOR CHECKING FOR OUTLIERS; REJECTION OF OUTLYING OR ABERRANT MEASUREMENTS

A measurement that is clearly questionable because of a failure in the assay procedure should be rejected, whether it is discovered during the measuring or tabulation procedure. The arbitrary rejection or retention of an apparently aberrant measurement can be a serious source of bias. In general, the rejection of measurements solely on the basis of their relative magnitudes is a procedure that should be used sparingly.

Each suspected potency measurement, or outlier, may be tested against the following criterion. This criterion is based on the variation within a single group of supposedly equivalent measurements from a normal distribution. On average, it will reject a valid observation once in 25 trials or once in 50 trials. Designate the measurements in order of magnitude from y_1 to y_N, where y_1 is the candidate outlier, and N is the number of measurements in the group. Compute the relative gap by using the table *Test for Outlier Measurements* and the formulas below:

$$G_1 = (y_2 - y_1) / (y_N - y_1)$$

When N = 3 to 7:

$$G_2 = (y_2 - y_1) / (y_{N-1} - y_1)$$

When N = 8 to 10:

$$G_3 = (y_3 - y_1) / (y_{N-1} - y_1)$$

When N = 11 to 13:

If G_1, G_2, or G_3, as appropriate, exceeds the critical value in the table *Test for Outlier Measurements* for the observed N, there is a statistical basis for omitting the outlier measurement(s).

Test for Outlier Measurements

In samples from a normal population, gaps equal to or larger than the following values of G_1, G_2, and G_3 occur with a probability $P = 0.01$, when outlier measurements can occur only at one end; or with $P = 0.02$, when they may occur at either end.

N	3	4	5	6	7
G_1	0.987	0.889	0.781	0.698	0.637
N	8	9	10		
G_2	0.681	0.634	0.597		
N	11	12	13		
G_3	0.674	0.643	0.617		

Example: Estimated potencies of sample in log scale = 1.561, 1.444, 1.517, 1.535.

Check lowest potency for outlier:

$$G_1 = (1.517 - 1.444)/(1.561 - 1.444) = 0.624 < 0.889$$

Therefore 1.444 is not an outlier.

Check highest potency for outlier:

$$G_1 = (1.561 - 1.535)/(1.561 - 1.444) = 0.222 < 0.889$$

Therefore 1.561 is not an outlier.

Outlier potencies should be marked as outlier values and excluded from the assay calculations. NMT one potency can be excluded as an outlier.■2S *(USP34)*