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⟨81⟩ ANTIBIOTICS—MICROBIAL ASSAYS

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
Amoxicillin	Cylinder-plate
Amphotericin B	Cylinder-plate
Bacitracin	Cylinder-plate
Bleomycin	Cylinder-plate
Capreomycin	Turbidimetric
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
Nafcillin	Cylinder-plate
Neomycin	Cylinder-plate
	Turbidimetric
Novobiocin	Cylinder-plate
Nystatin	Cylinder-plate
Oxytetracycline	Turbidimetric

Antibiotic	Type of Assay
Paromomycin	Cylinder-plate
Penicillin G	Cylinder-plate
Polymyxin B	Cylinder-plate
Tetracycline	Turbidimetric
Thiostrepton	Turbidimetric
Tylosin	Turbidimetric
Vancomycin	Cylinder-plate

[Note—Perform all procedures under conditions designed to avoid extrinsic microbial contamination. 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

For substances like the antibiotics quantified using the method in this chapter which are not easily characterized by chemical or physical means, it is still necessary to express quantities of biological activity in units of biological potency, each defined by an authoritative reference standard. 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 US Federal Master Standard for that antibiotic. This practice started because originally the 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. Since relative potency was used to assign this value and not the mass of the antibiotics themselves, such antibiotics had an "activity" equivalent to a given number of µg of the original reference standard (rather than a µg of mass as would be expected). Over time, less complex antibiotics or those with a single primary active substance were demonstrated to have µg of activity equivalent numerically to the µg (weight) of the pure substance. In these cases, the antibiotics have moved away from the microbial assay methods and are assigned by a mass balance approach; until that exercise is completed for a given antibiotic, manufacturers cannot assume that the µg of activity corresponds to the µg (weight) of the antibiotic substance. Since the US Federal Master antibiotic standards are no longer available, USP Reference Standards for antibiotics that are assigned relative potency by the methods in this chapter are calibrated either against the World Health Organization (WHO) International Standard for Antibiotics (ISA), if it exists, or the previous USP lot when a WHO ISA or another authoritative reference standard calibrated against an ISA does not exist. Once assigned, the International Units (IUs), when used, are converted to USP units by the previously agreed upon bridging value between the two when the respective Reference Standards were originally assigned. Over time, it may be possible to convert the assay methods of more antibiotics from the microbial bioassay to a physicochemical method much like that suggested in *Validation of Alternative Methods to Antibiotic Microbial Assays* ⟨1223.1⟩ or other suitable strategies that include validated methods with associated bridging data.

Apparatus

Labware used for the storage and transfer of test dilutions and microorganisms must be sterile and free of interfering residues (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, defibrinated 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 3 weeks. A single primary culture can be used to prepare working cultures only for as many as 7 days.

Working Cultures

Prepare working cultures by transferring the primary culture onto appropriate solid media to obtain isolated colonies. Incubate working cultures 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 3 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 2 treatments, the reference treatment (median level standard, i.e., S_3) and one of the other 4 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 three 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. The mean potency should include the results from all the valid independent assays. The latter is assessed by the width of the confidence interval (refer to *Confidence Limits and Combination of Assays Calculations*). The combined result of a series of smaller, independent assays 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

Use glass or disposable plastic Petri dishes (approximately 20 mm × 100 mm or other appropriate dimensions) with lids.

CYLINDERS

Use stainless steel or porcelain cylinders; 8 ± 0.1-mm o.d.; 6 ± 0.1-mm i.d.; 10 ± 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 ⟨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 (see *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

Antibiotic	Stock Solution					Test Dilution	
	Initial Solvent	Initial Concentration	Further Diluent	Final Concentration	Use Within (days)	Final Diluent	Median Concentration (S_3)[a,b]
Amoxicillin[c]	Water	—	—	100 µg/mL	7	B.3[d]	0.1 µg/mL
Amphotericin B[c,e]	Dimethyl sulfoxide	—	—	1 mg/mL	Same day	B.10[d]	1 µg/mL
Bacitracin[f]	0.01 N hydrochloric acid	—	—	100 U/mL	Same day	B.1[d]	1 U/mL
Bleomycin	B.16[d]	—	—	2 U/mL	14	B.16[d]	0.04 U/mL
Cloxacillin	B.1[d]	—	—	1 mg/mL	7	B.1[d]	5 µg/mL
Colistimethate[c]	Water	10 mg/mL	B.6[d]	1 mg/mL	Same day	B.6[d]	1 µg/mL
Colistin	Water	10 mg/mL	B.6[d]	1 mg/mL	14	B.6[d]	1 µg/mL
Dihydrostreptomycin[g]	B.3[d]	—	—	1 mg/mL	30	B.3[d]	1 µg/mL
Erythromycin	Methanol	10 mg/mL	B.3[d]	1 mg/mL	14	B.3[d]	1 µg/mL
Gentamicin	B.3[d]	—	—	1 mg/mL	30	B.3[d]	0.1 µg/mL
Nafcillin	B.1[d]	—	—	1 mg/mL	2	B.1[d]	2 µg/mL
Neomycin[g]	B.3[d]	—	—	1 mg/mL	14	B.3[d]	1 µg/mL
Novobiocin	alcohol	10 mg/mL	B.3[d]	1 mg/mL	5	B.6[d]	0.5 µg/mL

Antibiotic	Stock Solution					Test Dilution	
	Initial Solvent	Initial Concentration	Further Diluent	Final Concentration	Use Within (days)	Final Diluent	Median Concentration (S_3)[a,b]
Nystatin[c,h]	Dimethylformamide	—	—	1000 U/mL	Same day	B.6[d]	20 U/mL
Paromomycin	B.3[d]	—	—	1 mg/mL	21	B.3[d]	1 µg/mL
Penicillin G	B.1[d]	—	—	1000 U/mL	4	B.1[d]	1 U/mL
Polymyxin B[i]	Water	—	B.6[d]	10,000 U/mL	14	B.6[d]	10 U/mL
Vancomycin	Water	—	—	1 mg/mL	7	B.4[d]	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] The letter B refers to buffer. See *Buffers* for a description of each buffer listed in this table.

[e] Further dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8, 16, 20, 25, 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.

[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 *Media and Solutions*), using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass container. This is the harvest suspension.

To make the stock suspension, dilute an appropriate amount of the harvest suspension with sterile saline TS. Using the UV-Vis spectrophotometer, measure the percentage 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)
Amoxicillin	*Kocuria rhizophila*	9341	1	32–35	24 h	11	0.5
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
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	*Kocuria rhizophila*	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 Kudriavzevii*	2601	19	29–31	48 h	19	1.0
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
Vancomycin	*Bacillus subtilis*	6633	32	32–35	5 days	8	As required

[a] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (*www.atcc.org*).

[b] See *Media*.

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 (see *Table 5*) as directed for the given antibiotic (see *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)
Amoxicillin	11	21
Amphotericin B[b]	—	—
Bleomycin	35	10
Colistimethate	9	21
Colistin	9	21
Dihydrostreptomycin	5	21
Erythromycin	11	21
Gentamicin	11	21
Neomycin	11	21
Nystatin[b]	—	—
Paromomycin	11	21
Polymyxin B	9	21
Vancomycin	8	10
All others	2	21

[a] See *Media*.

[b] No base layer is used. [NOTE—The base layer may be warmed to facilitate a uniform seed layer.]

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*.

Drop 6 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 6 cylinders on each plate with the same volume of 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 6

Antibiotic	Incubation Temperature (°)
Amphotericin B	29–31
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
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 in *Standard Solutions* and *Sample Solutions* will be used in the assay. For deriving the standard curve, fill alternate cylinders on each of 3 plates with the median test dilution (S_3) of the Standard and each of the remaining 9 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 3 plates with the median test dilution of the Standard (S_3), and fill the remaining 9 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 the use of 580- or 530-nm filters. Alternatively, a variable-wavelength spectrophotometer can be used and set to a wavelength of 580 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

Use glass or plastic test tubes, e.g., 16 mm × 125 mm or 18 mm × 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 (see *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 (days)	Final Diluent	Median Concentration (S_3)[a]
Capreomycin	Water	—	—	1 mg/mL	7	Water	100 µg/mL
Chloramphenicol	Alcohol	10 mg/mL	Water	1 mg/mL	30	Water	2.5 µg/mL
Chlortetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	4	Water	0.06 µg/mL
Dihydrostreptomycin[b]	Water	—	—	1 mg/mL	30	Water	30 µg/mL
Gramicidin	Alcohol	—	—	1 mg/mL	30	Alcohol	0.04 µg/mL
Neomycin[b,c]	B.3[d]	—	—	100 µg/mL	14	B.3[d]	1.0 µg/mL
Oxytetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	4	Water	0.24 µg/mL
Tetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	1	Water	0.24 µg/mL
Thiostrepton	Dimethyl sulfoxide	—	—	100 U/mL	Same day	Dimethyl sulfoxide	0.80 U/mL
Tylosin	Methanol	10 mg/mL	B.16[d]	1 mg/mL	30	Methanol and B.3[d] (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] 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 0.1 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.

[d] The letter B refers to buffer. See *Buffers* for a description of each buffer listed in this table.

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) (when used for the gramicidin assay), 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 Standard (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 (h)	Medium[b]	Amount (mL/100 mL)
Capreomycin	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24	3	0.05
Chloramphenicol	*Escherichia coli*	10536	1	32–35	24	3	0.7
Chlortetracycline	*Staphylococcus aureus*	29737	1	32–35	24	3	0.1
Dihydrostreptomycin	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24	3	0.1
Gramicidin	*Enterococcus hirae*	10541	3	36–37.5	16–18	3	1.0
Neomycin	*Klebsiella pneumoniae*	10031	1	36–37.5	16–24	39	2
Oxytetracycline	*Staphylococcus aureus*	29737	1	32–35	24	3	0.1
Tetracycline	*Staphylococcus aureus*	29737	1	32–35	24	3	0.1
Thiostrepton	*Enterococcus hirae*	10541	40	36–37.5	18–24	41	0.2
Tylosin	*Staphylococcus aureus*	9144	3	35–39	16–18	39	2–3

[a] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (*www.atcc.org*).

[b] See *Media*.

Table 9

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

Antibiotic	Absorbance, NLT (a.u.)
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 5 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.

Place the tubes in test tube racks or other carriers. Include in each rack 1–2 control tubes containing 0.1 mL of the test diluent for gramicidin, thiostrepton, and tylosin, or 1 mL of the test diluent for all others (see *Table 7*), 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 36.0°–37.5° for the time specified in *Table 11*.

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

Table 11

Antibiotic	Incubation Time (h)
Capreomycin	3–4
Chloramphenicol	3–4
Cycloserine	3–4
Dihydrostreptomycin	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
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 directed 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 TS (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	Concentration of Dibasic Potassium Phosphate (g/L)	Concentration of Monobasic Potassium Phosphate (g/L)	Volume of 10 N Potassium Hydroxide TS (mL)	pH after Sterilization[a]
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 TS.

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 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 half-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 half-width. This maximum value should be determined during development and confirmed during validation or verification. If the calculated confidence interval half-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 half-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: Formulas for Manual Calculations of Regression and Sample Concentration* 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 15 plates, zones 1, 3, and 5 are for the reference concentration, (S_3), and the other three zones are for one of the other four standard concentrations, (S_1, S_2, S_4, and S_5) or the sample (U_3), 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 3 plates, average the 9 reference values and average the 9 Standard values.

Example: See Table 13.

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

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

For each set of 3 plates, determine the standard deviation of the 9 reference values and the standard deviation of the 9 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 3 replicate plates were the same as the value of the correction point:

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

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

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

$\overline{X}_R$ = reference mean

P = correction point

Example: For the first set of 3 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: Formulas for Manual Calculations of Regression and Sample Concentration*. [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)						Standard						Corrected Mean (mm)
			Zone 1 (mm)	Zone 3 (mm)	Zone 5 (mm)	Mean (mm)	SD	%RSD	Zone 2 (mm)	Zone 4 (mm)	Zone 6 (mm)	Mean (mm)	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

Standard	Concentration (U/mL)	Plate Replicate	Reference (S_3) Zone 1 (mm)	Zone 3 (mm)	Zone 5 (mm)	Mean (mm)	SD	%RSD	Standard Zone 2 (mm)	Zone 4 (mm)	Zone 6 (mm)	Mean (mm)	SD	%RSD	Corrected Mean (mm)
		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				
		1	15.6	15.6	15.5				17.3	17.0	17.0				
		2	15.6	15.7	15.5				17.3	17.4	17.2				
S_5	7.8125	3	15.9	15.8	15.8	15.667	0.141	0.9	17.3	17.3	16.7	17.167	0.224	1.3	17.222
						15.722[a]									
Sample			**Reference (S_3)**						**Sample**						
		1	15.7	15.8	15.7				15.3	15.8	15.7				
		2	15.9	15.7	15.7				15.8	15.8	15.5				
U_3	Unknown	3	15.5	15.8	15.3	15.678	0.179	1.1	15.2	15.1	15.1	15.478	0.307	2.0	15.522

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

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 3 plates used. Correct for plate-to-plate variation using the correction point determined above to obtain a corrected average for the unknown, $\overline{U}$. [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:

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

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

Natural log of the sample concentration:

$$L_U = (15.522 - 9.978)/3.551 = 1.561$$

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		

Standard	Concentration (µg/mL)	Replicate	Absorbance (a.u.)	Average (a.u.)	Standard Deviation
		3	0.5316		
U_3	Unknown	1	0.7130	0.7430	0.0460
		2	0.7960		
		3	0.7201		

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.

$$0.0062 = SD(0.8545, 0.8422, 0.8495)$$

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

$$0.0322 = \{[(0.0062)^2 + (0.0125)^2 + (0.0640)^2 + (0.0119)^2 + (0.0272)^2]/5\}^{1/2}$$

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 5, 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: Formulas for Manual Calculations of Regression and Sample Concentration*. [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 unknown, $\overline{U}$. 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 (see *Table 15*) = 0.7430.

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

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

Percentage of reference concentration = (93.2/100.0) × 100 = 93.2%

C_U = concentration of the 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.

Given a set of at least three determinations of the unknown potency, use the method of *Appendix 2: Procedure for Checking for Outliers; Rejection of Outlying or Aberrant Measurements* 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:

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

M = average

$t(0.05, N-1)$ = 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.]

SD = standard deviation

N = number of assays

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

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

Change to read:

APPENDICES

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:

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

k = 1, 2, 3, 4, 5

$\overline{S}$ = mean of the five $\overline{S}_k$ 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:]

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

$$Y_{high} = \tfrac{1}{5}(3\overline{S}_5 + 2\overline{S}_4 + \overline{S}_3 - \overline{S}_1)$$

$$Y_{low} = \tfrac{1}{5}(3\overline{S}_1 + 2\overline{S}_2 + \overline{S}_3 - \overline{S}_5)$$

X_{high} = L_5

X_{low} = L_1

Combine and simplify to:

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

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

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

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

$$b = [(4 \times 17.222) + (2 \times 16.511) - (2 \times 14.989) - (4 \times {}^{\blacktriangle}14.022_{\blacktriangle\,(\text{ERR 1-Jan-2024})})]/\{5[\ln(7.81) - \ln(3.2)]\} = 3.551$$

$$\overline{S} = ({}^{\blacktriangle}14.022_{\blacktriangle\,(\text{ERR 1-Jan-2024})} + 14.989 + 15.722 + 16.511 + 17.222)/5 = 15.693$$

$$\text{Natural log of sample concentration} = \ln(5) + [(15.522 - 15.693)/3.551] = 1.561$$

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

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. At a confidence level of 99%, a valid observation will be rejected once in 100 trials (when the suspected outlier can occur at only one end) or once in 50 trials (when the suspected outlier can occur at either end), provided that relatively few, if any, responses within the group are identical. Arrange the responses in order of magnitude from y_1 to y_N, where N is the number of observations in the group. Compute the relative gap by using *Table A2-1*, and the formulas below:

When N = 3–7:

$$G_1 = (y_2 - y_1)/(y_N - y_1)\ \text{Candidate Outlier is Smallest } (y_1)$$

$$G_1 = (y_N - y_{N-1})/(y_N - y_1)\ \text{Candidate Outlier is Largest } (y_N)$$

When N = 8–10:

$$G_2 = (y_2 - y_1)/(y_{N-1} - y_1)\ \text{Candidate Outlier is Smallest } (y_1)$$

$$G_2 = (y_N - y_{N-1})/(y_N - y_2)\ \text{Candidate Outlier is Largest } (y_N)$$

When N = 11–13:

$$G_3 = (y_3 - y_1)/(y_{N-1} - y_1)\ \text{Candidate Outlier is Smallest } (y_1)$$

$G_3 = (y_N - y_{N-2})/(y_N - y_2)$ Candidate Outlier is Largest (y_N)

If G_1, G_2, or G_3, as appropriate, exceeds the critical value in Table A2-1, for the observed N, there is a statistical basis for identifying the discordant measurement as an outlier and considering its removal.

Table A2-1. 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	0.988	0.889	0.780	0.698	0.637
N	8	9	10	—	—
G_2	0.683	0.635	0.597	—	—
N	11	12	13	—	—
G_3	0.679	0.642	0.615	—	—

EXAMPLE

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

Check the 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 the 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 1 potency can be excluded as an outlier.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
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