Procedure

Pour in the scrubbing solution to half the height of the gas scrubber E, and put 20 mL of the absorbing solution in the absorption tube J. Weigh accurately a sample corresponding to about 6.5 mg as methoxyl group (CH_3O: 31.03), transfer to the decomposition flask A, and add boiling tips and 6 mL of hydroiodic acid. Moisten the ground joint C of A with 1 drop of hydroiodic acid, and connect to the condenser D. Prepare the apparatus by connecting the ball joint G using a suitable silicone grease. Bubble nitrogen or carbon dioxide through the gas-introducing tube B, and adjust the bubbles appearing in E at the rate of 2 bubbles per second, using a suitable pressure-regulating device. Place A in an oil bath, heat the flask until the temperature of the bath becomes 150°C after heating for 20 or 30 minutes, and boil at the same temperature for further 60 minutes. Remove the oil bath, cool the flask to room temperature under the gas stream, cool, and separate G. Drain the contents of J into a 500 mL conical flask containing 10 mL of a solution of sodium acetate trihydrate (1 in 5), rinse the tube with water several times, adding the rinsings to the flask, and finally dilute with water to about 200 mL. Add formic acid dropwise with shaking until the red color of the bromine disappears, and add 1 mL of formic acid. Add 3 g of potassium iodide and 15 mL of dilute sulfuric acid, stopper, shake gently, allow to stand for 5 minutes, and titrate the liberated iodine with 0.1 mol/L sodium thiosulfate VS (indicator: 1 mL of starch TS). Perform a blank determination, and make any necessary correction.

Each mL of 0.1 mol/L sodium thiosulfate VS
= 0.5172 mg of CH_3O

34. Microbial Assay for Antibiotics

The Microbial Assay for Antibiotics is a method that uses microorganisms to determine the antimicrobial potency of antibiotics contained in medicines. There are three methods for this test: the cylinder-plate, perforated plate, and turbidimetric methods. The former two are based on the measurement of the size of the zones of microbial growth inhibition in a nutrient agar medium, and the turbidimetric method is based on the measurement of the inhibition of turbidity development in a fluid medium with microbial growth. Unless otherwise specified in the individual monograph, tests specified to be carried out by the cylinder-plate method may be conducted under the same test conditions using the perforated plate method instead. If necessary, first sterilize water, isotonic sodium chloride solution, buffer solutions, reagents, test solutions and essential parts of measuring instruments and appliances to be used for the test.

I. Cylinder-plate method

The cylinder-plate method is a method to determine the antimicrobial potency of the antibiotic to be tested, and is based on the measurement of the size of the zone of growth inhibition of a test organism by the use of cylinder-agar plates.

1. Test organisms

According to the specification of the individual monograph, unless otherwise specified, select the test organism from the examples listed below.

[1] *Staphylococcus aureus* ATCC 6538 P
[2] *Staphylococcus epidermidis* ATCC 12228
[3] *Micrococcus luteus* ATCC 10240
[4] *Micrococcus luteus* ATCC 9341
[5] *Bacillus subtilis* ATCC 6633
[6] *Mycobacterium smegmatis* ATCC 607
[7] *Escherichia coli* NIHJ
[8] *Escherichia coli* ATCC 27166
[9] *Klebsiella pneumoniae* ATCC 10031
[10] *Pseudomonas aeruginosa* NCTC 10490
[11] *Comamonas terrigena* ATCC 8461
[12] *Saccharomyces cerevisiae* ATCC 9763
[13] *Candida albicans* No. Yu 1200
[14] *Penicillium chrysogenum* ATCC 10002

2. Culture media

Unless otherwise specified, use media with the following compositions. When 'peptone' is indicated as an ingredient of a medium, either meat peptone or casein peptone is applicable. Use sodium hydroxide TS or 1 mol/L hydrochloric acid TS to adjust the pH of the medium to obtain the specified value after sterilization. In the case of the medium for *Bacillus subtilis* ATCC 6633, adjust the pH using ammonia TS, potassium hydroxide TS or 1 mol/L hydrochloric acid TS. A different medium to the one specified for each test organism may be used if it has both a similar composition and an equal or better growth efficiency of the test organism in comparison with the specified medium. Sterilize the media to be used in an autoclave.

(1) Agar media for seed and base layer

1) Medium for test organism [5]

i.

Peptone	5.0 g
Meat extract	3.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 7.8 to 8.0 after sterilization.

ii.

Peptone	5.0 g
Meat extract	3.0 g
Trisodium citrate dihydrate	10.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

2) Medium for test organism [12]

Glucose	10.0 g
Peptone	9.4 g
Meat extract	2.4 g
Yeast extract	4.7 g
Sodium chloride	10.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.0 to 6.2 after sterilization.

3) Medium for other organisms

i.

Glucose	1.0 g
Peptone	6.0 g
Meat extract	1.5 g
Yeast extract	3.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

ii. Glucose	1.0 g
Meat peptone	6.0 g
Casein peptone	4.0 g
Meat extract	1.5 g
Yeast extract	3.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

iii. Peptone	10.0 g
Meat extract	5.0 g
Sodium chloride	2.5 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

(2) Agar media for transferring test organisms

1) Medium for test organism [12]

Glucose	15.0 g
Peptone	5.0 g
Yeast extract	2.0 g
Magnesium sulfate heptahydrate	0.5 g
Potassium dihydrogen phosphate	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.0 to 6.2 after sterilization.

2) Medium for other organisms

i. Glucose	1.0 g
Meat peptone	6.0 g
Casein peptone	4.0 g
Meat extract	1.5 g
Yeast extract	3.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

ii. Peptone	10.0 g
Meat extract	5.0 g
Sodium chloride	2.5 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

(3) Liquid media for suspending test organisms

1) Medium for test organism [12]

Glucose	20.0 g
Peptone	10.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 5.6 to 5.8 after sterilization.

2) Medium for other organisms

Peptone	10.0 g
Meat extract	5.0 g
Sodium chloride	2.5 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 7.0 to 7.1 after sterilization.

3. Preparation of agar slant media

Unless otherwise specified, dispense approximately 9 mL of melted agar medium in each test tube (approximately 16 mm in inside diameter), and then prepare agar slant media from them.

4. Preparation of stock suspensions of test organisms or spores

Unless otherwise specified, prepare stock suspensions of test organism or spore cultures as follows:

(1) Preparation of a stock suspension of the test organism [1], [2], [3], [7], [8] or [9]

Inoculate the test organism onto the slant of the agar medium which has been prepared for transferring the test organisms specified in 2 (2) 2) i. Incubate the inoculated slant at 32 to 37°C for 16 to 24 hours. The subculture should be performed at least three times.

Inoculate the subcultured test organism onto another slant of the agar medium (described above), and incubate the slant at 32 to 37°C for 16 to 24 hours. After addition of 10 mL of isotonic sodium chloride solution to the agar slant in a test tube, scrape away and wash out the resulting growth from the agar surface into another test tube, and use this as a stock suspension of the test organism.

Store the stock suspensions of the test organisms at a temperature not exceeding 5°C. Use the stock suspension of the test organism [2] within 5 days, and those of other test organisms within 7 days.

(2) Preparation of a stock suspension of the test organism [4] or [10]

Inoculate the test organism onto the slant of the agar medium which has been prepared for transferring the test organisms specified in 2 (2) 2) i. In the case of test organism [4], incubate the inoculated slants either at 25 to 30°C for 24 to 48 hours or at 32 to 37°C for 16 to 24 hours, and incubate at 25 to 30°C for 40 to 48 hours in the case of the test organism [10]. Each of the subcultures should be performed at least three times.

Inoculate each of the subcultured test organisms onto another slant of the agar medium (described above). In the case of test organism [4], incubate the slant either at 25 to 30°C for 40 to 48 hours or at 32 to 37°C for 16 to 24 hours, and at 25 to 30°C for 40 to 48 hours in the case of test organism [10].

After addition of 10 mL of isotonic sodium chloride solution to the agar slant in a test tube, scrape away and wash out the resulting growth from the agar surface into another test tube, and use this as a stock suspension of the test organism.

Store the stock suspensions of the test organisms at a temperature not exceeding 5°C. Use the stock suspension of test organism [4] within 5 days, and that of test organism [10] within 2 days.

(3) Preparation of a stock suspension of the test organism [12]

Inoculate test organism [12] onto the slant of the agar medium which has been prepared for transferring test organism [12] specified in 2 (2) 1).

Incubate the inoculated slant at 25 to 30°C for 40 to 48 hours. The subculture should be performed at least three times.

Inoculate the subcultured test organism onto another slant of the agar medium (described above), and incubate the slant at 25 to 30°C for 40 to 48 hours. After addition of 10 mL of isotonic sodium chloride solution to the agar slant in the test tube, scrape away and wash out the resulting growth from the agar surface into another test tube, and use

this as a stock suspension of the test organism.

Store the stock suspension of the test organism at a temperature not exceeding 5°C, and use it within 30 days.

(4) Preparation of a stock spore suspension of test organism [5]

Inoculate the test organism onto the slant of the agar medium which was prepared for transferring the test organisms specified in 2 (2) 2) i.

Incubate the inoculated slant at 32 to 37°C for 16 to 24 hours. Suspend the test organism cultivated on the slant of agar medium in approximately 3 mL of isotonic sodium chloride solution.

Add the suspension dropwise onto the slant of the agar medium, which was prepared for transferring the test organisms specified in 2 (2) 2) ii, contained in a suitable vessel such as a Roux bottle. Spread the suspension of organisms evenly over the large agar surface prepared in a Roux bottle with the aid of sterile glass beads. Then incubate the Roux bottle at 32 to 37°C for not less than 1 week to prepare spores. Suspend the spores in approximately 100 mL of isotonic sodium chloride solution. Heat at 65°C for 30 minutes, and then centrifuge. Wash the spore sediment three times with 50 mL of isotonic sodium chloride solution by means of centrifugation. Re-suspend the spore sediment in 100 mL of isotonic sodium chloride solution, and heat again at 65°C for 30 minutes to prepare the stock spore suspension. Store the stock spore suspension at a temperature not exceeding 5°C, and use it within 6 months.

5. Preparation of agar base layer plates

Unless otherwise specified, dispense 20 mL of the melted agar medium for the base layer into each Petri dish, and in the case of a large dish, dispense a quantity of the agar medium to form a uniform layer 2 to 3 mm thick. Distribute the agar evenly in each dish on a flat, level surface, and allow it to harden.

6. Preparation of seeded agar layers

First, determine the volume of the stock suspension of the test organism or the stock spore suspension with which the employed standard solution shows a clear and definite zone of growth inhibition. Prepare the seeded agar layer by mixing thoroughly the previously determined volume of stock suspension of test organism (or stock spore suspension) with agar medium for the seed layer kept at 48 to 51°C. Usually, 0.5 to 2.0 mL of a stock suspension of the test organism or 0.1 to 1.0 mL of a stock spore suspension is added to 100 mL of the agar medium for the seed layer.

7. Preparation of cylinder-agar plates

Dispense 4 to 6 mL of the seeded agar layer, which is specified in the individual monograph, on an agar base layer plate in a Petri dish. In the case of large dishes, dispense a quantity of the agar medium to form a uniform layer 1.5 to 2.5 mm thick, and spread evenly over the surface before hardening. Place 4 cylinders on an agar plate in a Petri dish so that the individual cylinders are equidistant from the center of the plate and equally spaced from one another (the cylinders are set on the circumference of a circle of 25 to 28 mm radius). When large dish plates are used, place cylinders on each plate according to the method of preparation for Petri dish agar plates. A set of 4 cylinders on each large dish plate is considered to be equivalent to one Petri dish plate.

Use stainless steel cylinders with the following dimensions: outside diameter 7.9 to 8.1 mm; inside diameter 5.9 to 6.1 mm; length 9.9 to 10.1 mm. The cylinders should not interfere with the test. Prepare the cylinder-agar plates before use.

8. Standard solutions

Use both a standard solution of high concentration and one of low concentration, as specified in the individual monograph. Unless otherwise specified, prepare the standard solutions before use.

9. Sample solutions

Use both a sample solution of high concentration and one of low concentration, as specified in the individual monograph. Unless otherwise specified, prepare the sample solutions before use.

10. Procedure

Unless otherwise specified, use 5 cylinder-agar plates as one assay set when Petri dishes are employed. When large dishes are employed, the number of cylinders for one assay set should be equal to that defined when using Petri dishes.

Apply the standard solution of high concentration and that of low concentration to a pair of cylinders set opposite each other on each plate. Apply the high and low concentration sample solutions to the remaining 2 cylinders. The same volume of solution must be added to each cylinder. Incubate the plates at 32 to 37°C for 16 to 20 hours.

Using a suitable measuring tool, measure the diameters of circular inhibition zones with a precision that can discriminate differences of at least 0.25 mm. Each procedure should be performed quickly under clean laboratory conditions.

11. Estimation of potency

The following correlation between the potency (P) of solution in a cylinder and the diameter (d) of zone of inhibition is established.

$$d = \alpha \log P + \beta$$

where, α and β are constants.

If necessary, ascertain the values in the above equation.

Based on this equation, estimate the potency of the sample solutions by application of the following equation:

Amount (potency) of sample
$= A \times$ Potency of S_H per mL $\times$ Dilution factor of U_H

where:

$$\log A = \frac{IV}{W}$$

$I = \log$ (Potency of S_H/Potency of S_L)
$V = \Sigma U_H + \Sigma U_L - \Sigma S_H - \Sigma S_L$
$W = \Sigma U_H + \Sigma S_H - \Sigma U_L - \Sigma S_L$

The sum of the diameter (mm) of the inhibitory zone measured in each plate is designated as follows:

for standard solution of high concentration (S_H) = ΣS_H
for standard solution of low concentration (S_L) = ΣS_L
for sample solution of high concentration (U_H) = ΣU_H
for sample solution of low concentration (U_L) = ΣU_L

II. Perforated plate method

The perforated plate method is a method to determine the antimicrobial potency of an antibiotic, based on the measurement of the size of the zone of growth inhibition of a test organism by the use of perforated agar plates.

This method is carried out by the use of perforated agar plates in lieu of cylinder-agar plates used in Cylinder-plate method.

Proceed as directed below, but comply with the requirements of Cylinder-plate method, such as test organisms, media, preparation of agar slant media, preparation of stock suspensions of test organisms or spores, preparation of agar base layer plates, preparation of seeded agar layers, standard solutions, sample solutions, and estimation of potency.

1. Preparation of perforated agar plates

Dispense 4 to 6 mL of the seeded agar layer specified in the individual monograph on each agar base layer plate of the Petri dish. In the case of large dishes, dispense a quantity of the agar medium to form a uniform layer 1.5 to 2.5 mm thick, and spread evenly over the surface before hardening.

Using a suitable tool, prepare 4 circular cavities having a diameter of 7.9 to 8.1 mm on a Petri dish agar plate so that the individual cavities are equidistant from the center of the plate. The cavities spaced equally from one another on the circumference of a circle with radius 25 to 28 mm, and are deep enough to reach the bottom of dish. When large dish plates are used, prepare the circular cavities on each plate according to the method of preparation for Petri dish agar plates. A set of 4 cavities on each large dish plate is considered to be equivalent to one Petri dish plate.

Prepare the perforated agar plates before use.

2. Procedure

Unless otherwise specified, use 5 perforated agar plates as one assay set when Petri dishes are employed. When large dishes are employed, the number of cavities for one assay set should be equal to that defined when using Petri dishes.

Apply the high and low concentration standard solutions to a pair of cavities prepared opposite each other on each plate, and apply the high and low concentration sample solutions to the remaining 2 cavities. The same volume of solution must be added to each cavity. Incubate the plates at 32 to 37°C for 16 to 20 hours.

Using a suitable measuring tool, measure the diameters of the circular inhibition zones with a precision that can discriminate differences of at least 0.25 mm. Each procedure should be performed quickly under clean laboratory conditions.

III. Turbidimetric method

The turbidimetric method is a method to determine the antimicrobial potency of an antibiotic, based on the measurement of the inhibition of growth of a microbial culture in a fluid medium. The inhibition of growth of a test organism is photometrically measured as changes in turbidity of the microbial culture.

1. Test organisms

According to the specification of the individual monograph, unless otherwise specified, select the test organism from the examples listed below.

[1] *Staphylococcus aureus* ATCC 6538 P
[2] *Staphylococcus aureus* ATCC 9144
[3] *Staphylococcus aureus* ATCC 10537
[4] *Klebsiella pneumoniae* ATCC 10031

2. Culture media

Unless otherwise specified, use media with the following compositions. When peptone is indicated as an ingredient of a medium, either meat peptone or casein peptone is applicable. Use sodium hydroxide TS or 1 mol/L hydrochloric acid TS to adjust the pH of the medium to obtain the specified value after sterilization. A different medium to the one specified for each test organism may be used if it has both a similar composition and an equal or better growth efficiency of the test organism in comparison with the specified medium. Sterilize the media to be used in an autoclave.

(1) Agar media for transferring test organisms

1) Medium for test organisms [1] and [2]

Glucose	1.0 g
Peptone	6.0 g
Meat extract	1.5 g
Yeast extract	3.0 g
Sodium chloride	2.5 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

2) Medium for test organism [3]

Peptone	10.0 g
Meat extract	5.0 g
Sodium chloride	2.5 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

3) Medium for test organism [4]

Glucose	1.0 g
Meat peptone	6.0 g
Casein peptone	4.0 g
Meat extract	1.5 g
Yeast extract	3.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 6.5 to 6.6 after sterilization.

(2) Liquid media for suspending test organisms

Glucose	1.0 g
Peptone	5.0 g
Meat extract	1.5 g
Yeast extract	1.5 g
Sodium chloride	3.5 g
Potassium dihydrogenphosphate	1.32 g
Disodium hydrogenphosphate, anhydrous*	3.0 g
Water	1000 mL

Mix all the ingredients, and sterilize. Adjust the pH of the solution so that it will be 7.0 to 7.1 after sterilization.

*Dipotassium hydrogenphosphate (3.68 g) may be used in lieu of disodium hydrogenphosphate, anhydrous (3.0 g).

3. Preparation of agar slant media

Unless otherwise specified, dispense approximately 9 mL of melted agar medium in each test tube (approximately 16 mm in inside diameter), and then prepare agar slant media from them.

4. Preparation of stock suspensions of test organisms

Unless otherwise specified, inoculate the test organism onto the slant of the agar medium which was prepared for

transferring the specified test organism. Incubate the inoculated slant at 32 to 37°C for 16 to 24 hours. The subculture should be performed at least three times.

Inoculate the subcultured test organism onto another slant of the agar medium (described above), and incubate the slant at 32 to 37°C for 16 to 24 hours. After incubation, prepare the stock suspensions of the test organism as follows:

(1) Preparation of a stock suspension of test organism [1]

Suspend the test organism in approximately 10 mL of the liquid medium for suspending the test organism. Add approximately 150 mL of the same medium to give approximately 85% transmittance at a wavelength of 650 nm. Before use, prepare the suspension of the test organism by adding 4.0 mL of this suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

(2) Preparation of a stock suspension of the test organism [2]

Suspend the test organism in approximately 10 mL of the liquid medium for suspending the test organism. Add approximately 150 mL of the same medium to give approximately 70% transmittance at a wavelength of 650 nm. Before use, prepare a suspension of the test organism by adding 5.0 mL of this suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

(3) Preparation of a stock suspension of the test organism [3]

Suspend the test organism in a little amount of the liquid medium for suspending the test organism. Add approximately 150 mL of the same medium to give approximately 85% transmittance at a wavelength of 650 nm. Before use, prepare the suspension of the test organism by adding 6.0 mL of this suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

(4) Preparation of a stock suspension of the test organism [4]

Suspend the test organism in 5 mL of water, and add this suspension dropwise onto the slant of agar medium, which was prepared for transferring the specified test organism, contained in a suitable vessel such as a Roux bottle. Spread the suspension of organisms evenly over the agar surface with the aid of sterile glass beads, and then incubate the Roux bottle at 32 to 37°C for 16 to 24 hours.

Scrape away the resulting growth from the agar surface, and suspend in water, adjusting to give approximately 65% transmittance at a wavelength of 650 nm. Store the stock suspension at a temperature not exceeding 5°C, and use it within 14 days. Before use, prepare the suspension of the test organism by adding 6.0 mL of this stock suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

5. Standard solutions

Use the standard solutions specified in the individual monograph. Unless otherwise specified, prepare the standard solutions before use.

6. Sample solutions

Use the sample solutions specified in the individual monograph. Unless otherwise specified, prepare the sample solutions before use.

7. Procedure

Unless otherwise specified, proceed as follows:

Distribute 1.0 mL of each concentration of the standard solution, the sample solution, and water used as a control, into each set composed of 3 test tubes (about 14 mm in inside diameter and about 13 cm in length). Add 9.0 mL of the suspension of the test organism to each tube, cover with a suitable lid or a cotton plug, and then incubate in a water bath maintained at 35 to 37°C for 3 to 4 hours. After incubation, add 0.5 mL of dilute formaldehyde (1 in 3) to each tube, and read each transmittance or absorbance at a wavelength of 530 nm.

8. Estimation of potency

Average the transmittance or absorbance values of each concentration of the standard solution, the sample solution and water used as a control, respectively. Generate the standard curve based on the average values of transmittance or absorbance of each concentration of the standard solution, and estimate the potency of the sample solution from its average value of transmittance or absorbance using the obtained standard curve.

If the standard dilutions of five concentrations in geometric progression are used, calculate the L and H values from the following equations. Plot point L and point H on graph paper and construct a straight line for the standard curve.

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

where:

L = calculated value of transmittance or absorbance for the lowest concentration of the standard curve.

H = calculated value of transmittance or absorbance for the highest concentration of the standard curve.

a, b, c, d, e = average transmittance or absorbance values for each standard dilution, where a is the value from the lowest concentration standard solution, b, c and d are the values from each geometrically increased concentration standard solution, respectively, and e is the value from the highest concentration standard solution.

35. Microbial Limit Test

This chapter provides tests for the qualitative and quantitative estimation of viable microorganisms present in pharmaceutical articles. It includes tests for total viable count (bacteria and fungi) and specified microbial species (*Escherichia coli, Salmonella, Pseudomonas aeruginosa* and *Staphylococcus aureus*). Microbial limit test must be carried out under conditions designed to avoid accidental microbial contamination of the preparation during the test. When test specimens have antimicrobial activity, or contain antimicrobial substances, any such antimicrobial properties must be eliminated by means of procedures such as dilution, filtration, neutralization or inactivation. For the test, use a mixture of several portions selected at random from the bulk or from the contents of a sufficient number of containers. If test specimens are diluted with fluid medium, the test should be performed quickly. In performing the test, precau-