

Section 1 General Notices for microbiological assays

Water, reagents, and instruments to be used in microbiological assays shall be sterilized before use as occasion demands.

[Methods listed in the Feed Analysis Standards]

1 Plate method ^[1] [Feed Analysis Standards, Chapter 9, Section 1, 1]

A. Reagent preparation

1) Buffer solutions^[2]

Prepare buffer solutions as directed below, and sterilize in an autoclave at 121°C for 15 minutes. If pH adjustment is needed, use phosphoric acid (1 mol/L) or potassium hydroxide solution (1 mol/L).

i) Buffer No.1 (pH 4.5)

Weigh 13.6 g of potassium dihydrogen phosphate, dissolve in 750 mL of water, adjust the pH to 4.4 to 4.6, and add water to make 1,000 mL.

ii) Buffer No.2 (pH 6.0) ^[3]

Potassium dihydrogen phosphate: 3.5 g

Disodium hydrogenphosphate 12-water: 3 g

Weigh the above amounts, dissolve in 750 mL of water, adjust the pH to 5.9 to 6.1, and add water to make 1,000 mL.

iii) Buffer No.3 (pH 6.0)

Potassium dihydrogen phosphate: 7 g

Disodium hydrogenphosphate 12-water: 6 g

Weigh the above amounts, dissolve in 750 mL of water, adjust the pH to 5.9 to 6.1, and add water to make 1,000 mL.

iv) Buffer No.4 (pH 8.0)

Weigh 13.3 g of potassium dihydrogen phosphate, dissolve in 750 mL of water, add about 100 mL of potassium hydroxide solution (1 mol/L) to adjust the pH to 7.9 to 8.1, and add water to make 1,000 mL.

v) Buffer No.5 (pH 6.0)

Potassium dihydrogen phosphate: 80 g

Dipotassium hydrogen phosphate: 20 g

Weigh the above amounts, dissolve in 750 mL of water, adjust the pH to 5.9 to 6.1, and add water to make 1,000 mL.

vi) Buffer No.6 (pH 8.0) ^[4]

Potassium dihydrogen phosphate: 13.3 g

Sodium chloride :100 g

Weigh the above-mentioned amounts, dissolve in 750 mL of water, add about 100 mL of potassium hydroxide solution (1 mol/L) to adjust the pH to 7.9 to 8.1, and add water to make 1,000 mL.

vii) Buffer No.7 (pH 7.0)

Potassium dihydrogen phosphate: 6.4 g

Disodium hydrogenphosphate 12-water: 18.9 g

Weigh the above amounts, dissolve in 750 mL of water, adjust the pH to 6.9 to 7.1, and add water to make 1,000 mL.

viii) Buffer No.8 (pH 4.0)

Weigh 7.5 mL of lactic acid^[5], dissolve in 750 mL of water, add about 50 mL of sodium hydroxide solution (1 mol/L) to adjust the pH to 3.9 to 4.1, and add water to make 1,000 mL.

ix) Buffer No.9 (pH 8.0)

Potassium dihydrogen phosphate: 0.5 g

Dipotassium hydrogen phosphate: 16.7 g

Sodium hydrogen carbonate: 20 g

Weigh the above amounts, dissolve in 750 mL of water, adjust the pH to 7.9 to 8.1, and add water to make 1,000 mL.

x) Buffer No.10 (pH 9.2)

Phosphoric acid: 2.3 mL^[6]

Acetic acid: 2.3 mL^[7]

Boric acid: 2.5 g

Weigh the above amounts, dissolve in 1,000 mL of water, add about 700 mL of sodium hydroxide solution (0.2 mol/L) to adjust the pH to 9.1 to 9.3.

xi) Buffer No.11 (pH 4.0)

Weigh 15.7 g of 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt, dissolve in 750 mL of water, add 7.5 mL of lactic acid^[5] and about 50 mL of sodium hydroxide solution (1 mol/L) to adjust the pH to 3.9 to 4.1, and add water to make 1,000 mL.

xii) Buffer No.12 (pH 7.5)

Potassium dihydrogen phosphate: 3.5 g

Disodium hydrogenphosphate 12-water: 3 g

Weigh the above-mentioned amounts, dissolve in 750 mL of water, adjust the pH to 7.4 to 7.6, and add water to make 1,000 mL.

2) Standard solutions

Prepare^[8] standard solutions as directed in the individual monograph in Section 2.

Prepare^[10] the standard stock solution using working standards^[9] (working standards currently specified or conventionally specified in Appendix 2 6 (13) of the Ordinance Concerning Ingredient Specifications Ministerial Ordinance Concerning the Ingredient Specifications for Feed and Feed Additives (Ordinance No. 35, issued by the Ministry of Agriculture and Forestry in 1976)) in the environment at a relative humidity of 50% or lower, and store in a hermetic container at -20°C or lower^[11].

3) Culture media

Prepare culture media with the compositions and pH values listed in the following table^[12], and sterilize in an autoclave at 121°C for 15 minutes. If pH adjustment is needed, use hydrochloric acid (1 mol/L) or

sodium hydroxide solution (1 mol/L).

Compositions and pH values per 1,000 mL of the culture medium

Medium No.		F-1	F-2	F-3	F-4 ^[13]	F-5 ^[14]	F-6 ^[15]
Peptone ^[22]	(g)	10	10	5	6	10	7.2
Pancreatic digest of casein ^[23]	(g)						
Papaic digest of soybean ^[24]	(g)						
Papaic digest of liver ^[25]	(g)						
Meat extract ^[26]	(g)	5	5	3	1.5	2	1.8
Bovine heart infusion ^[27]	(g)					250	
Calf brain infusion ^[28]	(g)					200	
Yeast extract ^[29]	(g)				3		3.6
Glucose	(g)				1	2	7.2
Sodium chloride	(g)	2.5	2.5			5	17.4
Magnesium chloride	(g)						
Magnesium sulfate	(g)						
Dipotassium hydrogen phosphate	(g)						
Disodium hydrogenphosphate 12-water	(g)					2.5	
Potassium dihydrogen phosphate	(g)						
Polyoxyethylene sorbitan monooleate ^[30]	(mL)						
Polyglycol ether surfactant ^{Note1} solution (1 w/v%)	(mL)						
Agar ^{Note2[31]}	(g)	13-20		13-20	13-20	13-20	13-20
Water		Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount
pH after sterilization		6.4-6.6	6.9-7.1	7.9-8.1	6.4-6.6	7.9-8.1	5.9-6.1

Medium No.	F-7 ^[16]	F-8 ^[17]	F-9 ^[18]	F-10	F-11	F-12 ^[19]
Peptone ^[22] (g)	6	7.2		10		3.75
Pancreatic digest of casein ^[23] (g)			17			
Papaic digest of soybean ^[24] (g)			3			
Papaic digest of liver ^[25] (g)						0.63
Meat extract ^[26] (g)	1.5	1.8		10		
Bovine heart infusion ^[27] (g)						
Calf brain infusion ^[28] (g)						
Yeast extract ^[29] (g)	3	3.6			1	1.25
Glucose (g)	1	7.2	2.5		10	
Sodium chloride (g)		7.2	5	5		1.25
Magnesium chloride (g)						
Magnesium sulfate (g)						
Dipotassium hydrogen phosphate (g)			2.5			
Disodium hydrogenphosphate 12-water (g)						
Potassium dihydrogen phosphate (g)						
Polyocycethylene sorbitan monooleate ^[30] (mL)			10			
Polyglycol ether surfactant ^{Note1} solution (1 w/v%) (mL)					3	
Agar ^{Note2[31]} (g)	13-20	13-20	13-20	13-20	13-20	13-20
Water	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount
pH after sterilization	7.9-8.1	7.9-8.1	7.2-7.4	6.4-6.6	5.9-6.1	7.2-7.4

Compositions and pH values per 1,000 mL of the culture medium (cont.)

Medium No.	F-13	F-14	F-15	F-16	F-17 ^[20]	F-18
Peptone ^[22] (g)	5	5		2	6	
Pancreatic digest of casein ^[23] (g)						
Pancreatic digest of soybean ^[24] (g)						
Papaic digest of liver ^[25] (g)						
Meat extract ^[26] (g)	5	3			1.5	
Bovine heart infusion ^[27] (g)						
Calf brain infusion ^[28] (g)						
Yeast extract ^[29] (g)			2.5	2.5	3	2.5
Glucose (g)			10	1		10
Sodium chloride (g)	80		70			
Magnesium chloride (g)						10
Magnesium sulfate (g)			50			
Dipotassium hydrogen phosphate (g)						
Disodium hydrogenphosphate 12-water (g)	2					
Potassium dihydrogen phosphate (g)						
Polyocycethylene sorbitan monooleate ^[30] (mL)						
Polyglycol ether surfactant ^{Note1} solution (1 w/v%) (mL)						
Agar ^{Note2[31]} (g)	13-20	13-20	13-20	13-20	13-20	13-20
Water	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount
pH after sterilization	7.9-8.1	5.9-6.1	4.9-5.1	5.9-6.1	5.6-5.8	Not prepared

Medium No.		F-19	F-20	F-21	F-22	F-23 ^[21]	F-24
Peptone ^[22]	(g)			5		5	10
Pancreatic digest of casein ^[23]	(g)						
Papaic digest of soybean ^[24]	(g)		3				
Papaic digest of liver ^[25]	(g)						
Meat extract ^[26]	(g)			5		1.5	5
Bovine heart infusion ^[27]	(g)						
Calf brain infusion ^[28]	(g)						
Yeast extract ^[29]	(g)	2.5			2.5	1.5	
Glucose	(g)	10			10	1	
Sodium chloride	(g)			50		3.5	2.5
Magnesium chloride	(g)	10					
Magnesium sulfate	(g)				50		
Dipotassium hydrogen phosphate	(g)					3.68	
Disodium hydrogen phosphate 12 water	(g)			0.5			
Potassium dihydrogen phosphate	(g)					1.32	
Polyoxyethylene sorbitan monooleate ^[30]	(mL)						
Plyglycol ether surfactantNote1 solution (1 w/v%)	(mL)						
Agar ^{Note2[31]}	(g)	13-20	13-20	13-20	13-20	13-20	13-20
Water		Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount
pH after sterilization		6.9-7.1	5.9-6.1	5.9-6.1	5.9-6.1	6.9-7.1	7.9-8.1

Compositions and pH values per 1,000 mL of the culture medium (cont.)

Mean No.	F-25	F-111 ^{Note3}	F-112 ^{Note4}	F-201 ^{Note5}	F-202 ^{Note6}
Peptone ^[22] (g)	7.2	6	7.2	10	10
Pancreatic digest of casein ^[23] (g)		4			
Papaic digest of soybean ^[24] (g)					
Papaic digest of liver ^[25] (g)					
Meat extract ^[26] (g)	1.8	1.5	1.8		
Bovine heart infusion ^[27] (g)				500	250
Calf brain infusion ^[28] (g)					200
Yeast extract ^[29] (g)	3.6	3	3.6		
Glucose (g)	7.2	1	7.2		2
Sodium chloride (g)	37.2		7.2	5	5
Magnesium chloride (g)					
Magnesium sulfate (g)					
Dipotassium hydrogen phosphate (g)					
Disodium hydrogen phosphate 12 water (g)					2.5
Potassium dihydrogen phosphate (g)					
Polyoxyethylene sorbitan monooleate ^[30] (mL)					
Polyglycol ether surfactant ^{Note1} solution (1 w/v%) (mL)					
Agar ^{Note2[31]} (g)	13-20	13-20	13-20	13-20	
Water	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount
pH after sterilization	7.9-8.1	7.9-8.1	5.9-6.1	7.3-7.5	7.3-7.5

Note 1: Tergitol Type NP-10 (Sigma Aldrich) or an equivalent medium

2: Bacto-Agar (Difco) or an equivalent medium

3: Antibiotic Medium 11 (Difco) or an equivalent medium

4: Antibiotic Medium 12 (Difco) or an equivalent medium

5: Bacto-Heart Infusion Agar (Difco) or an equivalent medium

6: Brain Heart Infusion Broth (Difco) or an equivalent medium

4) Suspensions of test organisms or spores and the amount of addition

Prepare suspensions of the test organism^[32] or spores as directed below. The amount of addition shall be determined by referring to the amounts described in the individual monograph in Section 2 as a guide. Add the suspension of the test organism or spores to the culture medium specified in the individual monograph in Section 2. The amount of addition shall be such that the high- and low-concentration standard solutions produces a inhibition zone diameter of 20 to 25 mm and 15 to 20 mm, respectively, when the test is performed by the 2-2 dose method, or such that the reference standard solution produces an inhibition zone diameter of about 20 mm when performing the test by the standard response line method. Use Medium F-2 or Medium F-202 when diluting bacterial suspension, and use water or isotonic sodium chloride solution when diluting spore suspension^[34].

i) Suspension of *Bordetella bronchiseptica* ATCC^[35] 4617, *Escherichia coli* ATCC 27166, *Micrococcus*

luteus ATCC 9341, *Micrococcus luteus* ATCC 10240 or *Staphylococcus aureus* ATCC 6538P

Incubate the test organism in Medium F-1 or Medium F-201 at 35 to 37°C for 16 to 24 hours. The subculture^[36] is performed at least three times. Transfer a platinum loopful^[37] of the test organism to 20 mL of Medium F-2 or Medium F-202, and incubate with shaking^[38] at 35 to 37°C for 16 to 24 hours to prepare a bacterial suspension.

ii) Suspension of *Pseudomonas Fluorescens* NIHJ^[39] B-254 or *Brevibacterium citreum var. polynactinus*

Incubate the test organism in Medium F-1 or Medium F-201 at 29 to 31°C for 16 to 24 hours. The subculture^[36] is performed at least three times. Transfer a platinum loopful^[37] of the test organism to 20 mL of Medium F-2 or Medium F-202, and incubate with shaking^[38] at 29 to 31°C for 16 to 24 hours to prepare a bacterial suspension.

iii) Suspension of *Corynebacterium xerosis* NCTC^[40] 9755

Incubate the test organism in Medium F-1 or Medium F-201 at 35 to 37°C for 16 to 24 hours, and subculture^[36] at least three times. Transfer a platinum loopful^[37] of the test organism to 10 mL of Medium F-2 or Medium F-202, and incubate with shaking^[38] at 35 to 37°C for 4 hours to prepare a bacterial suspension.

iv) Suspension of *Escherichia coli* BS-10^[41]

Incubate the test organism in Medium F-1 or Medium F-201 at 29 to 31°C for 16 to 24 hours. The subculture^[36] is performed at least three times. Transfer a platinum loopful^[37] of this bacterium to 10 mL of Medium F-202, and incubate with shaking^[38] at 29 to 31°C for 16 to 24 hours.

Further, transfer 1 mL of the incubation solution to 10 mL of Medium F-202, and incubate with shaking^[38] at 29 to 31°C for 3 hours to prepare a bacterial suspension.

v) Suspension of the spores of *Bacillus brevis* ATCC 8185, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633 or *Bacillus subtilis* ATCC 11774

Incubate the test organism in Medium F-1 or Medium F-201 at 35 to 37°C for 16 to 24 hours. The subculture^[36] is performed at 3-month intervals. Inoculate the test organism onto Medium F-1 or Medium F-201, and incubate at 35 to 37°C for at least 1 week to prepare spores^[42]. Scrape away suspend^[43] the spores evenly in water or isotonic sodium chloride solution, centrifuge at 1,500×g for 10 minutes, discard the supernatant liquid, add water or isotonic sodium chloride solution, shake, and heat at 65°C for 30 minutes two times at an interval of 24 hours^[44]. Further, centrifuge at 1,500×g for 10 minutes, discard the supernatant liquid, and add water or isotonic sodium chloride solution to suspend spores to prepare a spore suspension^[45].

vi) Suspension of the spores of *Bacillus cereus* ATCC 19637

Subculture^[36] the test organism in Medium F-1 or Medium F-201 at 27 to 29°C for 16 to 24 hours, at 3-month intervals. Inoculate the test organism onto Medium F-1 or Medium F-201, and incubate at 27 to 29°C for at least 1 week to prepare spores^[42]. Scrape away and suspend the spores evenly in water or isotonic sodium chloride solution^[43], centrifuge at 1,500×g for 10 minutes, discard the supernatant liquid, add water or isotonic sodium chloride solution, shake, and heat at 65°C for 30 minutes two times at an interval of 24 hours^[44]. Further, centrifuge the suspension at 1,500×g for 10 minutes, discard the supernatant liquid, and add water or isotonic sodium chloride solution to suspend the

spores to prepare a spore suspension^[45].

5) Agar plates

Unless otherwise specified^[46] in the individual monograph in Section 2, prepare agar plates as directed below, and use within the day.

i) Cylinder- plate method

Melt the suspension of the test organism or spores that had been prepared as directed in 4), add to the culture medium^[47] specified in the individual monograph in Section 2, previously melted and maintained at 49-51°C, stir thoroughly, dispense^[48] 10 mL into a Petri dish (of plastic or hard glass; 90 mm in internal diameter and 20 mm in height) to spread uniformly, and allow to stand horizontally to solidify. Drop^[49] 4 cylinders (of stainless steel; 7.9 to 8.1 mm in external diameter, 5.9 to 6.1 mm in internal diameter, and 9.9 to 10.1 mm in height) on the agar plate vertically from a height of 10 to 13 mm so that individual cylinders are set on the circumference of a circle 25 mm in radius and spaced from one another by 90 degrees relative to the center.

ii) Agar well method

Melt the suspension of the test organism or spores that had been prepared as directed in 4), add to the culture medium^[47] specified in the individual monograph in Section 2, previously melted and maintained at 49-51°C, stir thoroughly, dispense^[48] 20 mL into a Petri dish (of plastic or hard glass; 90 mm in internal diameter and 20 mm in height) to spread uniformly, and allow to stand horizontally to solidify. Make 4 circular cavities 7.9 to 8.1 mm in diameter in the agar plate so that individual cavities are set on the circumference of a circle 25 mm in radius and spaced from one another by 90 degrees relative to the center^[50].

B. Preparation of sample solutions

Prepare the sample solution quickly as specified in the individual monograph in Section 2^[51].

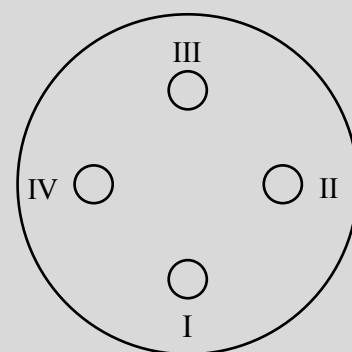
The concentration of the sample solution specified in the individual monograph in Section 2 is calculated from the labeled amount.

C. Quantification^[52]

Unless otherwise specified in the individual monograph in Section 2, proceed by the following method.

1) 2-2 dose method

Dispensing. Take 5 agar plates, and proceed as shown in the right figure. Dispense^[53] the high-concentration standard solution (S_H), high-concentration sample solution (U_H), low concentration standard solution (S_L), and low concentration sample solution (U_L) into cylinder or cavity I, II, III, and IV, respectively. The amount of addition is 0.25 mL per cylinder for the cylinder plate method and 0.1 mL per cavity for the agar well method.



Incubation. Allow each agar plate to stand under static conditions^[54]

at 10 to 20°C for 2 hours, place in an incubator^[55], and incubate at 35 to 37°C^[56] for 16 to 24 hours.

Measurement of inhibition zone diameter. Take the incubated agar plates out of the incubator, accurately

measure^[57] the diameter of each inhibition zone to a precision of 0.25 mm or less, and enter the results in the following form.

(in mm)				
No.	I High- concentration standard solution (S _H)	III LOW- concentration standard solution (S _L)	II High- concentration sample solution (U _H)	IV LOW- concentration sample solution (U _L)
Contents Agar plate				
1				
2				
3				
4				
5				
Total	S S _H	S S _L	S U _H	S U _L

Calculation^[58]. Calculate the ratio (θ)^[59] of the concentration (μg (potency) or unit/mL) of the antibiotic in the sample solution to that in the standard solution according to equation (1), and then estimate the concentration (g (potency) or unit/kg (g (potency) or unit/ton)) of the antibiotic in the sample according to equation (2).

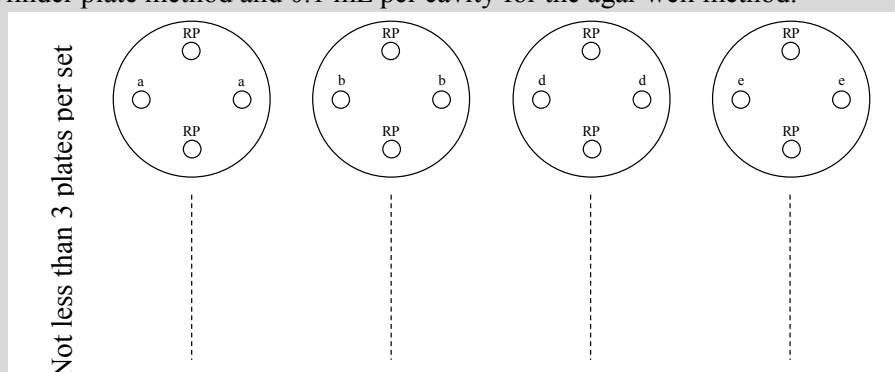
$$\log \theta = \frac{(\sum U_H + \sum U_L) - (\sum S_H + \sum S_L)}{(\sum U_H + \sum S_H) - (\sum U_L + \sum S_L)} \times \log X \dots (1)$$

X ^[60]: Ratio of the concentration of the antibiotic in the low-concentration standard solution to that in the high-concentration standard solution

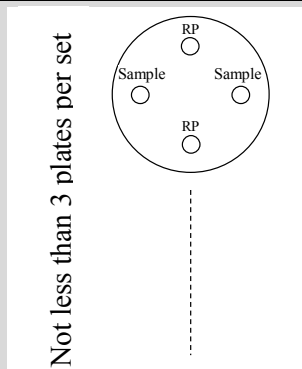
Concentration of the antibiotic in the sample = $\theta \times$ estimated concentration of the antibiotic in the sample... (2)

2) Standard response line method

Dispensing of the standard solution. Take not less than 3 agar plates for each standard solution. Denote the concentrations of the standard solution as $a, b, c, d,$ and e in a descending order from the highest concentration with e representing the concentration of the reference standard (RP), and dispense these concentrations as shown in the figure below. The amount of addition is 0.25 mL per cylinder for the cylinder plate method and 0.1 mL per cavity for the agar well method.



Dispensing of the sample solution. Take not less than three agar plates, and dispense^[53] the sample solution and reference standard solution as shown in the figure. The amount of addition is 0.25 mL per cylinder for the cylinder plate method and 0.1 mL per cavity for the agar well method.



Incubation. Allow each agar plate to stand under static conditions^[54] at 10 to 20°C for 2 hours, place in an incubator^[55], and incubate at 35 to 37°C^[56] for 16 to 24 hours.

Measurement of inhibition zone diameter. Take incubated agar plates out of the incubator, accurately measure^[57] the diameter of each inhibition zone to a precision of 0.25 mm or less, and enter the results in the following form.

		(in mm)									
Agar plate	Contents	RP	a	RP	b	RP	d	RP	e	RP	Sample
1											
1'											
2											
2'											
3											
3'											
.											
.											
Average inhibition zone diameter											
Corrected inhibition zone diameter											

Calculation^[61]. Based on the 4 sets of agar plates treated with the reference standard solution and the standard solution, calculate the average inhibition zone diameter for the reference standard solution and the standard solution from each set, and then the average inhibition zone diameter for the reference standard solution from all 4 sets (*C*: average value for correction).

If the average inhibition zone diameter for the reference standard solution calculated from each set is different from the average value for correction, correct the inhibition zone diameter for the standard solution of the same set by adding or subtracting the difference to or from the mean inhibition zone diameter of the standard solution of the same set.

Suppose, for example, that the average inhibition zone diameter of the standard solution in one set is 18.0 mm and that the average inhibition zone diameter of the reference standard solution in the same set is 19.8 mm. If the average value for correction is 20.0 mm, then correct the value for the

standard solution as $18.0 + (20.0 - 19.8)$ mm.

Next, create a semi-log graph with the logarithm of each concentration (μg (potency) or unit/mL) of the standard solution on the horizontal axis and the corrected inhibition zone diameter of each concentration of the standard solution on the vertical axis. Plot the average values for correction and the corrected inhibition zone diameter for each concentration of the standard solution, draw a straight line that passes nearest these plotted points, and use as the standard response line.

If the average value for correction and the corrected inhibition zone diameter for each concentration of the standard solution lie approximately on a straight line, plot the points of H and L obtained from the following equations on the semilog graph, and connect with a straight line.

$$H = \frac{3A + 2B + C - E}{5} \qquad L = \frac{3E + 2D + C - A}{5}$$

H : Inhibition zone diameter corresponding to concentration a of the standard solution

L : Calculated inhibition zone diameter corresponding to concentration e of the standard solution

$A, B, D,$ and E : Corrected diameters of the zones of inhibition for respective concentrations of the standard solution ($a, b, d,$ and e)

Further, calculate the average inhibition zone diameter for the reference standard solution and the average inhibition zone diameter for the sample solution based on the agar plates treated with the reference standard solution and the sample solution.

If the average inhibition zone diameter of the reference standard solution is different from the corrected inhibition zone diameter for the reference standard solution on the standard response line, calculate the corrected inhibition zone diameter of the sample solution by adding or subtracting the difference to or from the average inhibition zone diameter for the sample solution.

Finally, calculate the concentration of the antibiotic in the sample solution (μg (potency) or unit/mL) (n) from the points on the standard response line corresponding to the corrected values, calculate the ratio (θ) of n to the concentration of the antibiotic in the reference standard solution (μg (potency) or unit/mL), and calculate the concentration the antibiotic in the sample (g (potency) or unit/kg (g (potency) or unit/ton)) according to the following equation:

Concentration of the antibiotic in the sample = $\theta \times$ estimated concentration of the antibiotic in the sample.

«Notes and precautions»

[1] This method utilizes the property of antibiotics to diffuse into agar medium to estimate the concentration of an antibiotic by measuring the diameter of the zone of inhibited growth of a test organism susceptible to the antibiotic (which has a linear relationship with the logarithm of concentration of the antibiotic within a certain range).

Considering that the assay uses test organisms, water (purified water), reagents and instruments are disinfected before use, and glass tools are washed in an ultraviolet washing machine with a detergent for medical and laboratory instruments, rinsed thoroughly with water, and dried well in a drying machine. Disinfectants for particular purposes (those meeting the specifications of Japanese

Pharmacopoeia, such as ethanol for disinfection, phenol for disinfection, cresol soap solution for disinfection, and benzalkonium chloride solution) are made ready in the laboratory. Extreme care should be taken to prevent contamination and infection with microorganisms.

[2] The composition and pH value per 1,000 mL of each buffer solution used for preparing the standard solution, extract solvent or sample solution are shown in Table 9.1-1. Buffer solutions are prepared as follows:

Place about 750 mL (to 1,000 mL) of water in a suitable container (e.g., beaker), adjust the pH with stirring on a magnetic stirrer, and make the whole volume 1,000 mL. Place the amount in a suitable glass container (e.g., incubation bottle), gently plug (e.g., aluminum cap), and sterilize by autoclaving.

Do not use solutions that have been left to stand for a long period of time after preparation and that have suspended particles such as mold.

(Autoclave sterilization)

This is a method to sterilize buffer solutions, culture media, and reagents, or instruments and culture media contaminated with test organisms, usually by heating in an autoclave at 121°C for 15 to 30 minutes. If the heating can change their qualities, lower the sterilizing temperature or shorten the time of heating. After the sterilization is completed, decrease the pressure gradually to prevent the content from boiling over.

Table 9.1-1 Compositions and pH values per 1,000 mL of buffer solution

Buffer No.	1	2	3	4	5	6	7	8	9	10	11	12
Potassium hydrogen					20				16.7			
Potassium dihydrogen	13.6	3.5	7	13.3	80	13.3	6.4		0.5			3.5
Disodium hydrogenphosphate 12-water		3	6				18.9					3
Sodium chloride (g)						100						
Sodium hydrogen carbonate									20			
Phosphoric acid (g)										3.9		
Acetic acid (g)										2.4		
Boric acid (g)										2.5		
Lactic acid (g)								9			9	
1,2-Dihydroxybenzene-3,5- disulfonic acid sodium (g)												15.7
Potassium hydroxide solution (1 mol/L) (mL)				100		100						
Sodium hydroxide solution (1 mol/L) (mL)								50			50	
Sodium hydroxide solution (0.2 mol/L) (mL)										700		
Water (mL)	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	Suitable amount	1,000	Suitable amount	Suitable amount
pH after sterilization (±0.1)	4.5	6	6	8	6	8	7	4	8	9.2	4	7.5

[3] Buffer No.2 may be prepared by diluting Buffer No.3 with the same amount of sterile water and adjusting the pH.

[4] Buffer No.6 may be prepared by dissolving sodium chloride in Buffer No.4 and adjusting the pH.

- [5] The specific gravity of lactic acid is approximately 1.2; therefore, 7.5 mL of lactic acid corresponds to 9.0 g of lactic acid.
- [6] The specific gravity of phosphoric acid is approximately 1.69; therefore, 2.3 mL of phosphoric acid corresponds to 3.9 g of phosphoric acid.
- [7] The specific gravity of acetic acid is approximately 1.05; therefore, 2.3 mL of acetic acid corresponds to 2.4 g of acetic acid.
- [8] The method to prepare the standard solution, which is detailed in the individual monograph, is summarized as follows.

The following procedure is also recommended when a dilution procedure is required to prepare the sample solution (e.g., assays for premix).

For example, in order to prepare a 100 µg (potency)/mL standard solution from a 1,000 µg (potency)/mL standard stock solution, place accurately 18 mL of the dilution solvent specified in the individual monograph in a test tube about 25 mm in diameter and 200 mm in length, to which transfer a 2-mL portion of the standard stock solution (previously brought to room temperature) which had been dispensed in a small test tube (about 10 mL in capacity) using a Komagome pipette (capillary or Pasteur pipette etc.) while rinsing the tube with the solvent to achieve complete transfer, and mix. Care should be taken that some types of antibiotics can form bubbles during the dilution process (e.g., bacitracin, enramycin, colistin) and thus can affect the results of the assay. Further, when preparing a 25 µg (potency)/mL standard solution from the 100 µg (potency)/mL standard solution, proceed in the same manner as with the above-mentioned standard stock solution as follows: place accurately 15 mL of the dilution solvent in a test tube for dilution, to which add 5 mL of the 100 µg (potency)/mL standard solution using a one-mark pipette, and mix with a test tube mixer.

This preparation method, which does not require a volumetric flask, provides such benefits as saving of solvent and convenience for washing instruments; however, the one-mark pipettes should be made available from 1 to 30 mL with an increment of 1 mL.

- [9] Working standards listed in the Feed Analysis Standards are antimicrobial substances with specific serial numbers designated by the chairman of FAMIC as standards for use in the determination of the potency of antibiotics.

The definitions of working standards presently specified in the Ministerial Ordinance, Appendix 2 6 (13) Assays for antibiotics are shown in Table 9-2.

These working standards are available from FAMIC (for distribution criteria, visit FAMIC homepage (<http://www.famic.go.jp/>)).

For reference, the definition of conventionally specified working standards are also shown in Table 9-3. As the conventionally specified working standards are not available from FAMIC, use equivalent substances in the assay.

To prevent moisture and decrease in potency, it is preferable to store the working standards listed in Tables 9.1-2 and 9.1-3 in well-closed containers with a drying agent (silica gel etc.) in a freezer at -20°C or lower.

Table 9.1-2 Working standards specified in Appendix 2 6 (13)

Names of working standards	Definition of working standards
Avilamycin working standard	Avilamycin (Avilamycin A: $C_{61}H_{88}Cl_2O_{32}$, Avilamycin B: $C_{59}H_{84}Cl_2O_{32}$)
Efrotomycin working standard	Efrotomycin (Efrotomycin A1: $C_{59}H_{88}N_2O_{20}$, EfrotomycinAZ: $C_{59}H_{88}N_2O_{20}$, Efrotomycin B: $C_{59}H_{88}N_2O_{20}$)
Enramycin working standard	Enramycin monohydrochloride [Enramycin monohydrochloride A: $C_{107}H_{138}Cl_2N_{26}O_{31} \cdot HCl$ (58 %), Enramycin monohydrochloride B: $C_{108}H_{140}Cl_2N_{26}O_{31} \cdot HCl$]
Oxytetracycline working standard	Oxytetracycline hydrochloride ($C_{22}H_{24}N_2O_9 \cdot HCl$)
Chlortetracycline working standard	Chlortetracycline hydrochloride ($C_{22}H_{23}ClN_2O_8 \cdot HCl$)
Colistin working standard	Colistin sulfate (colistin sulfate A: $C_{53}H_{100}N_{16}O_{13} \cdot 5/2H_2SO_4$, Cholistin sulfate B: $C_{52}H_{98}N_{16}O_{13} \cdot 5/2H_2SO_4$)
Salinomycin working standard	Sodium salinomycin ($C_{42}H_{69}O_{11}Na$)
Sedecamycin working standard	Sedecamycin A ($C_{27}H_{35}NO_8$)
Semduramicin working standard	Sodium semduramicin ($C_{45}H_{75}O_{16}Na$)
Tylosin working standard	Tylosin A ($C_{46}H_{77}NO_{17}$)
Destomycin A working standard	Destomycin A ($C_{20}H_{37}N_3O_{13}$)
Narasin working standard	Narasin A ($C_{43}H_{72}O_{11}$)
Nosiheptide working standard	Nosiheptide ($C_{51}H_{43}O_{12}N_{13}S_6$)
Bacitracin working standard	Bacitracin (Bacitoracin A: $C_{66}H_{103}N_{17}O_{16}S$)
Virginiamycin working standard	Virginiamycin [Virginiamycin M ₁ : $C_{28}H_{35}N_3O_7$ (95 %), Virginiamycin S: $C_{43}H_{49}N_7O_{10}$ (5 %)]
Bicozamycin working standard	Bicozamycin ($C_{12}H_{18}N_2O_7$)
Flavophospholipol working standard	Flavophospholipol ($C_{65-75}H_{124-135}N_{6-7}O_{40-42}P$)
Monensin working standard	Sodium monensin (Sodium monensin A: $C_{36}H_{61}O_{11}Na$)
Lasalocid working standard	Sodium lasalocid ($C_{34}H_{53}O_8Na$)

Table 9.1-3 Working standards specified conventionally

Avoparcin working standard*4	Avoparcin sulfate [α -Avoparcin sulfate: (C ₈₉ H ₁₀₁ O ₃₆ N ₉ Cl) ₂ ·3H ₂ SO ₄ (29 %), β -Avoparcin sulfate: (C ₈₉ H ₁₀₀ O ₃₆ N ₉ Cl ₂) ₂ ·3H ₂ SO ₄ (71 %)]
Orietacin working standard*4	Orietacin A (C ₇₃ H ₈₉ ClN ₁₀ O ₂₆)
Oleandomycin working standard*3	Chloroform adduct of oleandomycin (C ₃₅ H ₆₁ NO ₁₂ ·CHCl ₃), Oleandomycin phosphate
Kasugamycin working standard*1	Kasugamycin hydrochloride (C ₁₄ H ₂₅ N ₃ O ₉ ·HCl·H ₂ O)
Kanamycin working standard*1	Kanamycin monosulfate (C ₁₈ H ₃₆ N ₄ O ₁₁ ·H ₂ SO ₄ ·H ₂ O)
Kitasamycin working standard*5	Leucomycin A (C ₃₉ H ₆₅ NO ₁₄)
Quebemycin working standard*1	Sodium Quebemycin (C ₆₇₋₇₀ H ₁₁₆₋₁₃₄ N ₅₋₆ O ₄₀₋₄₃ PNa ₂₋₃)
Spiramycin working standard *3	Spiramycin [C ₄₃ H ₇₄ N ₂ O ₁₄ (50 %), C ₄₅ H ₇₆ N ₂ O ₁₅ (25 %), C ₄₆ H ₇₈ N ₂ O ₁₅ (25 %)]
Thiopeptin working standard*5	Thiopeptin (Thiopeptin Ba: C ₇₁ H ₈₄ N ₁₈ O ₁₈ S ₆ , Thiopeptin Bb: C ₇₁ H ₈₂ N ₁₈ O ₁₈ S ₆ , Thiopeptin A ₁ a: C ₇₂ H ₈₆ N ₁₈ O ₁₈ S ₆ , Thiopeptin A ₁ b: C ₇₂ H ₈₄ N ₁₈ O ₁₈ S ₆ , Thiopeptin A ₃ a: C ₆₅ H ₇₉ N ₁₇ O ₁₅ S ₆ , Thiopeptin A ₃ b: C ₆₅ H ₇₇ N ₁₇ O ₁₅ S ₆ , Thiopeptin A ₄ a: C ₆₈ H ₈₂ N ₁₈ O ₁₆ S ₆ , Thiopeptin A ₄ b: C ₆₈ H ₈₀ N ₁₈ O ₁₆ S ₆)
Hygromycin B working standard*5	Hygromycin B (C ₂₀ H ₃₇ N ₃ O ₁₃)
Fradiomycin working standard*2	Fradiomycin sulfate (C ₂₃ H ₄₆ N ₆ O ₁₃ ·3H ₂ SO ₄)
Polynactin working standard*5	Polynactin (Tetranactin: C ₄₄ H ₇₂ O ₁₂ , Trinactin: C ₄₃ H ₇₀ O ₁₂ , Dinactin: C ₄₂ H ₆₈ O ₁₂)
Macarbomycin working standard*2	Macarbomycin ammonium (C ₆₈₋₇₉ H ₁₂₃₋₁₄₄ N ₆₋₇ O ₄₁₋₄₃ P· χ NH ₄ OH)

*1: Deleted as of January 1, 1984.

*2: Deleted as of April 1, 1986.

*3: Deleted as of September 1, 1990.

*4: Deleted as of March 18, 1997.

*5: Deleted as of October 12, 2004.

[10] The method to prepare the standard stock solution, which is detailed in the individual monograph, is summarized as follows:

In the case where preparatory drying conditions are specified, dry the working standard under reduced pressure according to the conditions before weighing. Then, from about 30 minutes before weighing, allow the dried working standard to become room temperature in a desiccator containing a drying agent (e.g., silica gel). Weigh to a precision of 0.1 mg into a weighing bottle on a precision balance placed in an environment at a relative humidity of not exceeding 50%. Calculate the amount of the solvent required to dissolve the working standard based on the concentration of the standard stock solution and the labeled potency of the working standard.

Place the calculated amount of the solvent in a stoppered Erlenmeyer flask. Transfer the working standard in the weighing bottle to the stopped Erlenmeyer flask using a Komagome pipette (capillary or Pasteur pipette etc.) while rinsing the bottle with the solvent to achieve complete transfer,

and mix on a magnetic stirrer for about 30 minutes. After the complete dissolution of the working standard, measure accurately 2 mL each into small tubes etc., and store in a hermetic container at -20°C or lower. Make sure to bring the standard stock solution to room temperature before use.

When a small amount of organic solvent is required for dissolving the working standard, first take an accurately measured portion of the solvent, e.g., 1 mL per 20 mg of the working standard, into a weighing bottle and dissolve the working standard. Then place the amount of the organic solvent remaining after subtracting the amount used for dissolution in a stoppered Erlenmeyer flask, and, in the same manner mentioned above, transfer the working standard in the weighing bottle to the stoppered Erlenmeyer flask using a Komagome pipette etc. while rinsing the bottle with the solvent to achieve complete transfer, and dissolve the contents completely to prepare the standard solution.

[11] It is recommended to use sealable screw-top vials as storage containers in lieu of small test tubes. Further, standard stock solutions to be stored unfrozen, which can change in volume due to evaporation, should be sealed with parafilm etc.

[12] Prepare culture media as follows:

Place about 750 mL of water (allow for loss by evaporation due to heating) in a suitable container (e.g., stainless steel pot that can be put over direct fire), add a specified amount of medium material (other than agar) and dissolve with heating, add 13 to 20 g (usually 18 g) of agar previously soaked in about 300 mL of water, and completely dissolve with stirring. With the whole container immersed in water bath, stir the medium to cool down to about 60°C , adjust the pH using a pH meter, transfer the medium to a suitable glass container (e.g., incubation bottle), plug with aluminum foil, and sterilize by autoclaving.

Where a change in the quality of the medium material due to heating can affect the sensitivity of the test organisms to the antibiotic, it is applicable to prepare the medium without heating. In this case, proceed as follows:

Place 1,000 mL of water in a suitable container, add a specified amount of medium material (other than agar) and completely dissolve with stirring on a magnetic stirrer, adjust the pH using a pH meter, transfer to a suitable glass container (e.g., incubation bottle) containing 13 to 20 g (usually 18 g) of agar, plug with aluminum foil, and sterilize by autoclaving.

If the pH of the medium has changed after autoclave sterilization, which can affect the growth of the test organism and thus assay results, re-adjust the pH.

[13] Medium F-4 may be replaced by a medium prepared by dissolving a required amount of Antibiotic Medium 4 (Difco) or an equivalent dry powder medium in 1,000 mL of water and sterilizing in an autoclave.

[14] Medium F-5 may be replaced by a medium prepared by dissolving 37 g of Brain Heart Infusion Broth (Difco, BBL, Oxoid, Eiken Chemical Co., Ltd.) or an equivalent dry powder medium and 2 g of meat extract in 1,000 mL of water, adjusting the pH to 7.9 to 8.1, add 18 g of agar, and sterilizing in an autoclave.

[15] Medium F-6 may be replaced by a medium prepared by dissolving 45 g of Antibiotic Medium 12 (Difco) or an equivalent dry powder medium and 10.2 g of sodium chloride in 1,000 mL of water, and sterilizing in an autoclave.

- [16] Medium F-7 may be replaced by a medium prepared by dissolving a required amount of Antibiotic Medium 4 (Difco) or an equivalent dry powder medium in 1,000 mL of water, and adjusting the pH to 7.9 to 8.1, or by dissolving a required amount of Antibiotic Medium 5 (Difco) or an equivalent dry powder medium and 1 g of glucose in 1,000 mL of water, and sterilizing in an autoclave.
- [17] Medium F-8 may be replaced by a medium prepared by dissolving 45 g of Antibiotic Medium 12 (Difco) or an equivalent dry powder medium in 1,000 mL of water, adjusting the pH to 7.9 to 8.1, and sterilizing in an autoclave.
- [18] Medium F-9 may be replaced by a medium prepared by dissolving a required amount of Antibiotic Medium 10 (Difco) or an equivalent dry powder medium in 1,000 mL of water, and sterilizing in an autoclave.
- [19] Medium F-12 may be replaced by a medium prepared by dissolving 10 g of Blood Agar Base No.2 (Oxoid) or an equivalent dry powder medium and 18 g of agar in 1,000 mL of water, and sterilizing in an autoclave.
- [20] Medium F-17 may be replaced by a medium prepared by dissolving a required amount of Antibiotic Medium 8 (Difco) or an equivalent dry powder medium in 1,000 mL of water, and sterilizing in an autoclave.
- [21] Medium F-23 can be replaced by a medium prepared by dissolving a required amount of Antibiotic Medium 3 (Difco) or an equivalent dry powder medium and 18 g of agar in 1,000 mL of water, and sterilizing in an autoclave.
- [22] a mixture of casein peptone and meat peptone, commercially available as Peptone (Difco), Polypeptone Peptone (BBL), Peptone Bacteriological (Oxoid), polypeptone (Nihon Pharmaceutical Co., Ltd.), etc.
- [23] Peptone obtained by pancreatic digest of casein, commercially available as Casitone, Tryptone (Difco), Trypticase Peptone (BBL), Tryptone, Tryptone T (Oxoid), etc.
- [24] Peptone obtained by papain digest of soy protein, commercially available as Phytone Peptone (BBL), Soytone (Difco), Peptone Soya (Oxoid), etc.
- [25] Peptone obtained by papaic digest of ox liver, commercially available as Liver Digest Neutralised (Oxoid).
- [26] Meat infusion condensed by heating, made from fish or beef. The former is commercially available as Wako Meat Extract (Wako Pure Chemical Industries, Ltd.) etc., and the latter, as "Lab-Lemco" Powder (Oxoid), Beef Extract (Difco, BBL) etc.
- [27] Bovine heart muscle infusion, not commercially available of its own, used as an ingredient of Heart Infusion Agar and Brain Heart Infusion Broth.
- [28] Calf brain infusion, not commercially available of its own, used as an ingredient of Brain Heart Infusion Broth.
- [29] Powder prepared by drying cold infusion of beer yeast or bread yeast at low temperatures, commercially available as Yeast Extract (Difco, Oxoid), powder yeast extract (Nihon Pharmaceutical Co., Ltd.), etc.
- [30] A polyoxyethylene ether of anhydrous sorbitol with a hydroxyl group partially esterified with oleic acid. It is nonionic surfactant and commercially available as a trade name of Tween 80 etc. The

chemical name is monooleic acid polyoxyethylene sorbitan.

[31] Power prepared by drying gel-like substance extracted from seaweeds (e.g., Ceylon moss, false Ceylon moss), commercially available as Bacto Agar (Difco), Agar Bacteriological (Agar No. 1) (Oxoid), etc.

[32] These test organisms may be stored as follows:

1) Storage in a slant medium

Allow Medium F-1 or F-201 to solidify in a test tube in a slant. Streak the test organism on the slant, incubate about 24 hours, and store at 4°C (or room temperature). Usually it can be stored for about 2 weeks to 3 months. Slant media are prepared as follows: dispense about 10 mL of Medium F-1 or F-201 into a test tube 18 mm in diameter and about 180 mm in length, plug with a silicon (or cotton) stopper, sterilize in an autoclave, and allow to solidify in a slant.

2) Storage in a semisolid medium

Use a medium prepared by diluting Medium F-1 or F-201 about 2-fold with water (or a commercially available semisolid medium). Dilute the medium about 2-fold with water, allow to solidify in a high layer in a test tube, stab the test organism, incubate for about 24 hours, stopper tightly, and store at 4°C (or room temperature). Usually it can be stored for several years.

3) Storage by freeze-drying

Place the test organism together with dispersing medium, such as skimmed milk, in ampoules, dry by vacuum-freezing, and store at -20°C or lower. Usually, it can be stored for 10 years or more.

The ampoules are opened as follows:

Wipe the neck of the ampoule with an alcohol swab, and make a scratch with a file. Wipe well the neck again with an alcohol swab, and apply a red-heated glass rod onto the neck a little distant from the scratch to make a crack. Wrap the head of the ampoule with sterilized gauze, and snap off. Disperse the content in suitable media, such as Medium F-2 or F-202.

4) Storage by freezing

Place the test organism in a heat-resistant tube together with a dispersing medium, such as 10% glycerin (protection agent), and store sealed at -70°C or lower. Usually, it can be stored for several years.

The melting procedure is performed rapidly by washing frozen tubes with water while shaking gently until ice crystals disappear.

[33] As the concentration of suspension of test organism can vary with incubation conditions (e.g., temperature, medium type, duration), it may not always necessary to strictly comply with the amount of addition specified in the individual monograph (suspensions of test organisms other than spores, are prepared by incubating with shaking in Medium F-202). It is recommended, however, to confirm the optimal amount of test organism by performing a preparatory test.

The amounts of typical test organisms incubated with shaking or under static conditions in medium F-202 are shown in Table 9.1-4.

Table 9.1-4 Concentrations of test organisms in culture media incubated with shaking or under static conditions

Type of test organism	Concentration of test organism incubated with shaking	Concentration of test organism incubated under static conditions
<i>Bordetella bronchiseptica</i> ATCC 4617	3.2×10^9 bacteria/mL	9.3×10^8 bacteria/mL
<i>Corynebacterium xerosis</i> NCTC 9755	1.8×10^8 bacteria/mL	1.1×10^8 bacteria/mL
<i>Escherichia coli</i> ATCC 27166	1.3×10^9 bacteria/mL	4.5×10^8 bacteria/mL
<i>Micrococcus luteus</i> ATCC 9341	5.2×10^8 bacteria/mL	5.9×10^7 bacteria/mL
<i>Micrococcus luteus</i> ATCC 10240	3.2×10^9 bacteria/mL	1.3×10^8 bacteria/mL
<i>Staphylococcus aureus</i> ATCC 6538P	1.8×10^9 bacteria/mL	1.2×10^8 bacteria/mL

[Incubation conditions]

Fluid medium: Medium F-202

Incubation with shaking: Incubate at 35 to 37 °C for 24 hours (except for *C. xerosis* NCTC 9755, which is incubated for 4 hours) at a shaking rate of 60 cycles/min

Static incubation: at 35 to 37°C for 24 hours incubation

[34] Suspensions of test organisms or spores are diluted as follows:

Place 1 mL of the stock suspension of the test organism, which has been incubated with shaking, into a test tube containing 9 mL of medium F-2 or medium F-202, mix, and use as a 10-fold dilution of the test organism suspension.

Dilute the stock suspension of the test spores, after confirming the number of spores, with a suitable amount of water or isotonic sodium chloride solution, and use as the spore suspension.

[35] ATCC stands for the American Type Culture Collection, a source of bacterial strains in the US.

[36] In the case of a test organism that is stored for a long period of time, one that subcultured about 3 times has more vitality and thus often produces better results. In the case of a test organism that is used routinely, it is preferable to repeat transfer at about one-month intervals. An example of the method of subculture is as follows:

Using a platinum loop sterilized with a flame (cooled on the tube wall etc.), collect an amount of the test organism that has been stored by a suitable method, and dip in the condensed water generated on a slant medium, carefully not to touch the tube wall or surface of the medium. Streak the test organism, firstly in a



Figure 9.1-1 Method of streaking

straight line and then in a finely undulating line from the bottom (Figure 9.1-1), and incubate.

On the next day, collect the bacterial lawn on the slant media using a platinum loop sterilized with a flame (cool the tip with the condensed water), and repeat this procedure 2 or 3 times.

(Flame sterilization)

This is a method to sterilize a platinum loop, the mouth of a test tube, etc. by heating in a flame of a Bunsen burner for a few seconds.

A platinum loop attached with bacterial lawn should be heated high above the flame, charred with reducing flame, and burn completely with oxidizing flame. Care should be taken to prevent the lawn from spattering.

[37] A platinum loop is made of platinum wire 0.5 to 0.8 mm in diameter, but usually nichrome wire about 0.6 to 0.7 mm in diameter and 50 to 70 mm in length may be used. Bend one end of the wire to make a loop about 3 mm in external diameter with the other end inserted in a commercially available platinum loop holder. Other types of platinum tools include a platinum coil, which is used for scraping away a large amount of bacterial lawn, and a platinum needle, which is used for stab-inoculation. Sterilized disposable platinum loops are also commercially available.

[38] The method of incubation with shaking, which differs with types of shaking incubators (e.g., reciprocal, see-saw, or rotary), is summarized as follows:

Place 10 to 20 mL of Medium F-2 or F-202 in a test tube, L-tube, or Erlenmeyer flask, etc., close with a silicon (or cotton) stopper, and sterilize in an autoclave. Collect the lawn growth on the slant media with a flame-sterilized platinum loop, scrape off the lawn on the boundary between the tube wall and medium, suspend in the medium, and load in an incubator.

Static incubation is applicable if there is no shaking incubator available. For the amount of addition for the suspension of the test organism, it is recommended to refer to the amounts of test organisms described in Table 9.1 to 4.

[39] NIHJ stands for National Institute of Health Japan, a national institute for infectious diseases.

[40] NCTC stands for National Collection of Type Cultures, a source of bacterial strains in the UK.

[41] A test organism developed by the Research Technology Headquarters of Fujisawa Pharmaceutical Co., Ltd. It is a mutant strain obtained by treating *Escherichia coli* ATCC 27166 with *N*-methyl-*N'*-nitro-*N*-nitroguanidine [$\text{CH}_3\text{N}(\text{NO})\text{C}(\text{NH})\text{NHNO}_2$] and is 100 times more sensitive to bicozamycin than the original strain. It is recommended that the test organism incubated in a slant medium be stored at room temperature. If the vitality of the test organism is considered to be weak, it is preferable to incubate with shaking in Medium F-2 or F-202 spiked with 0.3% yeast extract.

[42] It is preferable to streak on Medium F-1 or F-201 that has been allowed to solidify in slants in incubation bottles (about 10 bottles) or in plates in sterilized large Petri dishes. If a spore suspension has been prepared, the suspension (one with fewer spores is applicable) may be poured directly over the surface of the medium to prepare spores. An incubation period of 1 to 2 weeks is sufficient.

[43] Scrape away spores with a suitable tool such as a platinum coil or bacterial spreader, gather into an Erlenmeyer flask containing about 50 mL of water (or isotonic sodium chloride solution) and glass beads, gently shake to separate spores from agar, and filter through gauze in a funnel into a stopped centrifuge tube. For preparation of spore suspension, the tools and instruments should be sterilized by

suitable method before use.

[44] This procedure (intermittent sterilization) destroys proliferative bacteria, molds, etc. It is preferable to allow the culture media to stand at room temperature (not lower than 20°C).

(Low-temperature intermittent sterilization)

This is a method for sterilizing reagents, media, and other materials that can change in quality at high temperatures by repeating heating at 60 to 80°C for 30 to 60 minutes 2 to 5 times, usually once daily (24-hour intervals).

[45] The method for counting the number of spores is summarized as follows:

Place 9 mL of water (or isotonic sodium chloride solution) in each of about 10 medium-sized test tubes (capacity of about 25 mL), and sterilize by autoclaving. Add 1 mL of spore suspension to one of the tubes, and dilute. Serially dilute the dilution to prepare spore suspensions of $N \times 10^{-1}$ to $N \times 10^{-10}$ spores/mL (N : unknown number). Then, spread 10 mL of Medium F-201, previously melted and cooled to 49 to 51°C, evenly on Petri dishes (about 3 dishes per dilution), allow to stand horizontally to harden. Dispense 1 mL of each dilution and then 10 mL each of medium F-201 on each Petri dish to form a uniform layer, and allow to harden. Further, dispense 5 to 10 mL of Medium F-201 into each dish, and allow to harden. Incubate the dishes at 35 to 37°C (or 27 to 29°C) for 24 hours, count colonies on plates that have 30 to 300 colonies, and back-calculate from the mean value to estimate the number of spores in the spore suspension.

For example, if the average number of colonies on a plate treated with a spore dilution of $N \times 10^{-6}$ spores/mL is 120, the original number of spores is estimated to be 1.2×10^8 spores/mL.

[46] Care should be taken for avoparcin (premix and feed), alkyl trimethyl ammonium calcium oxytetracycline or oxytetracycline hydrochloride (feed), chlortetracycline (feed), and polynactins (feed) because their assay methods are specified otherwise.

[47] A medium that has been stored after preparation is re-melted by re-heating at 121°C for 5 to 10 minutes in an autoclave or by heating in a water bath, kept in a thermostat bath set at a temperature that does not kill the test organism or harden the medium (about 50°C). If the thermostat temperature is high, the test organism can die or not grow.

[48] Dispense the medium with a sterilized 10- or 20-mL measuring pipette (ordinary scale) into Petri dishes, promptly tilt the plate back and forth to uniformly spread, and allow to solidify on a horizontal plate to prevent an uneven layer. If it takes time to solidify due to high room temperature, it is applicable to use an air conditioner to make the agar solidity.

Use a measuring pipette whose tip is cut off (hole diameter about 3 mm) to facilitate the flow of culture media. For sucking up live bacteria, use a pipette with the mouth plugged with cotton wool. Sterilize the pipettes with dry heat before use. After use, place the pipettes in a container with disinfectant and sterilize in an autoclave after.

Use glass Petri dishes sterilized with dry heat before use. After use, sterilize the dishes in an autoclave, and wash off the agar. It is more convenient to use disposable plastic Petri dishes (those sterilized by radiation or gas are commercially available from Terumo, Nipro, etc.).

It is recommended that the horizontal board on which culture media are allowed to stand have a smooth surface in contact with Petri dishes and that the horizontality be checked with a level before

use. If the board is not horizontal, the agar layer becomes uneven, and the antibiotic does not diffuse uniformly in the media, preventing the formation of a circular zone of growth inhibition.

(Dry-heat sterilization)

A method to sterilize tools and instruments made of glass, metal, fibers, heat-resistant rubber, etc., which are wrapped with paper or placed in cans, by heating in dry air using a dry-heat sterilizer at 180 to 200°C for 30 minutes to 1 hour. Placing too many tools to be sterilized in the sterilizer can lead to a decrease in sterilization efficiency, and opening the sterilizer while the inside temperature is still high can damage the tools to be sterilized due to rapid cooling.

(Radiation sterilization)

A method to sterilize tools and instruments made of glass, metal, fibers, plastic, etc. by irradiating gamma ray from radiation source containing radioisotopes (^{60}Co or ^{137}Cs).

(Gas sterilization)

A method to sterilize tools and instruments made of glass, porcelain, metal, fibers, plastic as well as facilities, equipments, etc. with such gases as ethylene oxide and formaldehyde. Although usually a gas sterilizer is used, care should be taken for residual gas and byproducts.

[49] Sterilize cylinders (Toyo Sokki Co., Ltd., System Science Co., Ltd., etc.) with dry heat before use. It is preferable to place mechanically on agar plates using a cup dropper (Toyo Sokki Co., Ltd., System Science Co., Ltd., etc.); however, if there is no cup dropper available, it may be applicable to place the cylinders manually with a suitable tool such as sterilized tweezers.

[50] It is preferable to make cavities mechanically using a perforator with an apparatus for sucking up excess agar (Toyo Sokki Co., Ltd., System Science Co., Ltd., etc.). If there is no perforator available, it is applicable to make cavities as follows: stick a sterilized cork borer vertically into an agar plate and carefully remove the unnecessary pieces of agar one by one with a suitable tool such as a spatula; or place 4 cylinders on a Petri dish before dispensing agar medium and carefully pull out the cylinders with a suitable tool such as tweezers after the agar is hardened. Wash off agar attached to the used perforator or cork borer with ethanol for disinfection, etc., and then sterilize with ultraviolet light.

(Ultraviolet sterilization)

A method to sterilize facilities and equipments as well as the surfaces of tools made of glass, metals, fibers, plastic, etc., by irradiating 240 to 280 nm ultraviolet light for about 30 minutes. Special care should be taken not to look directly at or expose skin to the ultraviolet light.

[51] As some types of antibiotics can become unstable or lose their potency in an acidic or alkaline solution, it is recommended to promptly complete the process from extracting antibiotics from the sample through preparing the sample solution to dispensing onto agar plates. Do not use prepared solutions such as sample solution or standard solution the next day or later.

When a dilution procedure is required in the process of sample preparation (e.g., quantitative test for premixes), it is advisable to refer to [8] Preparation method for the standard solution.

[52] Two test methods are listed in the Feed Analysis Standards as microbiological assays: the 2-2 dose method and standard response line method. The former is used in quantitative tests for premixes and particular types of feeds, and the latter, for feeds. Both methods have their own characteristics. The 2-2 dose method is suitable when the concentration of the antibiotic in the sample is known, while the

standard response line method is suitable when the concentration of the antibiotic in the sample is unknown. Other test methods include microbioautography, liquid chromatography, and spectrophotometry.

[53] Dispense accurately a constant amount using a commercially available micropipette, carefully not to touch the cylinder or cavity. The dispensing of the standard and sample solutions should be completed as promptly as possible not to cause diffusion into the agar.

The dispensing amount of the standard solution and sample solution is 50 μL .

[54] Static incubation for about 2 hours at 10 to 20°C (temperatures that make it difficult for the test organism to grow) allows the test organism to penetrate and diffuse into the medium, making the size of the inhibition zone easier to measure. This procedure is necessary especially when the concentration of the antibiotic in the sample solution is low (e.g., quantitative test for feeds). If it is difficult to maintain the temperature at 10 to 20°C, the plates may be allowed to stand in a refrigerator or at room temperature.

[55] An incubator that has a wide range of adjustable temperature is preferable, but any is applicable if it covers the ranges of incubation temperatures specified in the Feed Analysis Standards, which are 35 to 37°C, 29 to 31°C and 27 to 29°C. After dispensing, place the Petri dishes on the same rack of the incubator and incubate under the same conditions wherever possible in order to minimize any difference in the size of the inhibition zone. It is convenient to use an incubator that is programmable for the temperature and time for diffusion, incubation, and storage.

[56] Incubation temperature of agar plates is 27 to 29°C for efrotomycin (feed), quebemycin (premix), flavophospholipol (premix) and macarbomycin (premix and feed), and 29 to 31°C for polynactins (feed).

[57] Usually, the diameter of the zone of inhibition is measured in units of 0.25 mm using a caliper, but it is acceptable to use a projection or image-processing inhibition zone meter (System Science Co., Ltd., etc.), if available. When the zone of inhibition is clearly insufficient due to leakage etc., it is recommended to exclude the value of this inhibition zone and substitute the average value of the other inhibition zones, or perform a re-test.

[58] For example, quantitative data for virginiamycin in a premix (the labeled amount: 5 g (potency)/kg; concentration of S_H , and U_H : 2 μg (potency)/mL; concentration of S_L and U_L : 0.5 μg (potency)/mL; $X = 4$) are shown in Table 9.1-5. From these values calculate the sum of the inhibition zone diameters for each concentration of the standard solution and for each concentration of the sample solution ($\sum S_H$, $\sum S_L$, $\sum U_H$ and $\sum U_L$). Further, substitute the calculated values into the equation shown below to calculate $\log \theta$, and calculate the ratio (θ) of the concentration of the antibiotic in the sample solution to that in the standard solution.

Table 9.1-5 Quantitative data for virginiamycin in a premix

(in mm)				
No.	I	III	II	IV
Agar plate	High-	Low-	High-	Low-
	concentration standard solution (S _H)	concentration standard solution (S _L)	concentration sample solution (U _H)	concentration sample solution (U _L)
1	22.5	16.7	23.1	16.9
2	23.1	16.7	22.9	16.8
3	23.2	16.8	23.4	16.8
4	23.3	17.1	23.4	17.0
5	22.9	17.1	23.7	17.3
Total	ΣS _H =115.0	ΣS _L = 84.4	ΣU _H =116.5	ΣU _L = 84.8

$$\begin{aligned} \log \theta &= \frac{(\sum U_H + \sum U_L) - (\sum S_H + \sum S_L)}{(\sum U_H + \sum S_H) - (\sum U_L + \sum S_L)} \times \log X \\ &= \frac{(116.5 + 84.8) - (115.0 + 84.4)}{(116.5 + 115.0) - (84.8 + 84.4)} \times \log 4 \\ &= \frac{1.9}{62.3} \times \log 4 \\ &= 1.84 \times 10^{-2} \\ \theta &= 1.043 \end{aligned}$$

In this case, the standard solution and sample solution are diluted and prepared to have the same concentration. Because the labeled amount of this sample is 5 g (potency)/kg, antibiotic concentration in the sample is calculated to be $1.043 \times 5 \text{ g (potency)/kg} = 5.215 \text{ g (potency)/kg}$.

[59] If the value of θ is not more than 0.5 or not less than 1.5, it is recommended to obtain a more accurate value by repeating the test by adjusting test conditions such as the amount of the sample or dilution factor by converting from the value of θ .

[60] The value of X for antibiotics listed in the Feed Analysis Standards is 4 except for semduramicin sodium (in premix or feed) and lasalocid sodium (in feed), for which the value is 2.

[61] For example, quantitative data for zinc bacitracin (labeled amount: 1,680,000 unit/ton; concentration of RP: 0.1 unit/mL) are shown in Table 9.1-6. From these data, calculate the average inhibition zone diameter for each concentration of the standard solution and the average inhibition zone diameter for RP from each set of concentration as shown in the table below, and then the average inhibition zone diameter for RP (average value for correction: C) from all 4 sets, which is calculated to be $C = 20.58$. From the value of C , as in the example of the main text, the corrected inhibition zone diameters (A , B , D , and E) are calculated to be $A = 25.14$, $B = 23.13$, $D = 18.16$, and $E = 15.65$.

Plot the values of A to E versus the logarithm of corresponding concentrations of the standard solution on a semilog graph paper (Figure 9.1-2), draw the straight line that passes nearest the plotted points, and use as the standard response line. As these points often lie approximately on a straight line, as seen in the following example, the standard response line is often prepared by substituting the values of A to E directly into the equation to calculate the values of H and L , and connecting these

points with a straight line.

Table 9.1-6 Quantitative data for zinc bacitracin in feeds

Content Agar plate	RP	0.4	RP	0.2	RP	0.05	RP	0.025	RP	Sample
1	20.7	25.2	20.5	23.2	20.7	18.2	20.7	15.6	20.7	20.2
2	20.7	25.2	20.3	22.8	20.7	18.3	20.5	15.8	20.3	20.2
3	20.7	25.2	20.5	23.0	20.5	18.2	20.5	15.6	20.5	19.8
4	20.7	25.4	20.4	23.0	20.6	18.3	20.6	15.6	20.5	20.1
5	20.7	25.8	20.4	22.8	20.7	18.3	20.6	15.6	20.5	20.0
6	20.7	25.2	20.4	22.8	20.7	18.0	20.5	15.8	20.6	20.2
7	20.7	25.1	20.5	22.8	20.7	18.0	20.5	15.7	20.8	20.2
8	20.7	25.1	20.3	23.1	20.6	18.2	20.7	15.7	20.5	20.5
9	20.7	25.2	20.5	23.0	20.5	18.2	20.7	15.6	20.3	20.4
10	20.7	25.2	20.2	23.0	20.5	18.3	20.5	15.5	20.5	20.4
Average inhibition zone diameter	20.70	25.26	20.40	22.95	20.62	18.20	20.58	15.65	20.52	20.20
Corrected inhibition zone diameter	/	A=	/	B=	/	D=	/	E=	/	Y=
		25.14		23.13		18.16		15.65		20.21

$$L = (3E + 2D + C - A) / 5$$

$$= (3 \times 15.65 + 2 \times 18.16 + 20.58 - 25.14) / 5$$

$$= 15.74$$

$$H = (3A + 2B + C - E) / 5$$

$$= (3 \times 25.14 + 2 \times 23.13 + 20.58 - 15.65) / 5$$

$$= 25.32$$

Based on the values of H and L , the corrected inhibition zone diameter for RP (C^*) on the standard response line is calculated to be $C^* = (H+L) / 2 = 20.53$. Based on the value of C^* , the corrected inhibition zone diameter (Y) for the sample solution is calculated to be $Y = 20.21$. Thus, the concentration of 0.0912 unit/mL corresponding to 20.21 mm on the standard response line is the antibiotic concentration of the sample solution (n) to be estimated.

When using a calculator etc., calculate the following equation for the standard response line for the above-mentioned values of H and L :

$$Y = \frac{H-L}{\log a - \log e} \times \log x + \left(L - \frac{H-L}{\log a - \log e} \times \log e \right)$$

$$= \frac{25.32 - 15.74}{4 \log 2} \times \log x + \left(15.74 - \frac{25.32 - 15.74}{4 \log 2} \times \log 0.025 \right)$$

$$= \frac{2.395}{\log 2} \times \log x + 28.49$$

and substitute $Y = 20.21$ into this equation:

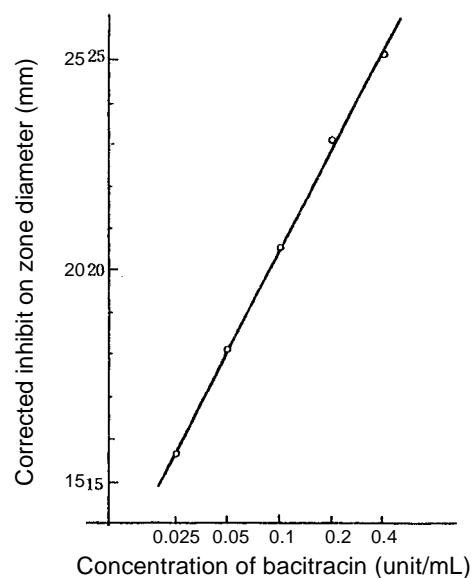


Figure 9.1-2 Standard response line for bacitracin

$$n = 10^{\frac{(20.21-28.49) \times \log 2}{2.395}} = 10^{-1.040} = 0.0912$$

In this case, RP and sample solution are diluted and prepared to have the same concentration. As the concentration of this reference standard solution is 0.1 unit/mL, θ is calculated to be $0.0912 / 0.1 = 0.912$. As the labeled amount of this sample is 1,680,000 unit/ton, antibiotic concentration in the sample is calculated to be $0.912 \times 1,680,000$ unit/ton = 1,530,000 unit/ton.

2 Microbioautography^[1] [Feed Analysis Standards, Chapter 9, Section 1 2]

A. Reagent preparation

- 1) Buffer solution. Proceed as directed in 1 A 1).
- 2) Standard solution. Proceed as directed in 1 A 2).
- 3) Culture medium. Proceed as directed in 1 A 3).
- 4) Suspension of test organism or spores and the amount of addition. Proceed as directed in 1 A 4).

B. Preparation of the sample solution

Prepare the sample solution quickly as specified in the individual monograph in Section 2.

C. Quantification

Unless otherwise specified in the individual monograph in Section 2, proceed by the following method. Thin-layer chromatography. Designate a line about 20 mm distant from the bottom of the thin-layer plate^[2] as the starting line, spot using a microsyringe 20 μ L each of the standard solution and sample solution, 25 mm apart from the both sides, accurately on this line, separated by 30 mm, and air dry the plate.

Place the thin-layer plate in a developing chamber^[5] previously saturated^[4] with gas of the developing solvent^[3] for not less than 1 hour, allow the developing solvent to ascend at 20 to 25°C. When the front of the developing solvent has ascended to the distance directed in the individual monograph in Section 2, stop the development and take out the plate. Allow the plate to stand at room temperature, dry the developing solvent, and remove^[6].

Preparation of agar plates. Place a thin-layer plate, with the glass side down, in an incubation box (of stainless steel or plastic; 210 mm in length, 210 mm in width, and approximately 15 mm in height), spray the medium specified in the individual monograph in Section 2, previously melted and maintained at 49 to 51°C, evenly over the whole surface of the thin-layer plate^[7]. Allow the plates to stand for several minutes, add the suspension of the test organism or spores prepared as described in A 4) to 100 mL of the medium, thoroughly stir, pour to spread uniformly, and allow to stand horizontally to solidify.

Incubation. Allow the incubation box to stand at 10 to 20°C for 3 hours^[8], place in an incubator, and incubate at 35 to 37°C for 16 to 24 hours.

Identification. After incubation, take the incubation box out of the incubator, pour the chromogenic reagent specified in the individual monograph in Section 2 over the whole surface of the medium to develop color, and calculate the Rf value^{note 1} for identification based on the inhibition zone diameters for the standard solution and sample solution.

Measurement of the inhibition zone diameter. Accurately measure the major axis (a mm) and minor axis (b

mm) of the zone of inhibition to a precision of 0.25 mm or less, and use the geometric mean (\sqrt{ab} mm) as the inhibition zone diameter.

Calculation. Plot the inhibition zone diameters obtained from the standard solution on graph paper to create a calibration curve, and calculate the concentration of the sample corresponding to the inhibition zone diameter for the sample solution based on the calibration curve.

Note 1: $R_f \text{ value} = \frac{\text{Distance from the starting line to the center of the inhibition zone}}{\text{Distance from the starting line to the solvent front}}$

«Notes and precautions»

- [1] Microbioautography is a method to identify and quantify ingredients with antibacterial properties by separating them from the sample solution using thin-layer chromatography.
- [2] Use a commercially available thin-layer plate specified in the individual monograph, and activate before use under the conditions specified in the individual monograph. Store the plate in a desiccator.
- [3] Measure solvents for development at the time of use with a volumetric cylinder etc. in the proportions specified in the individual monograph, place in a suitable container, and mix.
- [4] Place the developing solvent in a container (developing chamber), close the cover, and allow to stand for not less than 1 hour to become saturated with gas. The amount of the developing solvent should be such that when the thin-layer plate is placed in the container, the surface of the solvent comes under the starting line of the plate.
- [5] Usually, use those made of glass.
- [6] Sufficient drying is required as insufficient removal of solvent can affect the growth of test organisms etc. A dryer is applicable for those that are relatively stable against heat such as polyether antibiotics. Remove the solvent in a draft chamber etc. for the safety of the operator.
- [7] Spraying the medium can prevent silica gel from flaking off, which can happen when the medium is flowed over the plate. It is recommended to spray with a spray nozzle attached at the tip of the air compressor.
- [8] Three hours of diffusion results in a sufficient size of inhibition zone for measurement.

Supplementary notes

The names of antibiotics can be abbreviated as follows:

Zinc bacitracin	: BC
Avilamycin	: AVM
Avoparcin	: AV
Alkyl trimethyl ammonium calcium oxytetracycline	: OTC
Efrotomycin	: ET
Oxytetracycline hydrochloride	: OTC
Spiramycin embonate	: SP
Enramycin	: ER
Orienticin	: OR
Kitasamycin	: KT
Chloramphenicol	: CP
Chlortetracycline	: CTC
Quebemycin sodium	: QM
Salinomycin sodium	: SL
Sedecamycin	: SCM
Semduramicin sodium	: SD
Thiopeptin	: TP
Destomycin A	: DM-A
Narasin	: NR
Nosiheptide	: NH
Hygromycin B	: HM-B
Virginiamycin	: VM
Bicozamycin	: BZM
Flavophospholipol	: FV
Polystyrenesulfonic acid oleandomycin	: OM
Polynactin	: PN
Macarbomycin	: MC
Bacitracin manganese	: BC
Monensin sodium	: MN
Lasalocid sodium	: LS
Kanamycin sulfate	: KM
Colistin sulfate	: CL
Fradiomycin sulfate	: FM
Tylosin phosphate	: TS

[Reference]

«Literature on antibiotics»

- Related articles (excluding study reports on feeds etc.)

Institute of Medical Science University of Tokyo ed., “微生物学実習提要 第2版 (Manual to Practice Bacteriology, 2nd)”, Maruzen Co., Ltd. (1998)

The Pharmaceutical Society of Japan ed., “衛生試験法・注解 2005 (Methods of Analysis in Health Science 2005)”, Kanehara & Co., Ltd. (2005)

The Japanese Pharmacopoeia, Fifteenth Edition:

(<http://www.mhlw.go.jp/topics/bukyoku/iyaku/yakkyoku/>)

For details of culture media, refer to the following works:

Riichi Sakazaki: “新細菌培地学講座 上 第2版 (New Bacterial Culture Media I, 2nd)”, Kindai Shuppan Co., Ltd. (1986)

The Oxoid Manual 8th Edition: Oxoid LTD. (1998)

Difco™ & BBL™ Manual: <http://www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp>

For standard methods for counting the number of plated bacteria, refer to the following works:

Institute of Medical Science University of Tokyo ed., “微生物学実習提要 第2版 (Manual to Practice Bacteriology, 2nd)”, Maruzen Co., Ltd. (1998)

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Kiyoji Ninomiya: “動物の抗生物質 (Antibiotics for Animals)”, Yokendo Co., Ltd. (1987)

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