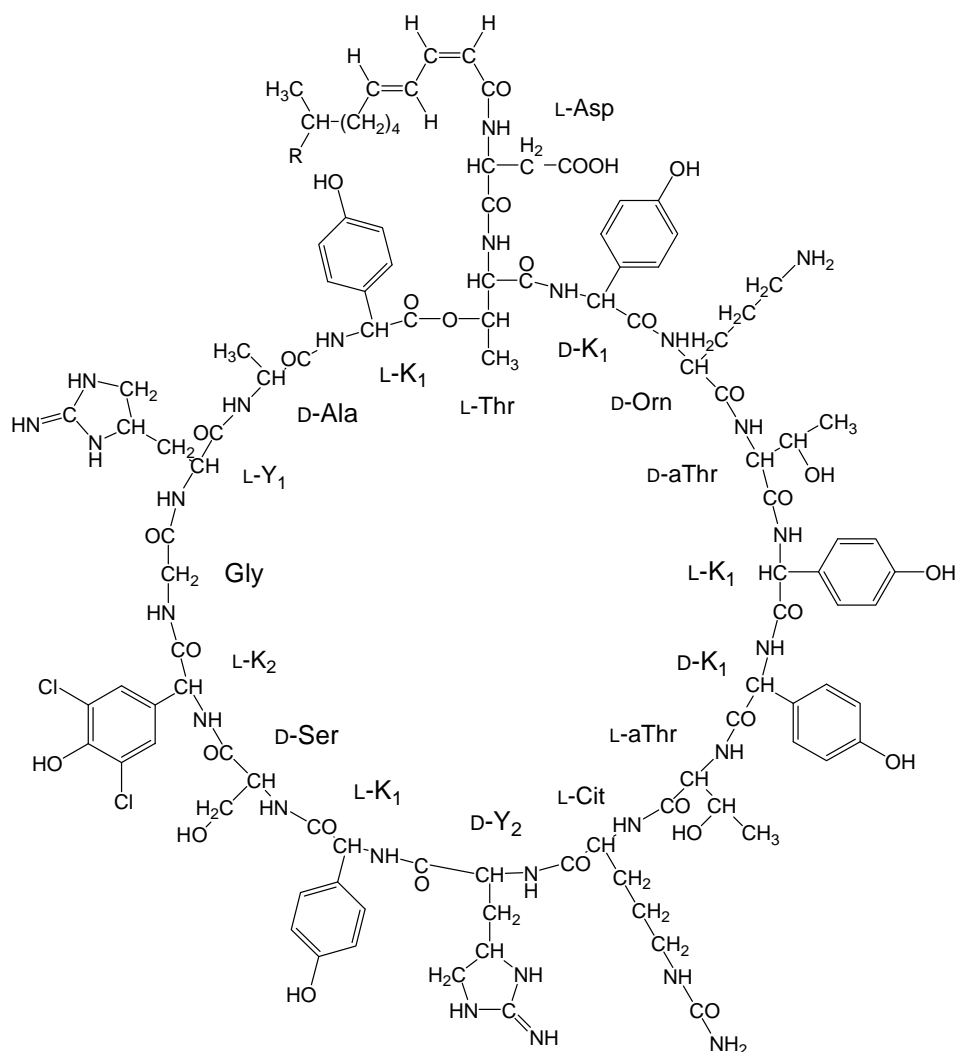


7 Enramycin



K₁: α -amino-4-hydroxyphenyl acetic acid

K₂: α -amino-3,5-dichloro-4-hydroxyphenyl acetic acid

Y₁: α -(S)-amino- β -4(R)-(2-iminoimidazolidinyl) propionic acid

Y₂: α -(R)-amino- β -4(R)-(2-iminoimidazolidinyl) propionic acid

ER-A R: CH₃ C₁₀₇H₁₃₈Cl₂N₂₆O₃₁ MW: 2355 CAS No.: 34438-27-2

ER-B R: C₂H₅ C₁₀₈H₁₄₀Cl₂N₂₆O₃₁ MW: 2369 CAS No.: 34304-21-7

[Summary of enramycin]

Enramycin (ER) is a polypeptide antibiotic obtained by the incubation of *Streptomyces fungicidicus*, and has the chemical structure shown above.

For physicochemical properties, ER technical occurs as a gray-brown to brown powder or particles, and slightly has a characteristic odor. It is freely soluble in dilute hydrochloric acid and in dimethylformamide, slightly soluble in water and in methanol, and very slightly soluble in acetone, in ethanol, in chloroform and in benzene.

ER has an antibacterial effect mainly on Gram-positive bacteria and a growth promoting effect on chickens (including broilers) and pigs.

A study of time-dependent changes of a feed spiked with ER has showed that it practically stable for 1 month when stored at 0°C but loses its potency by about 10% when stored for 1 month at 30°C or higher.

«Standards and specifications in the Act on Safety Assurance and Quality Improvement of Feeds»

ER is a feed-grade antibiotic that was designated as a feed additive as of July 24, 1976. The specifications for feeds containing this ingredient are specified in Appendix 1, 1-(1)-C of the Ordinance on Specifications for Ingredients, etc. of Feeds and Feed Additives.

Feed of interest	(in g(potency/t))			
	For chickens (except for broilers)	For broilers	For pigs	
	Starting chicks Growing chicks	Starting broilers Finishing broilers	Sucking piglets	Piglets
Added amount	1~10	1~10	2.5~20	2.5~20

The amount of ER added to a commercial premix is roughly 1 to 100 g (potency)/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test methods - Plate method

1.1 Premix

[Feed Analysis Standards Chapter 9, Section 2, 7.1.1]

A. Reagent preparation

- 1) Buffer solution: Buffer No.4
- 2) Enramycin standard solution. Dry a quantity of enramycin working standard^[1] under reduced pressure (not exceeding 0.27 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add a mixture of methanol and water (4:1) and dissolve to prepare an enramycin standard stock solution with a concentration of 1 mg (potency)/mL^[2].
At the time of use, accurately dilute a quantity of the standard stock solution with Buffer No.4 to prepare high- and low-concentration standard solutions with concentrations of 4 and 1 µg (potency)/mL, respectively^[3].
- 3) Culture medium: Medium F-111
- 4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240^[4] as the test organism. Add about 0.2 mL of a 100-diluted suspension of the test organism to 100 mL of the culture medium.
- 5) Agar plate. Proceed by the agar well method.
- 6) Extracting solvent: A mixture of phosphoric acid (0.075 mol/L)^[5] and acetone (2:1)

B. Preparation of sample solution

- 1) When the analysis sample does not contain SL, NR or MN
Weigh accurately 1 to 4 g of the analysis sample (equivalent to not more than 10 mg (potency) of ER), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, and adjust the pH to 7.9 to 8.1 with ammonia solution. Transfer the whole amount of this liquid with Buffer No.4 to a 100-mL one-mark flask, further add Buffer No.4 up to the marked line, and filter through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with Buffer No.4 to prepare high- and low-concentration sample solutions with concentrations of 4 and 1 µg (potency)/mL, respectively^[6].

2) When the analysis sample contains SL, NR or MN^[7]

Weigh accurately 1 to 4 g of the analysis sample (equivalent to not more than 10 mg (potency) as ER), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, adjust the pH to 1.0 or lower with hydrochloric acid, allow to stand for 1 hour, and again adjust the pH to 7.9 to 8.1 with ammonia solution. Transfer the whole amount of this liquid with Buffer No.4 to a 100-mL one-mark flask, add Buffer No.4 up to the marked line, and filter through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with Buffer No.4 to prepare high- and low-concentration sample solutions with concentrations of 4 and 1 µg (potency)/mL, respectively^[6].

C. Quantification

Proceed by the 2-2 dose method^{[8][9]}.

«Summary of analysis method»

This method is intended to determine the amount of ER in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of phosphoric acid (0.075 mol/L) and acetone (2:1) and diluting with Buffer No.4.

The flow sheet of this method is shown in Figure 9.2.7-1.

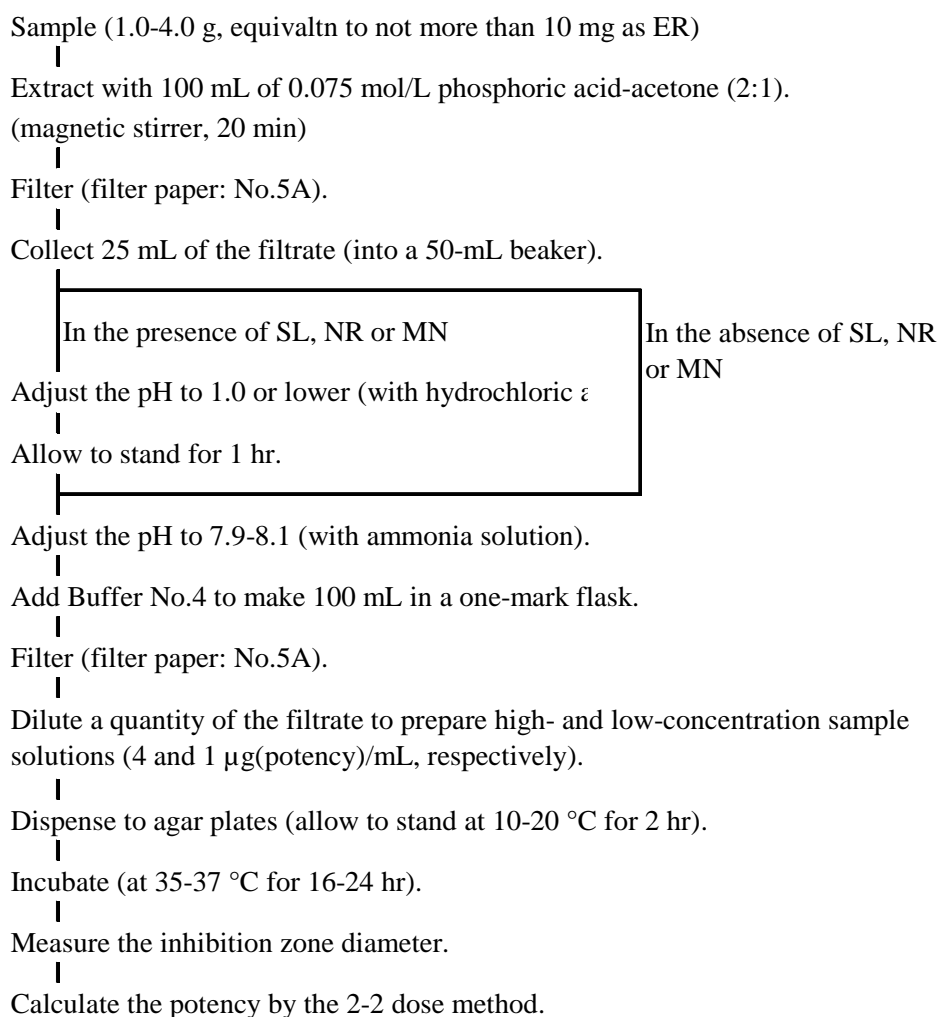


Figure 9.2.7-1 Quantitative test method for enramycin (premix)

References: Toshikazu komoritani, Fumihiro Abe, Yuji Fukumoto, Yukinobu Nakamura: Research Report of Animal Feed, 27, 47 (2002)

History in the Feed Analysis Standards [3] New, [25] Revision

«Validation of analysis method»

• Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Chicken premix	0.5~10	3	94.6~100.6	5.0
Chicken premix	0.5~10	3	97.0~99.9	4.3
Pig premix	0.5~10	3	93.6~95.7	2.5

• Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/kg)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Chicken premix	7	5	97.4	0.9	3.1

«Notes and precautions»

[1] For the definition etc. of enramycin working standard, refer to «Notes and precautions» [9] in

Section 1, 1 of this Chapter.

[2] For the method of preparation for the standard stock solution, refer to «Notes and precautions» [10] in Section 1, 1 of this Chapter.

Method of preparation: example (when the weighed amount is 50 mg)

When the labeled potency of the working standard is 1,091 µg (potency)/mg, 50 mg of the working standard contains 54,550 µg (potency) (i.e., 50 mg × 1,091 µg (potency)/mg). To prepare a standard stock solution with a concentration of 1,000 µg (potency)/mL, the required amount of the solvent is thus calculated to be 54.55 mL (i.e., 54,550 µg (potency) / 1,000 µg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 54.55 mL of a mixture of methanol and water (4:1), and dissolve to prepare a standard stock solution with a concentration of 1,000 µg (potency)/mL.

[3] For the method of preparation for the standard solution, refer to «Notes and precautions» [10] in Section 1, 1 of this Chapter.

An example method of preparation for enramycin standard solution is shown in Table 9.2.7-1.

[4] For the number of bacteria, refer to «Notes and precautions» [33] in Section 1, 1 of this Chapter.

[5] Dilute 4.72 mL of phosphoric acid (Guaranteed) with water to make 1 L.

[6] For the method of preparation for the sample solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation is shown in Table 9.2.7-1.

Table 9.2.7-1 Method of preparation for enramycin standard solution and sample solution

1) Method of preparation for enramycin standard solution (premix, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	5	5
Amount (mL) of Buffer No.4	23	16	15	15
Concentration (µg(potency)/mL)	80	16	4	1

Note: ②mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

2) Method of preparation for sample solution (premix, example)

When the analysis sample is weighed in an amount equivalent to 10,000 µg (potency) of ER, the concentration of ER in the filtrate is calculated to be 25 µg (potency)/mL.

Test tube No.	1	2
Amount (mL) of sample solution	④	5
Amount (mL) of Buffer No.4	21	15
Concentration (µg(potency)/mL)	4	1

Note: ④ mL" means "4 mL" of the filtrate (25 µg(potency)/mL)".

[7] When the sample solution contains not less than 10 µg (potency)/mL of SL or MN, test organism *Micrococcus luteus* ATCC 10240 become sensitive to SL or MN and thus affect the quantified results of ER. To remove this effect, treat the sample solution with hydrochloric acid to reduce the antibacterial activity of these ingredients.

References: Noriyuki Koyama: Research Report of Animal Feed, 6, 163 (1980)

[8] An example standard response line for ER is shown in Figure 9.2.7-2.

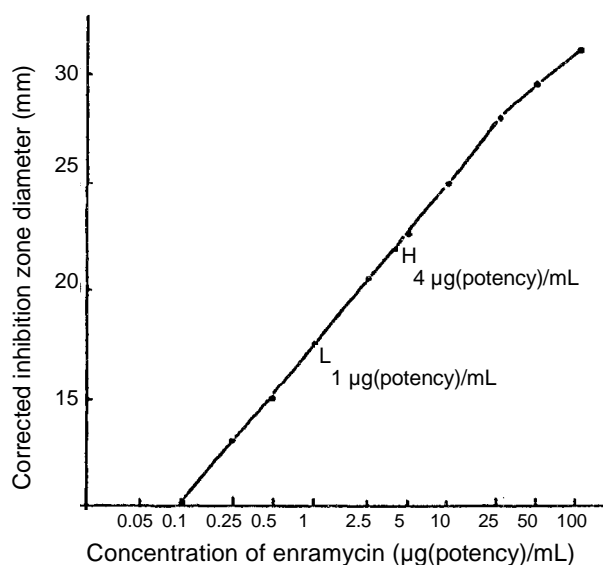


Figure 9.2.7-2 Standard response line for enramycin (premix, example)
(*Micrococcus luteus* ATCC 10240, Medium F-111, Agar well method)

[9] Refer to «Notes and precautions» [53] to [60] in Section 1, 1 of this Chapter.

1.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 7.2.1]

A. Reagent preparation

- 1) Buffer solution: Buffer No.3
- 2) Dilution solvent: A mixture of Buffer No.3 and acetone (7:3)
- 3) Enramycin standard solution. Dry a quantity of enramycin working standard under reduced pressure (not exceeding 0.27 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add a mixture of methanol and water (4:1) and dissolve to prepare an enramycin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with the dilution solvent to prepare standard solutions with concentrations of 1.6, 0.8, 0.4, 0.2 and 0.1 µg (potency)/mL^[1].

- 4) Culture medium: Medium F-21^[2]
- 5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240^[3] as the test organism. Add about 0.1 mL of a 10-fold diluted suspension of the test organism to 100 mL of the culture medium.
- 6) Agar plate. Proceed by the agar well method^[4].
- 7) Extracting solvent: A mixture of hydrochloric acid (0.5 mol/L) and acetone (7:3)^[5]

B. Preparation of sample solution

- 1) When the analysis sample does not contain SL or MN

Weigh accurately a quantity of the analysis sample (equivalent to 80 µg (potency) as ER), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×g for 5

minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, and adjust the pH to 5.9 to 6.1^[6] with ammonia solution (6 mol/L). Transfer the whole amount of this liquid with the dilution solvent to a 50-mL one-mark flask, further add the dilution solvent up to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.4 µg (potency)/mL.

2) When the analysis sample contains SL or MN^[7]

Weigh accurately a quantity of the analysis sample (equivalent to 80 µg (potency) as ER), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×g for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Measure accurately 25 mL of the filtrate, place in a 50-mL beaker, allow to stand for 1 hour, and adjust the pH to 5.9 to 6.1 with ammonia solution (6 mol/L). Transfer the whole amount of this liquid with the dilution solvent to a 50-mL one-mark flask, add the dilution solvent up to the marked line, filter through filter paper (No.5A), and prepare a sample solution with a concentration of 0.4 µg (potency)/mL.

C. Quantification

Proceed by the standard response line method^{[8][9]}.

«Summary of analysis method»

This method is intended to determine the amount of ER in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of hydrochloric acid (0.5 mol/L) and acetone (7:3) and adjusting the pH to 5.9 to 6.1.

SL or MN, which are approved for combined use with ER, can affect the quantification of ER. When the feed of interest contains these ingredients, allow the sample to stand for 1 hour after the extraction/filtration process in order to nullify the effect of these ingredients by exposing to hydrochloric acid present in the filtrate.

When the feed of interest contains a large amount of skimmed milk, such as formula feed for sucking piglets, application of this method can result in a decrease in ER recovery if ER content in the analysis sample is less than 5 g (potency)/t.

The flow sheet of this method is shown in Figure 9.2.7-3.

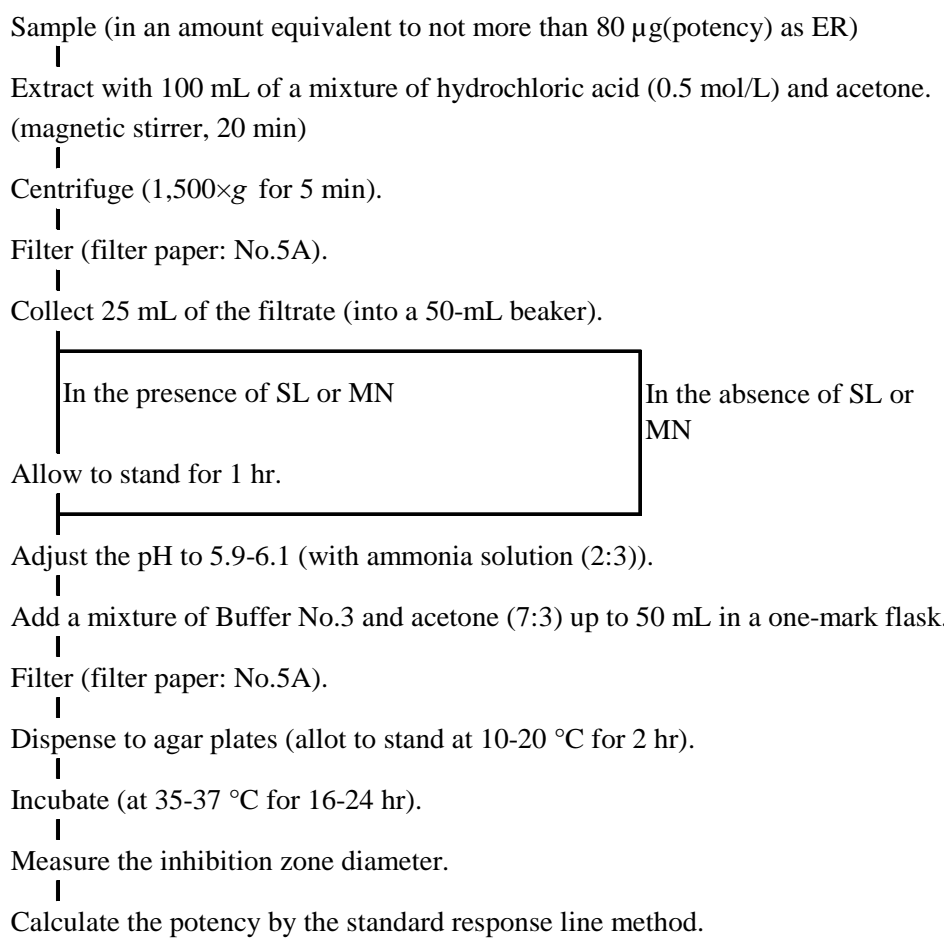


Figure 9.2.7-3 Quantitative test method for enramycin (feed)

References: Toyoko Kusama: Research Report of Animal Feed, 7, 140 (1981)

History in the Feed Analysis Standards [5] New

«Validation of analysis method»

• Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Starting chick formula feed	5~20	4	94.6~100.6	5.0
Growing chick formula feed	5~20	4	97.0~99.9	4.3
Sucking piglet formula feed	5~20	4	97.0~99.10	5.3
Growing piglet formula feed	5~20	4	93.6~95.7	2.5

• Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDr (%)
Sucking piglet formula feed	5	10	100.1	5.5	8.6

«Notes and precautions»

[1] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation for enramycin standard solution is shown in Table 9.2.7-2.

Table 9.2.7-2 Method of preparation for enramycin standard solution (feed, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	2	4	10	10	10	5
Amount (mL) of Buffer No.3- acetone (7:3)	23	18	16	10	10	10	5
Concentration (µg(potency)/mL)	80	8	1.6	0.8	<0.4>	0.2	0.1

Note: ② mL" means "2 mL" of standard stock solution (1 mg(potency)/mL)".

[2] This culture medium is an improved version of Medium 13, a medium intended for assay of ER formulations (composed of the same components as Medium F-13) specified in Appended Table 2 of the Ministerial Ordinance Concerning the Ingredient Specifications for Feeds and Feed Additives (Ordinance No. 35, issued by the Ministry of Agriculture and Forestry in 1976), with changes in the amount of sodium chloride and pH. The sensitivity to ER has been improved by increasing the sodium chloride content to inhibit the growth of the test organism, which, however, can result in an ill-defined edge of the zone of growth inhibition if the vitality of the test organism is low. In this case, it is recommended to prolong the incubation period to 24 hours.

When preparing a culture medium, avoid long-term heating by, e.g., melting sodium chloride after having melted the other ingredients with heat. It is also necessary to minimize the use of an autoclave.

When the culture medium results in poor growth of the test organism, it is recommended to decrease the amount of sodium chloride from 50 g to 30 g (per 1,000 mL of the culture medium) or change the pH from 5.9-6.1 to 7.9-8.1.

Medium F-21 can be replaced by a culture medium prepared by adding 45 g of Antibiotic Medium 12 (Difco) and 22.8 g of sodium chloride to 1,000 mL of water and dissolving. Care should be taken, however, that the growth of the test organism can be poor depending on the lot of the dry powder culture medium.

[3] For the number of bacteria, refer to «Notes and precautions» [33] in Section 1, 1 of this Chapter.

[4] Because of the low diffusion rate of ER in the agar culture medium, the agar well method was employed instead of the cylinder plate method because the former is more favorable for ER to diffuse. This resulted in a suitable size of the inhibition zone and a suitable slope of the standard response line.

[5] Corresponds to a mixture of water, acetone, and hydrochloric acid (22:10:1).

[6] To facilitate pH adjustment, it is advisable to add 5 to 10 mL of a mixture of Buffer No.3 and acetone (7:3) to 25mL of the filtrate in advance.

[7] When the sample solution contains not less than 10 mg (potency)/mL of SL or MN, test organism *Micrococcus luteus* ATCC 10240 becomes sensitive to these ingredients and thus produces a larger inhibition zone (Figure 9.2.1-2) . To remove this effect, treat the sample solution with hydrochloric acid when the analysis sample contains SL or MN in combination. Refer to 1. Zinc bacitracin or manganese bacitracin 1.1 «Notes and precautions» [8] in this Section.

[8] An example standard response line for ER is shown in Figure 9.2.7-4.

Linearity is observed in the range of quantification for ER (ER concentrations between 0.1 and 1.6 µg

(potency)/mL).

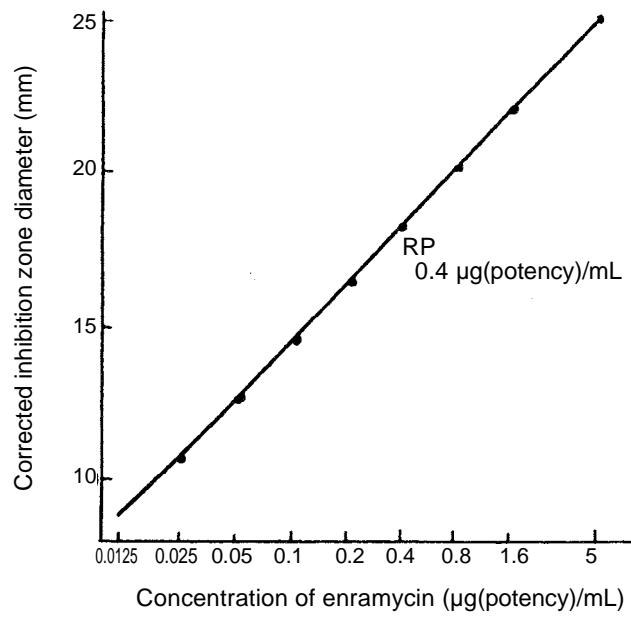


Figure 9.2.7-4 Standard response line for enramycin (feed, example)
(*Micrococcus luteus* ATCC 10240, Medium F-21, Agar well method)

[9] Refer to «Notes and precautions» [53] to [58] and [61] in Section 1, 1 of this Chapter.