Establishment of Evaluation Criteria for Feed Additives

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Director General of the Livestock Industry Bureau, MAFF
Director-General of the Fisheries Agency
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When the Minister of Agriculture, Forestry and Fisheries intends to designate a feed additive based on the provisions of Article 2, paragraph (3) of the Act on Safety Assurance and Quality Improvement of Feed (Act No. 35 of 1953), or intends to establish any standards or specifications based on the provisions of Article 3, paragraph (1) of the same Act, the Minister is required to hear the opinions of the Agricultural Materials Council under Article 2, paragraph (3) or Article 3, paragraph (2) of the same Act. With regard to the evaluation criteria, which serve as the indicators for the deliberations of the Council, a notice titled "Establishment of Evaluation Criteria for Feed Additives, etc." (dated April 5, 1977; circular notice by the Director General of the Livestock Industry Bureau, Ministry of Agriculture and Forestry, and the Director-General of the Fisheries Agency; 52-Chiku A No. 1200 and 52-Suigyo No. 1111) and a notice titled "Establishment of Evaluation Criteria for Live Microbial Agents as Feed Additives" (dated May 30, 1991; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 3-Chiku A No. 1169) had been issued. Regarding matters to note in conducting tests, etc., a notice titled "Establishment of Test Manuals Based on the Evaluation Criteria for Feed Additives" (dated February 4, 1980; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 54-Chiku A No. 5001 and 54-Suishin No. 3380) had been issued. This time, these circular notices are abolished and the Evaluation Criteria for Feed Additives as attached here are newly established. Relevant departments and bureaus are requested to note the following matters and strive to broadly disseminate these criteria among related parties under their jurisdiction.

Please note that the following notices were partially revised as indicated in the comparative tables, Attachments 1 to 7, upon the establishment of the new Evaluation Criteria: "Operation of the Act on Safety Assurance and Quality Improvement of Feed (dated June 27, 1977; circular notice of the Director General of the Livestock Industry

Bureau, Ministry of Agriculture and Forestry; 52-Chiku B No. 696), "Documents, etc. to be Submitted upon Designation of Feed Additives and other data" (dated February 4, 1980; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 54-Chiku A No. 5002 and 54-Suishin No. 3381), "Enforcement of the Ministerial Order, etc. Partially Amending Ministerial Orders concerning Compositional Standards and other standards of Feed and Feed Additives" (dated July 27, 1981; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 56-Chiku B No. 1594), "Establishment of Evaluation Criteria for Safety of Feed" (dated April 12, 1988; circular notice by the Director General of the Livestock Industry Bureau, MAFF; 63-Chiku B No. 617), "Standards on the Implementation of Animal Tests for Feed Additives" (dated July 29, 1988; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 63-Chiku A No. 3039), "Establishment of Evaluation Criteria for Safety of Feed for Cultured Aquatic Animals" (dated February 13, 1991; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 2-Chiku B. No. 2103), and "Submission of Documents and other data for Designating Live Microbial Agents as Feed Additives" (dated January 30, 1992; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 4-Chiku A No. 25).

Note

1. Purpose of the Revision, etc.

The current revision aims to integrate the existing evaluation criteria for feed additives and the test manuals based thereon, decrease the number of laboratory animals and measures from the perspective of animal welfare, and clarify tests to be conducted for each type of feed additive.

The new Evaluation Criteria for Feed Additives are established based on the scientific level at present. Examinations of individual feed additives are to be conducted, as in a conventional manner, in consideration of new knowledge on the safety and others available at that point in time and the characteristics of the relevant feed additives, etc., while referring to these Evaluation Criteria.

As feed additives are mainly added to feed at compound feed plants and are sold as feed products to many and unspecified persons for their use, effects and safety of feed additives should be checked while taking into consideration diverse feeding conditions of various types of livestock, etc.

2. Designation of Feed Additives

Designation of feed additives should continue to be limited to the minimum extent

necessary among additives that are highly necessary and are clearly effective and whose safety has been confirmed. Accordingly, any person who intends to produce, import or otherwise handle undesignated substances as feed additives must have sufficient consultations with the authority in advance and receive instructions of the authority.

3. Date of Application of the New Evaluation Criteria

Future tests should be conducted based on the new Evaluation Criteria for Feed Additives. However, tests that are commenced by September 30, 1992, may be conducted based on the former criteria.

Evaluation Criteria for Feed Additives

These Criteria provide for the basic concept and methods for the evaluation of the effects and safety of feed additives necessary for the Feed Committee of the Agricultural Materials Council (hereinafter referred to as the "Committee") to conduct deliberations for the designation of feed additives and the establishment of standards and specifications for feed additives based on the Act on Safety Assurance and Quality Improvement of Feed (Act No. 35 of 1953).

I. Basic Conditions of Feed Additives

1. Conditions concerning Effects

(1) Feed additives must have effects suited for the use specified in Article 1 of the Regulation for Enforcement of the Act on Safety Assurance and Quality Improvement of Feed (Order of the Ministry of Agriculture and Forestry No. 36 of 1976; hereinafter referred to as the "Enforcement Regulation").

(2) Effects of antimicrobial agents that are approved as feed additives should not exceed the following scope:
   (a) To prevent the deterioration of the feed quality due to mold, etc.;
   (b) To promote growth of livestock, etc. (meaning animals specified in Article 1 of the Order for Enforcement of the Act on Safety Assurance and Quality Improvement of Feed (Cabinet Order No. 198 of 1976; hereinafter referred to as the "Enforcement Order"); the same applies hereinafter) (limited to those in the juvenile period, in principle) or to improve the feed efficiency;
   (c) To prevent the deterioration in productivity of livestock, etc. during the juvenile period due to specific pathogenic parasites.

(3) When a new feed additive has similar effects as those of any already designated feed additive, the effects of the former must be equivalent or superior to those of the latter.

2. Conditions concerning Persistence

Among feed additives, antimicrobial agents, etc. must not be detected, by way of a quantitative method having considerable sensitivity, in products of livestock, etc. provided with any feed including the relevant feed additives.

3. Conditions concerning Safety

(1) Feed additives must not be such that the use of any feed including them results in producing harmful livestock products (meaning meat, milk or other edible products of livestock, etc. that may harm human health), or causes damage to livestock, etc. and

hinders the production of livestock products (meaning products relating to livestock, etc.).

(2) When a new feed additive has similar structure, behavior, etc. as those of any already designated feed additive, the new feed additive must exhibit equivalent or superior safety than the latter.

(3) Feed additives must have a considerable safety margin for livestock, etc.

(4) Feed additives must not be anything designated as a poisonous drug or dangerous drug under the Pharmaceutical Affairs Act (Act No. 145 of 1960) or a poisonous substance or deleterious substance under the Poisonous and Deleterious Substances Control Act (Act No. 303 of 1950), in principle.

(5) Feed additives must not be such that the provision of any feed including them exerts any adverse effect in the medical field.

4. Others

(1) Feed additives must be able to be quantitated from feed including them by any of the physical method, chemical method or biological method, in principle.

(2) Feed additives must not result in deteriorating the quality of the feed wherein they are included or decreasing their own effects due to inclusion in the relevant feed.

II. Matters Necessary for Evaluation

In order to prove whether or not feed additives satisfy the conditions set forth in I. above, the following matters need to be clarified.

When a feed additive does not fall under a deleterious substance or a poisonous substance, exhibits no problems through a residue test, has negative mutagenicity, and poses no cancer-causing risks based on existing knowledge, etc., a carcinogenicity test may be omitted. When it is judged unnecessary to check long-term repeated-dose toxicity based on the results of a short-term repeated-dose toxicity test and existing knowledge, etc., a long-term repeated-dose toxicity test may be omitted, and when no adverse effects on reproduction are suspected based on existing knowledge, etc., a multi-generation reproduction test may be omitted.

Additionally, the matters concerning safety of additives that have already been designated as food additives or used broadly in food may be omitted.

However, when any matters are omitted as mentioned above, the grounds therefor and the appropriateness of the omission need to be clarified.

1. Feed Additives Other than Live Microbial Agents

(1) Genesis or developments leading to discovery, permission as feed additives and use in foreign countries, etc.

(2) Matters concerning specifications
   (a) Name:
      a. Generic name
      b. Chemical name
   (b) Chemical structure
   (c) Production method
   (d) Biological and physicochemical properties
      a. Properties
      b. Identity test
      c. Purity test
      d. Content and quantitative method
   (e) Quantitative method for additives in feed
   (f) Change over time (stability of feed additives themselves and feed additives in feed)

(3) Matters concerning effects
   (a) Basic tests to prove effects
   (b) Field application tests to prove effects

(4) Matters concerning persistence

   Residue test using targeted livestock, etc.

(5) Matters concerning safety

(a) Toxicity tests
  a. General toxicity tests
    (i) Single-dose toxicity test
    (ii) Short-term repeated-dose toxicity test
    (iii) Long-term repeated-dose toxicity test
  b. Special toxicity tests
    (i) Multi-generation reproduction test
    (ii) Developmental toxicity test
    (iii) Carcinogenicity test
    (iv) Mutagenicity test
    (v) Other tests (local toxicity test, inhalation toxicity test, etc.)
  c. Pharmacological tests
  d. Tests on in vivo kinetics (absorption, distribution, metabolism, excretion, accumulation)
(b) Feeding tests using targeted livestock, etc.
(c) Tests on emergence of resistant bacteria
(d) Others
  a. Tests on impacts on the natural environment (phytotoxicity, fish toxicity, environmental pollution, etc.)
  b. Others

2. s Live Microbial Agents

(1) Genesis or developments leading to discovery, permission as feed additives and use in foreign countries, etc.
(2) Matters concerning specifications
  (a) Name:
    a. Generic name
    b. Scientific name
  (b) Production method
  (c) Bacteriological properties
    a. Properties
    b. Identity test (simplified identification method)
    c. Purity test (other microbes, etc.)
    d. Content (viable bacteria count) and quantitative method (viable count method)
  (d) Quantitative method for additives in feed
  (e) Change over time (stability of feed additives themselves and feed additives in feed)
  (f) Specification of seed bacteria for production
    a. Subculture method
    b. Storage method

(g) Quality control method
(h) Physical properties of products

(3) Matters concerning effects
(a) Basic tests to prove effects
(b) Tests on impacts caused by combined use with antimicrobial feed additives
(c) Field application tests to prove effects

(4) Matters concerning safety
(a) Taxonomic situation, etc. of bacteria
(b) Toxicity tests
a. Single-dose toxicity test
b. Short-term repeated-dose toxicity test
c. Tests on in vivo kinetics (distribution)
(c) Feeding tests using targeted livestock, etc.
(d) Tests on impacts on the natural environment

III. Documents for Evaluation

Documents sufficient to prove all matters necessary for evaluation set forth in II. above should be prepared. Documents must satisfy the following conditions.

(1) Tests for preparing documents must be conducted properly by a person with required knowledge and experience in facilities where tests can be conducted sufficiently, and the documents must contain records of the detailed data and the results of close and objective consideration taking into account feeding conditions in Japan. In particular, tests for which the "Standards on the Implementation of Animal Tests for Feed Additives" (dated July 29, 1988; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 63-Chiku A No. 3039) are applied must be conducted in line with these Standards.

(2) When the Committee finds it appropriate, part of the matters concerning evaluation set forth in II. above may be omitted or added.

(3) Methods of conducting principal tests for preparing documents are as outlined in the Attachments.
Attachments show standard test methods for evaluating effects and safety, etc. as feed additives. If test results sufficient for evaluation can be obtained, other methods may be employed.

Outline of Principal Test Methods

I. Tests on Effects

1. Objective

The tests aim to clarify that the test substance (or the test live microbial agent in the case of a live microbial agent; the same applies in this section) has effects suited for the use of the relevant feed additives.

2. Tests of Substances for Preventing the Deterioration of the Feed Quality

(1) Basic tests

The tests aim to clarify the effects of the test substance and ascertain the optimum additive amount.

(2) Tests to clarify the sustainability of effects

The test substance should be added to standard feed and its effects checked under natural conditions and severe conditions (light, temperature, humidity, etc.). The tests are to be conducted in the same manner as tests on stability of feed additives.

3. Tests of Substances for Supplementing Nutrients and Other Active Ingredients

The tests are to be conducted concerning the availability and other effectiveness of the test substance using laboratory animals or targeted livestock, etc.

Additionally, a comparison should be made with already designated feed additives as necessary.

4. Tests of Substances for Promoting Effective Use of Nutrients Contained in Feed

(1) Tests to check effects to promote growth or improve the feed efficiency (excluding live microbial agents)

The tests are to be conducted for antibiotics, synthetic antimicrobial agents, enzymes, etc.

(a) Basic tests

The tests aim to clarify or estimate the effects of the test substance. A control group using already designated feed additives with similar effects should be prepared separately.

a. In-vitro test

b. In-vivo test

Laboratory animals or targeted livestock, etc. are to be used.

(b) Field application tests

The tests use targeted livestock, etc. and aim to statistically check the effects of the

test substance to promote growth under field feeding conditions.

In addition to the method explained here, the randomized block method, split-plot method, etc. may be employed for enhancing the accuracy.

Regarding enzymes, the tests should be conducted in line with (2), (c) below. In that case, laboratory animals, number of replications, and number of facilities mentioned in (2), (c), a., (i) should be as follows: in the case of cows, at least 5 cows per group (1 cow × 5 replications × 1 facility); in the case of pigs, at least 20 pigs per group (4 pigs × 5 replications × 1 facility); in the case of chickens, at least 100 chickens per group (20 chickens × 5 replications × 1 facility); and in the case of cultured aquatic animals, at least 60 aquatic animals per group (30 aquatic animals × 2 replications × 1 facility). The provisions concerning the number of domestic facilities are not applied.

a. Test animals and number of replications

The tests should use targeted livestock, etc. to which the test substance is to be applied.

(i) Livestock and poultry

In principle, the number of animals for each dose group should be at least 1 in the case of cows, at least 4 in the case of pigs, and at least 20 in the case of chickens. Regarding the number of replications, the degree of freedom for error should be set at no smaller than 10 and preferably at 20 or over.

Locations of test groups in feeding facilities are to be at random.

(ii) Cultured aquatic animals

In principle, the number of cultured aquatic animals should be at least 30 for each dose group and the number of replications should be at least 2 per facility.

Locations of test groups in feeding facilities are to be at random.

Attention should be paid to the environmental conditions for conducting a test. Water temperature should basically be between 18 and 28°C for yellowtails, red sea bream, carp and eels, between 8 and 18°C for rainbow trouts and white salmons, between 15 and 25°C for sweetfish, and between 21 and 28°C for prawns.

Furthermore, regarding prawns, a preliminary test should be conducted to statistically estimate the effectiveness of the test substance, and due consideration should be given to the environmental conditions to prevent cannibalization and dying (in particular, when using water tanks, the number of cultured fish, the area, daylighting and locations of test groups in feeding facilities, etc.) and transportation conditions.

b. Administration period

Administration should be continued for the period during which the test substance is scheduled to be applied.

However, regarding cultured aquatic animals, if the administration period is prolonged, the period may be substituted with the period of time until the average weight of the control group triples, except for cases where the test animals are fish juveniles or prawns less than 1g of weight (excluding tests using water tanks). For tests regarding prawns using water tanks, the following period may be adopted as the administering period:

(i) When using prawns less than 1g of weight, at least four weeks;

(ii) When using prawns 1g to 5g of weight, at least eight weeks and until the average weight of the control group doubles.

c. Administration method

The test substance should be added to feed and administered continuously.

Feed to be used should be nutritionally complete and raw materials and composition ratios thereof should be clarified.

d. Doses

A test should be conducted beforehand in order to statistically estimate the dose-response relationship concerning the effects of the test substance and the optimum additive amounts are to be ascertained by comprehensively considering the results of such test, basic tests and persistence test, etc. Three levels of doses should be set, including the maximum amount and the minimum amount of the optimum additive amounts, in principle. A control group should be separately prepared.

Additionally, groups to which feed with regular amounts of already designated feed additives is administered should also be prepared as necessary.

e. Number of facilities

The tests should be conducted at least at three test facilities. For tests concerning antibiotics, synthetic antimicrobial agents, or organic acid, at least two test facilities must be located in Japan.

f. Observations and inspections

(i) Body weight, feed consumption (for cultured aquatic animals, feeding amount), consumption of the test substance (for cultured aquatic animals, doses of the test substance), and feed efficiency (the value obtained by dividing weight increase by feed consumption; the same applies hereinafter)

For livestock and poultry, measurements should be carried out upon commencement and completion of a test that lasts for around one week, at an interval of one week in the case of a test that lasts for around one month, or at an interval of two weeks in the case of a test that lasts two months or longer, respectively.

For cultured aquatic animals, measurements should be carried out upon commencement and completion of a test that lasts for around one week, and at an interval of two weeks in the case of a test that lasts one month or longer,

respectively. However, for prawns, measurements should be carried out upon commencement and completion of a test, and additionally around once a month in the case of a test that lasts several months.

(ii) General status

During the test period, general status of test animals is to be observed (for cultured aquatic animals, any abnormality in feed consumption status, behavior, body color, or body shape, etc.; the same applies hereinafter).

When feces is abnormally loose, water consumption should be roughly measured.

(iii) Pathological inspection

A pathological inspection, etc. should be conducted as necessary for test animals that show any health abnormalities or were dead.

g. Analysis of variance in test results

Variance analysis should be conducted for each test station, in principle, and the entirety of the test results should also be evaluated.

When test animals are fed in group, one group should be considered as one unit. However, when feed consumption (for cultured aquatic animals, feeding amount) is recorded individually and a feeding facility allows individual animals to live freely, independent of one another, the test results of such test animals fed in group may be treated as those of test animals fed separately.

(2) Tests to check effects to promote growth or improve the feed efficiency (live microbial agents)

The tests are to be conducted for a live microbial agent.

(a) Basic tests

The tests aim to check one or more of the effects of the test live microbial agent to maintain a normal intestinal flora or normalize an intestinal flora, to reduce intestinal harmful substances, or to promote growth, etc.

a. In-vitro test

b. In-vivo test

Laboratory animals or targeted livestock, etc. are to be used. It is preferable to also check the distribution and colonization, etc. of the test viable bacteria.

(b) Tests on impacts caused by combined use with antimicrobials

a. In-vitro test

Sensitivity of the test viable bacteria against already designated antimicrobial feed additives is to be examined.

b. In-vivo test

When an in-vitro test reveals the possibility that the test viable bacteria are highly sensitive and may not survive in the digestive tract when the test viable bacteria are used with an antimicrobial, suspected effects should be examined through

conducting a test as follows.

With regard to antimicrobial feed additives in the same system, a test should be conducted using a representative one.

(i) Test animals

The test should use targeted livestock, etc. to which the test viable bacteria are to be applied. The number of test animals should be at least 5 per group in the case of cows and pigs and at least 10 per group in the case of chickens.

(ii) Administration period

The administration period of the test viable bacteria with an antimicrobial feed additive should be at least one week.

Administration of the antimicrobial feed additive should be commenced after commencing the administration of the test viable bacteria and when the bacterial count collected from feces becomes constant.

(iii) Administration method

The test viable bacteria and the antimicrobial feed additive should be added to feed and administered continuously in principle.

(iv) Doses

The test viable bacteria should be administered at the intermediate amount between the maximum and the minimum amounts of the optimum additive amounts, while the antimicrobial feed additive should be administered at the maximum permissible amount.

(v) Matters to observe

i. Before administration of the antimicrobial feed additive

After commencing the administration of the test viable bacteria, feces should be collected at an interval of several days to confirm that the count of the test viable bacteria in 1g of feces has become constant.

In this case, whether the viable bacteria count has become constant should be confirmed by the fact that similar counts have been obtained at least three consecutive times.

ii. After administration of the antimicrobial feed additive

Feces should be collected every day while concurrently administering the antimicrobial feed additive to observe changes in the counts of the test viable bacteria.

(c) Field application tests

a. The effects of live microbial agents in the field are to be evaluated through conducting tests as follows, in principle.

A pathological inspection, etc. should be conducted as necessary for test animals that show any health abnormalities or were dead.

(i) Test animals, number of replications, and number of facilities

The tests should use targeted livestock, etc. to which the test live microbial agent is to be applied.

i. Livestock and poultry

In principle, the number of animals should be, in the case of cows, at least 15 cows per group (1 cow × 5 replications × 3 facilities, or three times at different timing for 1 cow × 5 replications × 1 facility), in the case of pigs, at least 60 pigs per group (4 pigs × 5 replications × 3 facilities, or three times at different timing for 4 pigs × 5 replications × 1 facility), and in the case of chickens, at least 300 chickens per group (20 chickens × 5 replications × 3 facilities, or three times at different timing for 20 chickens × 5 replications × 1 facility). At least one facility must be located in Japan.

ii. Cultured aquatic animals

In principle, the number of cultured aquatic animals should be at least 180 per group (30 aquatic animals × 2 replications × 3 facilities, or three times at different timing for 30 aquatic animals × 2 replications × 1 facility). At least one facility must be located in Japan.

Targeted livestock, etc. should be divided into two groups, i.e., a group fed in seawater and a group fed in fresh water. The tests should be conducted using at least one type of fish in these groups to which the test live microbial agent is to be applied. Attention should be paid to the environmental conditions for conducting a test. Water temperature should basically be between 18 and 28°C for yellowtails, red sea bream, carp and eels, between 8 and 18°C for rainbow trouts and white salmons, between 15 and 25°C for sweetfish, and between 21 and 28°C for prawns.

Furthermore, regarding prawns, a preliminary test should be conducted to statistically estimate the effectiveness of the test live microbial agent, and due consideration should be given to the environmental conditions to prevent cannibalization and dying (in particular, when using water tanks, the number of cultured fish, the area, daylighting and locations of test groups in feeding facilities, etc.) and transportation conditions.

(ii) Administration period

The same as explained in (1), (b), b

(iii) Doses

The group to administer the test substance at the amount estimated to be the optimum additive amount (hereinafter referred to as the "group administered at an estimated optimum additive amount") is to be the test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

(iv) Matters to observe

During the test period, the following matters should be observed, in principle.

i. Body weight

ii. Feed consumption (for cultured aquatic animals, feeding amount)

iii. Consumption of the test viable bacteria (for cultured aquatic animals, doses of the test viable bacteria)

iv. Feed efficiency

v. General status

b. When the tests mentioned in a. above showed no statistically significant differences, the evaluation may be made by conducting additional tests and taking into account the results thereof.

When conducting additional tests, the test scale should preferably be estimated through statistical processing of the results of the tests mentioned in a. above.

(Example of statistical processing)

$n > 2t^2 \cdot s^2/d^2$

n: Required number of replications

t: t-value obtained based on a t-distribution table with 2n-2 degrees of freedom and an α risk level

s^2: Error variance in the previous tests

d: Gap in mean values in the previous tests

(3) Tests to check the improvements in digestibility

The tests are to be conducted for enzymes, etc.

(a) Basic tests

a. In-vitro test

b. In-vivo test

(b) Field application tests

The tests aim to check, using targeted livestock, etc., whether the test substance works to improve digestibility of feed ingredients. Test animals should be targeted livestock, etc. to which the test substance is to be applied.

A pathological inspection, etc. should be conducted as necessary for test animals that show any health abnormalities or were dead.

a. Chickens

(i) Test animals

Chickens with an artificial anus should be used and the number is to be at least 4 per group.

(ii) Administration period

At least 6 days

(iii) Doses

The group administered at an estimated optimum additive amount is to be the

test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

(iv) Feed

Basal feed should be compound feed that contain nutrients included in the Japanese Feeding Standard (edited by the National Agriculture and Biooriented Research Organization) sufficiently in a well-balanced manner. When using chromium oxide as an indicator, chromium oxide should be mixed at 0.1% to 0.2%, and when using acid-insoluble ash as an indicator, cerite should be mixed at 1.0%, uniformly in both cases. The particle size should be in a powdery state as minute as possible so as not to be sorted out upon eating. Fat-enriched feed should be avoided.

(v) Sampling of feces

Test animals are to be put in metabolism cages, etc. individually and each should be fed approx. 80g of feed every day. While paying attention to constipation, feces should be collected individually for at least two days after the lapse of five days from the commencement of feeding. Collected feces should be dried at a temperature of approx. 60°C using a circulation dryer, and then air-dried and ground to make analysis samples.

(vi) Analysis

General ingredients should be analyzed by the method specified in the Official Specifications on Feed (Public Notice of the Ministry of Agriculture and Forestry No. 756 of 1976; hereinafter referred to as the "Official Specifications").

Chromium oxide and acid-insoluble ash should be analyzed by the method specified in 2.(4) of Appendix 3 "Handling of Total Digestible Nutrients or Metabolic Energy in Quality Labeling of Feed" attached to the Notice on the Enforcement of the Ministerial Order, etc. for Amending the Ministerial Order on the Ingredient or other Standards for Feed and Feed Additives (notice issued by the Director of the Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries, and the Director-General of the Fisheries Agency No. 56-Chiku B No. 1594 dated July 27, 1981; hereinafter referred to as the "Notice").

(vii) Calculation of digestibility

Digestibility should be calculated using an index method formula.

b. Pigs

(i) Index method using chromium oxide, acid-insoluble ash, or titanium oxide as an indicator

i. Test animals

25-50 kg fattening pigs are to be used. One group should consist of at least 4 pigs.

ii. Administration period

At least 9 days

iii. Doses

The group administered at an estimated optimum additive amount is to be the test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

iv. Feed

Basal feed should be powdery compound feed that contain nutrients included in the Japanese Feeding Standard sufficiently in a well-balanced manner. When using chromium oxide as an indicator, chromium oxide should be mixed at 0.1% to 0.2%, when using acid-insoluble ash as an indicator, cerite should be mixed at 1.0%, and when using titanium oxide, titanium oxide should be mixed at 0.1%, uniformly in all cases. Feed in a stiff kneaded state with the addition of water may be provided.

v. Sampling of feces

Test animals are to be put in metabolism cages, etc. individually and each should be fed with feed every day at an amount around 3% of body weight that would not cause weight loss, at once or in up to three times a day. Feces should be collected individually for at least five days after the lapse of five days from the commencement of feeding. Collected feces should be dried at a temperature of approx. 60°C using a circulation dryer, and then air-dried and ground to make analysis samples. Feces should be collected at least twice in the morning and evening at certain fixed times every day. Collected feces should all be dried, in principle, but analysis samples may be made by taking parts of the feces, mixing them uniformly and drying them.

vi. Analysis

General ingredients should be analyzed by the method specified in the Official Specifications.

Chromium oxide, acid-insoluble ash, and titanium oxide should be analyzed by the method specified in 2.(4) of Appendix 3 "Handling of Total Digestible Nutrients or Metabolic Energy in Quality Labeling of Feed" attached to the Notice.

vii. Calculation of digestibility

Digestibility should be calculated using an index method formula.

(ii) Total collection method (in the case of fibrous feed)

i. Test animals

Adult pigs older than 8 months are to be used. One group should consist of at least 4 pigs.

ii. Administration period

At least 10 days

iii. Doses

The group administered at an estimated optimum additive amount is to be the test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

iv. Feed

Basal feed should be powdery compound feed that contain nutrients included in the Japanese Feeding Standard sufficiently in a well-balanced manner. Feed in a stiff kneaded state with the addition of water may be provided.

v. Sampling of feces

Test animals are to be put in metabolism cages, etc. individually and each should be fed with feed every day at an amount no more than 3kg in the dry state so that none is left uneaten, at once or in up to three times a day. All feces should be collected individually for at least five days after the lapse of six days from the commencement of feeding. Collected feces should be dried at a temperature of approx. 60°C using a circulation dryer, and then air-dried and ground to make analysis samples. Feces should be collected at least twice in the morning and evening at certain fixed times every day.

vi. Analysis

General ingredients should be analyzed by the method specified in the Official Specifications.

vii. Calculation of digestibility

Digestibility should be calculated using a total collection method formula based on feed consumption, amount of feces and their analyzed values.

c. Cows

(i) Test animals

One group should consist of at least 4 cows.

Sheep or goats may be used instead of cows.

If the feeding style is to be changed radically, at least 14 days of preconditioning time will be necessary before the tests.

(ii) Administration period

At least 14 days

(iii) Doses

The group administered at an estimated optimum additive amount is to be the test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

(iv) Feed

Basal feed should contain no more than 60% of concentrated feed, at least 12% of crude protein content, and at least 15% of crude fiber content (all on a dry basis), as well as other nutrients sufficiently in a well-balanced manner. Hay should be used as roughage.

(v) Sampling of feces

Test animals are to be put in metabolism cages, etc. individually and each should be fed with feed containing energy enough for maintenance and crude protein more than required for maintenance. The feed amount should be adjusted so that none is left uneaten. All feces should be collected individually for at least seven days after the lapse of eight days from the commencement of feeding at a fixed time with an interval of not more than 24 hours. Collected feces should be weighed, and mixed well, and samples should be collected at a certain ratio against the total amount of feces. Collected samples should be sealed and stored in a freezer. After collecting all samples, they should be mixed, dried at a temperature of approx. 60°C using a circulation dryer, and then air-dried and ground to make analysis samples. With regard to water and crude protein content, fresh mixed samples are to be used for analysis, in principle.

When using sheep or goats, all feces should be collected individually and be dried at approx. 60°C using a circulation dryer. Then, dried samples should be restored to air-dry state, weighed, sealed and stored. After collecting all samples, they should be mixed well and a part thereof should be grounded and used for analysis.

(vi) Analysis

General ingredients should be analyzed by the method specified in the Official Specifications.

(vii) Calculation of digestibility

Digestibility should be calculated using a total collection method formula based on feed consumption, amount of feces and their analyzed values.

d. Cultured aquatic animals

(i) Test animals and number of replications

The number of test animals should be restricted so as not to prevent normal intake of feed.

The number of replications should be at least two, and there are two options: to

conduct tests using at least two water tanks at the same time, or to conduct tests using one tank at least twice at different times.

Attention should be paid to the environmental conditions for conducting a test. Water temperature should basically be between 18 and 28℃ for yellowtails, red sea bream, carp and eels, between 8 and 18℃ for rainbow trouts and white salmons, between 15 and 25℃ for sweetfish.

(ii) Administration period

At least 9 days

(iii) Doses

The group administered at an estimated optimum additive amount is to be the test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

(iv) Feed

Basal feed to be used should be nutritionally complete and raw materials and composition ratios thereof should be clarified. When using chromium oxide as an indicator, chromium oxide should be mixed at 0.5% to 1.0%, and when using acid-insoluble ash as an indicator, cerite should be mixed at 1.0%, uniformly in both cases.

For each type of fish, an indicator that has been used in the past should be selected.

(v) Sampling of feces

Test animals are to be put in feces sampling water tanks and naturally excreted feces should be collected for at least three days after the lapse of seven days from the commencement of feeding.

(vi) Analysis

Analysis of feces is to be conducted for each water tank, and general ingredients should be analyzed by the method specified in the Official Specifications.

Chromium oxide and acid-insoluble ash should be analyzed by the method specified in 2.(4) of Appendix 3 "Handling of Total Digestible Nutrients or Metabolic Energy in Quality Labeling of Feed" attached to the Notice.

(vii) Calculation of digestibility

Digestibility should be calculated using an index method formula.

(4) Tests to confirm improvement of feed palatability

The tests aim to confirm how the test substance (flavor and taste substances, etc.) improves feed palatability by using targeted livestock, etc.

(a) Test by free choice method

a. Test animals and number of replications

Test animals should be targeted livestock, etc. to which the test substance is to be applied. In principle, one group should consist of at least one cow or pig, or at least 10 chickens. The number of replications should be set with degrees of freedom caused by measurement error of at least 10 or at least 20, if possible.

Locations of test groups in feeding facilities are to be at random.

b. Administration period

Around 1 to 2 weeks

c. Administration method

Two types of feed, i.e., one containing the maximum amount of the optimum additive amounts of the test substance, and the other not containing the test substance, should be placed in a test chamber in a manner that test animals can choose and eat them freely.

In this case, due consideration should be given so that these two types of feed are placed in the test chamber under the same conditions. It is preferable to change the locations of feeders every day if possible.

Feed to be used should be nutritionally complete and raw materials and composition ratios thereof should be clarified.

d. Observations and inspections

(i) The consumption of feed with the test substance and feed without the test substance (for cultured aquatic animals, feeding amount) should be measured every day.

(ii) General status

During the test period, general status of the test animals should be observed.

A pathological inspection, etc. should be conducted as necessary for test animals that show any health abnormalities or were dead.

(b) Test by separation method

a. Test animals, number of replications and number of facilities

Test animals should be targeted livestock, etc. to which the test substance is to be applied. In principle, the number of animals should be, in the case of cows, at least 5 cows per group (1 cow × 5 replications × 1 facility), in the case of pigs, at least 20 pigs per group (4 pigs × 5 replications × 1 facility), in the case of chickens, at least 100 chickens per group (20 chickens × 5 replications × 1 facility), and in the case of cultured aquatic animals, at least 60 aquatic animals per group (30 aquatic animals × 2 replications × 1 facility).

Attention should be paid to the environmental conditions for conducting a test for cultured aquatic animals. Water temperature should basically be between 18 and 28°C for yellowtails, red sea bream, carp and eels, between 8 and 18°C for rainbow trouts and white salmons, and between 15 and 25°C for sweetfish.

b. Administration period

The same as explained in (1), (b), b

c. Doses

The group administered at an estimated optimum additive amount is to be the test group and a control group should be prepared separately.

Multiple groups administered with different doses (including the group administered at an estimated optimum additive amount) may be prepared.

d. Observations and inspections

During the test period, the following matters should be observed in principle.

(i) Body weight

(ii) Feed consumption (for cultured aquatic animals, feeding amount)

(iii) Consumption of the test substance (for cultured aquatic animals, doses of the test substance)

(iv) General status

During the test period, the general status of the test animals should be observed. A pathological inspection, etc. should be conducted as necessary for test animals that show any health abnormalities or were dead.

(5) Tests to confirm the effectiveness to prevent the deterioration in productivity caused by specific pathogenic parasites

The tests are to be conducted for antibiotics and synthetic antimicrobial agents, etc., which are used for preventing the deterioration in productivity caused by specific pathogenic parasites, by using targeted livestock, etc. The effectiveness of the test substance to prevent the deterioration in productivity due to specific pathogenic parasites is checked in the field.

Tests should be conducted in accordance with (1) above, in principle, but when making a test plan, due consideration should be given to ensure that the adopted test method can clearly evaluate the effectiveness of the test substance.

II. Residue Tests

1. Usual Addition

(1) Objective

The tests aim to clarify how the test substance administered to targeted livestock, etc. remains in their bodies and resulting livestock products.

(2) Sampling places

Samples are to be collected from test animals raised in at least two different places.

(3) Test animals

(a) The tests should use targeted livestock, etc. to which the test substance is to be applied as a feed additive. Test animals should be those with clear records of the

past use of feed and feed additives as well as feeding method, etc. before the tests.

(b) The number of test animals should be sufficient for collecting samples required for measuring the residual volume of the test substance and clarifying the changes of the test substance. Specifically, the number for each dose group per sampling time should be at least 2 in the case of cows, at least 3 in the case of pigs, and at least 3 in the case of chickens (preferably hens). In the case of cultured aquatic animals, the number should be sufficient for collecting required analysis samples and not disturbing proper feed intake.

(c) Attention should be paid to the environmental conditions for conducting a test for cultured aquatic animals. Water temperature should basically be between 18 and 24°C for yellowtails, red sea bream, carp, eels, and prawns, between 8 and 14°C for rainbow trouts and white salmons, and between 15 and 21°C for sweetfish.

(4) Administration period

The administration period should be the period during which the test substance is applied as a feed additive. However, when said application period extends over a long term, it may be allowed to conduct a preliminary test, etc. and set an appropriate administration period based on the results and other outcome thereof. In this case, the grounds need to be presented to prove that the residual volumes in the samples reach a certain level after consecutive administration for a certain period and remain unchanged thereafter.

(5) Administration method

The test substance is to be added to feed and administered consecutively.

It should be noted upon administration that feed is to be consumed by test animals uniformly.

(6) Doses

In principle, the highest concentration of the test substance to be applied should be considered as the minimum dose. Additionally, dose levels of several times to tens of times the minimum dose should be set. A control group should also be prepared separately.

The concentration of the test substance for the group administered with the highest dose should be a level that will not apparently reduce the feed consumption of test animals.

(7) Feed

Basal feed to be used for tests should be in conformity with the Official Specifications if applicable.

Addition of feed additives other than the test substance (excluding vitamins, minerals, amino acids, etc.) to basal feed should be avoided, but if it is inevitable for the test animals, feed additives that do not evidently hinder the analysis of the test substance should be added.

(8) Collection of samples

(a) The sampling time should be set as required to clarify the changes of the test substance.

(b) Samples should be collected, in principle, from edible parts so as to clarify the distribution of the test substance respectively in the muscle, fat, liver (hepatopancreas), kidney, small intestine, egg, and milk, etc.

(c) Samples should be stored at 0-5°C after collection and analyzed promptly. Long-term storage should be avoided, but when unavoidable, samples should be frozen. In this case, it should be confirmed that the test substance is not resolved during the process of freezing and thawing.

(9) Analysis

(a) For these tests, an adequately sensitive, accurate and reproducible analytical method should be established in advance. The adequate sensitivity, accuracy and reproducibility here mean the detection limit of 0.05ppm or lower, the recovery rate of at least 70% in the recovery test after adding 1-2ppm of substance, and the coefficient of variation (standard deviation/mean) of 0.1 or lower. However, when the residual limit for food is specified for the test substance and the limit is less than 0.05ppm, the detection limit must be no larger than said residual limit.

When the test substance is an antibiotic, a biological or chemical analysis method should be employed.

(b) Analysis should be made for residue of the active ingredients of the relevant feed additive, but when the persistence of metabolites needs to be made clear, the metabolites should also be analyzed.

(c) Analyzed values should be recorded as they are, without subtracting control values. The recovery rate should not be corrected.

(d) When the measurement results are less than the detection limit (X ppm), the results should be recorded as "< X ppm" not as "Not detectable."

(e) When measurement results include values less than the detection limit, mean values should not be calculated.

2. Micro-amount Addition

(1) Objective

The tests aim to clarify how the test substance, which is added at a micro amount to feed and provided to targeted livestock, etc., remains in their bodies and resulting livestock products.

These tests may be omitted when it is clear that the test substance is hardly absorbable.

(2) Sampling places

Samples are to be collected from test animals raised in one or more places.

(3) Test animals

(a) One or more types of targeted livestock, etc., including laying hens, are to be used. Test animals should be those with clear records of the past use of feed and feed additives as well as feeding method, etc. before the tests.

(b) The number of laying hens to be tested should be at least 6 per dose group, and for other types of test animals, the number should be as explained in 1, (3), b.

(4) Administration period

At least 4 weeks

(5) Administration method

The same as explained in 1, (5)

(6) Doses

The dose of around one-hundredth of a usual dose is to be considered as the minimum dose, in principle, and dose levels of several times to tens of times the minimum dose should be set. A control group should also be prepared separately.

(7) Feed

The same as explained in 1, (7)

(8) Collection of samples

The same as explained in 1, (8)

(9) Analysis

The same as explained in 1, (9)

III. Taxonomic Situation, etc. of Bacteria

Regarding live microbial agents, safety of bacteria is to be confirmed by clarifying the taxonomic situation, origin, history of use, and products of bacteria, etc.

IV. Single-dose Toxicity Test

1. Objective

This test aims to clarify the toxicity of the test substance (or the test live microbial agent in the case of a live microbial agent; hereinafter the same applies in this section) when a single dose thereof is orally administered to test animals.

2. Test Method

When possible toxic signs due to a single oral administration of the test substance are clear, a fixed-dose method should be used, and when the lethal dose can be estimated, a toxic class method should be used.

When possible toxic signs and the lethal dose are both known or are both unknown,

either of the above methods should be selected.

(1) Fixed-dose method

(a) Test animals

a. Animal types

Rodents (rats, etc.) should be used.

b. Conditions

Healthy young animals aged 8 to 12 weeks (whose weights are within a range of ±20% of the average) are to be used. Test animals should be habituated for at least five days prior to commencing administration.

Females (limited to nulliparous and non-pregnant ones) should be used in principle, but when there is any data suggesting that males are more sensitive, males should be used.

(b) Administration method

A forced single oral administration should be adopted. Before administration, animals should be fasted (for one night in the case of rats and for three to four hours in the case of mice; only drinking of water is permitted). When a single administration is impossible, the administration may be conducted in divided doses within 24 hours.

The total dose should not exceed 1ml/100g body weight, in principle. The test substance should be dissolved or suspended in water or in an appropriate solvent, as necessary. When using a solvent other than water, a solvent whose toxicity is already known must be selected.

(c) Setting of the number of animals

a. Estimation test

One animal for each dose

b. Major test

Each dose group should consist of five animals. With regard to doses for which the estimation test has been conducted, four animals are to be used for the major test so that the total becomes five, adding the one used in the estimation test.

(d) Test procedures

a. Estimation test

The estimation test should be conducted in accordance with the procedures shown in Appendix 1 for dose levels of 5mg/kg body weight, 50mg/kg body weight, 300mg/kg body weight, and 2,000mg/kg body weight, with the aim of deciding the initial dose to be adopted in the major test. The first dose for the estimation test should be the one that is expected to cause clear toxic signs. If there is no data relating to such dose, the test is to be preferably commenced with the dose level of 300mg/kg body weight. There must be at least a 24-hour administration interval.

If an animal dies with a dose level of 5mg/kg body weight, the test should be

terminated without conducting the major test, with the result being recorded as "LD50≤5mg/kg body weight."

b. Major test

The test should be conducted in accordance with the procedures shown in Appendix 2. However, for doses that caused death(s) in the estimation test, the major test is to be omitted, regarding that two or more animals died in the major test. Administration intervals should be decided based on the sustainability and seriousness of the toxic signs. Subsequent administrations should be suspended until it is confirmed whether the test animals previously administered with the test substance are dead or alive.

c. Limit test

When there was no death with a dose level of 2,000mg/kg body weight in the estimation test, and there was less than one death with the same dose level in the major test, further administration at a dose level over 2,000mg/kg body weight is unnecessary.

(e) Observations and inspections

The test animals should be fed at least for 14 days and the following matters should be observed and inspected.

a. General status

Test animals should be observed at least once within 30 minutes after administration of the test substance and at regular intervals thereafter for the whole day (to be observed especially carefully for four hours after administration), and from the following day onward, they should be carefully observed once every day. Observation records must contain changes in feces colors, etc. in order to enable judgment as to whether changes in the status of the test animals are due to procedural differences in the test, such as the manner of forced oral administration, or the toxicity of the test substance.

Additionally, types of all visually detected toxic signs, times when the signs appeared and disappeared and the time of death should be recorded separately for all individual animals.

b. Body weight

All test animals should be weighed individually once immediately before administration of the test substance, and then once a week thereafter. When an animal dies during the test, its body weight should be measured upon death. Test animals that are alive when the test is over are to be killed after measuring their body weight.

c. Pathological inspection

An autopsy is to be performed for all test animals and visual pathological findings are to be recorded. Regarding organs with any visual pathological findings of test

animals that were alive for 24 hours or longer after administration of the test substance, a histopathological examination should preferably be conducted.

(2) Toxic class method

(a) Test animals

The same as explained in (1), (a)

(b) Administration method

The same as explained in (1), (b)

(c) Setting of the number of animals

Three for each administration stage

(d) Test procedures

a. The first dose for the test should be selected from 5mg/kg body weight, 50mg/kg body weight, 300mg/kg body weight, and 2,000mg/kg body weight, and the test should be conducted in accordance with the procedures shown in Appendix 3. A dose that is expected to cause deaths of several administered test animals is to be selected as the first dose. If there is no data relating to acute toxicity of the test substance, the test is to be preferably commenced with a dose level of 300mg/kg body weight.

b. Administration intervals should be decided based on the sustainability and seriousness of the toxic signs. Subsequent administrations should be suspended until it is confirmed whether the test animals previously administered with the test substance are dead or alive.

c. When three test animals were administered with the test substance at a dose level of 2,000mg/kg body weight, and there was less than one death, the test substance at the same dose level is to be administered anew to three test animals. If there was less than one death after the second administration, further administration at a dose level over 2,000mg/kg body weight is unnecessary.

(e) Observations and inspections

The same as explained in (1), (e)

V. Short-term Repeated-dose Toxicity Test

1. Objective

This test aims to clarify the toxicity of the test substance (or the test live microbial agent in the case of a live microbial agent; hereinafter the same applies in this section) by continuously administering it to test animals for at least three months.

2. Test Animals

(1) Animal types

One or more types of animals including rodents (usually rats) are to be used. When data on the influence of exposure to chemical substances regarding non-rodents is required based on the results of another toxicity test or pharmacokinetic test using rodents, non-rodents (usually dogs) are to be used.

When the test is conducted as a preliminary test for a long-term repeated-dose toxicity test, it is preferable to use the same type and same species of animals in both tests.

(2) Conditions

For rodents, healthy young animals (whose weights are within a range of ±20% of the average for females and males, respectively) are to be used. Administration should be commenced as early as possible after the animals are weaned and then habituated for at least five days, before they become nine weeks old. Females should be nulliparous and non-pregnant.

For non-rodents, healthy young animals should be used. In the case of using dogs, administration should be commenced after they are habituated for at least five days and preferably when they are four to six months old (before they become nine months old at the latest).

(3) Number of animals

Each dose groups and control group should consist of at least ten males and ten females in the case of rodents and at least four males and four females in the case of non-rodents.

Depending on available knowledge on the test substance or similar substances, it should be considered to prepare satellite groups (rodents: five males and five females; non-rodents; four males and four females) for the control group and the highest dose group (meaning the group administered with the highest dose; the same applies hereinafter) in order to observe the sustainability of toxic changes, recovery therefrom, or delayed toxicity for an appropriate period of time after the final administration.

3. Administration Period

90 days

4. Administration Method

The test substance should be added to feed or drinking water and administered continuously, in principle. If this is difficult, the test substance may be administered continuously for five days a week through forced oral administration or administration in capsules.

5. Doses

Groups of at least three different stages of doses should be prepared in addition to a control group.

Dose stages should be set so that the entirety of the toxicity of the test substance can be clarified and the non-toxic amount can be estimated. The highest dose should be at a level that shows toxic impacts without causing many deaths and the lowest dose should be at a level that shows no toxic impacts (non-toxic amount). Other dose stages should be set so that a dose-response relationship can be observed.

The grounds for setting dose levels are to be also indicated.

When no toxic action was observed in one test administering the maximum dose technically feasible or a dose equivalent to 1,000mg/kg body weight/day, and no toxicity is expected based on the test results for structurally similar substances, there is no need to continue the test with larger doses.

When adding the test substance to feed, due consideration should be given not to cause any malnutrition, and there is no need to conduct the test with doses wherein the concentration of the test substance added to the feed exceeds 5% (W/W).

When no solvent and no other material is used, the control group should be a non-treated control group, and when any solvent or other materials is used, it should be a solvent control group. Test animals of the control group should be under the same conditions as those of the other groups, except for not being administered with the test substance. When using a solvent, etc. for administering the test substance, the control group should be administered with the same amount as that for the group administered with the largest amount of solvent. When using a solvent other than water, a solvent whose toxicity is already known must be selected.

6. Observations and Inspections

In order to sufficiently ascertain the toxicity of the test substance, at least the following matters should be observed and inspected.

(1) Body weight and feed consumption

Measurement should be conducted once prior to commencing administration and at an interval of one week after commencing administration.

(2) Consumption of the test substance

Measurement should be conducted at an interval of one week.

(3) Water consumption

When adding the test substance to drinking water, measurement should be conducted at an interval of one week.

Even when adding the test substance to feed or in the case of forced oral administration, if any changes in drinking behavior are expected during the test period, measuring of water consumption should be taken into consideration.

(4) Feed efficiency

Measurement should be conducted at an interval of one week.

(5) General status and mortality rate

A general clinical observation should be conducted at least once a day. Additionally, signs of pathologies and deaths should be checked at least twice a day.

(6) Hematological test

In principle, blood samples should be taken at the end of the short-term repeated-dose toxicity test (or at the mid-period and at the end of the test, if necessary) for rodents, and prior to commencing administration, at the mid-period (at an interval of one month, etc.) and at the end of the test for non-rodents to individually check hematocrit levels, hemoglobin concentrations, red blood cell counts, total white blood cell counts, white blood cell differential counts, blood platelet counts, and blood clotting time or blood's ability to clot.

(7) Serum chemistry

In principle, blood samples should be taken at the end of the short-term repeated-dose toxicity test (or at the mid-period and at the end of the test, if necessary) for rodents, and prior to commencing administration, at the mid-period (at an interval of one month, etc.) and at the end of the test for non-rodents. Animals excluding mice should preferably be fasted over night before taking blood samples.

For rodents, the test should cover levels of sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein, albumin, and at least two types of enzymes indicating hepatic function (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma-glutamyl transpeptidase (γGTP), and sorbitol dehydrogenase (SDH)), etc. Levels of other enzymes or bile acid may also be measured additionally to check functions of the liver or other organs.

For non-rodents, the test should cover levels of calcium, phosphorus, chlorides, sodium, potassium, fasting glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), ornithine decarboxylase, gamma-glutamyl transpeptidase (γGTP), urea nitrogen, albumin, blood creatinine, total bilirubin, and total serum protein, etc.

(8) Ophthalmologic test

An ophthalmologic test should be conducted at least for the highest dose group and the control group before administration of the test substance and at the end of the short-term repeated-dose toxicity test. If any abnormality is found, all test animals should be checked.

(9) Urine test

For rodents, urine samples should be collected optionally at the end of the short-term repeated-dose toxicity test to check the appearance and levels of urine volume, osmotic pressure or specific gravity, pH, protein, glucose, and blood or blood cell in the urine.

For non-rodents, urine samples should be collected prior to the commencement of the

test, at the mid-period and at the end of the test to check the appearance and levels of urine volume, osmotic pressure or specific gravity, pH, protein, glucose, and blood or blood cell in the urine.

(10) Pathological inspection

The inspection should be conducted regarding the following matters for as many organs as possible of all test animals (including the body surface, all orifices, skull cavity, chest cavity, abdominal cavity and contents in these organs). For test animals that died during the short-term repeated-dose toxicity test, causes of their deaths should be ascertained through an autopsy or other means.

(a) Visual observations

(b) Weight of organs

For rodents, the weight of the liver, kidney, adrenal gland, testis, epididymis, uterus, ovary, thymus gland, spleen, brain and heart of all test animals (excluding test animals that are dying or were killed during the short-term repeated-dose toxicity test) should be measured.

For non-rodents, the weight of the gallbladder and thyroid gland (including parathyroid) should additionally be measured.

(c) Preservation of organs and tissues

The following organs and tissues must be preserved even after the end of the short-term repeated-dose toxicity test so that a histopathological examination can be conducted in the future as necessary.

All visually detected lesions, skin, brain (major parts including cerebrum, cerebellum, cerebellopontine and medulla oblongata), pituitary gland, thyroid gland, parathyroid, thymus gland, esophagus, salivary gland, stomach, small intestine, large intestine (including Peyer's patch), liver, pancreas, kidney, adrenal gland, spleen, heart, trachea and lung (immersion preservation after injecting a fixative solution), aorta, gonad, uterus, accessory reproductive organ, mammary gland of female animals, prostate gland, urinary bladder, gallbladder, lymph node (one lymph node on the administration route, and one distant lymph node for checking the influence on the whole body), peripheral nerve (nerve close to muscles, such as sciatic nerve or tibial nerve), spinal cord (neck spinal cord, midthoracic spinal cord, and lumbar spinal cord), bone marrow and eyeball (when any abnormality is found in an ophthalmologic examination)

(d) Histopathological examination

For non-rodents, the examination should be conducted for preserved organs and tissues of all test animals of all dose groups.

For rodents, the examination should be conducted for preserved organs and tissues of all test animals of at least the control group and the highest dose group. Any organs or tissues that reveal changes suggesting a correlation with the

administration of the highest dose should be examined histopathologically also in the other groups.

Additionally, all visually detected lesions in test animals of all dose groups should be examined.

Preserved organs and tissues of all test animals that died or were killed during the short-term repeated-dose toxicity test should be examined as well.

When satellite groups were prepared, the examination should be conducted for animals in these groups regarding any organs or tissues where changes were observed in groups administered with the test substance.

VI. Long-term Repeated-dose Toxicity Test

1. Objective

This test aims to clarify the toxicity of the test substance by continuously administering it to test animals for a long term. When a carcinogenicity test is additionally required, the option to conduct the test explained in VIII in lieu of this test should be considered.

2. Test Animals

(1) Animal types

One or more types of animals including rodents (usually rats) are to be used. When data on the influence of exposure to chemical substances regarding non-rodents is required based on the results of other pharmacokinetic test using rodents, non-rodents (usually dogs) are to be used.

The same type and same species of animals as those used in the short-term repeated-dose toxicity test should be used.

(2) Conditions

Healthy young animals (whose weights are within a range of ±20% of the average for females and males, respectively) are to be used. Administration should be commenced as early as possible after the animals are weaned and then habituated for at least seven days, before they become eight weeks old. Females should be nulliparous and non-pregnant.

(3) Number of animals

Each dose group and control group should consist of at least 20 males and 20 females in the case of rodents and at least four males and four females in the case of non-rodents.

When performing an autopsy and sampling in the middle of the test, test animals are to be added as necessary. For example, when some test animals are killed in the middle (after six months, etc.), at least ten males and ten females should be added for each

dose group on each occasion. Additionally, it should be considered to prepare satellite groups (usually for the control group and the highest dose group) in order to observe the recovery from toxic changes, and also to prepare a monitor group (usually five males and five females), as necessary, in order to monitor the disease status.

3. Administration Period

12 months

4. Administration Method

The same as explained in V, 4

5. Doses

The same as explained in V, 5; Dose stages should be set based on the results of the short-term repeated-dose toxicity test. The highest dose should be at a level that does not cause many deaths but with which major target organs and toxic impacts can be identified.

6. Observations and Inspections

In order to sufficiently ascertain the toxicity of the test substance, at least the following matters should be observed and inspected.

(1) Body weight and feed consumption

Measurement should be conducted once prior to commencing administration, at an interval of one week until the lapse of 13 weeks after commencing administration, and at an interval of one month thereafter.

(2) Consumption of the test substance

Measurement should be conducted at an interval of one week until the lapse of 13 weeks after commencing the test, and at an interval of one month thereafter.

(3) Water consumption

When adding the test substance to drinking water, measurement should be conducted at an interval of one week until the lapse of 13 weeks after commencing the long-term repeated-dose toxicity test, and at an interval of one month thereafter.

Even when adding the test substance to feed or in the case of forced oral administration, if there are any changes in drinking behavior during the test period, measuring of water consumption should be taken into consideration.

(4) Feed efficiency

Measurement should be conducted at an interval of one week until the lapse of 13 weeks after commencing the test, and at an interval of one month thereafter.

(5) General status and mortality rate

A general clinical observation should be conducted at least once a day. Additionally, signs of pathological state and deaths should be checked at least twice a day.

(6) Hematological test

(a) Sampling targets

For rodents, blood samples should be taken from 10 males and 10 females each of all groups, and for non-rodents, the number of animals from which blood samples should be collected is to be less than the total number of test animals (for example, in the case of dogs, 4 males and 4 females for each dose group).

(b) Sampling time

After the commencement of the long-term repeated-dose toxicity test, blood sampling should be conducted upon the lapse of 3 months, 6 months, and 12 months (at the end of the test). For non-rodents, blood samples should be taken also prior to the commencement of administration.

However, if the results of the short-term repeated-dose toxicity test show no influence on the hematological indicators, blood sampling upon the lapse of 3 months is not necessary.

(c) Inspection items

Total white blood cell counts, white blood cell differential counts, red blood cell counts, blood platelet counts, hemoglobin concentrations, hematocrit levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), prothrombin time, and activated partial thromboplastin time, etc.

(7) Serum chemistry

(a) Sampling targets

The same as in the case of a hematological test

(b) Sampling time

Sampling time should be the same as in the case of a hematological test. However, if the results of the short-term repeated-dose toxicity test show no influence on the biochemical indicators, blood sampling upon the lapse of 3 months is not necessary. Animals excluding mice should preferably be fasted over night before collecting blood.

(c) Inspection items

Glucose, urea (urea nitrogen), creatinine, total protein, albumin, calcium, sodium, potassium, total cholesterol, at least two types of inspections for evaluating hepatic function (alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), and total bile acid), at least two types of inspections for evaluating hepatobiliary function (alkaline phosphatase, gamma-glutamyl transpeptidase (γGTP), 5'-nucleotidase, total bilirubin, and total bile acid), etc.

(8) Ophthalmologic test

An ophthalmologic test should be conducted at least for the highest dose group and the

control group before administration of the test substance and at the end of the long-term repeated-dose toxicity test. If any abnormality is found, all test animals should be checked.

(9) Urine test

(a) Sampling targets

10 males and 10 females for all groups

(b) Sampling time

Sampling time should be the same as in the case of a hematological test and serum chemistry. However, if the results of the short-term repeated-dose toxicity test show no influence on the urine test indicators, blood sampling upon the lapse of 3 months is not necessary.

(c) Inspection items

The appearance and levels of urine volume, osmotic pressure or specific gravity, pH, protein, and glucose, etc.

(10) Pathological inspection

The inspection should be conducted regarding the following matters for as many organs as possible of all test animals (including the body surface, all orifices, skull cavity, chest cavity, abdominal cavity and contents in these organs). For test animals that died during the long-term repeated-dose toxicity test, causes of their deaths should be ascertained through an autopsy or other means.

(a) Visual observations

(b) Weight of organs

The weight of the adrenal gland, brain, epididymis, heart, kidney, liver, ovary, spleen, testis, thyroid gland (including parathyroid) and uterus of all test animals (excluding test animals that are dying or were killed during the test) should be measured.

(c) Preservation of organs and tissues

The following organs and tissues must be preserved even after the end of the long-term repeated-dose toxicity test so that a histopathological examination can be conducted in the future as necessary.

All visually detected lesions, brain (including sections of the cerebrum, cerebellum, cerebellopontine and medulla oblongata), spinal cord (neck spinal cord, midthoracic spinal cord, and lumbar spinal cord), pituitary gland, thyroid gland, parathyroid, thymus gland, [tooth, tongue,] esophagus, salivary gland, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, kidney, adrenal gland, spleen, heart, trachea, lung, [upper respiratory tract (including nose, nasal concha, and nasal sinuses),] aorta, testis, ovary, uterus (including uterine cervix), vagina, seminal gland, coagulating gland, epididymis, mammary gland (indispensable for female animals), prostate gland, urinary bladder, [ureter,

urethra,] gallbladder (for animals other than rats), lymph node (superficial lymph node and deep lymph node), peripheral nerve, bone marrow, skin, eyeball (including retina), lacrimal gland (exorbital lacrimal gland), harderian gland, [breast bone, femur (including joint parts), olfactory bulb,] and skeletal muscle (*tissues in the square brackets are optional)

(d) Histopathological examination

The examination should be conducted for preserved organs and tissues of all test animals of at least the control group and the highest dose group. Any organs or tissues that reveal changes suggesting a correlation with the administration of the highest dose should be examined histopathologically also in the other groups.

Additionally, all visually detected lesions in test animals of all dose groups should be examined.

Regarding paired organs (kidneys and adrenal glands, etc.), both of them (left and right) should be examined.

Preserved organs and tissues of all test animals that died or were killed during the long-term repeated-dose toxicity test should be examined as well.

VII. Carcinogenicity Test

1. Objective

This test aims to clarify the carcinogenicity and other possibilities of the test substance, in particular, among its overall influence by continuously administering it to test animals throughout their lives. When a long-term repeated-dose toxicity test is additionally required, the option to conduct the test explained in VIII in lieu of this test should be considered.

2. Test Animals

(1) Animal types

One or more types of animals including rodents (usually rats) are to be used. When there are justifiable grounds proving the appropriateness of using non-rodents for the purpose of estimating health effects on livestock and human beings, non-rodents may be used.

It is preferable to use the same type and same species of animals as those used in the short-term repeated-dose toxicity test.

(2) Conditions

Healthy young animals (whose weights are within a range of ±20% of the average for females and males, respectively) are to be used. Administration should be commenced as early as possible after the animals are weaned and then habituated for at least seven days, before they become eight weeks old. Females should be nulliparous and non-

pregnant.

(3) Number of animals

Each dose group and control group should consist of at least 50 males and 50 females. When performing an autopsy and sampling in the middle of the test, test animals are to be added as necessary. For example, when some test animals are killed in the middle, at least ten males and ten females should be added for each dose group on each occasion. If any data on neoplastic changes has been obtained as a result of a repeated-dose toxicity test, it is unnecessary to kill test animals in the middle. It should also be considered to prepare a monitor group (usually five males and five females), as necessary, in order to monitor the disease status.

3. Administration Period

The administration period should be 24 months, and when setting a shorter or longer period, justifiable grounds therefor should be indicated. For some species of mice (AKR/J, C3H/J, C57BL/6J, etc.), a shorter administration period of 18 months would be appropriate.

When the percentage of surviving test animals in lower dose groups or the control group becomes less than 25%, termination of the test should be considered. When only test animals of higher dose groups died early due to the toxicity, the test should be continued. The number of surviving animals should be counted separately for males and females when making a judgment on whether or not to continue the test. The administration period should not be extended exceeding the scope necessary for making statistically significant evaluations.

4. Administration Method

The test substance should be added to feed or drinking water and administered continuously, in principle. If this is difficult, the test substance may be administered continuously for five days a week through forced oral administration or administration in capsules.

5. Doses

Groups of at least three different stages of doses should be prepared in addition to a control group.

Dose stages should be set based on the results of the short-term repeated-dose toxicity test. The highest dose should be at a level that can identify major targeted organs and toxic impacts without causing many deaths and the lowest dose should be at a level that shows no toxic impacts (non-toxic amount). Other dose stages should be set so that a dose-response relationship can be observed. The grounds for setting dose levels are to be also indicated.

When adding the test substance to feed, due consideration should be given not to cause

any malnutrition, and there is no need to conduct the test with doses wherein the concentration of the test substance added to the feed exceeds 5% (W/W).

When no solvent and no other material is used, the control group should be a non-treated control group, and when any solvent or other materials is used, it should be a solvent control group. Test animals of the control group should be under the same conditions as those of the other groups, except for not being administered with the test substance. When using a solvent, etc. for administering the test substance, the control group should be administered with the same amount as that for the group administered with the largest amount of solvent. When using a solvent other than water, a solvent whose toxicity is already known must be selected.

6. Observations and Inspections

During the test period, at least the following matters should be observed and inspected.

(1) General status, etc.

(a) Body weight

Measurement should be conducted once prior to commencing administration, at an interval of one week until the lapse of 13 weeks after commencing administration, and at an interval of one month thereafter.

(b) Feed consumption, consumption of the test substance, and feed efficiency should be measured at an interval of one week until the lapse of 13 weeks after commencing administration, and at an interval of one month thereafter.

When adding the test substance to drinking water, water consumption should be measured in the same manner. Even when adding the test substance to feed or in the case of forced oral administration, if there are any changes in drinking behavior during the test period, measuring of water consumption should be taken into consideration.

(c) General status

Signs of pathologies and deaths should be checked for individual animals of all groups (twice a day). Toxic signs should be checked once a day and tumorous lesions should be recorded, detailing changes in the statuses of respective tumors from their occurrence.

(d) Other necessary tests

A hematological test, serum chemistry, urine test, etc. should be conducted when a test director (meaning a person prescribed in Chapter I, Article 2, (6) of the Standards on the Implementation of Animal Tests for Feed Additives" (dated July 29, 1988; circular notice by the Director General of the Livestock Industry Bureau, MAFF, and the Director-General of the Fisheries Agency; 63-Chiku A No. 3039; the same applies hereinafter) finds it necessary.

(2) Pathological inspection

The inspection should be conducted regarding the following matters for as many organs as possible of all test animals (including the body surface, all orifices, skull cavity, chest cavity, abdominal cavity and contents in these organs). For test animals that died during the carcinogenicity test, causes of their deaths should be ascertained through an autopsy or other means.

(a) Visual observations

(b) Preservation of organs and tissues

The same as explained in VI, 6, (10), (c)

(c) Histopathological examination

The same as explained in VI, 6, (10), (d)

VIII. Long-term Repeated-dose Toxicity and Carcinogenicity Test

1. Objective

This test aims to clarify the toxicity of the test substance by continuously administering it to test animals for a long term, specifically aiming to clarify the long-term repeated-dose toxicity and carcinogenicity and other possibilities of the test substance at the same time.

2. Test Animals

(1) Animal types

One or more types of animals including rodents (usually rats) are to be used. When there are justifiable grounds proving the appropriateness of using non-rodents for the purpose of estimating health effects on livestock and human beings, non-rodents (usually dogs) may be used.

It is preferable to use the same type and same species of animals as those used in the short-term repeated-dose toxicity test.

(2) Conditions

Healthy young animals (whose weights are within a range of ±20% of the average for females and males, respectively) are to be used. Administration should be commenced as early as possible after the animals are weaned and then habituated for at least seven days, before they become eight weeks old. Females should be nulliparous and non-pregnant.

(3) Number of animals

Regarding groups for studying the long-term repeated-dose toxicity (hereinafter referred to as "repeated-dose toxicity study groups"), each dose group and control group should consist of at least 10 males and 10 females.

Regarding groups for studying the carcinogenicity (hereinafter referred to as

"carcinogenicity study groups"), each dose group and control group should consist of at least 50 males and 50 females.

When performing an autopsy and sampling in the middle of the test, test animals are to be added as necessary. For example, when some test animals are killed in the middle (after six months, etc. for repeated-dose toxicity study groups), at least ten males and ten females should be added for each dose group on each occasion. Additionally, it should be considered to prepare satellite groups (usually for the control group and the highest dose group) in order to observe the recovery from toxic changes, and also to prepare a monitor group (usually five males and five females) as necessary in order to monitor the disease status.

3. Administration Period

The administration period for repeated-dose toxicity study groups should be 12 months.

The administration period for carcinogenicity study groups should be 24 months, and when setting a shorter or longer period, justifiable grounds therefor should be indicated. For some species of mice (AKR/J, C3H/J, C57BL/6J, etc.), a shorter administration period of 18 months would be appropriate.

When the percentage of surviving test animals in lower dose groups or the control group becomes less than 25% in the test for carcinogenicity study groups, termination of the test should be considered. When only test animals of higher dose groups died early due to the toxicity, the test should be continued. The number of surviving animals should be counted separately for males and females when making a judgment on whether or not to continue the test. The administration period should not be extended exceeding the scope necessary for making statistically significant evaluations.

4. Administration Method

The test substance should be added to feed or drinking water and administered continuously, in principle. If this is difficult, the test substance may be administered continuously for five days a week through forced oral administration or administration in capsules.

5. Doses

Groups of at least three different stages of doses should be prepared in addition to a control group.

Dose stages should be set so that the entirety of the toxicity of the test substance can be clarified and the non-toxic amount can be estimated. The highest dose should be at a level that can identify major targeted organs and toxic impacts without causing many deaths and the lowest dose should be at a level that shows no toxic impacts (non-toxic amount). Other dose stages should be set so that a dose-response relationship can be observed. The grounds

for setting dose levels are to be also indicated.

Regarding repeated-dose toxicity study groups, when no toxic action was observed in one test administering the maximum dose technically feasible or a dose equivalent to 1,000mg/kg body weight/day, and no toxicity is expected based on the test results for structurally similar substances, there is no need to continue the test with larger doses.

When adding the test substance to feed, due consideration should be given not to cause any malnutrition, and there is no need to conduct the test with doses wherein the concentration of the test substance added to the feed exceeds 5% (W/W).

When no solvent and no other material is used, the control group should be a non-treated control group, and when any solvent or other materials is used, it should be a solvent control group. Test animals of the control group should be under the same conditions as those of the other groups, except for not being administered with the test substance. When using a solvent, etc. for administering the test substance, the control group should be administered with the same amount as that for the group administered with the largest amount of solvent. When using a solvent other than water, a solvent whose toxicity is already known must be selected.

6. Observations and Inspections

During the test period, at least the following matters should be observed and inspected.

(1) General status, etc.

(a) Body weight

Measurement should be conducted once prior to commencing administration, at an interval of one week until the lapse of 13 weeks after commencing administration, and at an interval of one month thereafter.

(b) Feed consumption, consumption of the test substance, and feed efficiency should be measured at an interval of one week until the lapse of 13 weeks after commencing administration, and at an interval of one month thereafter.

When adding the test substance to drinking water, water consumption should be measured in the same manner. Even when adding the test substance to feed or in the case of forced oral administration, if there are any changes in drinking behavior during the test period, measuring of water consumption should be taken into consideration.

(c) General status

Signs of pathologies and deaths should be checked for individual animals of all groups (twice a day). General status and toxic signs should be checked once a day.

For carcinogenicity study groups, tumorous lesions, in particular, should be recorded, detailing changes in the statuses of respective tumors from their occurrence.

(d) Hematological test

a. Repeated-dose toxicity study groups

For rodents, blood samples should be taken from 10 males and 10 females each of all dose groups, and for non-rodents, the number of animals from which blood samples should be collected is to be less than the total number of test animals (for example, in the case of dogs, 4 males and 4 females for each dose group).

The sampling time and inspection items should be as explained in VI, 6, (6), (b) and (c), respectively.

b. Carcinogenicity study groups

A hematological test should be conducted when a test director finds it necessary.

(e) Serum chemistry

a. Repeated-dose toxicity study groups

Blood samples should be taken as explained in (d) above.

The sampling time and inspection items should be as explained in VI, 6, (7), (b) and (c), respectively.

b. Carcinogenicity study groups

Serum chemistry should be conducted when a test director finds it necessary.

(f) Ophthalmologic test

Regarding repeated-dose toxicity study groups, an ophthalmologic test should be conducted at least for the highest dose group and the control group before administration of the test substance and at the end of the long-term repeated-dose toxicity and carcinogenicity test. If any abnormality is found, all test animals should be checked.

(g) Urine test

a. Repeated-dose toxicity study groups

Urine samples should be taken from 10 males and 10 females each of all dose groups.

The sampling time and inspection items should be as explained in VI, 6, (9), (b) and (c), respectively.

b. Carcinogenicity study groups

A urine test should be conducted when a test director finds it necessary.

(2) Pathological inspection

The inspection should be conducted regarding the following matters for as many organs as possible of all test animals (including the body surface, all orifices, skull cavity, chest cavity, abdominal cavity and contents in these organs). For test animals that died during the test, causes of their deaths should be ascertained through an autopsy or other means.

(a) Visual observations

(b) Weight of organs

Regarding repeated-dose toxicity study groups, the weight of the adrenal gland,

brain, epididymis, heart, kidney, liver, ovary, spleen, testis, thyroid gland (including parathyroid) and uterus of all test animals (excluding test animals that are dying or were killed during the test) should be measured (the measurement is not necessary for carcinogenicity study groups).

(c) Preservation of organs and tissues
The same as explained in VI, 6, (10), (c)

(d) Histopathological examination
The same as explained in VI, 6, (10), (d)

IX. Multi-generation Reproduction Test

1. Objective

This test aims to clarify the effects of the test substance on fertility and on subsequent generations by administering it to male and female test animals over several generations.

2. Test Animals

(1) Animal types
One or more types of animals including rodents (usually rats) are to be used.

(2) Conditions
For the parent generation (hereinafter referred to as the "P-generation"), the weight and age of test animals should be aligned as uniformly as possible.

(3) Number of animals
Each dose group should contain at least 20 pregnant females.

3. Number of generations

Two generations, in principle

4. Administration Method

The test substance should be added to feed or drinking water and administered continuously, or should be administered continuously through forced oral administration.

5. Doses

Groups of at least three different stages of doses should be prepared in addition to a control group.

Dose stages should be set so that the entirety of the toxicity of the test substance can be clarified and the non-toxic amount can be estimated. The highest dose should be at a level that shows toxic impacts without causing many deaths in the P-generation and the lowest dose should be at a level that shows no toxic impacts on both parents and child animals

(non-toxic amount). Other dose stages should be set so that a dose-response relationship can be observed. The grounds for setting dose levels are to be also indicated.

When no toxic action was observed in one test administering the maximum dose technically feasible or a dose equivalent to 1,000mg/kg body weight/day, and no toxicity is expected based on the test results for structurally similar substances, there is no need to continue the test with larger doses.

When adding the test substance to feed, due consideration should be given not to cause any malnutrition, and there is no need to conduct the test with doses wherein the concentration of the test substance added to the feed exceeds 5% (W/W).

When no solvent and no other material is used, the control group should be a non-treated control group, and when any solvent or other materials is used, it should be a solvent control group. Test animals of the control group should be under the same conditions as those of the other groups, except for not being administered with the test substance. When using a solvent, etc. for administering the test substance, the control group should be administered with the same amount as that for the group administered with the largest amount of solvent. When using a solvent other than water, a solvent whose toxicity is already known must be selected.

6. Mating

(1) P-generation

(a) After administering the test substance continuously for at least 10 weeks, a male and a female in the same dose stage are to be crossed with one on one (have them mate or live together for two weeks).

(b) Mated couples should be identified, and mating among siblings should be avoided.

(2) First (F1) generation

(a) At the time of weaning, one male and one female should be selected at random from among litters of each mother. After administering the test substance continuously for at least 10 weeks, a male and a female from other mothers in the same group are to be crossed.

(b) Children should not be crossed until they attain sexual maturity.

(c) Regarding couples that did not mate, the causes thereof should be examined. For that purpose, a histopathological examination on their reproductive organs, re-mating with another male or female, and checking of their heat cycles or spermatogenetic cycles are to be conducted.

7. Administration of the test substance

(1) P-generation

(a) Regarding the P-generation, administration of the test substance is to be commenced at the age of 5 to 9 weeks at the latest.

(b) For P-generation males, the test substance should be administered during their growing phase and for the whole period of at least one full spermatogenetic cycle (mice: approx. 56 days; rats: approx. 70 days).

(c) For P-generation females, the test substance should be administered during their growing phase and for the whole period of multiple sexual cycles.

(d) The test substance should be administered to both males and females during the mating period and to only females during the pregnancy period and lactational period.

(2) F1-generation

Regarding the F1-generation, administration of the test substance is to be commenced at the time of weaning and be continued throughout the period from the growing phase, mating period and pregnancy period until the time when the second (F2) generation is weaned.

8. Observations and Inspections

During the test period, at least the following matters should be observed and inspected.

(1) P-generation

(a) Body weight, feed consumption, feed efficiency, and consumption of the test substance; when adding the test substance to drinking water, water consumption (measurement should be conducted at an interval of one week from the day of commencing administration of the test substance)

(b) Regarding females, body weight on the 0th, 7th, 14th, and 20th or 21st days of the pregnancy period, during the lactational period (simultaneously upon weighing children), and on the day when they are killed

(c) General status (every day), and pathologies and deaths (at least twice a day)

(d) The length of females' heat cycles before mating and their normality (to be evaluated based on the status of the vaginal smear)

(e) Weight of the testis and epididymis, and shapes of sperm or other matters to evaluate the status of sperm; However, when all required matters concerning sperm have been already evaluated in the test explained in V, there is no need to repeat observations of the same matters.

(f) After the completion of the multi-generation reproduction test or upon deaths of test animals, an autopsy should be performed for all test animals used for mating to check structural abnormalities or lesions in the reproductive system, in particular. Additionally, implantation count should be checked for all primiparous females.

(g) After the completion of the multi-generation reproduction test, body weight, and the weight of the uterus, ovary, testis, epididymis, prostate gland, seminal gland, coagulating gland, brain, liver, kidney, spleen, pituitary gland, thyroid gland, adrenal gland, and other target organs should be measured.

(h) The following organs and tissues are to be preserved for a histopathological examination.

Vagina, uterus (including uterine cervix), ovary, testis on one side, epididymis on one side, seminal gland, prostate gland, and coagulating gland, and target organs of all test animals used for mating

(i) A histopathological examination should be conducted for all test animals of the highest dose group and the control group out of test animals used for mating. Any organs or tissues that reveal changes suggesting a correlation with the administration of the highest dose should be examined histopathologically also in the other dose groups.

Additionally, all visually detected lesions in test animals of all dose groups should be examined.

Regarding paired organs (kidneys and adrenal glands, etc.), both of them (left and right) should be examined.

(2) F1-generation

(a) Life or death and gender of all newborns, and their physical abnormalities and behavioral abnormalities

(b) Body weight of surviving children (on the day of birth (0-day), the 4th and 7th days, and once every week thereafter)

(c) Test animals that were not used for mating after weaning should be killed. At least one male and one female should be selected at random from among litters of each mother to check structural abnormalities or lesions in the reproductive system, in particular, through an autopsy and histopathological examination.

(d) Regarding test animals used for mating, observations should be conducted for the matters indicated in (a) to (e) in the section of "(1) P-generation" in the same manner as explained therein.

(e) After the completion of the multi-generation reproduction test or upon deaths of test animals, an autopsy should be performed for all test animals used for mating or those wherein abnormalities or clinical symptoms were found to check structural abnormalities or lesions in the reproductive system, in particular.

Additionally, implantation count should be checked for all primiparous females.

(f) Regarding all test animals wherein abnormalities or clinical symptoms were found, a histopathological examination should be conducted for their tissues and organs with visually detected abnormalities.

(g) After the completion of the multi-generation reproduction test, body weight, and the weight of the uterus, ovary, testis, epididymis, prostate gland, seminal gland, coagulating gland, brain, liver, kidney, spleen, pituitary gland, thyroid gland, adrenal gland, and other target organs of all test animals used for mating should be measured.

(h) The preservation of organs and tissues for histopathological examinations should be as explained in (h) and (i) in the section of "(1) P-generation."

(3) F2-generation

(a) Observations should be conducted for the matters indicated in (a) and (b) in the section of "(2) F1-generation" in the same manner as explained therein and test animals should be killed after weaning.

(b) After the completion of the multi-generation reproduction test or upon deaths of test animals, an autopsy should be performed for all test animals wherein abnormalities or clinical symptoms were found and at least one male and one female selected at random from among litters of each mother to check structural abnormalities or lesions in the reproductive system, in particular.

(c) A histopathological examination should be conducted for tissues and organs with visually detected abnormalities of all test animals wherein abnormalities or clinical symptoms were found and for at least one male and one female selected at random from among litters of each mother.

9. Matters to Note for Observations

(1) Pregnant females should be bred separately.

(2) When the delivery is approaching, nest-building materials should be prepared.

X. Developmental toxicity Test

1. Objective

This test aims to clarify the effects of the test substance on fetuses by administering it to pregnant females during the period of fetuses' organogenesis.

2. Test Animals

(1) Animal types

Rodents and non-rodents are to be used (preferably rats for rodents and rabbits for non-rodents). If any other types of animals are used, justifiable grounds therefor should be indicated.

(2) Conditions

Healthy young nulliparous females should be used after habituating them for at least five days.

(3) Number of animals

The number of test animals should be sufficient to find implantation in around 20 females in each dose group at the time of an autopsy. The number of females with an implanted uterus must not be below 16 for each group.

Additionally, the mortality rate of pregnant females should not exceed 10%.

3. Administration Period

The test substance should be administered from the time of implantation at the latest to one day prior to the day on which the relevant animal is scheduled to be killed (the day preceding the estimated delivery date). In principle, the administration is for examining the effects of the test substance during the period of fetuses' organogenesis (from the 5th day to the 15th day of pregnancy for rodents, and from the 6th day to the 18th day of pregnancy for rabbits). However, the administration may be continued throughout the pregnancy period from before implantation to one day prior to the day on which the relevant animal is scheduled to be killed in order to examine the effects from before implantation.

Regarding rodents, the day on which a vaginal plug or sperm was confirmed is considered to be the first day (0-day) of pregnancy, while regarding non-rodents, the day of mating or artificial insemination is considered to be the first day (0-day) of pregnancy.

4. Administration Method

Forced oral administration should be adopted in principle.

5. Doses

Groups of at least three different stages of doses should be prepared in addition to a control group.

Dose stages should be set so that the entirety of the toxicity of the test substance can be clarified and the non-toxic amount can be estimated. The highest dose should be at a level that shows toxic impacts without causing many deaths in mother animals and the lowest dose should be at a level that shows no toxic impacts on both mothers and fetuses (non-toxic amount). Other dose stages should be set so that a dose-response relationship can be observed. At least one intermediate dose should be the minimum concentration causing the onset of toxicity. The grounds for setting dose levels are to be also indicated.

When no toxic action was observed in one test administering the maximum dose technically feasible or a dose equivalent to 1,000mg/kg body weight/day, and no toxicity is expected based on the test results for structurally similar substances, there is no need to continue the test with larger doses.

When no solvent and no other material is used, the control group should be a non-treated control group, and when any solvent or other materials is used, it should be a solvent control group. Test animals of the control group should be under the same conditions as those of the other groups, except for not being administered with the test substance. When using a solvent, etc. for administering the test substance, the control group should be administered with the same amount as that for the group administered with the largest amount of solvent.

A solvent that does not cause developmental toxicity or has no influence on test animals' reproductive competence should be selected.

6. Observations and Inspections

During the test period, at least the following matters should be observed and inspected.

(1) Mothers
   (a) Body weight (on the day of commencing the developmental toxicity test (0-day) and the day of commencing the administration, during the administration period (every three days), and at the time of an autopsy)
   (b) Feed consumption (the same days of measuring body weight)
   (c) General status (every day) (mortality rate, pathologies, behavioral changes, and other toxic signs)
   (d) Test animals should be killed one day prior to the estimated delivery date and an autopsy should be performed to check the establishment of pregnancy, corpus luteum count, uterus weight, implantation count, numbers of dead fetuses and surviving fetuses, and levels of embryonic resorption (early or late).

(2) Fetuses
   (a) Body weight
   (b) Gender
   (c) Any external abnormalities
   (d) Any skeletal abnormalities
   (e) Any visceral abnormalities (in reproductive organs, in particular)

XI. Mutagenicity Test

1. Objective

This test aims to clarify the mutagenicity of the test substance.

2. Test Method

In principle, an in vitro reverse mutation test and chromosomal aberration test should be conducted, and if any abnormalities are found through these tests, a micronucleus test should be conducted. Other tests should also be conducted as needed.

(1) Reverse mutation test

This test aims to clarify the effects of the test substance on DNA base pairs by exposing bacterial strains requiring amino acid to the test substance and detecting point mutations.

   (a) Bacterial strains

At least five types of bacterial strains should be used. Recommended combinations

of bacterial strains are as follows.

a. *Salmonella* Typhimurium TA1535
b. *S*.Typhimurium TA1537, TA97 or TA97a
c. *S*.Typhimurium TA98
d. *S*.Typhimurium TA100
e. *Escherichia coli* WP2 uvrA, *E.coli* WP2 uvrA (pKM101) or *S*.Typhimurium TA102

(b) Dose stages

Groups of at least five different stages of doses should be prepared in addition to a control group.

The highest dose should be no larger than 5mg/plate, in principle.

(c) Control substances

Negative and positive controls should be prepared. In principle, the negative control is to be a solvent control, while the positive control is to be a known mutagenic substance.

(d) Metabolic activation

Test cells are to be exposed to the test substance under conditions with and without a metabolic activation system. Generally, S 9mix, which is prepared from rodent liver treated using an appropriate drug-metabolizing enzyme as an inducer, is to be used.

(e) Test method

Either of the pre-incubation method or the plate method should be adopted.

(f) Recording of the results

The test results should be recorded with regard to the following matters

a. Toxic signs
b. Precipitating signs
c. Number of colonies in each plate
d. Mean value and standard deviation of reverse mutation colonies per plate
e. Dose-response relationship (if any)
f. Statistical analysis (if necessary)
g. Concurrent control data of the negative control (solvent/diluent) and the positive control, and their scope, mean value and standard deviation
h. Historical control data of the negative control (solvent/diluent) and the positive control, and their scope, mean value and standard deviation

(2) Chromosomal aberration test

This test aims to clarify the structural effects of the test substance on chromosomes by exposing cultured mammalian cells to the test substance.

(a) Cells

Chinese hamster ovary cells (CHO), Chinese hamster lung cells (V79 or CHL/IU),

human cells (TK6), or other cell lines, or primary cultured cells including human or other mammalian peripheral blood lymphocyte are to be used.

(b) Dose stages

At least three different stages of doses should be set.

The highest dose should be set so as to show the cytotoxicity of 55±5% based on the relevant indicator. When no cytotoxicity is found, 10m M or 2mg/mL, whichever is lower, should be the upper concentration limit.

As a cytotoxicity indicator, the Relative Population Doubling (RPD) or Relative Increase Cell Count (RICC) should be used for cell lines, and the Mitotic Index (MI) should be used for primary cultured cells.

(c) Control substances

In principle, the negative control is to be a solvent control, while the positive control is to be a known mutagenic substance.

(d) Metabolic activation

Test cells are to be exposed to the test substance under conditions with and without a metabolic activation system. Generally, S-9mix, which is prepared from rodent liver treated using an appropriate drug-metabolizing enzyme as an inducer, is to be used.

(e) Search method

Chromosome samples should be collected on two occasions with an appropriate interval some time after treating the test substance. All sample slides including negative and positive controls should be encoded so that their treatment conditions are not identified. Cells with chromosomal aberrations and polyploid cells are to be searched in at least 300 well−spread metaphase cells for each dose group.

(f) Recording of the results

The test results should be recorded with regard to the following matters to the extent possible.

a. Information on the length of cell cycle, doubling time, or proliferation index (limited to the case of using cell lines)
b. The number of treated cells and collected cells for each culture (limited to the case of using cell lines)
c. Used cytotoxicity indicator and measured cytotoxicity values
d. Any precipitation and observation time
e. Definition of chromosomal aberrations
f. Types and numbers of cells showing chromosomal aberrations (including the number of polyploid cells)
g. Dose-response relationship
h. Statistical analysis and p-value
i. Concurrent control data of the negative control (solvent) and the positive control

(concentration and solution)

j. Historical control data of the negative control and positive control, and their scope, mean value, standard deviation, 95% confidence interval, and data amount

(3) Micronucleus test

This test aims to clarify the effects of the test substance on chromosomes or the mitotic apparatus of the erythroblast by administering the test substance to test animals once or several times continuously at one-day intervals and analyzing red blood cells in the bone marrow or peripheral blood.

(a) Test animals

a. Animal types

Preferably mice or rats

b. Number of animals

Each dose group should consist of at least five animals.

(b) Administration period

Single-dose administration or repeated-dose administration at one-day intervals

(c) Administration method

In consideration of assumed human exposure routes, a method to ensure proper exposure of targeted organs should be adopted.

(d) Doses

Groups of at least three different stages of doses should be prepared. The highest dose should be set so that the test reveals toxic signs but causes no deaths. If no toxic signs appear in the case of single-dose administration or repeated-dose administration for less than 14 days, the highest dose should be 2,000mg/kg. In the case of repeated-dose administration for over 14 days, the highest dose should be 1,000mg/kg.

Separately, negative and positive control groups should also be prepared. The negative control should be a solvent control in principle, and a known micronucleus inducer should be used as the positive control.

(e) Search method

In the case of single-dose administration, sampling should be conducted on two occasions each within 24 to 48 hours and within 36 to 72 hours after administration for the bone marrow and for the peripheral blood, respectively. In the case of repeated-dose administration, sampling should be conducted on two occasions each within 18 to 24 hours and within 36 to 48 hours after the final administration for the bone marrow and for the peripheral blood, respectively. However, in both cases, when a preliminary test was conducted, sampling may be conducted only once.

All sample slides including negative and positive controls should be encoded so that their treatment conditions are not identified.

Micronuclei are to be searched in at least 4,000 immature erythrocytes (meaning

polychromatophilic erythrocytes or reticulocyte) for each test animal.

(f) Recording of the results

The test results should be recorded with regard to the following matters to the extent possible.

a. Standard of the positive reaction or negative reaction
b. Status of test animals (including toxic signs) before and during the test
c. Percentage of immature erythrocytes among total erythrocytes
d. Number of immature erythrocytes with micronuclei
e. Mean number and standard deviation of immature erythrocytes with micronuclei for each group
f. Dose-response relationship
g. Statistical analysis and method thereof
h. Historical data of the negative control and positive control, and their scope, mean value, standard deviation, 95% confidence interval, and number of tests
i. Data proving the exposure of the bone marrow
j. Data indicating whether the micronuclei are from the entirety or a section of the chromosome

3. Other Tests

(1) Tests focused on induction of gene mutation as an indicator
  (a) Gene mutation test using cultured mammalian cells
  (b) Test using drosophila
  (c) Spot test using mice
  (d) Specific locus test using mice
(2) Tests focused on induction of chromosomal aberration as an indicator
  (a) Chromosomal aberration test using rodent reproductive cells
  (b) Dominant lethal test using rodents
  (c) Reciprocal translocation test using mice
(3) Tests focused on DNA-damaging effects as an indicator
  (a) Prophage induction test using bacteria
  (b) DNA repair test using bacteria
  (c) Unscheduled DNA synthesis (UDS) test using mammalian cells
  (d) Sister chromatid exchange (SCE) test using mammalian cells
(4) Other tests
  (a) Somatic cell recombinant test and gene exchange test using yeast
  (b) Sperm aberration test using mice

XII. Tests on In Vivo Kinetics

1. Substances other than Live Microbial Agents

(1) Objective

The test aims to ascertain in vivo kinetics of the test substance by administering it to test animals and check absorption, distribution, accumulation, metabolism, excretion, etc. thereof.

(2) Test animals

Targeted livestock, etc. is to be used, and matured rats, rabbits or other animals should be added as test animals as needed. The number of test animals should be sufficient to obtain knowledge for making evaluations.

(3) Administration method

In principle, the test substance should be orally administered once, but it is preferable to consider the necessity for continuous administration. For an accumulation test, the test substance should be administered continuously over a sufficient period of time. Additionally, an in situ test and in vitro test should also be conducted as necessary.

(4) Doses

Doses should be the minimum necessary to achieve the objective of the test at a level that ensures appropriate amounts of the test substance or metabolite found in each tissue or feces after administration, in light of the used analysis method.

(5) Search

(a) Absorption and excretion

Blood levels, intestinal residues, fecal and urinary excretion levels, etc. of the test substance and major metabolites (hereinafter referred to as the "test substance, etc.") should be measured at appropriate time intervals to determine the rate of absorption via the digestive tract, the excretory route and excretory rate of the test substance.

When administering an isotope-labeled compound as the test substance, chemical types of recovered isotopes should be checked.

(b) Distribution

A distribution test should be conducted when an absorption and excretion test has revealed the absorption of the test substance, aiming to ascertain distribution over time of the test substance, etc. in as many organs and tissues as possible and to calculate their biological half-lives to the extent possible.

The test should cover the liver (hepatopancreas), kidney, heart, lung (gill), spleen, muscle, digestive tract, brain, skin, gonad, adrenal gland, thyroid gland, thymus, and pituitary gland, etc. Changes over time should be obtained for major organs and tissues, but in the case of using small animals, measurement of changes over time in the endocrine gland may be omitted.

Additionally, autoradiography or other method after administration of a labeled

compound should also be conducted as necessary.

(c) Accumulation

With regard to organs and tissues wherein the test substance or other material is likely to be accumulated based on the results of the distribution test, accumulation over time should be examined. In this case, it is preferable to administer the test substance continuously until the cumulative amount reaches a plateau and examine the changes in that amount after discontinuing the administration.

(d) Metabolism

When the test substance is metabolized in vivo, its major metabolites should be identified and their rates of formation in principal organs and tissues involved in metabolism should be determined as needed.

Metabolism should be examined in vitro using organs and tissues mainly involved in metabolism for the determination of the formation rates of each metabolite. When there are species differences in the formation rates of each metabolite, it is preferable to further conduct another test using other species.

2. Live Microbial Agents

(1) Objective

The test aims to ascertain in vivo kinetics of the test viable bacteria by administering it to test animals and check its distribution in the digestive tract, excretion, and possible penetration, etc. into tissues other than the digestive tract.

(2) Test animals

In principle, targeted livestock, etc. should be used.

(3) Administration method

The test viable bacteria should be orally administered continuously over a sufficient period of time until fecal excretory amount of the test viable bacteria reaches a fixed level in principle.

(4) Doses

Doses should be the minimum necessary to achieve the objective of the test.

(5) Search

(a) Rootage and excretion of the test viable bacteria in and from the digestive tract, and its fate after discontinuing administration should be examined.

(b) Distribution of the test viable bacteria should be examined in as many organs and tissues as possible.

XIII. Feeding Tests Using Targeted Livestock, etc.

1. Objective

The tests aim to clarify the effects of the test substance (or the test live microbial agent in the case of a live microbial agent; hereinafter the same applies in this section) by continuously administering it to targeted livestock, etc. for which it is used as a feed additive, in light of the status of usage of feed additives at the feeders', breeder’s or raiser’s level.

2. Test Animals

Targeted livestock, etc. to which the test substance is to be applied as a feed additive should be used. The number of test animals for each dose group should be around 3 to 10 in the case of cows or pigs, around 20 to 30 in the case of chickens, and at least 30 in the case of cultured aquatic animals. However, when it is difficult to feed 30 or more animals due to the size of the test animals and the test facilities, etc., each dose group should consist of at least 20 animals.

Regarding a test for live microbial agents, targeted livestock, etc. should be divided into four categories as prescribed in Article 1, item (i) to item (iv) of the Enforcement Order and the test should be conducted, in principle, for at least one type of animals in the category to which targeted livestock, etc., belongs.

Attention should be paid to the environmental conditions for conducting a test for cultured aquatic animals. Water temperature should basically be between 22 and 28℃ for yellowtails, red sea bream, carp, eels, and prawns, between 12 and 18℃ for rainbow trouts and white salmons, and between 19 and 25°C for sweetfish.

3. Administration Period

The administration period should be set in light of the period during which the test substance is to be actually used. However, regarding cultured aquatic animals, if the administration period is prolonged, the period may be substituted with the period of time that is longer than one-half of the scheduled application period of the test substance and during which the average weight of the control group triples in principle.

4. Administration Method

The test substance should be added to feed and continuously administered to test animals.

5. Doses

At least two different stages of doses should be set in the range from the maximum amount of the optimum additive amounts to around 10 times that amount, and a control group should be prepared separately.

6. Observations and Inspections

During the test period, at least the following matters (1) to (3) should be observed, and when any abnormality is found, the following (4) to (6) should be conducted as necessary.

(1) Body weight
(2) Feed consumption and consumption of the test substance (for cultured aquatic animals, feeding amount and doses of the test substance)
(3) General status
(4) Hematological test (not required for prawns)
(5) Serum chemistry
(6) Pathological inspection

XIV. Tests on Emergence of Resistant Bacteria

1. Objective

The tests aim to qualitatively and quantitatively examine and clarify the effects of the use of antimicrobials on the emergence of drug-resistant bacteria out of the entirety of their effects on microbes.

2. Tests on Antibacterial Spectrums

(1) Sensitivity test using preserved bacterial strain in the laboratory

(a) Test bacterial strains

Test bacterial strains should be at least 20 types, including the following. Standard bacterial strains (ATCC strain, etc.) that enable easy comparisons with other test results are preferable.

a. Gram positive bacterial strains

(i) *Staphylococcus aureus*
(ii) *Staphylococus epidermidis*
(iii) *Streptococcus agalactiae*
(iv) *Streptococcus pyogenes*
(v) *Streptococcus suis*
(vi) *Bacillus cereus*
(vii) *Clostridium perfringens*
(viii) *Actinomyces pyogenes*
(ix) *Erysipelothrix rhusiopathiae*

b. Gram negative bacterial strains

(i) *Bordetella bronchiseptica*
(ii) *Escherichia coli*
(iii) *Avibacterium paragallinarum*
(iv) *Actinobacillus pleuropneumoniae*

(v) *Pseudomonas aeruginosa*
(vi) *Pasteurella multocida*
(vii) *Salmonella* Typhimurium
(viii) *Salmonella* Enteritidis
(ix) *Salmonella* Pullorum

(b) Test method

The minimal inhibitory concentrations of the test substance against test bacterial strains are to be determined according to the latest "Standard Methods of the Japanese Society of Chemotherapy."

(2) Sensitivity test using field bacterial strains

(a) Test bacterial strains

Around 50 each of fresh bacterial strains of the following categories that are isolated from livestock and poultry should be used.

It is preferable to select test bacterial strains from among those collected from as broad areas as possible.

a. When active ingredients of the test substance mainly affect gram positive bacterial strains:
(i) *Staphylococcus aureus*
(ii) *Streptococcus pyogenes*

b. When active ingredients of the test substance mainly affect gram negative bacterial strains:
(i) *Escherichia coli*
(ii) *Salmonella* Typhimurium

c. When active ingredients of the test substance affect both gram positive and negative bacterial strains:
Types of bacterial strains indicated in a. and b.

(b) Test method

The same as explained in (1), (b)

3. Tests on Effects on Other Antimicrobials

(1) Test using bacterial strains that have already acquired resistance to known representative antimicrobials in each system

The effectiveness of the test substance and its biochemical mechanism of resistance development should be examined using an R plasmid-carrying organism or chromosomally resistant bacteria, etc. whose drug resistance has already been clarified.

(a) Test bacterial strains

Bacterial strains of the following categories should be used.

a. When the test substance shows antimicrobial activity mainly against gram positive bacterial strains

Staphylococcus aureus that shows single and multiple drug resistance to aminobenzylpenicillin, cefazolin, dihydrostreptomycin, gentamycin, oxytetracycline, chloramphenicol, erythromycin, sulfa drugs, etc.

b. When the test substance shows antimicrobial activity mainly against gram negative bacterial strains

Escherichia coli that shows single and multiple drug resistance to aminobenzylpenicillin, cefazolin, dihydrostreptomycin, gentamycin, oxytetracycline, chloramphenicol, sulfa drugs, etc.

c. When the test substance has wide spectrum effects on both gram positive and negative bacterial strains

Types of bacterial strains indicated in a. and b.

(b) Test method

The same as explained in 2, (1), (b)

(2) Test using field bacterial strains that have cross-resistance or other type of resistance to the test substance

This test should be conducted only when field bacterial strains that show resistance to the test substance have been collected. The minimal inhibitory concentrations of known antimicrobials in each system are to be measured using such resistant field bacterial strains.

(a) Test bacterial strains

Test bacterial strains are to be the types that showed resistance through the test explained in 2, (2).

(b) Test drug

A drug that contains at least one of the following ingredients should be selected, except for drugs that mainly affect protozoa.

In the case of the test substance that mainly affects gram positive bacterial strains, erythromycin should be added.

a. Aminobenzylpenicillin
b. Cefazolin
c. Dihydrostreptomycin
d. Gentamycin
e. Oxytetracycline
f. Chloramphenicol
g. Sulfa drug (1 type)

(c) Test method

The minimal inhibitory concentrations of the test drug against each test bacterial strain are to be measured as explained in 2, (1), (b).

4. Test on Acquisition of Resistance

The incidence of the appearance of resistant bacteria and the degree of resistance to the test substance are to be examined.

(1) In vitro test

The mechanism of resistance acquisition should be examined using standard bacterial strains that are succeeded in test tubes.

(a) Test bacterial strains

Bacterial strains of the following categories should be used.

a. When the test substance mainly affects gram positive bacterial strains

(i) *Staphylococcus aureus*

(ii) *Streptococcus pyogenes*

b. When the test substance mainly affects gram negative bacterial strains

(i) *Escherichia coli*

(ii) *Salmonella* Typhimurium

c. When the test substance has wide spectrum effects on both gram positive and negative bacterial strains

Types of bacterial strains indicated in a. and b.

(b) Test method

A subculture test using liquid culture media containing the test substance (with increased and fixed amounts of the test substance) should be conducted.

The subculture should be succeeded by at least 20 generations. When resistance acquisition is observed, subculture should be conducted using liquid culture media that do not contain the test substance to clarify the state of resistance disappearance.

(2) In vivo test

The practical concentrations and effects of the test substance on intestinal bacteria, protozoa, etc. in excreted feces during the administration period should be examined using targeted animals. At least Escherichia coli should be covered. Using plates containing the test substance and major known antimicrobials of each system, the ratio of resistant E. coli count to the total E. coli count per gram of rectal feces should be identified over time. At the same time, the mechanism and the degree of resistance should also be clarified.

(a) Test animals

Test animals should be targeted livestock, etc. to which the test substance is to be applied. The number of test animals for each dose group should be at least 5 in the case of cows and pigs, and at least 10 in the case of chickens. At least three groups, i.e., the maximum concentration group, the minimum concentration group, and the control group, should be set to apply the test substance.

(b) Test period

The test period should be the period adding at least 7 days to the period during which the test substance is scheduled to be applied.

(c) Test drugs and their concentrations

Test drugs should be selected from among those indicated in the following table.

Test drugs	Concentrations
Aminobenzylpenicillin	25.0μg (titer)/mL
Dihydrostreptomycin	12.5μg (titer)/mL
Gentamycin	25.0μg (titer)/mL
Oxytetracycline	25.0μg (titer)/mL
Chloramphenicol	25.0μg (titer)/mL
Test substance	The resistance limit concentration of E. coli measured in the tests explained in 1, (1) and (2)

(d) Test method

Rectal feces should be collected from test animals of each test group once before the use of the test drug and at least once a week thereafter. Collected feces samples should be diluted with sterile saline and be coated on plates with and without the test drug. The number of E. coli per 1g of feces should be measured by counting the number of colonies that have grown on the plates.

XV. Tests on Impacts on the Natural Environment

Regarding live microbial agents, it is necessary to confirm or estimate that the use of the test substance does not have impacts on the ecology of bacteria, etc. in the environment based on the origin of the test viable bacteria and their distribution in the natural environment, etc.

Additionally, existence of the test viable bacteria in the environment should be examined through field or laboratory tests to evaluate their impacts on the natural environment.

XVI. Tests on Stability of Feed Additives

1. Objective

The tests aim to clarify the stability of the test substance (or the test live microbial agent in the case of a live microbial agent; hereinafter the same applies in this section) under various conditions in consideration of situations where feed additives are actually used. Tests may be added or omitted as necessary.

2. Test Method

(1) Room temperature storage test

With regard to at least three lots each of raw materials and products, adequate amounts of the test substance should be put in containers ordinarily used and stored in an indoor warehouse to check the stability of the test substance upon the lapse of 0, 3, 6, 9, 12, 18 and 24 months (storage period can be extended or shortened depending on a predetermined validity term or use period). Container sizes may be reduced as needed.

(2) Heat tolerance test

With regard to at least three lots each of raw materials and products, adequate amounts of the test substance should be put in sealed glass bottles or airtight containers and stored at 40°C to check the stability of the test substance upon the lapse of 0, 1, 2, 3 and 6 months (storage period can be extended or shortened depending on the environment under which the test substance is treated as a feed additive or its physical properties).

(3) Humidity tolerance test

With regard to at least three lots each of raw materials and products, adequate amounts of the test substance should be placed in open petri dishes and stored at a fixed temperature between 25 and 30°C under at least two different levels of relative humidity to check the stability of the test substance upon the lapse of 0, 1, 2, 3 and 6 months (storage period can be extended or shortened depending on the environment under which the test substance is treated as a feed additive or its physical properties). At least two different relative humidity levels should be selected within a range where no clear reduction of quality, such as change of appearance, occurrence of fungi, deterioration, deliquescence, consolidation, etc., was caused due to a preliminary test. Selected relative humidity levels should include the one close to the upper limit of that range.

(4) Photostability test

With regard to at least three lots each of raw materials and products, adequate amounts of the test substance should be put in lidded petri dishes sealed with adhesive tape or paraffin and stored at room temperature (1 to 30°C) under fluorescent lighting of 500 lux to check the stability of the test substance upon the lapse of 0, 1, 2, 3 and 6 months (storage period can be extended or shortened depending on the environment under which the test substance is treated as a feed additive or its physical properties).

(5) Acceleration test

With regard to at least three lots each of raw materials and products, adequate amounts of the test substance should be put in containers ordinarily used and stored at 40°C, under a relative humidity level of 75% and in an indoor warehouse (at room temperature), in principle, to check the stability of the test substance upon the lapse of 0, 1, 3 and 6 months (storage period can be extended or shortened depending on the environment under which the test substance is treated as a feed additive or its physical properties). Container sizes may be reduced as needed.

(6) Stability test of the test substance in feed

The test substance (products) should be added to at least three types of feed ordinarily produced at the regular concentration, and adequate amounts thereof should be put in containers ordinarily used and stored in an indoor warehouse to check the stability of the test substance upon the lapse of 0, 0.5, 1, 2 and 3 months (and 6 months as necessary).

The method to determine the quantity of the test substance in feed should conform to the following requirements, in principle.

(a) Average recovery rate should be at least 90%, and accuracy of reproducibility (standard error is calculated by adding the error caused by repeat laboratory tests to the error caused by bias among laboratories) should be less than 0.1 as the coefficient of variation.

A recovery test should be conducted to determine the average recovery rate and accuracy of reproducibility by adding products of the test substance to feed at the regular concentration. The test should be conducted three times, in principle, at three or more laboratories using a two-point parallel line bioassay on a different test date.

(b) The quantitation limit should be that the quantity of the test substance at 10% or less of the regular concentration to be added to feed can be determined.

(c) The amount of active ingredients may be identified from that of degradants or other foreign substances.

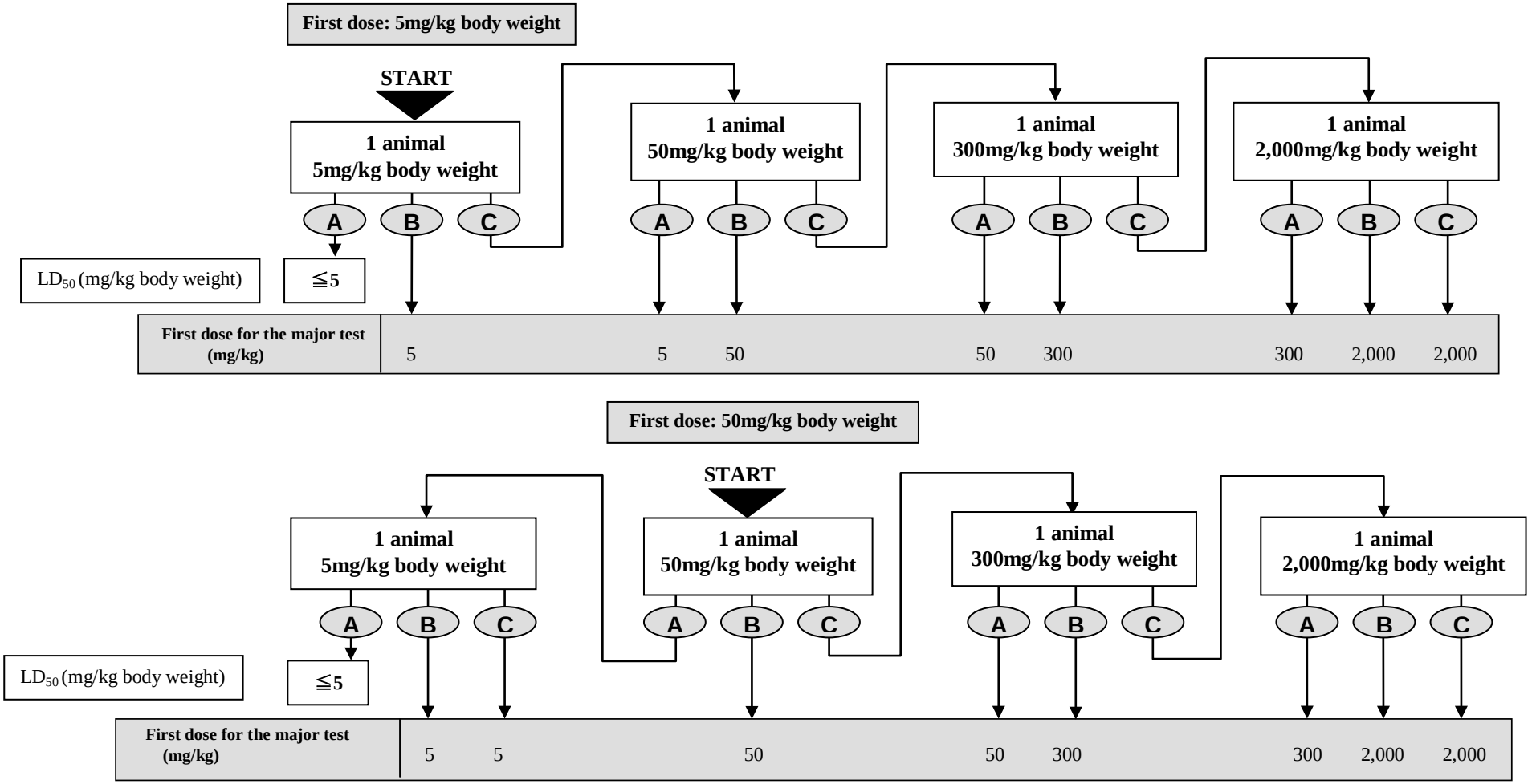
3. Measurement Items

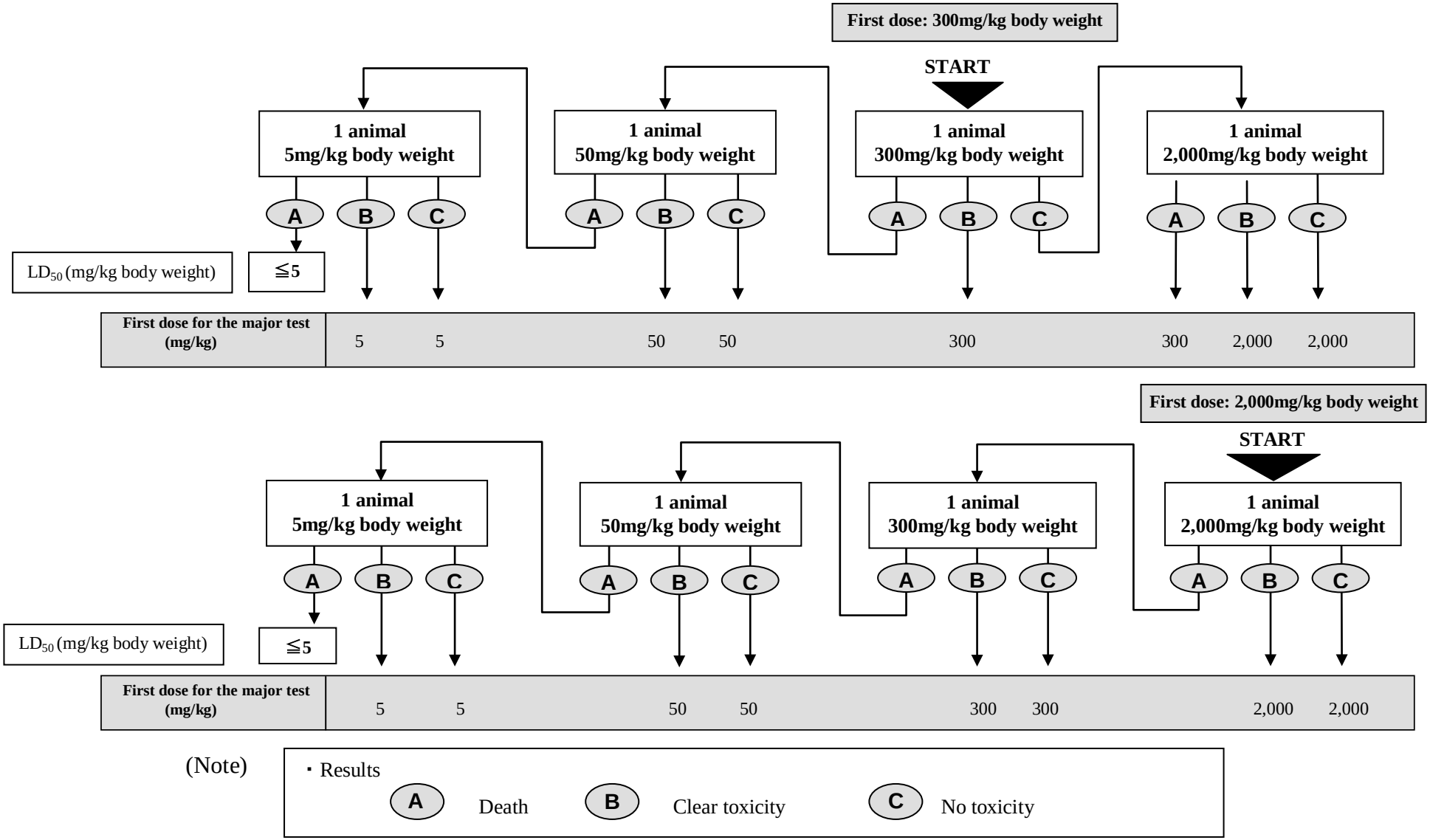
All items scheduled to be set as specifications of the ingredients should be measured on at least three occasions, including the time of starting and ending the tests. At other points in time, observations of appearance and measurements of the amounts of active ingredients should be conducted. With regard to a stability test in feed, room temperature storage test, and humidity tolerance test, the amount reduced by drying or water content should also be measured. Measurement items should be added in respective tests as necessary.

4. Statistical Analysis of Test Results

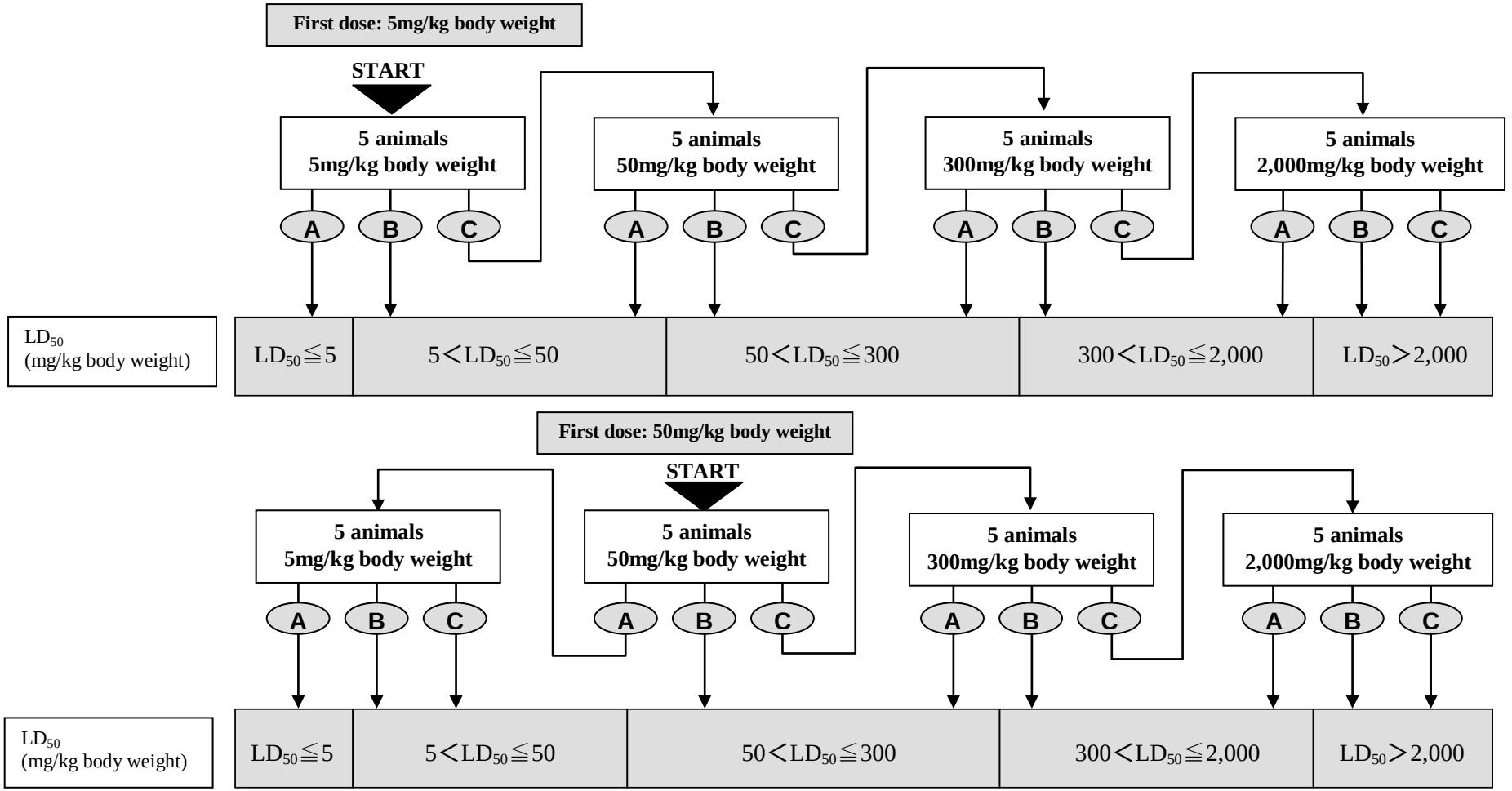
Regression analysis should be conducted to calculate 90% confidence limit of population mean by applying the relation between test periods and measured amounts of active ingredients to a model considered to be most appropriate.

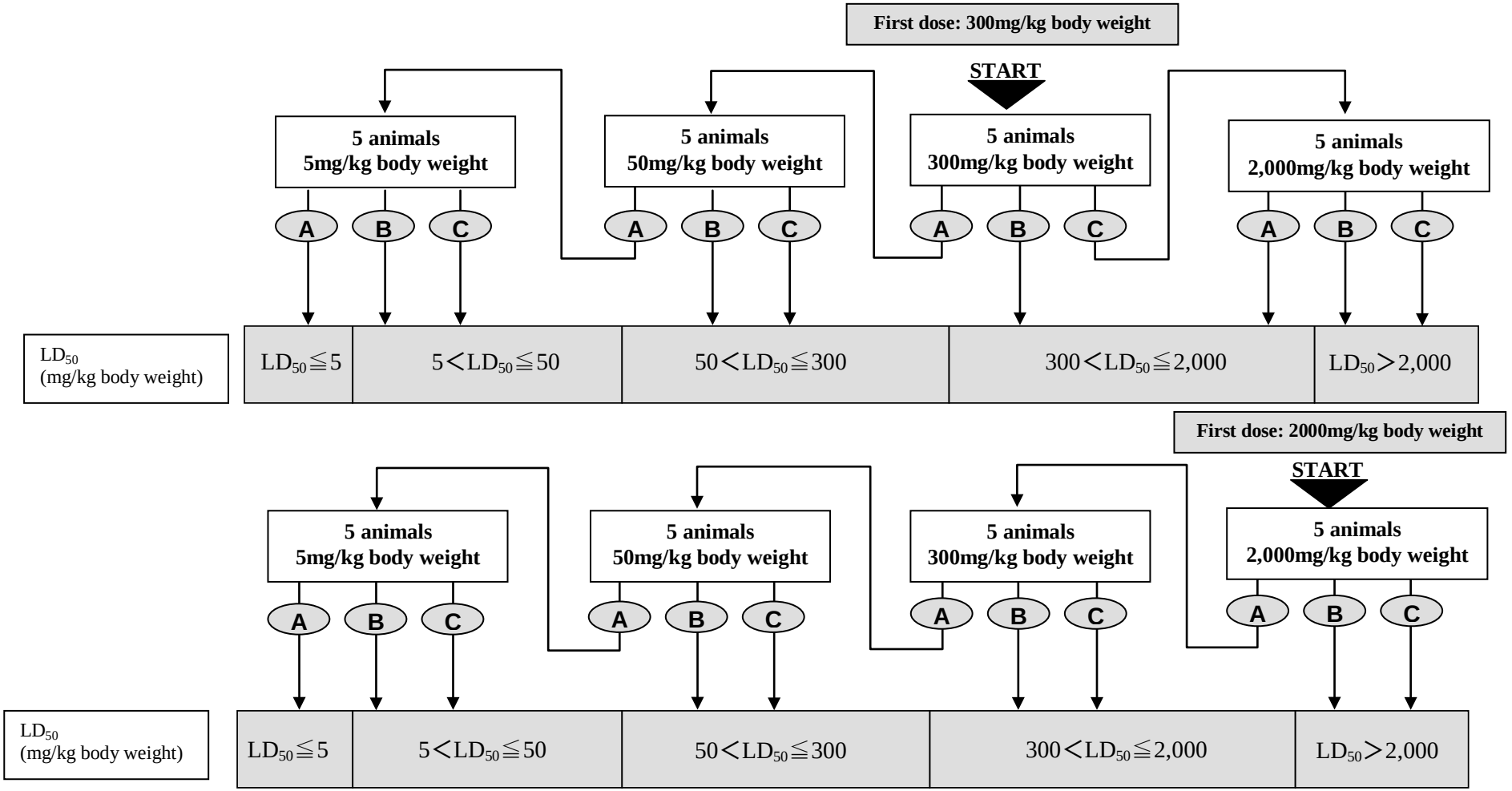
Appendix 1: Procedures for Fixed-dose Method/Estimation Test




First dose: 300mg/kg body weight
START
1 animal
5mg/kg body weight
1 animal
50mg/kg body weight
1 animal
300mg/kg body weight
1 animal
2,000mg/kg body weight
A
B
C
LD50 (mg/kg body weight)
≦5
First dose for the major test (mg/kg)
5
5
50
50
300
300
2,000
2,000
First dose: 2,000mg/kg body weight
START
1 animal
5mg/kg body weight
1 animal
50mg/kg body weight
1 animal
300mg/kg body weight
1 animal
2,000mg/kg body weight
A
B
C
LD50 (mg/kg body weight)
≦5
First dose for the major test (mg/kg)
5
5
50
50
300
300
2,000
2,000
(Note)
・Results
A Death
B Clear toxicity
C No toxicity

Appendix 2: Procedures for Fixed-dose Method/Major Test





(Note)

・Results

A Two or more deaths

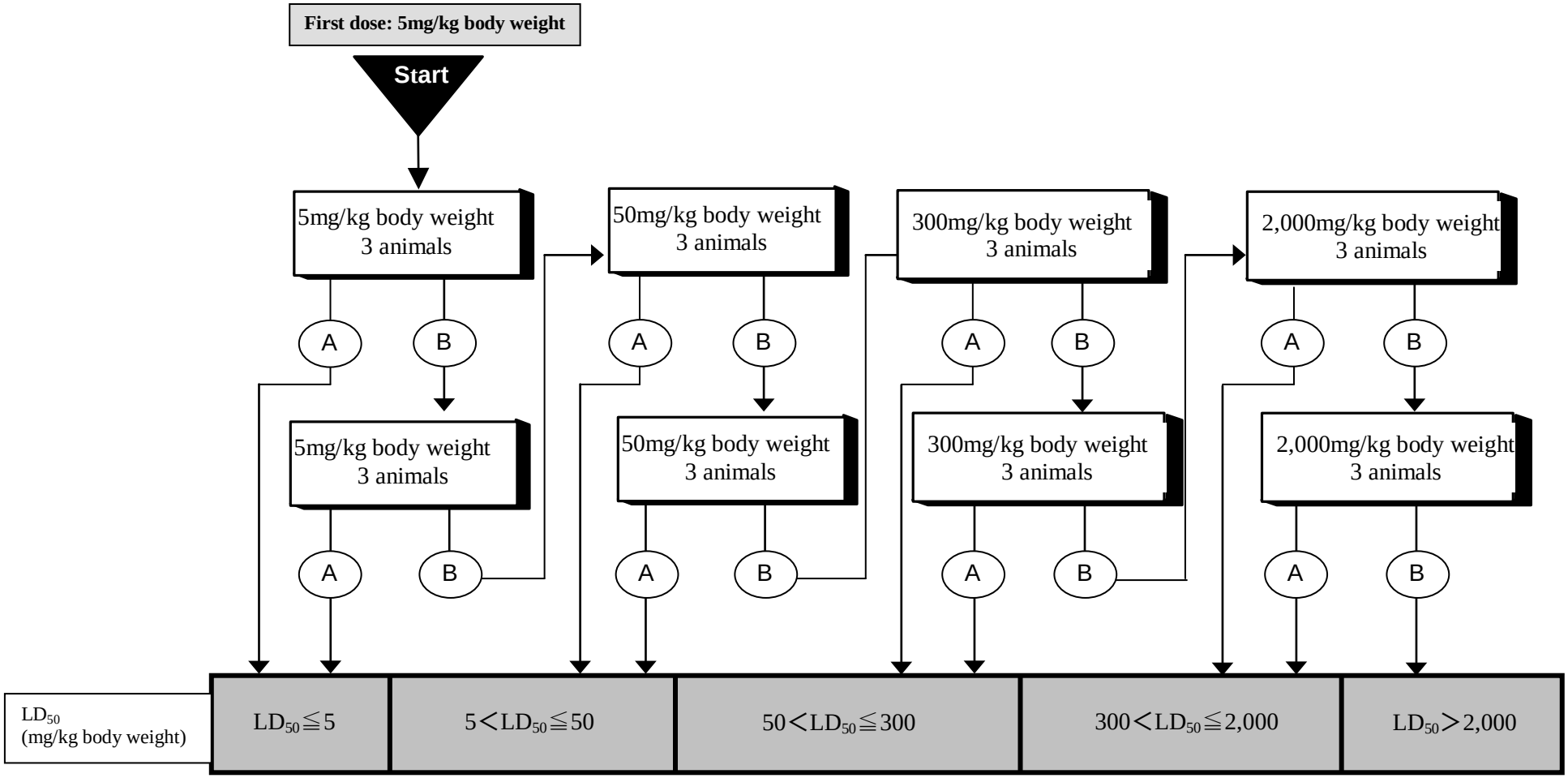
B One death,
Clear toxicity observed in one or more, or
One death and clear toxicity observed in one or more

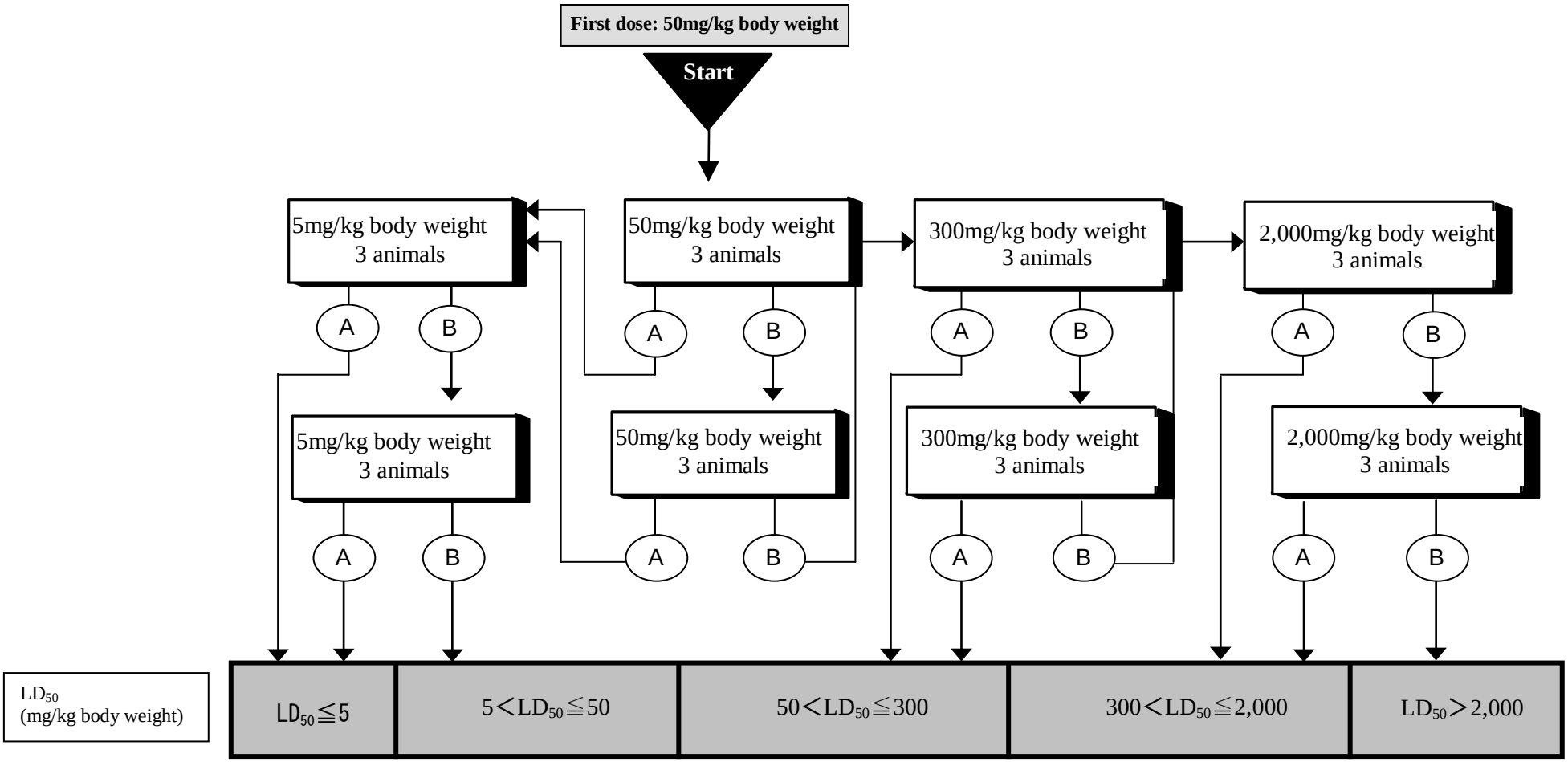
C No toxicity

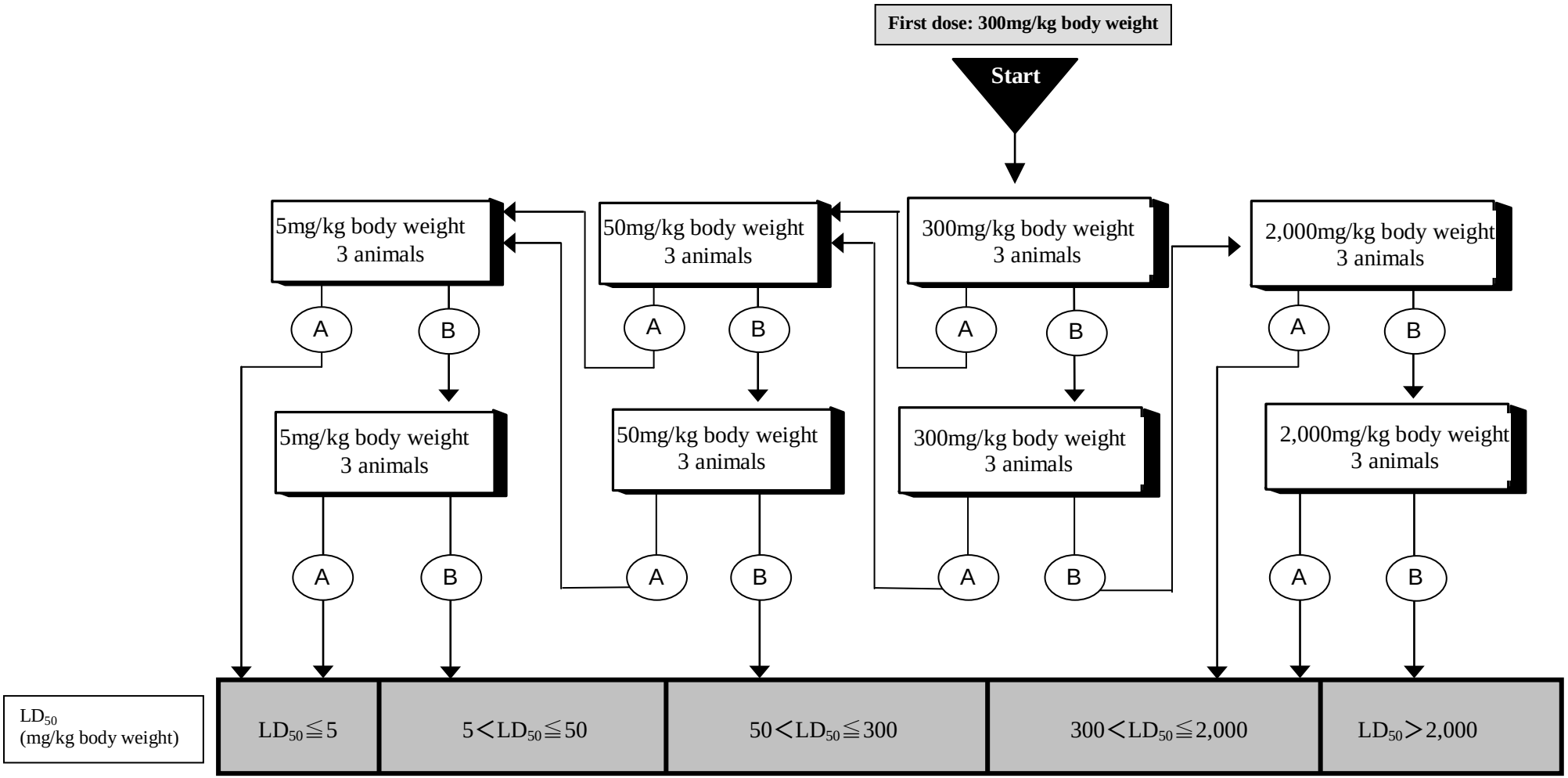
・Four animals are to be used for the major test so that the total becomes five, adding the one used in the estimation test. The results are to be evaluated for the five animals including the one used in the estimation test.

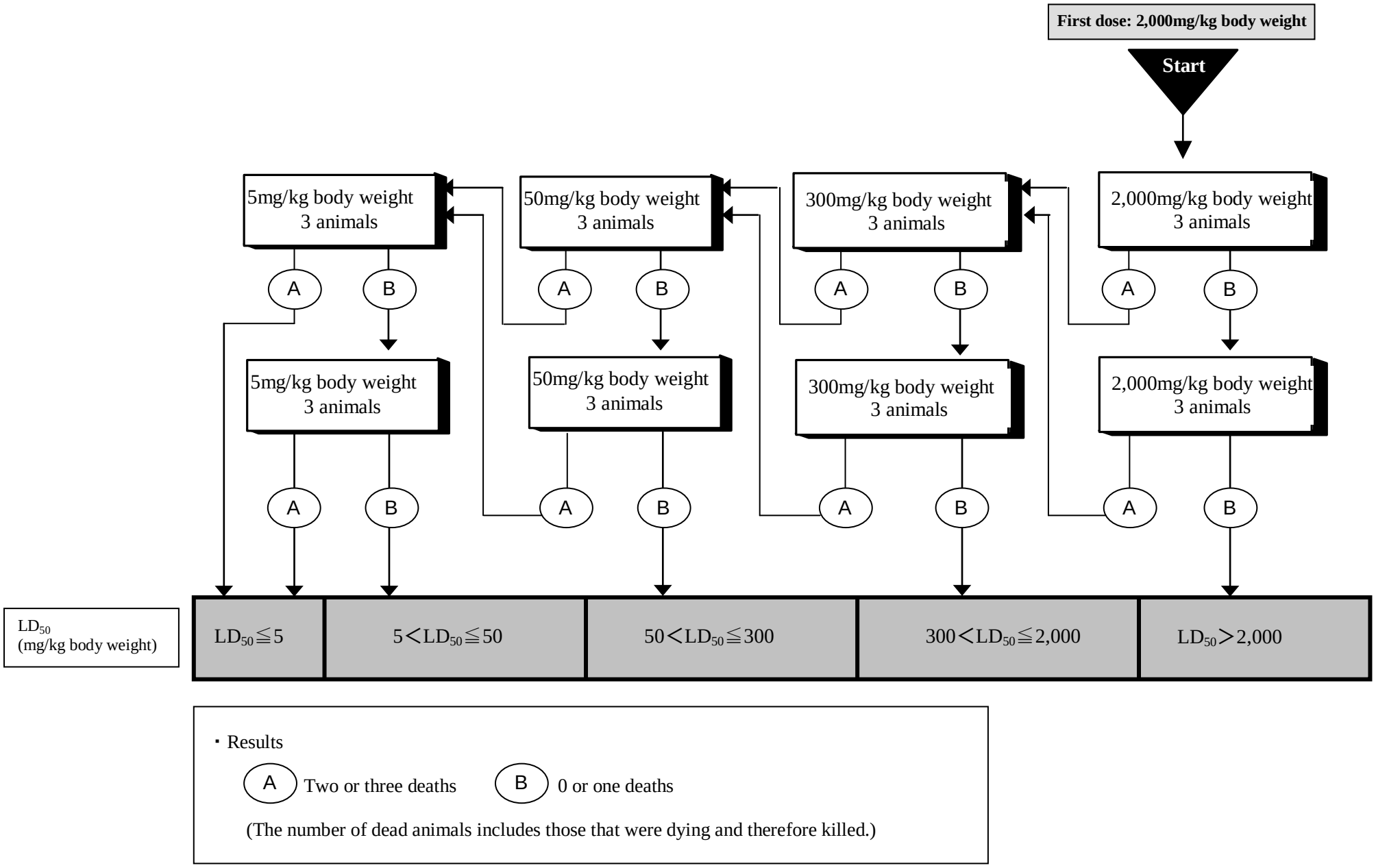
・For doses that caused death(s) in the estimation test, the major test is to be omitted, regarding that two or more animals died in the major test.

Appendix 3: Procedures for Toxic Class Method




First dose: 50mg/kg body weight
Start
5mg/kg body weight
3 animals
50mg/kg body weight
3 animals
300mg/kg body weight
3 animals
2,000mg/kg body weight
3 animals
A
B
5mg/kg body weight
3 animals
50mg/kg body weight
3 animals
300mg/kg body weight
3 animals
2,000mg/kg body weight
3 animals
LD50
(mg/kg body weight)
LD50≦5
5＜LD50≦50
50＜LD50≦300
300＜LD50≦2,000
LD50＞2,000


First dose: 300mg/kg body weight
Start
5mg/kg body weight
3 animals
50mg/kg body weight
3 animals
300mg/kg body weight
3 animals
2,000mg/kg body weight
3 animals
A
B
5mg/kg body weight
3 animals
50mg/kg body weight
3 animals
300mg/kg body weight
3 animals
2,000mg/kg body weight
3 animals
LD50
(mg/kg body weight)
LD50≦5
5＜LD50≦50
50＜LD50≦300
300＜LD50≦2,000
LD50＞2,000


First dose: 2,000mg/kg body weight
Start
5mg/kg body weight
3 animals
50mg/kg body weight
3 animals
300mg/kg body weight
3 animals
2,000mg/kg body weight
3 animals
A
B
5mg/kg body weight
3 animals
50mg/kg body weight
3 animals
300mg/kg body weight
3 animals
2,000mg/kg body weight
3 animals
LD50
(mg/kg body weight)
LD50≦5
5＜LD50≦50
50＜LD50≦300
300＜LD50≦2,000
LD50＞2,000
・Results
A Two or three deaths
B 0 or one deaths
(The number of dead animals includes those that were dying and therefore killed.)