Handbook of Feed Additive Designation

(Edition of Chemical Substances)

May 2016

Animal Products Safety Division

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Supplements

I Purpose of the Handbook

1. Introduction

Animal feed must be safe to humans, in terms of the safety of the livestock products made from the livestock that consumes the feed, not to mention the safety to the livestock themselves that consume the feed. For the same reason, feed additives, which must serve purposes such as preventing the quality deterioration of feed as a matter of course, must be safe to humans as well as to livestock.

The feed additive designation is granted by the Minister of Agriculture, Forestry and Fisheries through consultations with the Agricultural Materials Council, under the policy of a substantial necessity, only to those substances that are in high-demand and have been proved to be apparently effective and safe. The designation is not a matter of an approval issued in response to an application.
In other words, any substances that fall into the following categories cannot be designated as feed additives: substances having no apparent efficacy as a feed additive; substances lacking safety proofs; and substances with poor levels of demand.

When the production or distribution, etc., of a non-designated feed additive is planned, the documents required for the deliberation by the council will have to be prepared and submitted to the Animal Products Safety Division, Food Safety and Consumer Affairs Bureau of the Ministry of Agriculture, Forestry and Fisheries (hereinafter referred to as “the authorities”). Those submissions which are judged worthy of a hearing by the council are then sent to the council for deliberations.
The Agricultural Materials Council will conduct deliberations regarding the substance’s efficacy as a feed additive and its impact on livestock and humans. When preparing the documents, it should be borne in mind that the abstract that summarizes the test results of the efficacy of the substance as a feed additive and its safety, etc., is a particularly important document for the purposes of the deliberation. There may be difficulties in preparing the documents that meet the requirements of the deliberation and in offering an explanation in front of the deliberation council because these documents contain technical subjects, such as scientific test results and manufacturing process of the substances, and so on.

Based on the above consideration, this handbook explains the procedures and points of caution in preparing the required documents (in both abstract and specific terms) for the deliberations on a feed additive by the Agricultural Materials Council in reference to the process for a feed additive designation. However, note that this handbook is written for a supposed typical case of the feed additive designation of a chemical substance, and that the required documents for seeking a designation of live microbial agents or antibiotics, etc., are quite different from those explained herein. Please additionally note that following the procedures described in this handbook for the preparation of the required documents does not necessarily ensure success in the granting of a designation.

2. About Feed Additives

Definition of a Feed Additive

A feed additive is a substance that is used with animal feeds in the form of an addition, mixture, infiltration, etc. under the “Act Concerning the Safety Assurance and Quality Improvement of Feed” (hereinafter referred to as the “Feed Safety Act”) for preventing the feed quality deterioration and for other purposes specified in the ordinances of the Agriculture, Forestry and Fisheries Ministry, and as designated by the Minister for Agriculture, Forestry and Fisheries through consultations with the Agricultural Materials Council.
(Legal basis: Article 2, Paragraph 3 of the Feed Safety Act)

Intended Use of Feed Additives

The intended use of feed additives is limited to the following three uses outlined in the Ordinance for Enforcement of the Feed Safety Act. Any substances intended for other uses than these three are not designated as feed additives:

1) Preventing feed quality deterioration
2) Supplementing feeds with nutrients and other active ingredients
3) Facilitating the effective utilization of the nutrient ingredients of the feed

(Legal basis: Article 2, Paragraph 3 of the Feed Safety Act, Article 1 of the Ministerial Ordinance for Enforcement of the Act Concerning the Safety Assurance and Quality Improvement of Feed)

A feed additive designation is granted, within the limits of the minimum necessity, to those substances that are in high-demand and have proved to be apparently effective and safe, as has been conventionally exercised. Therefore, those who are planning the manufacturing, importation, etc., of a non-designated feed additive as a feed additive need to consult with the authorities in full, well before any actions are taken, and receive instructions from them.

Relevant laws, ordinances and others

- Law: the Act Concerning the Safety Assurance and Quality Improvement of Feed (Law No. 35 issued on April 11, 1953)
- Cabinet Order: the Order for Enforcement of the Act Concerning the Safety Assurance and Quality Improvement of Feed (Cabinet Order No. 198 issued on July 16, 1976)
- Ordinance of the Ministry: the Ministerial Ordinance for Enforcement of the Act Concerning Safety Assurance and Quality Improvement of Feed (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 36 issued on July 24, 1976), and the Ministerial Ordinance on the Specifications and Standards of Feed and

Feed Additives (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 35 issued on July 24, 1976)

- Public Announcement: the Ministerial Public Announcement Regarding the Designation of Feed Additives (Ministry of Agriculture, Forestry and Fisheries Public Announcement No. 750 issued on July 24, 1976)
- Notification : the Notification of the Establishment of the Standard for the Evaluation of Feed Additives (Ministry of Agriculture, Forestry and Fisheries, Food Safety and Consumer Affairs Bureau Director-General Notification No. 4-Chiku-A-201 issued on March 16, 1992), and the Notification Concerning the Documents, etc., Required for the Feed Additive Designation (Ministry of Agriculture, Forestry and Fisheries, Division Notification No. 54-Chiku-A-5002, 54-Suishin-3381 issued on February 4, 1980)

Note that substances that have been designated as a food additive cannot be used as feed additives unless such a use has been designated by the Minister of Agriculture, Forestry and Fisheries in accordance with Article 2, Paragraph 3 of the Feed Safety Act and Article 1 of the Ordinance for Enforcement of the Feed Safety Act (refer to the above listed laws, etc.).

[Reference 1] Glossary

Explanations of the technical terminologies are provided as follows.

Source: "Glossary concerning Food Safety" posted on the website of the Food Safety Commission (http://www.fsc.go.jp)
"Guideline for Total Diet Studies" posted on the website of the Ministry of Agriculture, Forestry and Fisheries (http://www.maff.go.jp)

- **Safety factor**

  A factor applied to the no-observed-adverse-effect-level (NOAEL), for the determination of the Acceptable Daily Intake (ADI), etc., of a substance from the perspective of further safety considerations. The ADI is given as a quotient of the NOAEL/Safety factor. A safety factor is determined in consideration of the species differences between animals and humans, and the differences in individual humans, and is given as a product of the species difference and the individual difference. Typically, a factor of 10 times for the species difference and the same 10 times for the individual difference is applied; thereby, the product of those values (100) is used as the safety factor.

- **Acceptable daily intake (ADI)**

The estimated amount of a substance per day that a human can take every day during her/his life span without incurring any supposed adverse health impacts

- **General toxicity**
  Toxicity which can be assessed by general test methods (blood tests, histopathological tests, etc.) in acute toxicity tests and chronic toxicity tests

- ***In vivo* test**
  A test conducted in living organisms, typically meaning animal tests.

- ***In vitro* test**
  A test conducted in test tubes

- **Ames test**
  A test using salmonella to assess the incidence of gene mutations caused by a substance

- **Acute toxicity**
  Toxicity that emerges in a short period of time (from the same day, to within about two weeks) after a single dose of a substance or multiple doses have been administered in a short period

- **Acute toxicity test**
  A test to assess the signs of acute toxicity by administering a substance to animals

- **Limit of detection (LOD)**
  The minimum detectable concentration of a substance in a method used for the analysis of the substance. There are several definitions of the LOD, including the following:
  i. AOAC (Association of Official Analytical Chemists) International: a concentration equivalent to the sum of the mean blank value and 3σ (wherein σ is a standard deviation of the distribution of a blank measurement)
  ii. IUPAC (International Union of Pure and Applied Chemistry): a concentration equivalent to the sum of the mean blank value and kσ (wherein k is a factor determined based on the reliability requirement, and σ is a standard deviation of the distribution of a blank measurement)
  It must be clearly expressed in the test report which definition is being used for the calculation. Note that the formula “LOD = LOQ” is scientifically false.

- **Maximum residue limit (MRL)**

The maximum permissible concentration of agricultural chemicals, feed additives, etc., remaining in food.

- **Good Laboratory Practice (GLP)**
  A set of principles and rules to be complied with in conducting tests concerning the safety and persistence of feed additives, formulated to achieve a higher reliability of the documents, and a more accurate and strict performance of the safety assessment. (For details, please refer to the Reference 6 “Conditions to be Complied with in Conducting Animal Tests” on page 28).

- **Micronucleus test**
  One form of mutagenicity tests, to assess the chromosome aberrations by examining the emergence of a micronucleus (cellular fragment)

- ***In vivo* kinetics test**
  A test to analyze the kinetics of the substance in the body of the animals (its absorption, distribution, metabolism, excretion, etc.) by administering the substance to animals

- **Transgenerational reproductive test (Transgenerational reproductive toxicity test)**
  A test to assess the reproductive toxicity of a substance by administering it to animals

- **Single dose toxicity test**
  A test that administers only a single dose of a substance to animals

- **Limit of quantitation (LOQ)**
  The minimum concentration of a substance that can be quantitated by the method used for the analysis of the substance with an appropriate accuracy and precision. There are several definitions of the LOQ, including the following:
  i. AOAC International: a concentration equivalent to the sum of the mean blank value and 10σ (wherein σ is a standard deviation of the distribution of a blank measurement)
  ii. Codex (a definition in the criteria approach): a concentration equivalent to the sum of the mean blank value and 6σ, or the sum of the mean blank value and 10σ (wherein σ is a standard deviation of the distribution of a blank measurement)

- **Spike recovery test**

A test to spike a predetermined amount of an objected substance to analyze and examine whether the spiked amount can be accurately quantitated

- **Special toxicity**
  Toxicity which is assessed by a special administration method (inhalation, transdermal delivery, etc.) and with special observation items (mutagenicity, carcinogenicity, etc.)

- **Carcinogenicity**
  Toxicity that causes cancers inside living organisms and can facilitate cancer progression by the intake of a substance

- **Developmental toxicity (Teratogenicity)**
  Toxicity that causes an impact on a fetus by way of the mother's ingestion of the substance

- **Developmental toxicity test (Teratogenicity test)**
  A test to assess the developmental toxicity (teratogenicity) of a substance by administering the substance to animals. The doses are administered during the organogenesis stage.

- **Reproductive toxicity (Reproduction toxicity)**
  Toxicity that causes damage to the reproductive potential, embryo and fetus

- **Lethal dose 50 (LD_{50})**
  An index of acute toxicity, where the amount of the substance is estimated to kill statistically 50% of the animals exposed to the substance

- **Repeated dose toxicity test**
  A test that administers repeated doses to animals

- **Repeatability**
  Errors between the values of measurements, each of which is obtained from the measurement of the samples which are identified as the same, conducted by the same method and by the same experimenter, and under conditions that are controlled to produce independent results within a short period of time

- **Mutagenicity (Genotoxicity)**

Toxicity that affects genes and chromosomes and causes mutations and damage to genes and chromosomal aberrations. These aberrations can result in carcinogenicity.

- **Mutagenicity test (Genotoxicity test)**
  A test to assess the mutations and damage to genes and chromosomal aberrations

- **Positive list system**
  A system to ban the sale, etc., of a food in principle, in which the residue of feed additives (and other agricultural chemicals, veterinary medicinal products, etc.) are found to be in excess of the predetermined level (0.01 ppm). As to a substance for which the standard of a food ingredient is prescribed, the criteria specified in that standard will apply. Otherwise, the value of 0.01 ppm (a concentration causing no potential harm to human health) is applied as the regulatory value for any substances which are out of the scope of any specific standards.

- **Chronic toxicity**
  Toxicity that is caused by the continuous or repeated administration of a substance over an extended period of time (typically 6 months or more)

- **Chronic toxicity test**
  Test to assess the signs of chronic toxicity by the administration of a substance to animals

- **No-observed-effect-level (NOEL)**
  The maximum dose of a substance at which no biological impact is found

- **No-observed-adverse-effect-level (NOAEL)**
  The maximum dose of a substance at which no adverse impact is found

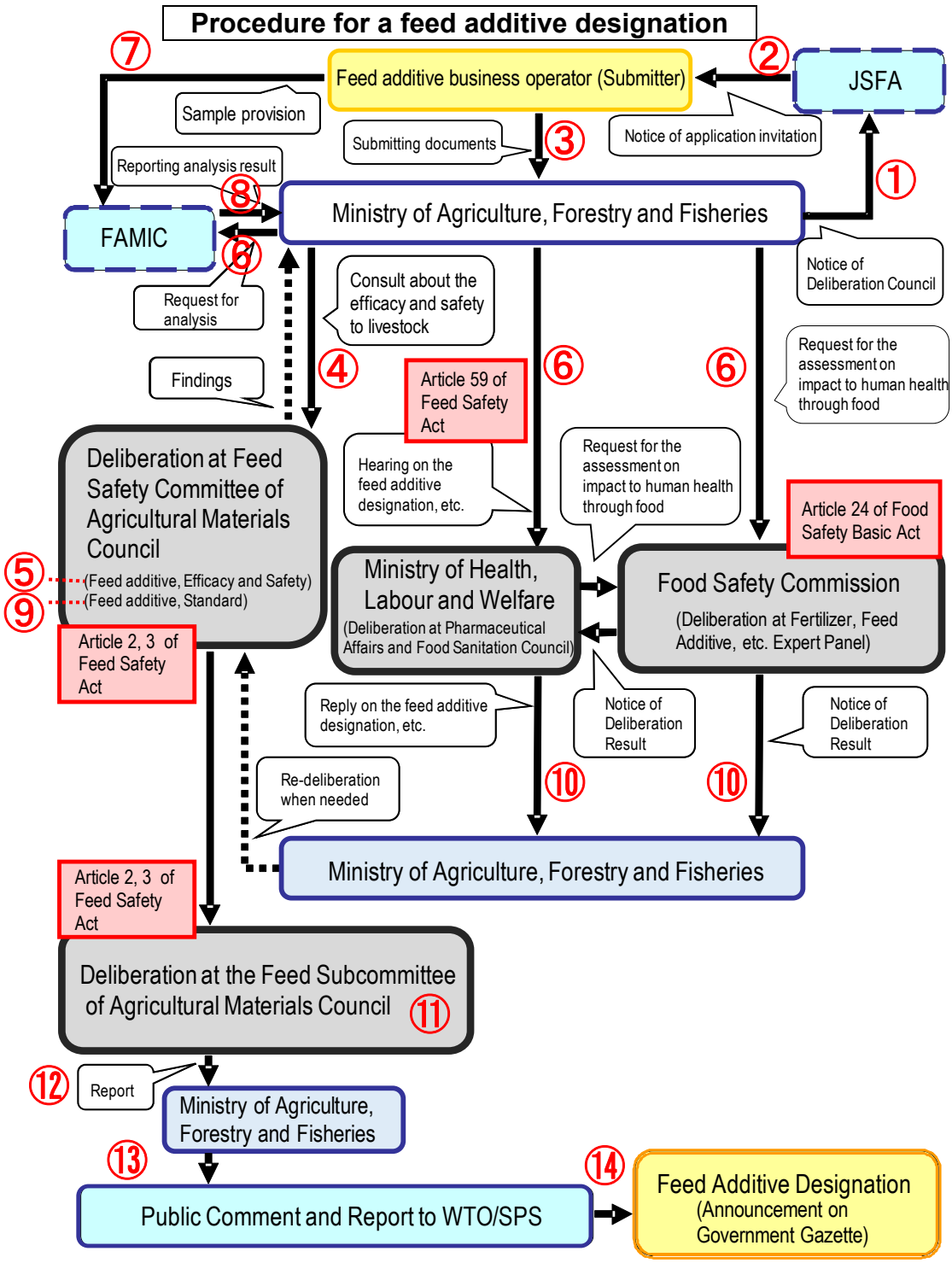
- **Pharmacological test**
  A test to clarify the actions of a substance that has been administered to living organisms

- **Lot**
  A set of foods which are regarded as having been produced under the same conditions based on the characteristics of the place of origin, place of production, variety, packer, packing form, label, consignee, etc., within a group of traded goods in a single delivery

II Basic Ideas in the Preparation of the Required Documents

1. Flow of the Procedure for a Feed Additive Designation

The flow of the procedure for a new feed additive designation is illustrated below. The designation is made through the deliberations by the Agricultural Materials Council (Ministry of Agriculture, Forestry and Fisheries), the Ministry of Health, Labour and Welfare and the Food Safety Commission.



A submitter first needs to prepare the documents required for the deliberations by the Agricultural Materials Council, etc. Refer to the Section “II-2 Outline of the Required Documents” on page 15 and the following for details of the required documents. The flow of the procedure from the preparation of the required documents to the deliberation council is as follow:

〈Preliminary consultation〉

It is essential for a business operator who is planning to start manufacturing or importing any non-designated feed additives to consult with the authorities thoroughly beforehand. To receive a consultation, a document that outlines the objected substance, including descriptions of what livestock are involved and which of the three effects provided for in the Feed Safety Act is expected, shall be prepared, at a minimum.

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〈Preparation of the required documents〉

While the consultation with the authorities is available anytime, any substances other than those whose applications have been received within the application acceptance period for the deliberation council won’t be subjected to the discussions concerning the application’s eligibility for a deliberation council.

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〈Application acceptance period for the deliberation council〉 The application shall be submitted to the authorities

The authorities will invite the application for a deliberate council through the Japan Scientific Feed Association (JSFA). Please make sure that all required documents have been properly prepared before submitting the application to the authorities. Note that the deliberation will not be conducted for the following cases:

- The required documents have deficiencies
- An application contains many subjects pertaining to matters of re-deliberation from previous deliberations.
- The objected substance does not fulfill the requirements for a feed additive designation, etc.

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When the authorities decide to deliberate in the deliberation council

〈Period between the acceptance of the application and before the deliberation council〉

The submitted documents shall be scrutinized. The copies of the documents for the deliberation council (about 20 sets) will have to be prepared before the deliberation.

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〈Deliberation council at the Feed Safety Committee of the Agricultural Materials

Commission (efficacy and safety)〉

Explanation (within 10 minutes), and questions and answers (about 10 minutes) in the deliberation council

With regard to a new substance, (i) the efficacy and safety of the feed additive will be deliberated by the Feed Safety Committee of the Agricultural Materials Commission.*

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〈Deliberation Council of the Feed Safety Committee of the Agricultural Materials Commission (standards)〉

After the efficacy and safety of the objected substance have been endorsed by the Feed Safety Committee, (ii) the standards of the feed additive will be deliberated in the deliberation council of the same committee at a later date.*

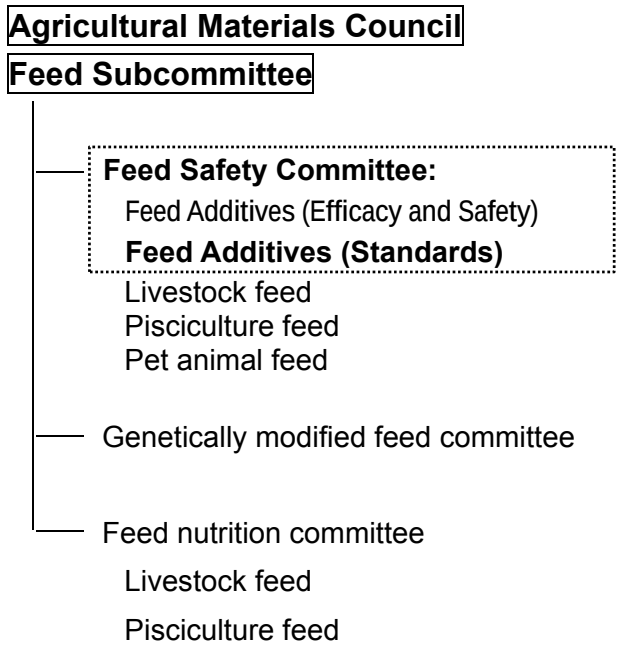
*The submitter will have to prepare responses to the findings of the council members if questions are raised about the contents of the documents in the deliberation council, and the subject matter will be included in the continuous deliberation. When the responses are ready, the deliberation will be conducted again.

After the endorsement of the substance by the Feed Safety Committee, discussions on the residue limit in foods and human dietary risk assessments will be conducted by the Ministry of Health, Labour and Welfare (Pharmaceutical Affairs and Food Sanitation Council) and the Food Safety Commission (Fertilizer, Feed, etc., Expert Panel). The Ministry of Health, Labour and Welfare and the Food Safety Commission will also refer to the documents prepared by the submitter in their deliberations. Although the submitted documents won't be made public as they are, the submitter is asked to black out any portions containing confidential information beforehand when the assessment reports are published, because the assessment reports, etc. drawn up by the deliberation council, etc., may contain information that is relevant to patents and the submitter's secrets.

Ministry of Agriculture, Forestry and Fisheries (Agricultural Materials Council)

When the Minister of Agriculture, Forestry and Fisheries makes a decision on the feed additive designation and thereby makes a change in the specifications and standards, etc., in accordance with the Feed Safety Act, the opinions of the Agricultural Materials Council shall be heard. After the deliberations of the Feed Safety Committee of the Agricultural Materials Council (Feed Additives: Efficacy and Safety) and (Feed Additives: Standards), the feed additive documents will be sent to the discussion at the Feed Subcommittee.

The deliberations of the council will be conducted based on the submitted documents prepared by the submitter. The council members will discuss in a scientific manner whether the efficacy and safety of the objected substance are assured in their practical use as a feed additive, and to what extent such efficacy and safety is assured, etc. With regard to the standards, the adequacy of the quantitative methods, etc., that have been employed will be discussed.



Based on the submitted documents, the authorities will scrutinize whether the objected substance is eligible for deliberations in light of the evaluation standard of the feed additive and whether the contents of the submitted documents have fulfilled the requirements.

Furthermore, if the objected substance is a substance (enzyme, etc.) made from recombinant microorganisms (bacteria, etc.), it must be subjected to further deliberations regarding genetically modified feed additives (at a genetically modified feed committee) in addition to the normal deliberations (on the efficacy and safety, standards, etc.).

Feed Subcommittee

This subcommittee will consolidate the deliberation results of the Feed Safety Committee (Feed Additives: Efficacy and Safety, as well as Standards), will hold a hearing in public and will issue a report.

Feed Safety Committee: Feed Additives (Efficacy and Safety)

This committee will conduct closed-door deliberations regarding the efficacy and safety to livestock of the feed additive.

Feed Safety Committee: Feed Additives (Standards)

This committee will conduct closed-door deliberations about the adequate specifications and standards for the feed additive based on the deliberation results of the Feed Safety Committee (Feed Additives: Efficacy and Safety).

Ministry of Health, Labour and Welfare, Pharmaceutical Affairs and Food Sanitation Council

The Ministry of Health, Labour and Welfare sets the residue limits and the standards for foods, etc., and oversees these in accordance with the Food Sanitation Act.

The Ministry conducts deliberations about the maximum residue limit (MRL) of the feed additive in foods as part of the Pharmaceutical Affairs and Food Sanitation Council, which is an advisory body to the Ministry of Health, Labour and Welfare Food Safety, based on the results of the dietary risk assessment (ADI) provided by the Food Safety Commission. When necessary, the Ministry may set the MRL of the feed additive in foods.

Food Safety Commission (Fertilizers, Feed, etc., Expert Panel)

The Food Safety Commission was established on July 1, 2003 in accordance with the Food Safety Basic Act. It conducts risk assessments regarding the impact of hazardous elements, such as additives and agricultural chemicals, etc., which may be contained in foods, on human health based on the scientific knowledge and from an objective, neutral and fair stand point (based on dietary risk assessment: settings of the ADI, etc.).

Particularly, subjects such as what intake amount of a hazardous element can cause a serious adverse impact on human health and to what degree of probability this will occur, are scientifically assessed. The dietary risk assessment is a requisite for the designation of new feed additives, as well as changes of standards for labeling and manufacturing, etc., and changes in the standards of purity, etc.

Incorporated Administrative Agency, the Food and Agricultural Materials Inspection Center (FAMIC)

The FAMIC assures the safety of consumers and foods, as well as fertilizers and feeds, registers agricultural chemicals and oversees their safety, and oversees the labeling of food products. In terms of the feed additive designation, the FAMIC examines the methods of analysis based on the submitted documents. More specifically, it determines whether the methods of analysis have been adequately described in the items relating to the standards of the feed additive. In addition, it may conduct an inspection of the facility to determine whether it complies with the principles of Good Laboratory Practice (GLP) on an as needed basis. (Refer to Reference 6 “Conditions to be Complied with in Conducting Animal Tests” on page 29 for information about GLP inspections.)

General Incorporated Association, the Japan Scientific Feed Association (JSFA)

The JSFA is an entity which conducts experiments and research for the manufacturing

and supply of “safe and high quality feed” through the rational and economical utilization of scientific feeds, and by doing so it aims to promote the development of technologies and the broader use of such technologies. It is organized by the members of companies, etc., which manufacture feeds and feed additives.
The announcement of the invitation of an application by the deliberation council is made by the authorities through the JSFA.

2. Outline of the Required Documents

(1) The test items required for a feed additive designation are specified in the "Standard for the Evaluation of Feed Additives."[*1] In addition, the proof of an appropriate test performance is required from the facility used for the toxicity tests and residue tests.*[2]

(2) Once the test results are obtained, an abstract will be drawn up by filling out the designated forms with the summary of the test results.[*3] The following documents shall be submitted:

i. Abstract
ii. Original papers included in the abstract
iii. Checklist*

Note that if any findings are pointed out by the council members in the deliberation, the responses to the findings shall be presented in the following deliberation. The required documents for the following deliberation are as follows:

i. Summary of the response paper to the findings
ii. Original papers included in the response paper to the findings
iii. Revised abstract based on the findings
iv. Original papers included in the abstract
v. Checklist*

Note 1: Notification of the Establishment of the Standard for the Evaluation of Feed Additives (Ministry of Agriculture, Forestry and Fisheries, Fisheries Agency Director-General Notice No. 4-Chiku-A-201 issued on March 16, 1992)
http://www.famic.go.jp/ffis/feed/tuti/4_201.html

Note 2: Notification of the Standard Concerning the Performance of Animal Tests for Feed Additive Assessments (Ministry of Agriculture, Forestry and Fisheries, Fisheries Agency Director-General Notification No. 63-Chiku-A-3039 issued on July 29, 1988)
http://www.famic.go.jp/ffis/feed/tuti/1_3039.html

Note 3: Notification Concerning the Documents, etc., Required for a Feed Additive Designation (Ministry of Agriculture, Forestry and Fisheries, Division Notification No. 54-Chiku-A-5002)
http://www.famic.go.jp/ffis/feed/tuti/54_a5002.html

In order to facilitate the smooth deliberation of the council, the authorities will check the submitted documents before holding the deliberation council, and will confirm whether the items are described in conformity with the Standard for the Evaluation of Feed Additives and are objectively described based on the test results. Insufficient descriptions in the submitted documents will cause an extended time for the document checks by the authorities. In addition, the submitters may be requested to revise the documents. Please be advised that such circumstances may result in a delay of the deliberation by the council and of the feed additive designation.

*Although the submission of the checklist attached at the end of this handbook (VI Checklist for the Submission of the Required Documents) on page 76 and following is not mandated by the notification or an equivalent, the submitters are kindly asked to submit this checklist in order to shorten the time for the document checks by the authorities.

In the following chapter, the procedures for the preparation of the required documents

(especially the abstract) are explained with reference to examples.

III Items to be Described in the Required Document (Abstract)

This chapter provides concrete explanations about the items to be described in the abstract, with reference to the “Notification Concerning the “Documents, etc., Required for the Feed Additive Designation” and the “Notification of the Establishment of the Standard for the Evaluation of Feed Additives”. In this handbook, a substance for which the feed additive designation is being sought is referred to as an “objected substance” for purposes of convenience. Before entering this chapter, please be advised that the “Requirements” only summarize the main items to be described, and the “Example” merely represents one of many examples.

1. Origin or Background of the Discovery, status of Authorization and Use as a Feed Additive in Foreign Countries, etc.
2. Items Concerning Standards
   - (1) Names
     - i. General name
     - ii. Chemical name
     - iii. Trade name
   - (2) Chemical structure
   - (3) Manufacturing process
   - (4) Biological and physicochemical properties
     - i. Physical and chemical properties
     - ii. Identification test
     - iii. Purity test
     - iv. Content and quantitative method
   - (5) Quantitation in feed
   - (6) Changes with time
     - i. Ambient temperature storage test
     - ii. Heat resistance test
     - iii. Humidity resistance test
     - iv. Light resistance test
     - v. Accelerated test
     - vi. In-feed stability test
3. Items Concerning Efficacy
   - (1) Basic tests to prove efficacy
     - i. *In vitro* test
     - ii. *In vivo* test
   - (2) Field application tests to prove efficacy
4. Items Concerning Residue
   Residue tests in targeted animals, etc.
5. Items Concerning Safety
   - (1) Toxicity tests
     - i. General toxicity tests
       - a. Single dose toxicity test
       - b. Repeated dose toxicity test (Short term)
       - c. Repeated dose toxicity test (Long term)
     - ii. Special toxicity tests
       - a. Transgenerational reproductive test
       - b. Developmental toxicity test
       - c. Carcinogenicity test
       - d. Mutagenicity test
       - e. Other tests
     - iii. Pharmacological tests
     - iv. In vivo kinetics tests
   - (2) Feeding tests using targeted animals, etc.
   - (3) Tests concerning the emergence of resistant bacteria
   - (4) Other tests

[Reference 2] Considerations for descriptions of the test data (omission of tests, use of new knowledge, etc.)

The notification of the “Establishment of the Standard for the Evaluation of Feed Additives” states the following: items concerning the safety of a substance that is designated as a food additive, or is widely used in foods, can be omitted; also, some of the toxicity tests can be omitted if the conditions are met. However, the reasons and adequacy of an omission must to be presented. Please bear in mind that reports and literature to support the reasons and adequacy must be submitted to explain the adequacy of the omission

In addition, with regard to substances already used as food additives or foods, if new knowledge about the substance (such as toxicity data for a newly found metabolite)

have been found at the time of seeking the feed additive designation, which was not known at the time of the substance's designation as a food additive, the data concerning the safety must still be described. Therefore, please always try to collect the latest available knowledge.

1. Origin or Background of the Discovery, Status of Authorization and Use as a Feed Additive in Foreign Countries, etc.

[Requirements]

○ Origin or background of the discovery: Describe the origin or background of the discovery of the objected substance that has resulted in the idea to use it as a feed additive. Describe what advantages it will bring about and how the current conditions will improve when the feed additive designation is granted to the objected substance. Also, write about the following things: (1) Which of the three effects provided for in the Feed Safety Act is achieved; (2) Which livestock animals are targeted and at which administration stage (period of the dose); (3) What is the recommended dose (method of use).

○ Status of authorization and use as a feed additive in foreign countries, etc. : If the objected substance is used as a feed additive in Europe and America, etc., describe the reasons for its authorization (targeted animals, doses, etc.). (see the [Example] below)

○ Status of the manufacturing and distribution authorization, and its importation as a veterinary medicinal product : If the objected substance is used for any purpose, including but not limited to its use as a veterinary medicinal product, describe the substance's intended use, method of use and dose, etc.

○ Comparison to related substances (generic substances having the same effect) : Write about the substances that are already designated as a feed additive and have similar attributes as the objected substance, such as having the same structure, same effect, etc. (see the [Example] below)

[Example]

The status of the substance as a feed additive in foreign countries is summarized in the table below.

Table 1 Status of the substance's designation in foreign countries, etc.

Country	Status of studies and authorization	Target livestock animals
United States of America	Designated in xxxx	Pigs and cattle
EU	Pending (submitted in xxxx)	Pigs (planned)
(omitted)		

[Example]

Related substance:

Weigh out 0.01 g (0.005–0.014g) of the substance and dissolve it in 1.0 mL of methanol to make a solution. Measure 10μL of the solution and spot it onto a thin layer of a chromatography silica gel plate. Then, spread out the spots into a span of about 10 cm using the mixture of ether and chloroform (50:50) as a spreading solvent, and dry the plate in the air. … (omitted)…

[Reference 3] Case of a Discussed/Authorized Substance in Foreign Countries as an Additive

The documents used in the assessment for the substance's authorization as an additive in foreign countries may substitute for the documents required for the

deliberation of its designation in Japan, depending on the test items. When using these documents, submitters are often requested to also provide information about the status of the discussion/authorization (date of the designation, standards, etc.) in foreign countries, as well as the status of the standard setting and assessment at international institutes, such as the Codex. Therefore, review such information thoroughly and be prepared for such requests. Note that because the deliberation is conducted according to the Standard for the Evaluation of Feed Additives of Japan, the documents used for the substance's designation in foreign countries may not suffice.

2. Items Concerning Standards

To ensure the safety of livestock and humans, any hazardous impurities which can be contained in feed additives and the purity of the product must be specified.
The specifications and standards specified in the Ministerial Ordinance on the Specifications and Standards of Feeds and Feed Additives shall be described in line with the writing style and definition of the terminology set out in the Appendix Table 2-1 “General rules for feed additives” attached to the above-mentioned ordinance. Therefore, please draw up the required documents according to the rules set out in the general rules. ([Reference 8] Style Reference for the Description of Items Concerning Standards, on page 53 and following.)

(1) Name

i. General name

Describe the general name as it is used in the feed additive listing in the Ministry ordinance.

ii. Chemical name

The IUPAC name shall be described.

iii. Trade name

The name in foreign countries shall be described, if the substance is sold overseas, etc.

(2) Chemical structure

The chemical formula shall be described for an organic compound. (If a substance has a chemical structure which cannot be expressed, such as an enzyme, it is allowable to omit the description of the formula with a note.) In addition, the molecular formula shall be described. For the calculation of the molecular weight, the Table of Standard Atomic Weights 2007 shall be referred to.

(3) Manufacturing process

The manufacturing process of the ingredients used for the manufacturing and formulations shall be described in enough detail including the following items, so that the detailed manufacturing process can be replicated: test laboratory, place where the test was conducted, manager responsible for the test, test method, lot number and manufactured date. A detailed flowchart of the manufacturing process shall also be attached so that the production of by-products as well as the purity of the ingredients for manufacturing, etc. can be understood. The use of a recombinant technique or production bacteria in the manufacturing process shall be noted with detailed descriptions.

[Example]
Oxidize Substance A (99.5%) with air using a platinum catalyst to produce Substance B. Initiate hydrolysis by adding sodium hydroxide in the special grade ethanol to produce unrefined xxxx. Refine this through a solvent extraction using hexane, then dehydrate to yield the objected substance xxxx.

Manufacturing Process Flowchart

Substance A (99.5%)
↓ platinum catalyst
Substance B
↓ sodium hydroxide
↓(special grade ethanol)
xxxx(unrefined), (by-product C)
↓ solvent extraction by hexane
objected substance xxxx

[Reference 4] Considerations for the Description of the Manufacturing Process
Information about the by-products derived from the ingredient for manufacturing, or produced by mixing with diluents, is essential because the ingestion of certain by-products produced in the manufacturing process may affect the health of the livestock.

(4) Biological and physicochemical properties

i. Physical and chemical properties

Information about the objected substance shall be described.
Describe the appearance and physical and chemical properties of the objected substance. ・ Appearance of the objected substance itself (color; state: powder, liquid, etc.) ・ Solubility in a solvent (degree of dissolution in an organic solvent or water) ・ Information about the denaturation of the objected substance (deliquesce, melting point, photo-degradation, etc.)

[Example]
Physical and chemical properties
a. Appearance: colorless or white crystal, or white crystalline powder
b. Chemical properties: freely soluble in water, soluble in methanol, or practically insoluble in hexane

… (omitted) …

ii. Identification test

A test to identify the objected substance in the analyte by conducting tests based on its specific properties.
For a new objected substance, the possibility of employing previously prescribed methods (methods described in the “List of the Specifications and Standards of Feed Additives”) shall be discussed.

[Example]
Identification test
This substance shows an IR absorption peak at near 1,750 cm^{-1} of a wavenumber in the infrared absorption spectrum by the potassium bromide tablet method of analysis. The aqueous solution of this substance (1→10) shows a quantitative reaction with xx salt.
… (omitted) …

iii. Purity test

A test to detect impurities contained in the analyte other than the objected substance
For the test to detect other items (lead, arsenic, etc.) for which standards have been specified in relation to other substances, the possibility of employing previously prescribed methods shall be discussed.

[Example]
Purity test
a. Clarity and color of a solution: An aqueous solution prepared with 1.0 g (0.95–1.04 g) of this substance dissolved in 20 mL of water shows a light tan color and is practically

clear.

b. Chloride limit: When conducting a chloride limit test with 1.0 g (0.95–1.04 g) of this substance, the turbidity of the test solution should not exceed that of the control solution prepared with 0.5 mL of 0.01 mol/L hydrochloric acid.

… (omitted) …

iv. Content and quantitative method

A method to quantitate the objected substance contained in the analyte

[Example]
Content: This substance contains 99.5% or more of the objected substance (describe the chemical formula of the objected substance) in the quantitation after drying.
Quantitative method: Dry the substance, weigh out 0.5 g of the material, dry down to 0.001 g and record the reading. Dissolve the weighed substance in 50 mL of water, and add 5 mL of formalin and titrate with 0.5 mol/L sodium hydroxide solution (use three drops of the phenolphthalein indicator). Perform a blank test with the same method for correction.
1 mL of 0.5 mol/L sodium hydroxide solution = equivalent weight in mg, chemical formula of the objected substance

… (omitted) …

(5) Quantitation in feeds

The information about the quantitation limit and the result of the spike recovery test shall be provided so that the accuracy of the analysis can be demonstrated. The identification method (method of analysis) is not prescribed for the quantitation in feed, as it is not a matter specified in the composition standard. However, from the standpoint of efficacy and safety, the quantitation in feed is an important matter in order for feed manufacturers to practice quality control of the proper additive amount when the additive amount needs to be controlled.

[Method to prove the adequacy of the quantitative method]

The following method is intended for the instrumental analysis method.

○Selectivity: Process a sample that does not contain the objected substance (the substance intended for analysis) as the blank sample, to confirm that there is no peak that will disturb the quantitative method. A compound feed to which the objected substance is planned to be added, the main basic ingredients and the feed additives included in the same compound feed are studied as the blank sample. The test data such as a chromatograph, obtained in the studies, shall be attached to the test report because it is important to determine the adequacy of the analysis method.

○Accuracy and repeatability: Perform the spike recovery test and calculate the mean recovery rate and the relative standard deviation in order to obtain the accuracy and repeatability. Note that the spike recovery test shall be performed in accordance with the following conditions, in principle:

・Sample: three kinds of compound feeds (choose those that correspond to the species of the targeted livestock for the objected substance)

・Additive concentration: arrange at minimum two levels of concentration based on the additive concentration of the objected substance in the feed (the recommended additive concentration).

・Number of repeats: three times

○Limit of the quantitation: Perform the spike recovery test with the sample that has one-tenth or less of the minimum of the objected substance concentration in the feed (recommended additive concentration), then calculate the mean recovery rate and relative standard deviation to obtain the accuracy and repeatability.

(6) Changes with time

Tests are needed to assess the stability of the objected substance based on the conditions of its actual use and handling. The changes in the properties of the objected substance (formulation) under severe conditions, as well as in the ambient temperature, shall be tested. From the results of this test, the suitable storage conditions shall be discussed.

[Requirements]

The test results of the ingredient for its manufacturing and formulation shall be described respectively. The information about the ingredients, etc., tests, and other things shall be described, including at least the following:

○Test conditions, place of storage (temperature, humidity, container, etc.): Describe which testing laboratory in Japan or overseas was used, and what storage conditions were set for the tests.
○Test period: Conduct the tests for the corresponding period and apply this to each test item.
○Information about the test samples (purity, lot number, etc.): The lot number shall be always recorded, as the purity may vary greatly depending on the lot. Tests shall be performed with samples from different lots.
○Method of analysis for the quantitation of the content: The method of analysis for the quantitation of the content shall be described, because the substance may decompose with time.
○Test results: The tests results shall be described in a properly arranged manner and using tables, as there are many test items.

i. Storage test in an ambient temperature

The actual storage shall be conducted in an ambient temperature (1–30℃) to assess the stability of the substance under normal conditions.

Storage tests shall be conducted using samples from at least three different lots of the ingredient, for manufacturing and for the formulation respectively. Put an appropriate amount of the objected substance in the packaging containers that are regularly used and store these in an indoor warehouse for each of the following periods, to assess the stability over the corresponding storage period: 0, 3, 6, 9, 12, 18 and 24 months (these test periods can be extended or shortened depending on the specifications of the shelf lives or the expiration date). Examine any quality deteriorations of the substance as a feed additive. Note that the size of the packaging container used for tests can be scaled-down as needed.

[Example] (A case where the objected substance is a white powder)
Table 2 Storage tests in an ambient temperature
Test conditions: air temperature 35℃; humidity 40% (for 24 months); in a 20 kg paper bag

Lot No.	Parameter	At the start	3 mos.	6 mos.	9 mos.	12 mos.	24 mos.
A	Appearance	white powder	white powder	white powder	white powder	white powder	white mud[1]
	Identification test	fit	fit	fit	fit	fit	fit
	Purity test	fit	fit	fit	fit	fit	fit

	Amount of active ingredient (g)	214.6	212.1	210.4	208.3	205.4	207.9
	Loss on drying (g)	5.2	4.9	3.7	5.0	6.3	4.7
	Amount of moisture (g)	32.3	30.0	29.4	35.1	33.8	35.3
B	Appearance	white powder	white powder	white powder	white powder	white powder	white powder
(omitted)							

Note 1: “White mud” indicates a white paste condition.
Although the appearance of the substance turned to a white mud (paste condition) after 24 months, it passed both the identification test and the purity test. In addition, no significant changes in the amount of the active ingredient were found. No other problems were found; thus, it can be concluded that the substance remains stable for at least 24 months.

ii. Heat resistance test

The outdoor air temperature rises, especially in the summer season. The indoor air temperature may also become high. Therefore, test the stability of the objected substance in such high temperatures, and ensure that no adverse changes are caused in its quality as a feed additive.

Storage tests shall be conducted with samples from at least three different lots of each of the ingredients for the manufacturing and the formulation. Put an appropriate amount of the objected substances in a sealed glass container or a tight container and keep them at 40℃ for each of the following periods to assess their stability over the corresponding period: 0, 1, 2, 3 and 6 months (these test periods can be extended or shortened depending on the conditions or the physical properties under which the objected substance will be used as a feed additive).

iii. Humidity resistance test

The reaction of the objected substance to humidity (moisture) shall be tested, in addition to the reaction to temperature, to ensure its stability and thereby no adverse changes in its quality as a feed additive.

Storage tests shall be conducted with samples from at least three different lots of each of the ingredients for the manufacturing and the formulation. Put an appropriate amount of the objected substances in separate Petri dishes and keep them at a constant temperature anywhere between 25–30℃, with two or more levels of relative humidity at that temperature and without the lid on the Petri dishes, for each of the following periods to assess their stability over the corresponding period: 0, 1, 2, 3 and 6 months (these test periods can be extended or shortened depending on the conditions or the physical properties under which the objected substance will be used as a feed additive). With regard to the level of relative humidity, more than two levels of humidity are set within the range in which the substance shows no apparent quality deterioration, such as changes in its appearance, growth of mold, degradation, deliquescence, consolidation, etc., in the preliminary test, and one of those levels will be determined to be near the upper-limit of the said range.

iv. Light resistance test

The reaction of the objected substance to light shall be tested. In cases where excess reactions against light are found, light shielded storage shall be arranged for quality assurance.

Storage tests shall be conducted with samples from at least three different lots of each of the ingredients for the manufacturing and the formulation. Put an appropriate amount of the

objected substances in separate Petri dishes, cover the Petri dishes with lids and seal the joint parts with tape or paraffin, then expose them to 500-lux fluorescent light at an ambient temperature (1–30℃) for each of the following periods to assess their stability over the corresponding period: 0, 1, 2, 3 and 6 months (these test periods can be extended or shortened depending on the conditions or the physical properties under which the objected substance will be used as a feed additive). After each period of time, check if any adverse changes have been caused in its quality as a feed additive.

[Example] (A case where the objected substance is a white powder)

Table 3 Light resistance test

Test conditions : air temperature 25℃; humidity 50% (for 6 months); kept under a 500-lux fluorescent light

Lot No.	Parameter	At the start	1 mo.	2 mos.	3 mos.	6 mos.
A	Appearance	white powder	white powder	white powder	white powder	white powder
	Identification test	fit	fit	fit	fit	fit
	Purity test	fit	fit	fit	fit	fit
	Amount of active ingredient (g)	210.6	205.2	204.6	202.3	200.4
B	Appearance	white powder	white powder	white powder	white powder	white powder
(omitted)						

The test results are summarized in the table above. The amount of the active ingredient decreased after 6 months of the substance's exposure to the light. According to the paper by xx et al. (xxxx), the amount of the active ingredient will decrease to below the limit of quantitation after 12 months. Based on the above results and information, it can be said that the amount of the active ingredient does not decrease following up to three months of exposure to the light, and thereby the substance is stable. Nonetheless, we have specified the use of light-shielded storage in the standards.

v. Accelerated test

The deterioration of a substance may be accelerated under conditions of high temperature and high humidity (especially in the summer season). Therefore, the stability of the objected substance under such severe conditions (in principle, 40℃ air temperature with a 75% relative humidity) shall be tested. After the test period, check if any adverse changes have been caused in its quality as a feed additive.

Storage tests shall be conducted on samples from at least three different lots of each of the ingredients for the manufacturing and the formulation. Put an appropriate amount of the objected substances in the packaging containers that are regularly used to store them at 40℃ with a 75% relative humidity, in principle, as well as in an indoor warehouse (ambient temperature) for each of the following periods to assess their stability over the corresponding storage period: 0, 1, 3 and 6 months (these test periods can be extended or shortened depending on the conditions or the physical properties under which the objected substance will be used as a feed additive). Note that the size of the packaging container used for the tests can be scaled-down as needed.

vi. In-feed stability test

Feed additives are added to feeds for their use. Therefore, the stability of the feed additives in feeds is essential. Any possible interactions between the feed additive and the feed, or with other feed additives, shall be tested.

The objected substance (formulation) is added to at least three kinds of typically

manufactured feeds at the regularly applied concentration. Put an appropriate amount of the samples of the feeds with the objected substance in the regularly used packaging container and store them in the indoor warehouse for each of the following periods to assess their stability over the corresponding storage period: 0, 0.5, 1, 2 and 3 months (and 6 months when needed). The method used for the quantitation of the objected substance in the feeds shall meet the following three conditions, in principle:

a. The mean recovery rate is 90% and higher. Repeatability (the value of the standard deviation plus the error of repeated tests in a laboratory plus the error of interlaboratory bias) is 0.1 or less in the coefficient of variation. Note that the recovery tests are conducted with the sample of feeds added with the object formulation at a regularly applied concentration in at least three laboratories, three times for each laboratory and with a parallel implementation in two laboratories on different days, to obtain the mean recovery rate and repeatability.
b. The limit of the quantitation shall be precise enough to quantitate the content equivalent to one-tenth or less of the regularly applied concentration in the feeds.
c. The amount of the active ingredient can be discriminated from degradation products and other impurities.

[Example]

Table 4 In-feed stability test

Test conditions: Put Feed I, Feed II and Feed III in separate regularly used packaging containers (packaging paper bags) and store in an indoor warehouse (air temperature 25℃, humidity 50%) for 3 months. (Data are the ratio to the content at the start of the test expressed in a 100-point scale.)

Sample	Run	At the start	0.5 mo.	1 mo.	2 mos.	3 mos.
Feed I	1	100	99.8	98.3	97.9	97.1
	2	100	99.2	98.3	96.9	96.2
	Average	100	99.5	98.3	97.4	96.6
Feed II	1	100	98.8	97.5	97.6	95.1
(omitted)						

For all the tested feeds, the residue rates remained at 95% and more. It can therefore be concluded that the objected substance is highly stable, even in feeds, for up to 3 months.

Item of measurement

All items that are planned to be specified in the composition standard shall be measured at three different time points or more, including at the start and the end of the test. At other time points, the amount of the active ingredient shall be measured in addition to the observation of any abnormalities in the appearance. Furthermore, for the in-feed stability test, ambient temperature storage test and humidity resistance, other items of measurement may be added for each test, such as measuring the loss on drying or moisture, as needed.

Statistical analysis of the test results

A model that is thought to be the best for characterizing the relation between the test period and the amount of active ingredient shall be used for the analysis. A regression analysis shall be used to calculate the 90% confidence interval for the population mean.

3. Items Concerning Efficacy

The following items concerning the efficacy of the objected substance shall be assessed.
(1) Efficacy as a feed additive: which of the three effects specified in the Feed Safety Act is attained.
(2) Targeted livestock animals and the administration stage (time of feeding)
(3) Most effective dose (method for using)

(1) Basic tests to prove the efficacy

i. *In vitro* test

A test to prove, or to extrapolate the efficacy of the objected substance in a test tube. In addition, the test aims to determine the optimum amount of the addition. A comparison with previously designated feed additives shall be made on an as-needed basis.

ii. *In vivo* test

A test to assess whether the expected efficacy can be brought about, even in the body of livestock, using laboratory animals or targeted livestock animals, etc.

(2) Field Application tests to prove efficacy

A test to assess whether the expected efficacy (effectiveness) can be brought about under the conditions of the actual use of the objected substance. Any livestock animals for which the efficacy is not confirmed cannot be designated as a target animal.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in.
○Test animals: Specify the species, age, etc. of the livestock animals for which the objected substance was planned to be applied. Also, describe the information about the test, such as the number of animals in a group.
○Method of administration and dose : Describe the additive amount of the objected substance contained in the sample feeds (mg/kg-feed), the method and period of the administration, as well as the daily intake amount of the objected substance per unit of body weight consumed by the test animals (mg/kg-BW/day; calculated from the body weight of the test animal and the daily consumption of feed). In principle, arrange three different dose groups (except for control groups) including doses that are equivalent to the maximum and minimum of the optimum additive amount range. If there was a non-exposure, describe this accordingly. Also, arrange a period of administration that is equal to the planned application period of the objected substance.
○Test results and observations: Describe the body weight (live weight gain), the intake amount of feeds, feed efficiency, survival rate, etc., for each of the different dose groups separately. The efficacy of the administration of the objected substance shall be assessed using an analysis of variance for each testing laboratory, in principle. Consolidate the results for concluding the assessment with a statement confirming the significant differences. Provide a summary of the test results for each test group in the abstract, while describing the results in each individual test animal in the original paper. Refer to the Appendix Form 1 for details of the observations. Note that the date shall be clearly stated if blood and enteruria, etc. were sampled, or if a slaughter was conducted.

As a summary of the tests concerning efficacy, estimate the optimum additive amount based on the results from the tests and describe it.

[Example] A case of an application to newly weaned piglets
A field application test to prove the efficacy was conducted at the xx laboratory in xx prefecture. Weaned piglets reared in an indoor pig house (Strain: xx, four pens each of males and females; 25–30 days old; average body weight: 10.3 kg) were fed feeds continuously with the objected substance added at the following different concentrations: 20, 40, 80, 100, 150 and 200 mg/kg (each concentration corresponding to the following daily intake amounts of the objected substance per unit of body weight: 0.4, 0.8, 1.6, 2.0, 3.0 and 4.0 mg/kg-BW/day) for four weeks, which is an intended period in the actual use. The consumption of water and feeds by the test animals was discretionary.

[Results]
The test results are shown in the form below. (Appendix Form 1) The live weight gain significantly increased at the concentrations of 40 mg/kg and higher ($p<0.05$). The live weight gain at 100 mg/kg did not show any difference to that at 80 mg/kg. Thus, 80 mg/kg can be thought to be the most effective concentration, even from an economical perspective.
Succeeding tests were conducted at a concentration of 80 mg/kg as optimum additive amount. With regard to the intake amount of feed, this peaked at 100 mg/kg and the proportional increase to the concentration of the objected substance was not marked. None of test animals died during this test.

[Reference 5] Considerations for the Description of the Test Results
The council deliberates about target animals for which the efficacy is proved, the period of feeding, concentrations, etc., based on the submitted documents (test data, etc.). Therefore, test results that demonstrate the conditions in which the objected substance produces effects shall be described in a well-organized manner. Any livestock animals for which the efficacy of the objected substance has not been proved cannot be designated as a target animal.
Furthermore, a daily intake amount of the objected substance per unit body weight (mg/kg-BW/day), as well as the additive concentration to the feed (mg/kg-Feed) in the feeding tests, shall be described for easy comparisons to the toxicity tests. In cases where body weights or the intake amount of the feeds are not known, refer to the Japan Feeding Standard and describe the cited data clearly.

Appendix Form 1 Tests concerning efficacy (Livestock)

Document No.	Testing laboratory, Testing place and period	Test animal		Test group assignment					Test results							Notes[*4]
		Species	No. of animals in a group	Test group	Dose of test substance[*1] (mg/kg-Feed)	No. of repeats	Total no. of animals	Feeding period	Average live weight gain[*3] (actual no.) (kg)	(%)	Average intake amount of feed (actual no.) (kg)	Average feed demand rate[*3] (actual no.)	(%)	Survival rate (%)	Pathological test findings	
	xx laboratory Fr. xx xx, xxxx To xx xx, xxxx	xx strain	8	Control 20 40	0 20 40	5	35	4 weeks	0.38a 0.49a 0.51b[*2]	100 129 134	0.60c 0.62c 0.69d	1.58e 1.27f 1.35f	100 124 117	100 100 100	- - -	
				(omitted)												
(omitted)																

Note 1: Describe the dose of the tested live microbial agent for the live microbial agent.
2: Test data denoted by a different superscript indicates that the differences in those data are significant.
3: Describe the ratio of the data of the samples to the data of the control group in a 100-point scale as well.
4: Describe the conditions of feeding and other noticeable observations, etc.

[Reference 6] Conditions to be Complied with in Conducting Animal Tests

The Notification of the "Standards Concerning the Performance of Animal Tests for Feed Additives Assessment" sets out the conditions to be complied with in conducting tests concerning the safety and persistence of feed additives. This standard was established to ensure a higher reliability of the required documents, and thereby ensure the accurate and strict performance of safety assessments by the Agricultural Materials Council in their deliberations for the designation of feed additives and the amendment of specifications and standards, etc. The standards are intended for the following tests:

General toxicity tests (single dose toxicity test, repeated dose toxicity test),
Special toxicity tests (transgenerational reproductive toxicity test, developmental toxicity test, carcinogenicity test, mutagenicity test, other tests)
Feeding tests using targeted livestock animals, etc.
Residue tests using targeted livestock animals, etc.

To prepare the required documents, the submitter must collect the data in the animal tests conducted by him/herself at the animal test laboratories, or from the tests contracted out to a third party. The data must be collected only for the tests that are conducted in conformity with the provisions of this standard, or the principles of the GLP developed by the OECD (Organization for Economic Co-operation and Development), which this standard conforms to. The laboratories used for the animal tests must be GLP compliant facilities.

Furthermore, the Ministry of Agriculture, Forestry and Fisheries, or an Incorporated Administrative Agency, the Food and Agricultural Materials inspection Center (FAMIC) will conduct inspections of the test laboratories used for the tests, according to the Notification of the Establishment of Inspection Proceeding Guidelines based on the Standard Concerning the Performance of Animal Tests for Feed Additive Assessments[*1] to confirm whether the tests concerning the safety and persistence of the feed additive have been conducted in conformity with the GLP.

The feed additive GLP compliant laboratories which have undergone inspections during the past three years are posted on the website of FAMIC.[*2]

Note 1: Notification of the Establishment of Inspection Proceeding Guidelines based on the Standard Concerning the Performance of Animal Tests for Feed Additive Assessments (Ministry of Agriculture, Forestry and Fisheries, Fisheries Agency Director-General Notice, Gen-chiku A No. 4 3441 issued on January 16, 1990)
http://www.famic.go.jp/ffis/feed/tuti/1_3441.html

Note 2: List of Feed Additive GLP Compliant Laboratories
http://www.famic.go.jp/ffis/feed/sub11_glp.html

4. Items Concerning Residue

Residue tests using targeted livestock animal, etc. (Subject to the Feed additive GLP)

When a livestock animal ingests a chemical substance, a certain amount of the chemical substance may remain within the animal's body, such as in its muscles, fat and liver, and not be discharged in the excreta. In the residue tests, the objected substances shall be administered to livestock animals and the amounts of the objected substance that remain in each organ, etc. of the animal shall be assessed.
Although the feed additives are ingested by livestock animals, humans may also ingest them through livestock products if they remain in the animal's body. For this reason, the residue test is one of the most important tests for proving the additive's safety to humans.
Besides the residue test, a test to analyze the kinetics of the objected substance in the animal's body (III-5 (1) iv. *In vivo* kinetics tests on page 47) is required as well. By conducting the kinetics test prior to the residue test, it can be roughly determined whether the objected substance has remained in the body of the livestock animal. If it is confirmed that the objected substance ingested by the animal has been completely discharged in the excreta and does not remain in the animal's body through the *in vivo* kinetics test, the residue test may be omitted.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in.

○Test animals: Specify the species, age, etc. of the livestock animals for which the objected substance was planned to be applied. Also, describe the information about the test, such as the number of animals in a group.

○Method of administration and dose : Describe the additive amount of the objected substance contained in the sample feeds (mg/kg-feed), the method and period of administration, as well as the daily intake amount of the objected substance per unit of body weight consumed by the test animal (mg/kg-BW/day; calculated from the body weight of the test animal and the daily consumption of feed). The minimum dose for the test shall be set as equal to the maximum level of the dose in the actual use, and the dose shall be administered several dozens of times higher than the said minimum dose in the test (except for the control group).

○Method of analysis: Sampling locations will be arranged in the edible parts (muscles, fat, liver, eggs, milk, etc.) in principle, and the sampling will be conducted so that the distribution of the objected substance is demonstrated. Although the analysis is intended to focus on the objected substance, an analysis of the metabolite may be needed as well in cases where the persistence of the metabolite needs to be examined. Describe the information about the metabolites (name, whether they have bioactive effects, etc.). In order to discuss the persistence of the substance in livestock bodies, the method for quantitating both the substance remaining in the body and the substance excreted to outside of the body is needed. Besides the sensitivity, high accuracy and repeatability are required in the method of analysis. In particular, the following specifications are required: limit of quantitation ≤ 0.05 mg/L; recovery rate in the spike recovery test on 1–2 mg/L sample ≥ 70%; and the coefficient of variation (standard deviation divided by the mean value) ≤ 0.1. These specifications need to be described. However, note that the limit of detection must be equal to, or lower than, the residue limit of the objected substance in foods if the said residue limit is set at below 0.05 mg/kg.

○Results of the analysis: If the results indicate that the amount of residue is smaller than

the limit of quantitation, describe as “< limit of quantitation.”

[Example] A case of an application to weaned cows
A field application test to assess the persistence of the objected substance was conducted using cows at the xx laboratory in xx prefecture. The cows reared in the indoor cow house of the laboratory (strain: xx; ten pens each of males and females; 2 years old; average body weight: 450 kg) were given feeds continuously with the objected substance added at the following different concentrations: 100, 300 and 1,000 mg/kg (each concentration corresponding to the following daily intake amounts of the objected substance per unit of body weight: 0.3, 0.9 and 3.0 mg/kg-BW/day) for four weeks. (The consumption of water and the feeds by the test animals was discretionary.) After four weeks of feeding, the cows were slaughtered for an analysis of the residue in each part of the anatomy. (There was no non-exposure period.) The tissues of the muscles (skeletal muscles), liver, kidneys and fat (from the abdominal area) were sampled and analyzed by the liquid chromatograph mass spectrometer (LC/MS) method. The limit of quantitation of this method (LC/MS) was 2.41 ng/g for the muscles, fat, liver, kidneys and milk. The average recovery rate was 85.7%, and the coefficient of variation was 0.1.
The details of the LC/MS method are as follows:
Equipment: xx (time-of-flight type), ultraviolet detector (280 nm)
Column: octadecylsilyl silica gel (inside diameter: 5–6 mm; length: 200–300 mm; particle diameter: 5 μm)
Column temperature: 25℃
Mobile phase: mixture of water and ethanol (90:10); gradient: 0 min. (90:10) → 30 min. (60:40); flow: 0.5 mL/min.; sample: 10 μL; ionization method: ESI (+), 5.0 kV, 500℃
… (omitted) …

[Result]
The results of the analysis are summarized in the table below (Table 5). The residue in the milk and muscles (skeletal muscles) were below the limit of quantitation even in the samples from the cows that had consumed the feed with 1,000 mg/kg of the additive amount. The maximum residue, 8 mg/kg, was detected in the liver and kidneys from the cows that had consumed the feed with 1,000 mg/kg of the additive amount. In the fat of the abdominal area, residue was identified in the cows which had consumed the feed with 300 mg/kg and more of the additive amount. However, the amount of residue was equal to or less than the limit of quantitation in all parts of the body of the cows that had consumed the feed with 100 mg/kg of the additive amount, which is the maximum additive amount to produce the efficacy of the objected substance.

Table 5 Animal residue test of the objected substance in cows

Concentration of the objected substance in the feed (mg/kg)	Analyzed body part				
	Muscle (skeletal muscle)	Liver	Kidneys	Fat (abdominal area)	Milk
100	<2	<2	<2	<2	<1
300	<2	<2	<2	10–15	<1
1,000	<2	5–7	5–8	40–50	<1

Limit of quantitation: 1μg/kg for milk and 2μg/kg for other parts

5. Items Concerning Safety

(1) Toxicity tests

Toxicity tests in laboratory animals such as mice and rats are very important, as they provide a tool to assess the toxicity of the objected substance with a high degree of accuracy because the tests are conducted under a genetic control (inbred line), microbiological control and environmental control. The toxicity tests are conducted to obtain the following information:

- Information about the effect of the initial dose on livestock animals
- Information about the safe administration period
- Information about the toxic effects (symptoms, toxic dose, etc.)

i. General toxicity tests (subject to the feed additive GLP)

a. Single dose toxicity test

A test to assess the toxic effects from both a quality and a quantity aspect by administering a single dose of the objected substance to the test animals and estimating the median lethal dose LD50. Also the level of intoxication and the appearance period of the symptoms shall be observed, and thereby the whole picture of the toxicity shall be clarified.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in.
○Test animals: Use young and healthy rodents (such as rats). Specify the strain, age in weeks, sex, body weight at the beginning of test, etc., of the test animals. Also, describe the information about the test, such as the number of animals in a group.
○Method of administration and dose: Administer a single dose by an oral gavage and specify the dose. Conduct an exploratory test to get a whole picture of the toxicity and approximate the lethal dose. Set the initial dose as equal to a dose by which an apparent sign of toxicity is expected to be caused, within 2,000 mg/kg-BW of the maximum limit.
○Test results and observations: Feed the test animals for at least 14 days and estimate the approximate lethal dose. Observe the level of intoxication and the appearance period of the symptoms, transitions, reversibility, etc. Record any kinds of signs of toxicity that were observed by the gross observation and the appearance period of symptoms for every single test animal, for all the involved animals. Refer to the Appendix Form 2 for details of the items in the observation.

[Example] A case of a single dose toxicity test in mice

A single dose toxicity tests in mice was conducted at the xx laboratory in xx prefecture. The mice (strain: xx; eight mice each of males and females; eight weeks old; average body weight: 21.2 g) were administered a single dose of the objected substance by an oral gavage. The doses were arranged in the following different concentrations: 100, 200, 400, 1,200 and 2,000 mg/kg-BW. The consumption of the water and feeds by the test animals was discretionary. Careful observations were made of the test mice for a period of two weeks to check whether general symptoms and abnormalities, such as death, were shown. The interval of the observations was 30 minutes during the period immediately after the start of test, then every 12 hours every day.

[Results]

On the seventh day of the test, a female mouse in the group of 200 mg/kg-BW was found

dead. On the day before the death, symptoms of anorexia and hypopraxia were observed in the mouse. Also, three dead cases (one male and two females) in the group of 1,200 mg/kg-BW, and six dead cases (three males and three females) in the 2,000 mg/kg-BW group occurred. Based on these results, the LD50 is estimated to be between 1,200 and 2,000 mg/kg-BW. The details of the results are summarized in the form below (Appendix Form 2).

Appendix Form 2 Single dose toxicity test

Document No.		(omitted)
Testing laboratory and testing period	xx laboratory Fr. xx xx, xxxx to xx xx, xxxx	
Test method		
Species of animal (name of strain, etc.)	Mice : xx strain	
Method of administration	Oral gavage	
Purity of the test substance	99.8%	
Dose (mg/kg-BW)	100–2,000	
Observation period	2 weeks	
Approximate lethal dose (mg/kg-BW)	1,200–2,000	
General symptoms	On the 3rd day, one male showed poor feeding (no body weight gain), and was confirmed dead on the 5th day (omitted)	
Period of occurrence, prosperity and decline of the signs of toxicity, and period of death	Dead cases 7th day: 1 fe. in 200 mg/kg-BW gr. 2nd day: 1 m. and 1 fe. in 1,200 mg/kg-BW gr. 4th day: 1 fe. in 1,200 mg/kg-BW gr. 2nd day: 2 fes. in 2,000 mg/kg-BW gr. 5th day: 1 m. in 2,000 mg/kg-BW gr. 7th day: 1 m. in 2,000 mg/kg-BW gr. 9th day: 2 fes. in 2,000 mg/kg-BW gr. (omitted)	
Notes		

Note: “-” indicates no abnormality was observed.

b. Repeated dose toxicity test (short term)

A test to estimate the dose that will evoke apparent toxic changes and to clarify the details of toxic changes by administering the objected substance to the test animals continuously for 3 or more months

c. Repeated toxicity test (long term)

A test to assess the toxicity by administering the objected substance to the test animals continuously for an extended period

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in. Test periods are 90 days for short-term tests and 12 months for long-term tests.
○Test animals: Use young and healthy animals. Specify the strain, age in weeks, sex, body weight at the beginning of test, etc., of the test animals. A sooner start of the test is desirable.
○Method of administration and dose: Add the objected substance to feed or water, in principle, and administer the feed by a continuous oral gavage. Arrange at least three different dose groups, including a dose that causes no effect in the animals and another dose that will cause a certain sign of toxicity. If solvents are not used, a non-treated group is the control. When a solvent is used, a group fed the solvent is the control. Note that the concentration of the additive amount shall be 5 w/w% or lower.
○Test results and observations: Measure the items listed in the appendix to clarify the whole picture of the toxicity. Refer to the Appendix Form 3 for the details of the items in the observation.

[Example] A case of a long term repeated dose toxicity test in mice
A long term repeated dose toxicity test in mice was conducted at the xx laboratory in xx prefecture. The mice (strain: xx; 16 mice each for males and females; 5 weeks old; average body weight: 20.7 g) were fed for 12 months. The objected substance was added to the feeds at the following variant concentrations: 100, 200, 500, 1,000 and 2,000 mg/kg. The consumption of the water and feeds by the test animals was discretionary. After the test period, clinical tests and pathological tests were conducted. For dead cases, a dissection was performed.

[Results]
The details of the results are summarized in the form below (Appendix Form 3).
The following dead cases occurred in the groups of 1,000 mg/kg and higher concentrations: one male in the 1,000 mg/kg group; one male and three females in the 2,000 mg/kg group. A tumor was found in the female (in the lungs) in the 1,000 mg/kg group. A discussion was conducted on whether the tumor was caused by the objected substance or not. Any dose-response relationships were not confirmed. In one of the three dead females in the 2,000 mg/kg group, symptoms of anorexia and akinesis were observed two days before its death. A dissection examination was performed, which provided no noticeable findings in the gross observation. It was therefore thought to be a natural death. In the higher dose groups (1,000 mg/kg and higher), the test animals showed a constant intake of the feeds and did not show a significant difference in live weight gain among the higher dose groups. Clinical tests were performed for each test animal, in addition to dissections for the dead animals, which provided no noticeable differences (no significant differences). Based on

these results, the NOAEL is estimated 500 mg/kg.

Appendix Form 3 Repeated dose toxicity test (short term or long term)

Document No.	Testing laboratory and testing period xx laboratory Fr. xx xx, xxxx to xx xx, xxxx	Species of animal (strain, etc.) Mice : xx strain	No. of animals per group 32 (16/16)	Method of administration Mixed feeding	Purity of the test substance 99.8%

Test group and dose (mg/kg-feed; mg/kg-BW/day)		0 (Control group)	(omitted)
General symptoms and death rate		General symptom Male: Bleeding on the 57th day (bite wound), cured a few days later Male: Loss of fur was found on the 112th day (omitted)	
Average live weight gain (g/day)		0.6	
Feed	Average intake of feed (g/day)	0.8	
	Feed efficiency	0.67	
Total dose administered of the test substance (mg/animal)		0	
Clinical test findings	Hematological test	-	
	Blood biochemical test	-	
	Urine test	-	
Pathological test findings	Gross observation	-	
	Weight of the organ	-	
	Histological test	-	
NOAEL and toxic dose		-	
Notes			

Note: “-” indicates no abnormality was observed.

ii. Special toxicity tests (subject to the feed additive GLP)

a. Transgenerational reproductive toxicity test

A test to assess the effects on the reproductive potential, as well as the trans-generational effects, by administering the objected substance to both male and female test animals over multiple generations.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in.
○Test animals and number of generations: Specify the strain, age in weeks, sex, body weight at the beginning of test, etc., of the test animals. Conduct the test over two generations, in principle, and extend the test to three generations if necessary. Also, describe the information about the test, such as the number of animals in a group.
○Method of administration and dosage: Add the objected substance to the feeds or water and administer the feed/water by a continuous oral gavage. Arrange at least three different dose groups to assess the dose-response relationship. The maximum dose shall be set as equal to the dose that invokes a sign of toxicity in the parent generation but does not kill them. The minimum dose shall be set as the dose that causes no sign of toxicity in both the parents and children. If solvents are not used, a non-treated group is the control. When a solvent is used, the group fed the solvent is the control. Note that the concentration of the additive amount shall be 5 w/w% or lower.
○Test results and observations: Measure the items listed in the appendix to clarify the whole picture of the toxicity. Refer to the Appendix Form 4 for the details of the items in the observation.

[Example] A case of a transgenerational reproductive toxicity test in mice
A transgenerational reproductive toxicity test in mice was conducted at the xx laboratory in xx prefecture. The mice (Generation: p; strain: xx; 30 females; 6 weeks old; average body weight: 20.4 g) were fed over two generations (F_1 and F_2 generations). The objected substance was added to the feeds at the following variant concentrations: 100, 500, 1,000 and 2,000 mg/kg. Among the mice prepared for the test, 22 mice became pregnant. These 22 pregnant females were used for the test.

[Results]
The details of the results are summarized in the form below (Appendix Form 4). The addition of the objected substance to the feed showed a contribution to a body weight gain and the increase of the intake of feed in the P generation mice ($p<0.1$). In the F_1 generation, a significant difference was indicated in both the initial body weight and the live weight gain ($p<0.01$, $p<0.02$). The intake of feed also increased ($p<0.02$). Similar results were obtained in the F_2 generation ($p<0.01$). There was no significant difference between the F_1 and F_2 generations. With regard to the performance of reproduction, no significant differences were indicated except for an increase in the average body weight of the newborns ($p<0.01$). A female of the second generation died at the age of one week old. This individual was dissected and examined. No noticeable findings were obtained in the microscopic and clinical tests. Therefore, it can be thought that this was a natural death.
Based on the above, it was confirmed that the administration of the objected substance causes a transgenerational effect, promotes live weight gain and affects the reproductive potential.

Appendix Form 4 Transgenerational reproductive toxicity test

Document No.	Testing laboratory and testing period xx laboratory Fr. xx xx, xxxx to xx xx, xxxx	Species of animal (strain, etc.) Mice : xx strain	No. of animals per group 30 (female)	Method of administration Mixed feeding	Purity of the test substance 99.8%

Generation		P Feeding period: 300 days				F_1 Feeding period: days	F_2 Feeding period: days
Test group and dose (mg/kg-feed; mg/kg-BW/day)		0	100	500	(omitted)	(omitted)	(omitted)
General feeding parameters	General symptoms	-	-	-			
	Death rate	0	0	0			
	Average live weight gain (g)	1.2	1.8	2.3			
	Average intake of feed (g/day)	2.6	2.7	3.1			
	Average feed efficiency	0.46	0.67	0.74			
	Findings	-	-	-			
Reproductive parameters	Period of the estrous cycle before copulation, normality (Female)	25	25	25			
	No. of copulations	14	16	15			
	Rate of copulation	88	100	94			
	No. of pregnancies	10	13	11			
	Rate of pregnancy	63	81	73			
	No. of live births	82	90	88			
	Average body weight of the newborns	0.5	0.7	0.8			
	No. of stillborns	0	0	0			
	Birth rate	100	100	100			
	Average litter size	6	10	10			
	Average live weight gain (at 21 days of age; g)	10.2	11.2	10.8			
	Surviving rate of the babies at 21 days of age	100	100	100			
	Sex distribution (%)	64	64	73			
	Findings	-	-	-			
Notes							

Note: "-" indicates no abnormality was observed.

b. Developmental toxicity test

A test to assess the effects of the substance on the birth of fetuses, especially on their teratogenicity, by administering the objected substance to pregnant animals in the organogenetic period of the fetus.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in.
○Test animals: Specify the strain, age in weeks, sex, body weight at the beginning of the test, etc. of the test animals. Also, describe the information about the test, such as the number of animals in a group. Young and healthy nulliparous female animals shall be used.
○Period of administration: Administer the objected substance in the organogenetic period of the fetus.
○Method of administration and dosage: Administer the objected substance by an oral gavage, in principle. Arrange at least three different dose groups to assess the dose-response relationship. The maximum dose shall be set within the extent of the confinement that the physicochemical properties define, as equal to the dose that invokes a certain sign of toxicity in the mother animals, such as an inhibition of the body weight gain. The minimum dose shall be set as the dose that causes no damage in both the mother and fetus. If solvents are not used, a non-treated group is the control. When a solvent is used, the group fed with the solvent is the control. The solvent shall not have an effect on the developmental toxicity and reproduction. Note that the dose should desirably be determined through the preliminary test and be within 1,000 mg/kg-BW of the maximum limit.
○Test results and observations: Measure the items listed in the appendix to clarify the whole picture of the toxicity. Refer to the Appendix Form 5 for the details of the items in the observation.

[Example] A case of a developmental toxicity test in mice
A developmental toxicity test in mice was conducted at the xx laboratory in xx prefecture. The mice (strain: xx; 40 mice each for males and females; 6 weeks old; average body weight: 21.3 g) were administered the objected substance on the tenth day of pregnancy by an oral gavage. The objected substance was added at the following variant concentrations: 50, 100, 400 and 1,000 mg/kg. The consumption of the water and feeds by the test animals was discretionary. Among the 40 mice of each males and females (total 80 mice) prepared for the test, 27 mice succeeded in an implantation and were used for the test.

[Results]
No noticeable changes were observed in either the mother animals or the fetuses. A dead case of a fetus occurred in a female in the 400 mg/kg-BW dose group. A dissection examination was conducted, the result of which suggested a crushing death, which was not the result of the intake of the objected substance.
Based on the above, it can be concluded that the dose administered during the pregnancy period caused no effects in either the mother or the fetuses. The details of the results are summarized in the form below (Appendix Form 5).

Appendix Form 5 Developmental toxicity test

Document No.	Testing laboratory and testing period xx laboratory Fr. xx xx, xxxx to xx xx, xxxx	Species of animal (strain, etc.) Mice : xx strain	No. of animals per group 80 (40/40)	Method of administration Oral gavage	Purity of the test substance 99.8%

Test group and dose (mg/kg-feed; mg/kg-BW/day)		0 (Control group)	(omitted)
No. of mother animals		25	
General symptoms		(omitted)	
Average body weight (g)		20.4	
Average intake of feed (g/day)		3.3	
Average feed efficiency		0.62	
Death rate		0	
No. of corpus luteum per dam		15.9	
Implantation findings	No. of implantation/dam Average implantation no. Rate of live fetuses (%) Average no. of live fetuses Fetal resorption Dead fetuses Macerated fetuses Others	14.2 8.2 78 6.4 1 0 0 0	
Fetal sex distribution (percentage of males)		48 (male)	
Fetal body weight Average ± standard deviation (g)		0.5±0.08	
External abnormalities		Hydrocephalus (male)	
Skeletal abnormalities		-	
Internal organ abnormalities		-	
Developmental abnormalities of newborns		-	
Notes			

Note: “-” indicates no abnormality was observed.

c. Carcinogenicity test

Administer the objected substance across the life span of animals and assess the oncogenicity in specific among other effects that may be caused.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in.
○Test animals: Specify the strain, age in weeks, sex, body weight at the beginning of the test, etc., of the test animals. Also, describe the information about the test, such as the number of animals in a group.
○Period of administration: Typically, the objected substance is administered for a period of 24 months. Adequate reasons for a change shall be explained if the period is changed.
○Method of administration and dose: Add the objected substance to the feeds or water, in principle, and administer these continuously via an oral route. Arrange at least three different dose groups to assess the dose-response relationship. The maximum dose shall be set as equal to the dose that is expected to cause an apparent effect on the frequency of tumor occurrence, and thereby on the lifespan of the test animals. Note that the concentration of the additive amount must be 5 w/w% or lower.
○Test results and observations: Measure the items listed in the appendix to clarify the whole picture of the toxicity. Refer to the Appendix Form 6 for the details of the items in the observation.

[Example] A case of a carcinogenicity test in mice
An oncogenicity test in mice was conducted at the xx laboratory in xx prefecture. The mice (Strain: xx; 50 mice each of males and females; 5 weeks old; average body weight: 20.5 g) were fed for 24 months. The objected substance was added in the following variant concentrations: 100, 1,000 and 2,000 mg/kg.

[Results]
Two dead cases occurred (one each for males and females) during the test. A tumor was found in the dead female (in the stomach). No tumor, etc., was found in the dead male, which suggests a natural death. In addition to these results, no change was indicated in the death rate. Based on the above, it can be concluded that the objected substance does not have an oncogenicity effect. The details of the results are summarized in the form below (Appendix Form 6).

Appendix Form 6 Carcinogenicity test

Document No.	Testing laboratory and testing period xx laboratory Fr. xx xx, xxxx to xx xx, xxxx	Species of animal (strain, etc.) Mice : xx strain	No. of animals per group 100 (50/50)	Method of administration Mixed feeding	Purity of the test substance 99.8%

Test group and dose (mg/kg-feed; mg/kg-BW/day)	0 (Control group)	(omitted)
Cumulative death rate	0	
Average live weight gain (g/day)	1.5	
Average intake of feed (g/day)	2.3	
General symptoms	(omitted)	
Weight of the organs (g) (Average value)	Body 22.1 Brain 0.42 Heart 0.12 Lungs 0.15 Kidney 0.27 (left) 0.13, (right) 0.14 Liver 0.95	
Histopathological findings	(omitted)	
Incidence of tumors	0	
(and incidence of a specific tumor)	(0)	
Findings of other tests	185th day: dead (1 m.) 241st day: dead (1 fe.)	
Note		

d. Mutagenicity test

A test to assess the mutagenicity of the objected substance by conducting *in vitro* reverse mutation tests and *in vitro* chromosome aberration tests. A micronucleus test shall be conducted if any abnormalities are found in the foregoing tests.

(d-1) Reverse mutation test

A test to assess the mutagenicity by way of examining the gene mutation inducibility (effect on the DNA base pairs) using Salmonella typhimurium and Escherichia coli bacteria.

[Requirements]

○Test strains: Conduct the tests using five or more kinds of strains including the following typical examples: Salmonella typhimurium—TA1535, TA1537, TA1538, TA98, TA100; Escherichia coli bacteria—WP2uvrA.

○Doses used in the test: Arrange five or more different dose groups. The maximum dose is 5 mg/plate, in principle. Prepare both a negative control and a positive control. The negative control is the group administered a solvent, and the positive controls are the groups administered a known mutagenic agent. For the positive control groups, both the substance requiring the presence of the S9 mix and the substance not requiring the S9 mix are prepared. Conduct the test using suitable metabolic activation methods (S9 mix) and observe the results.

○Observations: Record the actual numbers of revertant colonies and their mean value.

[Example] A case of a reverse mutation test using Salmonella typhimurium and Escherichia coli bacteria

Conduct the test for gene mutation inducibility using six Salmonella typhimurium strains (TA98, TA100, TA102, TA1535, TA1537 and TA1538) and Escherichia coli bacteria WP2uvrA. Six dose levels of the objected substance were arranged with the 5,000 μg/plate as the maximum dose. The test strains were cultured at 37℃ for three days. Three plates were tested for each of all possible combinations of the following contents and conditions: test strains each with the objected substance added at pre-arranged six dose levels; with and without the presence of the S9 mix metabolic activation. The negative control and positive control groups (three plates each) were included as well. After the culture, the revertant colonies were counted for each individual plate.

[Results]

The results are summarized in the table below (Table 6).

Table 6 Number of revertant colonies of Salmonella typhimurium

<table>
<tr><th rowspan="2">S9mix</th><th rowspan="2">Concentration (μg/plate)</th><th colspan="4">Salmonella typhimurium</th><td rowspan="11">(omitted)</td></tr>
<tr><th>TA98</th><th>TA100</th><th>TA102</th><th>TA1535</th></tr>
<tr><td rowspan="8">(-)</td><td>0[*1]</td><td>13</td><td>160</td><td rowspan="8" colspan="2">(omitted)</td></tr>
<tr><td>1.6</td><td>15</td><td>155</td></tr>
<tr><td>8.0</td><td>14</td><td>152</td></tr>
<tr><td>40.0</td><td>13</td><td>162</td></tr>
<tr><td>200.0</td><td>15</td><td>161</td></tr>
<tr><td>1,000.0</td><td>14</td><td>158</td></tr>
<tr><td>5,000.0</td><td>11</td><td>169</td></tr>
<tr><td>Positive control</td><td>12</td><td>163</td></tr>
<tr><td>(+)</td><td>0[*1]</td><td colspan="4">(omitted)</td></tr>
<tr><td colspan="6">(omitted)</td></tr>
</table>

(*1) Negative control
TA98: 2-nitrofluorene (5.0 μg/plate)
TA100: sodium azide (2.0 μg/plate)
TA1535: sodium azide (2.0 μg/plate)
TA1537: 9-aminoacridine (50.0 μg/plate)
TA102: Mitomycin (0.2 μg/plate)
TA98: benzo[α]pyrene (10.0 μg/plate)
TA100: 2-aminoanthracene (5.0 μg/plate)
TA1535: 2-aminoanthracene (5.0 μg/plate)
TA1537: 2-aminoanthracene (5.0 μg/plate)
WP2uvrA: 2-aminoanthracene (20.0 μg/plate)

No increase in the number of revertant colonies was found in any of the dose groups, including the maximum dose group, regardless of the presence of S9 mix for metabolic activation.
Based on the above results, it can be concluded that the objected substance does not have mutagenicity.

(d-2) Chromosome aberration test

Chromosome aberrations caused by damage to the DNA and the proteins can be detected by a microscopic observation in the metaphase of the cell division.

[Requirements]

○Test cells: Primary or passage cultured cells of mammals, including humans
○Doses used in test: Arrange at least three different dose groups with the maximum dose equivalent to the concentration at which a proliferation or division of the cells is inhibited by ≥ 50%. In a case where cell cytotoxicity is not identified, a 10 mm equivalent or 5 mg/mL of the concentration is the maximum limit. The negative control is the group administered the solvent, in principle, and the positive controls are the groups administered known chromosome aberration inducing agents. Conduct the test using suitable metabolic activation methods (S9mix) and observe the results.
○Observations: Observe the incidence of chromosomally aberrant cells and the incidence of polyploids, and record these values.

[Example] A case of a chromosome aberration test using CHL cells
<Method>
A test was conducted for chromosome aberrations using the CHL cells of a Chinese hamster. CHL cells were disseminated on 10 mm dishes at 5×10^4 cells/mL of the concentration, and were cultured at 37℃. In the continuous treatment method, the objected substance was added on the third day from the dissemination and was treated for 24 hours. In the short time treatment method, the cells were treated with and without the S9 mix for metabolic activation for 12 hours on the third day from the dissemination, and were further cultured.
Four dose levels of the objected substance (500, 1,000, 2,000 and 5,000 μg/plate) were arranged, with the 5,000 μg/plate as the maximum dose. The test cells were each given an additional pre-arranged dose of the objected substance and were cultured at 37℃ for three days, with and without the presence of the S9 mix for metabolic activation, along with the negative and positive control groups. All test pieces were treated with both the continuous treatment method (Test 1-1) and the short treatment method (Test 1-2). The structural chromosome aberrations (chromatid gaps, chromosome gaps, etc.) were examined for each dish.
(Test 2)
Prior to the determination of the four dose levels, a cytostatic test was conducted to assess

the concentration at which the proliferation of cells is inhibited by ≥ 50 %. The CHL cells were treated with the objected substance at concentrations of 500–2,000 μg/plate. The numbers of cells were counted on the fifth day from the start of the treatment.

<Results>
Test 2
The results of the cytostatic test are summarized in the table below (Table 7). The ratio of the cell proliferation rate to that of the negative control (survival rate [ratio in % against the negative control]), which was calculated from the number of live cells, was used as the index. From the results, the concentration at which the proliferation of cells was inhibited by ≥ 50 % was 1,000 μg/mL ≤. Based on this result, 1,000 μg/mL of the concentration was used for Test 1.
Table 7 Results of the cytostatic test

Observations	Additive concentration of the objected substance (μg/mL)			
	0*[1]	500	1,000	
Live cell no. ($\times 10^4$ cells)	72	56	40	(omitted)
Dead cell no. ($\times 10^4$ cells)	74	28	55	
Surviving rate (%)	98.6	66.7	42.1	
Surviving rate (ratio in % against the negative control)	100	67.6	42.7	

Test 1-1
The results of the chromosome aberration test (short time treatment method) are summarized in the table below (Table 8). Regardless of the presence of the S9 mix for metabolic activation, neither structural chromosome aberrations nor abnormal cells were detected.
Table 8 Incidence of chromosome aberrations

S9 mix	Treat-ment period (h)	No. of cells observed (cells)	Concentration (μg/dish)	Incidence of structural chromosome aberrations (%)							Judgment (*8)
				gap (*2)	ctb (*3)	cte (*4)	csb (*5)	cse (*6)	f (*7)	Total	
(-)	12	200	0(*1)	0	0	0	0	0	0	0	-
		200	500	1	0	0	0	0	0	1	-
		200	1,000	0	0	0	0	0	0	0	-
		200	2,000	0	0	1	0	1	1	3	-
		200	5,000	0	0	0	2	0	0	2	-
		200	Positive control	0	1	0	0	0	1	2	-
(+)	12	200	0(*1)	0	0	0	0	0	0	0	-
		(omitted)									
(omitted)											

S9 mix	Treat-ment period (h)	No. of cells observed (cells)	Concentration (μg/dish)	Incidence of chromosomally aberrant cells (%)		Polypoid	Judgment (*8)
				Chromatid gap			
				(-)	(+)		
(-)	12	200	0(*1)	0	0	0.0	-
		200	500	1	0	0.0	-
		200	1,000	0	0	0.0	-
		200	2,000	0	1	0.0	-

		200	5,000	0	0	0.0	-
		200	Positive control	0	1	0.0	-
(+)	12	200	0(*1)	0	0	0.0	-
		(omitted)					
(omitted)							

(*1) negative control, (*2) gap: chromatid gap, (*3) ctb: chromatid break, (*4) cte: chromatid exchange,
(*5) csb: chromosome break, (*6) cse: chromosome exchange, (*7) f: fragmentation,
(*8) Judgment (-): negative
… (omitted) … Results of Test 1-2, Continuous treatment method, are omitted as well.

(d-3) Micronucleus test

Test to assess the inducibility of chromosome aberrations by examining the incidence of micronucleated immature erythrocytes in mammalian bone marrow smears as an index of the toxicity.

[Requirements]

○Test animals: Mice or rats
○Method of administration and dosage: Use an intraperitoneal administration, or an oral administration (oral gavage, in principle). Administer a single dose, as well as a repeated dose 4–5 times. Prescribe an appropriate dose for the repeated dose. Arrange at least three different dose groups. Set the maximum dose as equal to the dose that causes any signs of toxicity, such as an inhibition of the body weight gain. Use 2,000 mg/kg as the maximum dose if no toxic signs occur. In addition, prepare negative and positive controls. The negative control is the group administered the solvent, in principle, and the positive controls are the groups administered the known micronucleus-inducing agents.
○Observations: After the administration, kill all the test animals and collect the bone marrow from each of them to prepare smears. In principle, count the micronuclei in a minimum 2,000 of immature erythrocytes per test animal. At the same time, examine the incidence of polychromatophilic erythrocytes to all the erythrocytes.

[Example]

The mice (strain: xx; 5 mice each of males and females; 8 weeks old; average body weight: 23.1 g) were administered a single dose of the objected substance (by oral gavage). Three different doses (500, 1,000 and 2,000 mg/kg) were arranged, with 2,000 mg/kg as the maximum dose. Bone marrow was collected at 24 hours and at 48 hours from the time of the administration, and 2,000 immature erythrocyte cells were examined per test mouse to detect the micronuclei.

[Results]

The results are summarized in the table below (Table 9).
The incidence of micronucleated cells was calculated (by the number of micronuclei/number of examined cells x 100).
No significant difference was identified in the incidence of polychromatophilic erythrocytes between the treated groups and the negative control group. Also, there were no significant differences between the treated groups and the positive control group.
Based on the above, it can be concluded that the objected substance does not have inducibility of micronuclei.
Table 9 Incidence of micronucleated erythrocytes

Dose (mg/kg-BW)	Treatment period (h)	No. of cells observed (cells)	No. of polychromatophilic erythrocytes (cells)	No. of orthochromatic erythrocytes (cells)	No. of micronuclei (cells)	Incidence of micronuclei (%)
0 (controls)	24	2,000	1,898	102	1.0	0.10
	48	2,000	1,914	86	1.6	0.08
(omitted)						

e. Other tests

(e-1) Tests to assess the inducibility of gene mutation as an index

・Gene mutation test using cultured mammalian cells

・Test in drosophila

・Spot test in mice

・Specific-locus test in mice

(e-2) Test to assess the inducibility of chromosome aberrations as an index

・Chromosome aberration test using rodent germ cells

・Dominant lethal test in rodents

・Test of interphase chromosome locus displacement in mice

(e-3) Test to assess the damage to DNA as an index

・Bacteriophage test using bacteria

・DNA repair test using bacteria

・Test of unscheduled DNA synthesis using bacteria

・Test of sister chromatid exchanges using mammalian cells

(e-4) Other tests

・Somatic recombination and gene exchange test using fermentum

・Sperm morphology aberration test in mice

iii. Pharmacological test

Test conducted to assess the pharmacological effect of the objected substance when it is anticipated. Unless it is anticipated, this test can be omitted by indicating so. Any antimicrobials which were proved to be effective in other things (disease treatments, for example) than the inhibition of the productivity in livestock, caused by the particular pathogenicity organisms in their juvenile period, cannot be designated as feed additives.

iv. Test concerning *in vivo* kinetics

Test to clarify the *in vivo* kinetics by tracing the objected substance in its absorption, distribution, metabolism, excretion, etc., when the animals ingest the objected substance.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in. The test period shall be equal to the period of applying the objected substance as a feed additive.

○Test animals: Use the targeted animals of the objected substance, and add rats and rabbits, etc., when needed.

○Method of administration and dose: Administer a single dose via an oral route, in principle. If possible, consider a continuous administration as well. Select a dose that is suited to

the method of analysis so that the test substance, etc., can be quantitated in the body tissues and/or in the excreta.
○Method of analysis: Employ an adequate method capable for the analysis of the *in vivo* kinetics.
○Results: The items to be observed are described in detail later. ([Reference 7] Tests for Analyzing *In vivo* Kinetics, on page 49.)

[Example] A case of an *in vivo* kinetics test in pigs

A field application test was conducted to prove the efficacy of the substance at the xx laboratory in xx prefecture. Weaned piglets reared in an indoor pig house (strain: xx; 12 pens each of males and females; 14 days old; average body weight: 11.7 kg) were administered a single dose of the objected substance at a 100 mg/kg concentration via an oral route (gavage). ^{12}C of the objected substance was replaced by ^{14}C, and the dynamic states of the radio isotopes were traced inside the body for a three-day period from the time of the administration.

[Results]
The results of the quantitative analysis of the discharged ^{14}C in the excreta are summarized in Table 10 below. The data shows that approximately 78% of ^{14}C was excreted within 24 hours. In addition, 83% was discharged to the outside of the body as the three day total. The test results indicate that the persistence of the objected substance inside the body is low, and that the objected substance is excreted mainly in the feces.
Table 10 Results of the quantitative analysis of ^{14}C in the feces in the single dose administration test of the ^{14}C labeled objected substance via an oral route (ratio in % to the total administered ^{14}C)

	Elapsed time (hrs.)			3 day total
	24	48	72	
Urine	0.48	0.66	0.32	1.46
Feces	77.29	2.34	1.91	81.54
Daily total	77.77	3.00	2.23	83.00

A quantitative analysis was also performed on the objected substance contained in the excreta. (Table 11)
Table 11 Ratio of the ^{14}C labeled subject substance in the feces. (Ratio in % to the total administered ^{14}C)

Objected substance	After 24 hours
	5
Metabolite A (N-hydroxide)	13
Metabolite B (Demethylated substance)	56

The ratio of ^{14}C in the feces was 56% at 24 hours from the time of the administration. Besides the objected substance, mainly Substance A and Substance B were detected. It is considered that these substances were produced by the metabolization process in the liver after the administration. These substances didn't have a physiological activation effect. Based on these results, it is clarified that the objected substance is metabolized within 24 hours after it is administered and loses its physiological activation effect.

[Reference 7] Tests for Analyzing *In vivo* Kinetics

To analyze the kinetics inside the body of livestock animals, such as whether metabolization takes place and what amount of the substance is excreted, the objected substance is labeled by a radioactive material such as ^{14}C: that is, the isotope-labeled compound produced by replacing a part of ^{12}C of the objected substance with ^{14}C, is administered to animals and the radioactive materials are traced in the organs and the excreta. Even if the structural formula has changed, the kinetics can be analyzed by tracing the ^{14}C.

○ Absorption and excretion test, the analysis of the distribution inside the livestock animal's body

Measurements are conducted of the blood concentration, residual amount in the digestive tracts and the amount excreted in the urine and feces of the objected substance and its main metabolites, and the change of those values with time, and an analysis is performed of the absorption rate in the digestive tracts, route of excretion and the rate of excretion based on the data. In addition, an analysis is performed of the distribution of those substances in the muscles, fat, liver, kidneys, and the other organs and anatomies, and its time-dependent changes, and the biological half-life is calculated on an as-needed basis. (For example, the combined use of autoradiography, etc., after the administration of the radio isotope-labeled compound is also effective.) Furthermore, the chemical type of the isotope-labeled compound recovered in urine, feces, each organ etc. can be identified. (There may be a possibility of the compound having been metabolized, if the identified chemical type differs from that of the original.)

○Identification of metabolites

Identify the main metabolites and analyze the production rate of those metabolites when the metabolization of the objected substance in the animal's body is confirmed. (Typically the *in vitro* test using the cells of the organs and tissues that are involved in the metabolization is conducted for the analysis.) When a difference in the production rate of the main metabolites is found between animal species, it is desirable to conduct further similar tests in other species of animals.

(2) Feeding tests using targeted animals, etc. (Subject to the feed additive GLP)

Based on the target animal and the effective dose, both of which are supposed to act on the efficacy of the objected substance as a feed additive, conduct a test to administer the objected substance to the targeted animal practically and continuously, and assess the effects on the animal.

[Requirements]

○Testing laboratory and period, testing place and conditions: Describe which testing laboratory in Japan or overseas was used, and what conditions the animals were fed in. Set the test period as equal to the applicable period of the objected substance when used as a feed additive.

○Test animals: Use livestock animals for which the objected substance is planned to be applied. Specify the strain, age, etc., of the test animals. Also, describe the information about the test, such as the number of animals in a group.

○Method of administration and dosage: Describe the additive amount of the objected

substance contained in the sample feeds (mg/kg-feed), the method and period of the administration, as well as the daily intake amount of the objected substance per unit of body weight ingested by the test animals (mg/kg-BW/day; calculated from the body weight of the test animal and its daily consumption of feed). Arrange at least two dose groups including a dose equivalent to the maximum of the optimum dose range, and another dose of approximately 10 times said dose (except for the control groups).

Method of analysis:

○Test results and observations: Describe the body weight (live weight gain), the intake amount of the feeds, the feed efficiency, surviving rate, etc. for each dose group separately. When any abnormalities are found in these parameters, perform a hematological test, biochemical test, pathological test, etc., on an as-needed basis. Refer to the Appendix Form 7 for the details of the items in the observation.

[Example] A case of an application to weaned piglets

A field application test was conducted to prove the efficacy at the xx laboratory in xx prefecture. Pigs reared in an indoor pig house (strain: xx; 15 pens each of males and females; 30 days old; average body weight: 8.2 kg) were given feeds continuously for four weeks, which is the intended period in actual use. The objected substance was added to the feeds at the following different concentrations: 50 mg/kg; 80 mg/kg as the optimum dose, which is equivalent to 0.4 mg/kg-BW/day of a daily intake amount of the objected substance per unit of body weight; 160 mg/kg as two times the optimum dose, which is equivalent to 0.8 mg/kg-BW/day; 400 mg/kg as five times the optimum dose, which is equivalent to 2.0 mg/kg-BW/day; and 800 mg/kg as ten times the optimum dose, which is equivalent to 4.0 mg/kg-BW/day. The consumption of the water and feeds by the test animals was discretionary.

[Results]

The results are summarized in the table below (Appendix Form 7). The live weight gain of the 80 mg/kg group was 15.4 kg larger than that of the control group ($p<0.01$). Although the increase in both the live weight gain and the intake of feeds was shown in the 800 mg/kg (10 times the optimum dose) group, a significant difference was not indicated between this group and the group without the additive. Two female pigs became anorexic, while no noticeable changes were observed as general symptoms. One of these two pigs showed a mild gastric erosion, which was cured in a week or so. After the cure, the live weight gain increased. The other pig did not show any noticeable symptoms.

Appendix Form 7 Feeding test on targeted animals, etc. (livestock)

Document No.	Testing laboratory, Testing place and period	Test animal		Test group assignment					Test results								Notes[*4]
		Species	No. of animals in a group	Test group	Dose of the test substance[*1] (mg/kg-feed)	No. of repeats	Total no. of animals	Feeding period	Average live weight gain[*3] (actual no.) (%)		Average intake amount of feed (actual no.)	Average feed demand rate[*3] (actual no.) (%)		Surviving rate (%)	Hematological test findings	Blood biochemical findings	Pathological test findings	
	xx laboratory Fr. xx xx, xxxx To xx xx, xxxx	xx breed	30 (15/15)	0 50 80 160	0 50 80 160	2	60	4 weeks	(kg) 0.41a 0.43a 0.55b 0.62c	100 105 134 151	(kg) 0.71d 0.68de 0.61de 0.60e	1.73f 1.58g 1.11h 0.97i	100 91 64 56	100 100 100 100	(omitted)			
(omitted)																	
(omitted)																	

Note: Test data noted with a different superscript indicate that the differences between those data are significant.

(3) Tests concerning the emergence of resistant bacteria

Perform quality and quantity assessments on the items concerning the emergence of drug-resistant strains among the effects caused by the use of antimicrobial substances.

(4) Other tests

Perform assessments on the effects of the use of the objected substance on the natural environment by way of the excreta, etc., of livestock animals.

[Reference 8] Style Reference for the Description of Items Concerning Standards
Once the objected substance has been designated as a feed additive, the items set forth in “III-2 Items Concerning Standards”, on page 19 and following, are listed in the Ministerial Ordinance on the Specifications and Standards of Feed and Feed Additives (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 35 issued on July 24, 1976) as composition standards. The instructions for the use of the symbols, etc., set forth in the Ministerial Ordinance on the Specifications and Standards of Feed and Feed Additives shall be referred to for the description of these items. The descriptions for previously designated feed additives, which can be found in the “List of the Specifications and Standards of Feed Additives,” may be referred to as well.
- e-gov: Electronic information provision services concerning laws (Ministerial Ordinance on the Specifications and Standards of Feed and Feed Additives)
http://law.e-gov.go.jp/htmldata/S51/S51F00601000035.html
- List of the Specifications and Standards of Feed Additives (13th Edition)

Source: Appendix 2-1 to the “Ministerial Ordinance on the Specifications and Standards of Feed and Feed Additives”

(1) Inspections of feed additives are made in accordance with the composition specifications and the standards of the manufacturing process, etc., of each feed additive (hereinafter referred to as “each article”), and in line with the test methods prescribed in the general rules on feed additives and the test method for the feed additives (hereinafter referred to as the “general test method”). Note that the physicochemical properties, such as the odor, taste, crystalline form, solubility, acidity or alkalinity of a solution, stability, light absorbance, freezing point, refractive index, optical rotation, viscosity, specific gravity and the melting point described in the pertinent section are for reference purposes only and do not constitute the elements of the inspection criteria. Note also that stabilizing agents, lubricating agents, binding agents, moistening agents, emulsifying agents, covering agents, dispersing agents, disintegrating agents, preserving agents, or solubilizing agents can be used for the formula prescribed in each article to increase the efficacy or the stability of each feed additive.

(2) The substance name followed by the molecular formulas in parentheses ( ) indicates a chemically pure substance.

(3) The following signs are used for the main measurement units.

meter	m	centimeter	cm
millimeter	mm	micrometer	μm
nanometer	nm	square centimeter	cm^2
liter	L	milliliter	mL
microliter	μL	ton	T (1,000 kg)
kilogram	kg	gram	g
milligram	mg	microgram	μg
kilopascal	kPa	mole	mol
micromole	μmol	mole per liter	mol/L
degrees Celsius	℃		

(4) The mass percentage is denoted using a percentage sign %. In addition, the following signs are used for each measurement: % w/v for the content of the substance (g) in

100 mL of solution; % v/v for the content of the substance (mL) in 100 mL of solution; % v/w for the content of the substance (mL) in 100 g of solution.

(5) With regard to the expression of the amount of antibiotics and enzymes, the potency of the discussed antibiotics and the enzyme per liter unit of the discussed enzyme are used, respectively.

(6) The term “standard temperature” indicates a temperature of 20℃. Similarly, “ordinary temperature” is a temperature of 15–25℃, “ambient temperature” is a temperature of 1–30℃, and “lukewarm” is a temperature of 30–40℃. The term “cold place” indicates a place of ≤15℃, unless otherwise specified. Likewise, “cold water” indicates water of ≤10℃; “lukewarm water” indicates water of 30–40℃; “warm water” indicates 60–70℃; and “hot water” indicates water of approximately 100℃. The term “heat on or in a water bath” indicates heating in a boiling water bath or a steam bath at approximately 100℃, unless otherwise specified.

(7) Purified water shall be used for the tests of the feed additives, unless otherwise specified.

(8) A dropping device which delivers 20 drops of purified water weighing 0.90–1.10g at 20℃ shall be used for measuring the number of drops.

(9) The value of the "n+ 1" figure shall be rounded off for obtaining a value of the "n" figures.

(10) The table of the “Standard Atomic Weights 2007” shall be referred to for the atomic masses. The molecular weight shall be calculated using the values from this table and shall be rounded off to two decimal points.

(11) The term “reduced pressure” indicates a pressure not exceeding 2.0 kPa, unless otherwise specified.

(12) The acidity or alkalinity of a solution is determined by the use of blue or red litmus papers, unless otherwise specified. The pH value shall be used for a precise expression.

(13) Solutions expressed with the word “solution” following the name of the solute, and not stating the name of the solute, indicates a water solution.

(14) Solutions denoted using the style of (1→3), (1→10) or (1→100) indicate that each solution has a concentration corresponding to 3 ml, 10 ml and 100 mL of solution, all of which contain 1 g of the dissolved solid solute or 1 mL of the dissolved liquid solute, respectively. Mixtures denoted by (1:10), (5:3:1), etc., are mixtures containing two kinds of liquids at a 1:10 ratio, and three kinds of liquids at a 5:3:1 ratio, respectively.

(15) The tests of the feed additives shall be conducted at an ordinary temperature, and the observations shall be made immediately after the operation, unless otherwise specified. When examining temperature-sensitive matters, the conditions at the standard temperature shall be examined.

(16) The term “white” used in the item of physicochemical properties indicates a white or practically white color. Similarly, the term “colorless” indicates a colorless or practically colorless item. Unless otherwise specified, the test of the color tone is performed by placing 1 g of the test feed additive on a sheet of white paper, or in a watch glass placed on white paper for solid feed additives. A liquid feed additive is put into a colorless test tube measuring 15 mm as the inside diameter, and is observed in front of a white background through a 30 mm liquid layer. For the clarity test of liquid feed additives, the above-mentioned procedure is used with either a black or a white background. Only a black background shall be used for testing the fluorescence of a liquid feed additive.

(17) The term “odorless” used in the item of physicochemical properties indicates an odorless or practically odorless item. Unless otherwise specified, the test of odor shall be performed by placing 1 g of the solid or liquid feed additive in a 100 ml beaker.

(18) The terms used to indicate the solubility in the item of physicochemical properties are defined in the table below. Unless otherwise specified, solubility means the degree of dissolution of a feed additive, previously powdered in the case of a solid feed additive, within 30 minutes of its immersion in a solvent at 20 ± 5℃, with 30 seconds of vigorous shaking repeated at 5-minute intervals.

Term	Volume of solvent required for dissolving 1 g or 1 mL of the solute	
Very soluble		< 1 mL
Freely soluble	1 mL ≤	< 10 mL
Soluble	10 mL ≤	<30mL
Sparingly soluble	30 mL ≤	<100mL
Slightly soluble	100 mL ≤	<1,000mL
Very slightly soluble	1,000 mL ≤	<10,000mL
Practically insoluble, or insoluble	10,000 mL ≤	

(19) In the test of feed additives, the term “dissolve in a solvent” or “mix with a solvent” indicates that the feed additive is dissolved in, or mixed with the solvent to form a clear solution or mixture. A fraction of fibers or dust should be considered as within the allowance.

(20) The identification test is the test to identify feed additives or the main ingredients of feed additives.

(21) The purity test aims to detect impurities in the feed additives. The test is intended to specify the purity of feed additives, together with the other test items specified in each article. It is typically performed to control the type and amount of impurities. The impurities subject to the impurity test shall include those anticipated to become mixed in during the manufacturing process or the storage of the feed additives, as well as hazardous impurities such as heavy metals and arsenic. If foreign substances are used or are predicted to be added, this test shall be performed on those substances.

(22) The terms “clear”, “practically clear”, “very slightly turbid”, “slightly turbid” and “turbid” are standardized by the following procedures respectively.

Turbidity standard stock solution: Add water to 14.1 mL of 0.1 mol/L of hydrochloric

acid to make 50 mL of solution. One ml of this solution contains 1 mg of chlorine (Cl).
Turbidity standard solution: Measure 10 ml of the turbidity standard stock solution, and add water to make 1,000 mL of solution. One ml of this solution contains 0.01 mg of Cl.

i. Clear: Measure 0.2 ml of the turbidity standard solution, and add water to make 20 ml of solution. Add 1 ml of diluted nitric acid (1 → 3), 0.2 ml of 2% w/v dextrin solution, and 1 ml of 2% w/v silver nitrate solution. Allow the solution to stand for 15 minutes to prepare the reference solution. To be described as “clear” the test solution shall have the same or lower turbidity than that of the reference solution. Note that impurities such as floating substances should not be observed.

ii. Practically clear: Measure 0.5 ml of the turbidity standard solution, and add water to make 20 ml of solution. Add 1 ml of diluted nitric acid (1 → 3), 0.2 ml of 2% w/v dextrin solution, and 1 ml of 2% w/v silver nitrate solution. Allow the solution to stand for 15 minutes to prepare the reference solution. To be described as “practically clear” the test solution shall have the same turbidity as that of the reference solution. Note that impurities such as floating substances should not be observed.

iii. Very slightly turbid: Measure 1.2 ml of the turbidity standard solution, and add water to make 20 ml of solution. Add 1 ml of diluted nitric acid (1 → 3), 0.2 ml of 2% w/v dextrin solution, and 1 ml of 2% w/v silver nitrate solution. Allow the solution to stand for 15 minutes to prepare the reference solution. To be described as “very slightly turbid” the test solution shall have the same turbidity as that of the reference solution.

iv. Slightly turbid: Measure 6 ml of the turbidity standard stock solution, and add water to make 20 ml of solution. Add 1 ml of diluted nitric acid (1 → 3), 0.2 ml of 2% w/v dextrin solution, and 1 ml of 2% w/v silver nitrate solution. Allow the solution to stand for 15 minutes to prepare the reference solution. To be described as “slightly turbid” the test solution shall have the same turbidity as that of the reference solution.

v. Turbid: Measure 0.3 ml of the turbidity standard solution, and add water to make 20 ml of solution. Add 1 ml of diluted nitric acid (1 → 3), 0.2 ml of 2% w/v dextrin solution, and 1 ml of 2% w/v silver nitrate solution. Allow the solution to stand for 15 minutes to prepare the reference solution. To be described as “turbid” the test solution shall have the same turbidity as that of the reference solution.

(23) In drying or ignition, the term “constant mass”, unless otherwise specified, means that the difference of the masses measured before and after an additional 1 hour of drying or ignition is not more than 0.10% of the preceding mass of the dried substance or ignited residue. In the cases of differences of masses ≤ 0.5 mg in a chemical balance, ≤ 0.05 mg in a semi-microbalance, and 0.005 mg in a microbalance, those masses are regarded as the constant mass.

(24) The quantitative method is the method of testing to determine the composition of feed additives, the content or concentrations, etc., of the ingredients by the use of physical, chemical or biological procedures.

(25) The quantity of test samples or standard substances preceded by the word “approximate” indicates a quantity that is within ±10% of the specified mass. The word “dry” indicates drying under the same conditions as are specified in each article, or in the section of the drying loss of the standard substances when it is simply used in conjunction with the test samples or standard substances. Similarly, the word “ignite” indicates igniting under the same conditions as are specified in the section of the igniting loss of each article.

(26) For the content of an ingredient determined by the quantitative method specified in each article, when it is expressed simply as "equal to or more than a certain percentage" without indicating its upper limit, 101.0% is understood as the upper limit. For example, content specified with the expression “contains a pure substance of a content equal to 90–110% of the labeled content” indicates that the substance is prepared so as to contain chemically pure substances or their equivalent at a 100% concentration, and the quantitation provides the percentage point within said range. Content expressed as “contains a potency of 85–125% of the labeled potency” indicates that the substance is prepared so as to maintain the labeled potency during its shelf life, and the quantitation provides the percentage point within said range.

(27) Any test methods may be employed to substitute for the general test methods and methods specified in each article when the same level of or a higher accuracy and precision are achieved by those methods; however, it is provided that the test using the specified methods shall be performed for the final determination if any questions have arisen as to the results.

(28) The term “container” indicates the container of the feed additives and includes all parts that constitute the container such as the cover and the lid.

(29) A “sealed container” indicates a container that is capable of protecting the contained feed additives from extraneous solids and from the loss of the feed additives under the ordinary or customary conditions of handling and storage. Where a sealed container is specified, it may be replaced by a tightly sealed container or by a hermetically sealed container.

(30) A “tightly sealed container” indicates a container that is capable of protecting the contained feed additives from extraneous solids, liquids or moisture, from the loss of the contents, and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling and storage. Where a tightly sealed container is specified, it may be replaced by a hermetically sealed container.

(31) A “hermetically sealed container” indicates a container that is impervious to any gases and microbes under the ordinary or customary conditions of handling and storage.

(32) A “light-shielded container” indicates a container that is made to shield light or having a light shielding encasement.

IV Exemplary Abstract

Referring to the chapter “III Items to be Described in the Required Documents (Abstract”, on page 16 and following, an abstract shall be drawn up following the style exhibited on page 58 and following.

The exemplary abstract, exhibited on page 58 and following, contains blank spaces that are marked with “…(omitted) …” where descriptions of the required items are omitted to simplify the explanation. In an actual abstract prepared for submission, all of the required items shall be described. If any items are omitted the reasons and grounds shall be presented.

Insufficient descriptions in the submitted documents, such as in the abstract, will cause an extended time for the document checks by the authorities, which may result in a delay of the deliberation in the council and the designation of the feed additive. Even if the deliberation is conducted in the council, the submitters may be requested to submit additional tests or documents, which will also defer the process. The abstract submitted as the very first action of the application is very important to avoid the above-mentioned situations.

In the preparation of the abstract, the cited test reports, academic papers, etc., shall be numbered by material numbers and submitted as attachments to the abstract.
Additionally, please refer to the chapter “VI Checklist for the Preparation of the Required Documents”, on page 76 and following, which summarizes the notes of caution for the wording and descriptions of the required items. Keep this checklist handy and make use of it during the preparation of the abstract. The checklist shall also be submitted together with the abstract after each of the items has been checked.

Abstract of the Test Results, etc., of the Objected Substance (xxxx)

mmmm, dd, yyyy

Feed Additive Business Operator

General name …(omitted)…	Chemical name …(omitted)…	Trade name …(omitted)…
Intended use and dosage …(omitted)…	Chemical structure …(omitted)…	

List of Items to be Described in the Abstract (page)

Document No.	Item	Specific item	Examined item and summary of results
	1. Origin or Background of the Discovery, Status of Authorization and Use as a Feed Additive in Foreign Countries, etc.	(1) Origin or background of the discovery (development) (2) Status of its authorization and use as a feed additive in foreign countries, etc. (3) Status of its manufacturing and distribution authorization, and its importation as a veterinary medicinal product (4) Comparison to related substances (generic substances having the same effect)	(1) Origin or background of the discovery (development) … (omitted) … (2) Status of the substance's authorization and use as a feed additive in foreign countries, etc. … (omitted) … (3) Status of the substance's manufacturing and distribution authorization, and its importation as a veterinary medicinal product … (omitted) … (4) Comparison to related substances (generic substances having the same effect) … (omitted) …
	2. Items Concerning Standards	(1) Name i. General name ii. Chemical name iii. Trade name	(1) Name i. General name … (omitted) … ii. Chemical name … (omitted) … iii. Trade name … (omitted) …

		(2)Chemical structure	(2) Chemical structure Definition … (omitted) … Potency … (omitted) … Structural formula, molecular formula, molecular weight, … (omitted) …
		(3) Manufacturing process	(3) Manufacturing process Oxidize Substance A (99.5%) with air using a platinum catalyst to produce Substance B. Initiate hydrolysis by adding sodium hydroxide in the special grade ethanol to produce the unrefined xxxx. Refine it through a solvent extraction using hexane, then dehydrate it to yield the objected substance xxxx Substance A (99.5%) ↓ platinum catalyst Substance B ↓ sodium hydroxide (in special grade ethanol) xxxx (unrefined) ,(by-product C) ↓ solvent extraction by hexane objected substance xxxx
		(4) Biological and physicochemical properties	(4) Biological and physicochemical properties
		i. Physical and chemical properties	i. Physical and chemical properties Physical and chemical properties a. Appearance: colorless or white crystal, or a white crystalline powder b. Chemical properties: freely soluble in water, soluble in methanol and practically insoluble in benzene. … (omitted) …
		ii. Identification test	ii. Identification test This substance shows an IR absorption peak at near 1,750 cm^{-1} of the wavenumber in the

			infrared absorption spectrum by the potassium bromide tablet method. The aqueous solution of this substance (1→10) shows a quantitative reaction with xx salt. … (omitted) …
		iii. Purity test	iii. Purity test a. Clarity and color of the solution: an aqueous solution prepared by dissolving 1.0 g (0.95–1.04 g) of this substance in 20 mL of water shows a light tan color and is practically clear. b. Chloride limit: When conducting a chloride limit test with 1.0 g (0.95–1.04 g) of this substance, the turbidity of the test solution does not exceed that of the control solution prepared with 0.5 mL of 0.01 mol/L hydrochloric acid. … (omitted) …
		iv. Content and quantitative method	iv. Content and quantitative method Content: This material contains 99.5% or more of the objected substance (describe the chemical formula of the objected substance) in the quantitation after drying. Quantitative method: Dry the material, weigh out 0.5 g of the material, dry down to 0.001 g and record the reading. Dissolve the weighed material in 50 mL of water, and add 5 mL of formalin and titrate with 0.5 mol/L sodium hydroxide solution (use three drops of the phenolphthalein indicator). Perform a blank test using the same method for correction. 1 mL of 0.5 mol/L sodium hydroxide solution = equivalent weight in mg, chemical formula of the objected substance … (omitted) …
		(5) Quantitation in feed	(5) Quantitation in feed Micronize the feed with the added substance, then weigh out 10 g (9.5–10.4 g) of the micronized feed. Dissolve the weighed material in 100 mL of a mixture of chloroform and ether (1:9). Extract the solution three times with 50 mL of a mixture of ethanol and methanol (6:4). Add 500 mL of purified water, then measure 100 mL of the obtained solution to yield the test solution. Add 2 mL of formalin to 10 mL of the test solution and titrate with 0.5 mol/L sodium hydroxide solution (use three drops of the phenolphthalein indicator). Perform a blank test using the same method for correction. … (omitted) …
		(6) Changes with time	(6) Changes with time

i. Storage test in an ambient temperature

i. Storage test in an ambient temperature

Pack the substance in a typical package (such as a plastic bag), then store the package in an indoor warehouse for 24 months at 25℃, 50% humidity to assess the stability. The observed changes with time are shown below.

[Results]

Table 1 Storage test in an ambient temperature

Test conditions: air temperature 25℃; humidity 50% (for period of 24 months); in a 20 kg paper bag

Lot No.	Parameter	At the start	3 mos.	6 mos.	9 mos.	12 mos.	24 mos.
A	Appearance	white powder	white powder	white powder	white powder	white powder	white mud[1]
	Identification test	fit	fit	fit	fit	fit	fit
	Purity test	fit	fit	fit	fit	fit	fit
	Amount of active ingredient (g)	214.6	212.1	210.4	208.3	205.4	207.9
	Drying loss(g)	5.2	4.9	3.7	5.0	6.3	4.7
	Amount of moisture (g)	32.3	30.0	29.4	35.1	33.8	35.3
B	Appearance	white powder	white powder	white powder	white powder	white powder	white powder
(omitted)							

Note 1: “White mud” indicates a white paste condition.

Although the appearance of the substance turned to a white mud (paste condition) after a period of 24 months, it passed both the identification test and the purity test. In addition, no significant changes in the amount of the active ingredient were found. No other problems were found; thus, it can be concluded that the substance remains stable for at least 24 months. … (omitted) …

ii. Heat resistance test

ii. Heat resistance test

		iii. Humidity resistance test iv. Light resistance test v. Accelerated test vi. In-feed stability test	… (omitted) … iii. Humidity resistance test … (omitted) … iv. Light resistance test … (omitted) … v. Accelerated test … (omitted) … vi. In-feed stability test … (omitted) …
	3. Items Concerning Efficacy	(1) Basic tests to prove the efficacy i. *In vitro* test ii. *In vivo* test (2) Field application tests to prove efficacy	(1) Basic tests to prove the efficacy i. *In vitro* test … (omitted) … ii. *In vivo* test … (omitted) … (2) Field application tests to prove efficacy Testing laboratory and place: xx laboratory in xx prefecture (indoor pig house) Test animals: Weaned piglets (Strain: xx; 25–30 days old; average body weight: 10.3 kg), four pens each of males and females in a group Dose and method of administration: The test animals were given feeds continuously with the objected substance added at the following different concentrations: 20, 40, 80, 100, 150 and 200 mg/kg, for four weeks which is an intended period in actual use. There was no non-exposure period. After four weeks, all of the test animals were slaughtered and subjected to a dissection examination. The test animals were given feeds continuously with the objected substance added at the

			following different concentrations: 20, 40, 80, 100, 150 and 200 mg/kg (each concentration corresponding to the following daily intake amounts of the objected substance per unit of body weight: 0.4, 0.8, 1.6, 2.0, 3.0 and 4.0 mg/kg-BW/day) for four weeks, which is an intended period in actual use. [Results] The test results are shown in the form below. (Appendix Form 2) The live weight gain significantly increased at the concentration of 40 mg/kg and higher ($p<0.05$). The live weight gain at 100 mg/kg did not show any difference to that at 80 mg/kg. Thus, 80 mg/kg can be thought to be the most effective concentration, even from an economical perspective. Succeeding tests were conducted at a concentration of 80 mg/kg as optimum additive amount. With regard to the intake amount of feed, this peaked at 100 mg/kg and the proportional increase to the concentration of the objected substance was not marked. … (omitted) …
	4. Items Concerning Residue		4. Items Concerning Residue Testing laboratory and place: xx laboratory in xx prefecture (indoor cow house) Test animals: Cows (Strain: xx; 2 years old; average body weight: 450 kg), 10 pens each of males and females in a group Dose and method of administration: The objected substance was mixed in the feed and fed continuously to the animals for 4 weeks. Feeds were given two times a day with the objected substance added at the following different concentrations: 100, 500, 1,000 and 5,000 g/kg. There was no non-exposure period. After four weeks, all of the test animals were slaughtered and subjected to a dissection examination. Method of analysis: The analysis was performed using the liquid chromatograph mass spectrometer (LC/MS) method. The limit of quantitation of this method (LC/MS) was 2.41 ng/g for the muscles, fat, liver, kidneys and milk. The average recovery rate was 85.7%, and the coefficient of variation was 0.1. The details of the method used are as follows. … (omitted) … [Result] The results of the analysis are summarized in the table below (Table 3). The residue in the milk and muscles (skeletal muscles) were below the limit of quantitation even in the samples from the cow that had consumed the feed with 1,000 mg/kg of the additive amount. The maximum residue, 8 mg/kg, was detected in the liver and kidneys from the cows that had

<table>
<tr>
<td></td>
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<td>consumed the feed with 1,000 mg/kg of the additive amount. In the fat of the abdominal area, residue was identified in the cows which had consumed the feed with 300 mg/kg and more of the additive amount. However, the amount of residue was equal to or less than the limit of quantitation in all parts of the body of the cows that had consumed the feed with 100 mg/kg of the additive amount, which is the maximum additive amount to produce the efficacy of the objected substance.

Table 3　Animal residue test of the objected substance in cows
<table>
<tr><td rowspan="2">Concentration of the objected substance in the feed (mg/kg)</td><td colspan="5">Analyzed body part</td></tr>
<tr><td>Muscles (skeletal muscles)</td><td>Liver</td><td>Kidneys</td><td>Fat (abdominal area)</td><td>Milk</td></tr>
<tr><td>100</td><td><2</td><td><2</td><td><2</td><td><2</td><td><1</td></tr>
<tr><td>300</td><td><2</td><td><2</td><td><2</td><td>10–15</td><td><1</td></tr>
<tr><td>1,000</td><td><2</td><td>5–7</td><td>5–8</td><td>40–50</td><td><1</td></tr>
</table>
Limit of quantitation: 1μg/kg for milk and 2μg/kg for other parts
… (omitted) …</td>
</tr>
<tr>
<td></td>
<td>5. Items Concerning Safety</td>
<td>(1) Toxicity tests
i. General toxicity tests
a. Single dose toxicity test</td>
<td>(1) Toxicity tests
i. General toxicity tests

a. Single dose toxicity test

Testing place and environment: xx laboratory in xx prefecture (indoor rearing facility)
Test animals: Mice (Strain: xx; 3 weeks old; average body weight: 7.9 g), 12 mice each of males and females in a group
Method of administration and dosage: Consumption of the water and feeds by the test animals was discretionary. The objected substance was administered at 100–2,000 mg/kg-BW by an oral gavage. General symptoms and any abnormalities, such as death, were observed for 2 weeks after the administration.
[Results]
On the seventh day of the test, a female mouse in the group of 200 mg/kg-BW was found dead. On the day before the death, symptoms of anorexia and hypopraxia were observed in the mouse. …. (omitted) …
Based on these results, the LD50 is estimated to be between 1,200 and 2,000 mg/kg-BW.</td>
</tr>
</table>

			The details of the results are summarized in the form below (Appendix Form 4) … (omitted) …
		ii. Repeated dose toxicity test (short term)	ii. Repeated dose toxicity test (short term) … (omitted) …
		iii. Repeated toxicity test (long term)	iii. Repeated toxicity test (long term) … (omitted) …
		ii. Special toxicity tests	ii. Special toxicity tests
		a. Transgenerational reproductive toxicity test	a. Transgenerational reproductive toxicity test (three generations) … (omitted) …
		b. Developmental toxicity test	b. Developmental toxicity test … (omitted) …
		c. Carcinogenicity test	c. Carcinogenicity test … (omitted) …
		d. Mutagenicity test	d. Mutagenicity test Conduct the test for gene mutation inducibility using six Salmonella typhimurium strains (TA98, TA100, TA102, TA1535, TA1537 and TA1538) and Escherichia coli bacteria WP2uvrA. Six dose levels of the objected substance were arranged with the 5,000 μg/plate as the maximum dose. Three plates for each test strains were cultured with the respective dose levels of the objected substance, and each of those plates were conditioned with and without the presence of the S9 mix for metabolic activation, along with negative control and positive control groups, at 37℃ for 3 days. After the culture, the revertant colonies were

counted for each individual plate.

[Results]
The results are summarized in the table below (Table 5
Table 5 Number of revertant colonies of Salmonella typ

S9mix	Concentration (μg/plate)	Salmonella t	
		TA98	TA100
(-)	0[*1]	13	160
	1.6	15	155
	8.0	14	152
	40.0	13	162
	200.0	15	161
	1,000.0	14	158
	5,000.0	11	169
	Positive control	12	163
(+)	0[*1]	(omi	
(omitted)			

(*1) Negative control
No increase in the number of revertant colonies wa
including the maximum dose group, regardless of the
activation.
Based on the above results, it can be concluded that t
mutagenicity.

e. Other tests

… (omitted) …

e. Other tests
Other tests were not conducted.

iii. Pharmacological test

iv. Test concerning the *in vivo* kinetics and metabolism

iii. Pharmacological test

The pharmacological test was omitted because the objected substance does not have any pharmacological effects.

iv. Test concerning the *in vivo* kinetics and metabolism

A single dose of the objected substance ^{12}C was replaced by ^{14}C, and was administered via an oral route to a mouse, cow, dog and a human. All of the urine and feces excreted within 24 hours from the time of the administration were collected for the analysis of the dynamic state of ^{14}C inside the bodies. Information about the tested individual mouse, cow dog and human is as follows. … (omitted) …

[Results]

Mouse: The ^{14}C excretion in 24 hrs was 0.48% in the urine and 89.2% in the feces; almost all the ^{14}C was excreted into the excreta in 72 hrs.; and 97.5% of the total excreted radioactive substance in the excreta was in the form of metabolites with 1.5% of the original substance at the 72 hr time point from the administration. The metabolite had experienced an O-demethylation reaction and had bioactivity. The level of the metabolite bioactivity was measured at 0.01, compared to that of the objected substance being 1.0. Almost all of the objected substance (approximately 98%) was metabolized in the liver and excreted as metabolites. Similar data was obtained in the cow: 98% of the objected substance was metabolized in the liver and was excreted as metabolites.

Table 6 Results of the quantitative analysis of ^{14}C in the feces in the single dose administration test of the ^{14}C labeled objected substance via an oral route (ratio in a 100-scale against the total administered ^{14}C)

Animal species	Test sample	Elapsed time (hrs.)			3 days total
		24	48	72	
Mouse	Urine	0.48	0.66	0.32	1.46
	Feces	89.29	4.34	3.91	97.54
Cow	Urine	0.61	0.52	0.10	1.23
	Feces	89.44	5.78	2.98	98.20
(omitted)					

Scintigraphy for the analysis of the dynamic state of the radioactive substance inside the body, as well as an infrared spectroscopy and nuclear magnetic resonance spectroscopy for

		(2) Feeding test using targeted animals	the identification of the chemical structure were used. The details of the method are as follows. … (omitted) … (2) Feeding test using targeted animals Testing place and environment: xx laboratory in xx prefecture (indoor pig house) Test animals: Pigs (Strain: xx; 30 days old; average body weight: 8.2 kg), 15 pens each of males and females in a group Dose and method of administration: Continuous administration was given by mixing the objected substance in the feeds for 3 months. Consumption of the water and feeds by the test animals was discretionary. The dose was 50–800 mg/kg (with 80 mg/kg as the optimum dose). … (omitted) … [Results] The results are summarized in the table below (Appendix Form 7). The live weight gain of the 80 mg/kg group was 15.4 kg larger than that of the control group ($p<0.01$). Although an increase in both the live weight gain and the intake of feeds was shown in the group given a 10 times larger dose than the optimum dose, a significant difference was not indicated when compared to the group without the additive. Two female pigs became anorexic, while no noticeable changes were observed as general symptoms. One of these two pigs showed a mild gastric erosion, which was cured in a week or so. After the cure, the live weight gain increased. … (omitted) …
		(3) Test concerning the emergence of resistant bacteria	(3) Test concerning the emergence of resistant bacteria This test was not conducted because the objected substance is not an antimicrobial agent, nor a live microbial agent.
		(4) Other tests	(4) Other tests This test was not conducted because no effects are anticipated by the use of the objected substance as a feed additive.

List of the original papers used for the preparation of the Abstract

No. of original paper	Title of original paper
1	(omitted)
2	(omitted)
3	(omitted)
4	(omitted)
5	(omitted)
(omitted)	

1

Title of Original Paper 1

*The original papers (attachments), etc., cited in the abstract are exhibited on the next page and the following pages.

V Closing

This handbook has been drawn up for those who are preparing the documents required for the designation of a mainly chemical substance as a feed additive for the first time.
For a feed additive designation to be granted, substantial documents are required to prove the substance's efficacy, safety, etc. Additional documents may be requested during the professional deliberations conducted by the council members. Therefore, it should be borne in mind that the submission of the documents that are prepared in full conformity to the requirements of this handbook does not necessarily guarantee the substance's successful designation, even if the intended substance is a chemical substance. If the intended substance is an antibiotic or a live microbial agent, significantly different documents are required from those needed for chemical substances.
Please contact the authorities or the Japan Scientific Feed Association with any questions.

In closing, we hope that this handbook will come in useful for those who are preparing such documents.
We would like to thank the Japan Scientific Feed Association and many other groups and individuals for their support and advice during the preparation of this handbook.

Contact

Feeds Safety Standard Group,
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Food Safety and Consumer Affairs Bureau,
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Postal Code: 100-8950
1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo
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General Incorporated Association
Japan Scientific Feed Association
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2-6-16 Shinkawa, Chuo-ku, Tokyo
Bajichikusan Kaikan Bldg.
Phone: 03-3297-5631

VI Checklist for the Submission of the Required Documents

Review and make sure that following items are properly completed, then enter a check mark in each check box (bold-outlined box) and submit it with the abstract.
Leave check boxes blank (no check mark) for items that are not relevant or are not confirmed.

Checked by: _____

Check box	Items to be checked
[In writing the abstract]	
	Ensure unity of the character style (Japanese characters, alphanumeric characters), with no mixing of one-byte and two-byte characters.
	Use a 12 point or larger font size, and do not use characters that are extremely difficult to read.
	Ensure there are no typographical errors, omissions, or transcription errors.
	Use proper punctuation (spaces, paragraphs, line feeds, etc.) for better readability. Split long sentences into two sentences.
	Use the unit signs commonly used in Japan (if not, convert these). [Example] pound→kilogram; ° → ℃; ppm→mg/kg or μg/mL
	Use general names, not trade names.
	Ensure uniformity of the writing style. [Example] Use general names; xx sodium salt (hereafter referred to as “xx”); xx salt of hydrochloric acid or hydrochloric acid xx salt
	Use tables and figures effectively for easy-to-understand explanations. [Example] Number tables and figures (Table 1, Figure 1, etc.).
	Clearly distinguish facts from presumptions. Where facts are stated, supporting documents have to be attached.
	The abstract must have a Table of Contents with a page index.
	Bind the documents with a front cover and back cover, both having the title of the document and the company name.
	The abstract is laid out in line with the “Notification on the Documents, etc., Required for the Submission for the Designation of Feed Additives” (issued on February 4, 1980).
	If there are findings from a previous deliberation, those findings and the response papers have to be included.
	Any data and the descriptions of items which are the same as previously submitted documents must be noted accordingly,

	and the relevant documents will still have to be attached and included.
[Attachments – Original papers, reports, etc.]	
	Make sure that there are no potential copyright infringements in the use of original papers.
	Number original papers (attachments) with serial numbers, create a list of these, and bind them using indexes, file separators, etc., for easy-to-read documents.
	Make sure that the document numbers correspond accurately to the original papers (attachments). Make sure that there are no mistakes nor gaps in the numbering.
	Make sure that cited texts from original papers (attachments) are marked using underlines, highlights, etc., for easy searching. (This is required only for the documents submitted to the authorities. The documents prepared for committee members that are otherwise instructed, or the documents for the deliberation by the council do not require this marking.)
	Make sure that all cited original papers (attachments) are included.
	Make sure that all technical terms written in English are translated into the correct Japanese terms.
	Make sure that the information and contents of the original papers (attachments), etc. are not misconstrued.
[Standards]	
	Check whether the tone of the description of items concerning the standards for a new substance accord with those for known substances. [Reference] List of the Specifications and Standards of Feed Additives (General Incorporated Association, Japan Scientific Feed Association)
	Are the identification tests simple enough to be repeated in an actual operating environment?
	Has the possibility of performing the identification test by a known identification test method been discussed?
	Are the manufacturing processes of the ingredients for the manufacturing and the formulation described in detail?
	Has the provision of samples to the FAMIC been arranged?
[Tests]	
	Have the tests that are subject to the “feed additive GLP” been performed in accordance with these principles?
	Are the facts and discussions clearly separated in the description of the test results?
	Is the test program described in enough detail to be duplicated?
	Are the values of the concentration in the feeds converted into the daily intake amount per unit of body weight? [Example] ppm, mg/kg → mg/kg-BW/day [Reference] Cite the data of the daily feed intake and the body weight of the livestock animals from the literature, etc., (or

<table>
<tr><td></td><td>from the Japan Feed Standard if no relevant literature can be found) and then calculate the converted value on an as-needed basis: concentration in feeds (mg/kg-feed) x daily feed intake/body weight.
Example of the feeds added at a concentration of 100mg/kg (daily feed intake: 2.89 kg/day; animal body weight: 93 kg-BW), 100 mg/kg×2.89 kg/day/93 kg-BW = 3.11 mg/kg-BW/day (round off the value on an as-needed basis)</td></tr>
<tr><td></td><td>Have the test results been statistically analyzed? When a significant difference is shown, the details of the analysis must be described.
[Example]…(omitted)… a significant difference was shown (t-test: $p<0.05$)</td></tr>
<tr><td></td><td>Are appropriate significant digits used for the data description?
Difference between “1” and “1.0”: “1” indicates a value in the range of 0.5–1.4; “1.0” indicates a value in the range of 1.05–1.14.</td></tr>
<tr><td></td><td>Check for any missing items to be described in each test: number of test animals, period of administration, tests omitted even if an omission not permissible, conformity to the evaluation standards, etc.
<table>
<tr><th colspan="2">Items Concerning Standards, Changes with Time</th><th colspan="2">Items Concerning Efficacy</th><th>Items Concerning Residue</th></tr>
<tr><td>Each storage test</td><td>Stability tests</td><td>Basic tests</td><td>Field application tests</td><td>Residue test</td></tr>
<tr><td colspan="5">Items Concerning Safety</td></tr>
<tr><td>Single dose toxicity test</td><td>Repeated dose toxicity test (short term)</td><td>Repeated dose toxicity test (long term)</td><td>Pharmacological test</td><td>In vivo kinetics test</td></tr>
<tr><td>Transgenerational reproductive toxicity test</td><td>Developmental toxicity test</td><td>Carcinogenicity test</td><td>Mutagenicity test</td><td>Feeding test</td></tr>
</table></td></tr>
<tr><td></td><td>In cases where tests, etc., have been omitted, has the adequacy of the omission been presented, or is the submitter ready to present this when requested?</td></tr>
</table>