Data Requirements for Supporting Registration of Agricultural Chemicals

(The Notification, Ref. No. 12-Nousan-8147, issued on November 24, 2000 by the Director-General, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan)

The Notification, Ref. No. 13-Seisan-1739, partly revised on June 26, 2001
The Notification, Ref. No. 14-Seisan-7269, partly revised on December 10, 2002
The Notification, Ref. No. 16-Shoan-6197, partly revised on November 24, 2004
The Notification, Ref. No. 16-Shoan-9260, partly revised on March 16, 2005
The Notification, Ref. No. 18-Shoan-14851, partly revised on April 2, 2007
The Notification, Ref. No. 19-Shoan-14966, partly revised on March 31, 2008
The Notification, Ref. No. 22-Shoan-10015, partly revised on April 1, 2011
The Notification, Ref.No.25-Shoan-630, partly revised on May 31, 2013
The Notification, Ref.No.26-Shoan-532, partly revised on May 15, 2014

(Appendix)

Section I Regarding specific details of test results

The specific details of test results regarding efficacy, phytotoxicity, toxicity and persistence of an agricultural chemical that an applicant (hereinafter referred to as "the applicant") shall submit for registration of an agricultural chemical (excluding those having microorganisms as active ingredients; the same shall apply hereinafter) pursuant to Article 2, paragraph (2) (including cases where it is applied mutatis mutandis pursuant to Article 15.2, paragraph (6)) and Article 6.2, paragraph (1) (including cases where it is applied mutatis mutandis pursuant to Article 15.2, paragraph (6); the same shall apply hereinafter) of the Agricultural Chemicals Regulation Law (No. 82, 1948; hereinafter referred to as "the Law") shall be as follows.

(1) Test results regarding efficacy

Test results regarding efficacy against the applicable diseases or pests (test results regarding efficacy to the applicable crops, in the case of an agricultural chemical used to promote or suppress physiological functions of crops)

(2) Test results regarding phytotoxicity

(a) Test results regarding phytotoxicity to applicable crops
(b) Test results regarding phytotoxicity to adjacent crops
(c) Test results regarding phytotoxicity to succeeding crops

(3) Test results regarding toxicity

(a) Test results regarding acute oral toxicity
(b) Test results regarding acute dermal toxicity
(c) Test results regarding acute inhalation toxicity
(d) Test results regarding skin irritation
(e) Test results regarding eye irritation
(f) Test results regarding skin sensitization
(g) Test results regarding acute neurotoxicity
(h) Test results regarding acute delayed neurotoxicity
(i) Test results regarding 90-day repeated dose oral toxicity
(j) Test results regarding 21-day repeated dermal toxicity
(k) Test results regarding 90-day repeated inhalation toxicity
(l) Test results regarding repeated dose oral neurotoxicity
(m) Test results regarding 28-day repeated administration delayed neurotoxicity
(n) Test results regarding 1-year repeated dose oral toxicity
(o) Test results regarding carcinogenicity
(p) Test results regarding reproductive toxicity
(q) Test results regarding teratogenicity
(r) Test results regarding mutagenicity
(s) Test results regarding pharmacology
(t) Test results regarding metabolism in animals
(u) Test results regarding metabolism in plants
(v) Test results regarding metabolism in livestock
(w) Test results regarding behavior in soil
(x) Test results regarding behavior in water

(y) Test results regarding toxicity on aquatic animals and plants
(z) Test results regarding toxicity on beneficial organisms other than aquatic animals and plants
(aa) Test results regarding the properties, stability, degradability, etc. of active ingredients
(ab) Test results regarding derivation of predicted environmental concentration

(4) Test results regarding persistence

(a) Test results regarding residues in crops
(b) Test results regarding residues in livestock
(c) Test results regarding persistence in soil

Section II Regarding conditions relevant to preparation of test results

The test results listed in Section I shall be obtained by conducting the tests listed in the "Test items" column of Appendix Table 1, under the conditions listed in the corresponding "Conditions necessary for conducting tests" column of the said table. The respective test methods used shall be provided in Annex entitled "Guidelines on Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals". The test results regarding "monitoring agricultural chemical concentration in the rivers" among test results regarding derivation of predicted environmental concentration shall only apply to existing registered agricultural chemicals.

Section III Regarding the submission of test results

Regarding the submission of test results listed in Section I, the applicant shall submit a list of the individual test results submitted, reports as to quality check for individual test results, comprehensive summaries and assessments of individual test results, and a completed form for the checking of dossiers for completeness, together with the individual test results. Necessary matters for the submission of these documents shall be provided for separately by the Director of Plant Products Safety Division of the Food Safety and Consumer Affairs Bureau.

Section IV Exceptions as regards submission of test results

In the cases listed in Appendix Table 2 and other cases where there are reasonable grounds on which the submission of some parts of test results is unnecessary in consideration of the type of active ingredients, the formulation type, the application method etc. of the relevant agricultural chemical, the applicant may submit documents that state the said grounds instead of the said test results, notwithstanding the provisions of Section I.

Section V Regarding substitutes for test results

(1) If some parts of the test results that are to be submitted with an application for registration of an agricultural chemical have already been submitted with another application for registration, and if it is found to be able to use those test results as the test results of the agricultural chemical pertaining to said application, the applicant may submit a Test Results Substitution Request (see Appended Form) instead of the relevant test results.

(2) In this case, if the person who has submitted the test results to be used is not the applicant, the applicant shall attach a document in which the person who has submitted the test results to be used states that he/she has no objection to the applicant's using the test

results.

If some parts of the test results (limited to test results listed in Section I, paragraph (3), items (a) to (c) and items (t) to (x), and paragraph (4), items (a) to (c)) that are to be submitted with an application for registration of an agricultural chemical have already been submitted with another application for registration 15 or more years previously, and if it is found that the agricultural chemical pertaining to the present application for registration is equivalent in its components, physical and chemical properties, toxicity to humans and livestock and other characteristics to the existing registered agricultural chemical for which 15 or more years have elapsed from its initial registration, the applicant may submit a Test Results Substitution Request pursuant to Appended Form instead of the relevant test results.

Section VI Regarding requests for additional test results, etc.

The applicant may be requested to submit additional test results etc. in regard to the agricultural chemical pertaining to the application, when it is found that those test results are necessary for the evaluation for registration of agricultural chemicals conducted pursuant to Article 2, paragraph (3) of the Law.

Section VII Regarding submission of reports on toxicity of agricultural chemicals

In order to ensure the quality and safety of the agricultural chemical pertaining to the application, the applicant shall endeavor to submit information on toxicity that was obtained from sources other than the test results listed in Section I, item (3), to the Minister of Agriculture, Forestry and Fisheries as much as possible. The same shall apply after registration of the agricultural chemical.

Section VIII Regarding handling of findings on agricultural chemicals

The applicant shall endeavor to disclose findings obtained from tests on the efficacy, phytotoxicity, toxicity and persistence of the agricultural chemical submitted at the time of application in an academic society, academic journal, or web page within three years after registration, in principle.

Supplementary Provisions (March 16, 2005)

This notification shall apply to the test results regarding efficacy, phytotoxicity, toxicity and persistence of agricultural chemicals that are submitted on and after April 1, 2005. However, “Test facility standards” pertaining to the test items (2), (4), (6), (7), (8) and (9) relating to test results regarding the toxicity on aquatic animals and plants listed in the Appendix Table 1 shall not apply to the tests initiated on or before 31 March, 2005.

Supplementary Provisions (April 2, 2007)

Amendments in accordance with this notification shall apply to the test results regarding efficacy, phytotoxicity, toxicity and persistence of agricultural chemicals that are submitted on and after April 2, 2008. However, “Test facility standards” pertaining to *bioconcentration test*, *test on residues in soil* and *test on residues in succeeding crops* listed in the Appendix Table 1, and Annex “Guidelines on Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals” shall apply to the tests initiated on and after October 2, 2007. An applicant may submit the test results in accordance with the notification revised by this notification before April 2, 2008.

Supplementary Provisions (March 31, 2008)

1. Amendments in accordance with this notification shall apply to the test results regarding efficacy, phytotoxicity, toxicity, and persistence of agricultural chemicals that are submitted on and after April 1, 2008. However, "Test facility standards" in Appendix Table 1, and Annex "Guidelines on Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals" shall apply to the tests initiated on and after April 1, 2008.

2. Regarding the "Test facility standards" pertaining to test results regarding residues in crops listed in Appendix Table 1, and Annex "Guidelines on Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals", if the tests were initiated on or before March 31, 2011 and conducted by official or semi-official test and research facilities according to the provisions of the former notification, those test results shall be deemed as test results conducted by test facilities which comply with the GLP standards for agricultural chemicals prescribed in this notification.

3. In the case of the preceding paragraph, regarding the specified number of replicates relevant to sample analysis conducted by a test facility which is deemed as a test facility conforming to the GLP standards for agricultural chemicals, the provisions prior to amendments by this notification shall apply. However, this shall not apply to cases where a sample analysis laboratory of a test facility which has already been certified for compliance with the GLP standards for agricultural chemicals regarding test results on residues in crops conducts the analysis under entrustment by the test facilities referred to in the preceding paragraph.

Supplementary Provisions (April 1, 2011)

1. The amendments in accordance with this notification shall apply to the test results regarding efficacy, phytotoxicity, toxicity, and persistence that are submitted on and after April 1, 2011. However, the amendments defined in the following items shall apply to the tests or test results stipulated in each item.
   (1) The amendments pertaining to item (3) of paragraph 8 of "3-1-1" (including cases where there are provisions described as "Follow the provisions of test on residues in crops" in "3-2-2") of Section 4 of Annex "Guidelines for Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals": Tests initiated on and after October 1, 2011.

   (2) The amendments pertaining to "Number of trials/Type of test crops, test animals, etc." and "Test facility standards" of "Test results regarding residues in crops" listed in Appendix Table 1 of Section II, and the amendments pertaining to Appendix Table 1 of item (1) of paragraph 6 of "3-1-1" of Section 4 of Annex "Guidelines for Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals": The test results regarding residues in crops which are submitted on and after April 1, 2014 and fall under the following sub-item ① or ②:
   ① The test results pertaining to tests used for newly setting a standard value stipulated in "Specifications and Standards for Food, Food Additives, etc" (Ministerial

Notification No. 370 of the Ministry of Health and Welfare, dated December 28, 1959) (referred to as "the standard value" in sub-item ②)

② The test results pertaining to tests used for changing the existing standard value

2. With regard to the test results which do not fall under sub-item ① nor ② of item (2) of the preceding paragraph, the provisions of the former notification shall remain applicable.

Supplementary Provisions (May 31, 2013)

The amendments in accordance with this notification shall apply to the test results regarding efficacy, phytotoxicity, toxicity, and persistence that are submitted on and after May 31, 2013. However, the amendments pertaining to "2-7-6" of Section 3 of Annex "Guidelines on Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals" shall apply to the tests initiated on and after December 1, 2013. An applicant may submit test results in accordance with the notification revised by this notification before December 1, 2013.

Supplementary Provision (May 15, 2014)

1. The amendments in accordance with this notification shall apply to the test results that are submitted when an application for registration of an agricultural chemical is made on and after May 15, 2014(hereinafter referred to as "the effective date"). However, the amendments other than those pertaining to Section III shall apply to the test results that are submitted when an application for registration of an agricultural chemical is made on and after the day on which three years have elapsed from the effective date (excluding test results pertaining to an application for re-registration of an agricultural chemical currently being registered).

2. Notwithstanding the provisions of the preceding paragraph, with regard to the test results pertaining to an application for registration of an agricultural chemical which is made before the day on which one year has elapsed from the effective date, or the test results pertaining to an application for registration of an agricultural chemical which contains the same active ingredient as the existing registered agricultural chemical for which the test results had been submitted pursuant to the provisions of Section III of the notification prior to the revision by this notification, the applicant may submit all or part of the test results in accordance with the provisions then in force, as specified separately by the Director of Plant Products Safety Division of the Food Safety and Consumer Affairs Bureau.

3. Notwithstanding the provisions of the proviso in paragraph 1, in accordance with the notification revised by this notification(hereinafter referred to as "the new notification"), an applicant may submit the test results regarding Section I, item (3)(v) and item (4)(b) of the new notification before the day as prescribed in the proviso of that paragraph.

4. Notwithstanding the provisions of the proviso in paragraph 1, with regard to the test facility standards as prescribed in Appendix Table 1 for the tests regarding Section I, item (3)(v) and item (4)(b) that are commenced before the day on which six months have

elapsed from the effective date, the test facility standards of Appendix Table 1 of the new notification shall be possible not to apply.

5. Notwithstanding the provisions of the proviso in paragraph 1, with regard to agricultural chemicals which are currently registered or for which registrations are currently being applied for on the day as prescribed in the proviso of that paragraph, if they meet requirements as provided for separately by the Director of Plant Products Safety Division of the Food Safety and Consumer Affairs Bureau, the provisions of Section I, item (3)(v) and item (4)(b) of the new notification shall apply and the relevant test results shall be submitted by the day on which six years have elapsed from the effective date. However, with regard to agricultural chemicals for which the Director of Plant Products Safety Division of the Food Safety and Consumer Affairs Bureau separately gives notice of a time limit for the submission, the relevant test results shall be submitted by that time limit.

(Appended Form)

Test Results Substitution Request

Year Month Date

To: The Minister of Agriculture, Forestry and Fisheries of Japan

Address:

Name: (Corporate name, if any and name of representative) Personal seal

I hereby request substitution of test results pertaining to application for registration of agricultural chemicals pursuant to Section V of Data Requirements for Supporting Registration of Agricultural Chemicals (The Notification Ref. No. 12-Nousan-8147, issued on November 24, 2000 by the Director-General, Agricultural Production Bureau).

1. The type and name of the agricultural chemical pertaining to the application for registration (In addition, state the registration number of the agricultural chemical in the case it is currently being registered.).

2. Test results which are subject to substitution, and the type and name of the agricultural chemical pertaining to the test results to be used as substitution (In addition, state the registration number of the agricultural chemical in the case it is currently being registered.).

(Japanese Industrial Standards A4)

Note: Use of a personal seal may be omitted if the applicant (or representative, in the case of a corporate applicant) uses his/her signature.

(Appendix Table 1)

Test Results	Test items	Conditions necessary for conducting tests			
		Type of test substance	**Number of trials /Type of test crops, test animals, etc.**	**Test facility standards**	**Test method number (see Annex)**
Test results regarding efficacy against the applicable diseases or insect pests (test results regarding efficacy to the applicable crops, in the case of an agricultural chemical used to promote or suppress physiological functions of crops.)	Efficacy test (Note 1)	Formulation (Note 6)	Shown in Appended Table 1.	Test facilities capable of adequate conduct of efficacy test	1-1-1
Test results regarding phytotoxicity to the applicable crops	(1) Phytotoxicity test (Note 2)	Formulation (Note 6)	Shown in Appended Table 1.	Test facilities capable of adequate conduct of phytotoxicity test	1-1-1
	(2) Limit test for phytotoxicity	Formulation	2 trials on each applicable crop (In cases where the applicable crop is a crop group, the type of crop to be tested and the number of trials shall follow the rules set by the Director of Plant Products Safety Division.)	No particular provisions	1-1-2
	(3) Test on residual odor in tea	Formulation	2 trials	Test facilities capable of adequate conduct of phytotoxicity test	1-1-3
	(4) Tobacco taste test	Formulation	2 trials (3 trials in cases where foliage is directly exposed to the relevant agricultural chemical, or where the active ingredients of the relevant agricultural chemical are absorbed and translocated via the roots.)	Test facilities capable of adequate conduct of phytotoxicity test	1-1-4
Test results regarding phytotoxicity to adjacent crops	(1) Test on phytotoxicity due to drift and scattering	Formulation	Select 1 representative type of crop from each of the following crop family, on the basis of applicable crops and applicable sites: the Solanaceae, the Cucurbitaceae, the Brassicaceae, the Leguminosae, the Poaceae, etc.	No particular provisions	1-2-1
	(2) Test on phytotoxicity due to runoff from paddy water	Formulation	Select 1 representative type of crop from rush, lotus root, *Kuwai* (*Sagittaria trifolia* var. *edulis*) etc.	No particular provisions	1-2-2

	(3) Test on phytotoxicity due to volatilization	Formulation	Select 1 type of crop that is thought to be highly susceptible to the relevant agricultural chemical.	No particular provisions	1-2-3
Test results regarding phytotoxicity to succeeding crops	Succeeding crops phytotoxicity test	Formulation	From among crops that are potentially cultivated after the applicable crops, select 1 type that is thought to be highly susceptible to the relevant agricultural chemical.	No particular provisions	1-3
Test results regarding acute oral toxicity	Acute oral toxicity test	Technical grade active ingredient (TGAI) and formulation	1 type of test animal for each test substance (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-1
Test results regarding acute dermal toxicity	Acute dermal toxicity test	TGAI and formulation	1 type of test animal for each test substance (usually rats, rabbits or guinea pigs)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-2
Test results regarding acute inhalation toxicity	Acute inhalation toxicity test	TGAI and formulation	1 type of test animal for each test substance (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-3
Test results regarding skin irritation	Skin irritation test	Formulation (or TGAI, if formulation is difficult to use)	1 type of test animal (usually rabbits)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-4
Test results regarding eye irritation	Eye irritation test	Formulation (or TGAI, if formulation is difficult to use)	1 type of test animal (usually rabbits)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-5
Test results regarding skin sensitization	Skin sensitization test	TGAI and formulation	1 type of test animal for each test substance (usually guinea pigs)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-6
Test results regarding acute neurotoxicity	Acute neurotoxicity test	TGAI	1 type of test animal (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-7
Test results regarding acute delayed neurotoxicity	Acute delayed neurotoxicity test	TGAI	1 type of test animal (usually chickens)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-8
Test results regarding 90-day repeated dose oral toxicity	90-day repeated dose oral toxicity test	TGAI	2 types of test animals (usually rats and dogs) (However, 1 type of test animal if the relevant agricultural chemical is found to be safe because there is no risk that humans will ingest its components for a long period, in light of its formulation type, its application method etc.)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-9

Test results regarding 21-day repeated dermal toxicity	21-day repeated dermal toxicity test	TGAI	1 type of test animal (usually rats, rabbits or guinea pigs)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-10
Test results regarding 90-day repeated inhalation toxicity	90-day repeated inhalation toxicity test	TGAI	1 type of test animal (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-11
Test results regarding repeated dose oral neurotoxicity	Repeated dose oral neurotoxicity test	TGAI	1 type of test animal (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-12
Test results regarding 28-day repeated administration delayed neurotoxicity	28-day repeated administration delayed neurotoxicity test	TGAI	1 type of test animal (usually chickens)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-13
Test results regarding 1-year repeated dose oral toxicity	1-year repeated dose oral toxicity test (1-year repeated dose oral toxicity/ carcinogenicity combined test) (Note 3)	TGAI	2 types (usually rats and dogs) 1 type may be concurrently used in carcinogenicity test.	Test facilities conforming to GLP standards for agricultural chemicals	2-1-14 (2-1-16)
Test results regarding carcinogenicity	Carcinogenicity test (1-year repeated dose oral toxicity/ carcinogenicity combined test) (Note 4)	TGAI	2 types of test animal (usually rats and mice) 1 type may be concurrently used in 1-year repeated dose oral toxicity test.	Test facilities conforming to GLP standards for agricultural chemicals	2-1-15 (2-1-16)
Test results regarding reproductive toxicity	Reproductive toxicity test	TGAI	1 type of test animal (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-17
Test results regarding teratogenicity	Teratogenicity test	TGAI	2 types of test animals (usually rats and rabbits) If reproductive toxicity test are conducted, 1 type shall be of the same species and strain as the test animal used in that test.	Test facilities conforming to GLP standards for agricultural chemicals	2-1-18
Test results regarding mutagenicity	(1) Reverse mutation test	TGAI	1 trial (Conduct test by using bacteria.)	Test facilities conforming to GLP standards for agricultural chemicals	2-1-19-1
	(2) Chromosomal aberration test	TGAI	1 trial (Conduct test by using cultured mammalian cells.)		2-1-19-2
	(3) Micronucleus test	TGAI	1 trial (Conduct test by using mammals.)		2-1-19-3
Test results regarding pharmacology	Pharmacology test	TGAI	1 trial (Conduct test by using species of animals appropriate for each inspection item)	Test facilities conforming to GLP standards for agricultural chemicals	2-2-1
Test results regarding metabolism in animals	Test on metabolism in animals	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes	1 type of test animal (usually rats)	Test facilities conforming to GLP standards for agricultural chemicals	2-3-1

Test results regarding metabolism in plants	Test on metabolism in plants	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes	Shown in Appended Table 1	Test facilities conforming to GLP standards for agricultural chemicals	2-4-1
Test results regarding metabolism in livestock	Test on metabolism in livestock	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes	2 types of test animals (1 type of ruminant and 1 type of poultry)	Test facilities conforming to GLP standards for agricultural chemicals	2-4-2
Test results regarding behavior in soil	(1) Test on behavior in flooded aerobic soil	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-5-1
	(2) Test on behavior in aerobic soil	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes; however, test shall also be conducted in regard to major metabolites that were detected in the test on behavior in flooded aerobic soil, if it is deemed necessary on the basis of that test results.	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-5-2
	(3) Test on behavior in anaerobic soil	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes; however, test shall also be conducted in regard to major metabolites that were detected in the test on behavior in aerobic soil, if it is deemed necessary on the basis of that test results.	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-5-3
Test results regarding behavior in water	(1) Test on hydrolytic behavior	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-6-1
	(2) Test on photolytic behavior in water	Active ingredients, etc. that are either labeled or unlabeled with radioactive isotopes	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-6-2
Test results regarding toxicity on aquatic animals and plants	(1) Fish acute toxicity test	TGAI and formulation	1 trial for each test substance (In regard to TGAI, conduct test by using common carp or rice fish (*Oryzias latipes*).)	Test facilities conforming to GLP standards for agricultural chemicals	2-7-1-1
		TGAI	Optional species (Conduct test by using optional species from among bluegill, rainbow trout, guppy, zebra-fish and fathead minnow).		

	(2) Fish (larvae) acute toxicity test	TGAI	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-7-1-2
	(3) *Daphnia spp* acute immobilization test	TGAI and formulation	1 trial for each test substance (In regard to TGAI, conduct test by using *Daphnia magna.*)	Test facilities conforming to GLP standards for agricultural chemicals	2-7-2-1
	(4) *Daphnia* (adult daphnids) acute immobilization test	TGAI	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-7-2-2
	(5)*Daphnia spp* reproduction test	TGAI	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-7-2-3
	(6) Test on effects of coexistent organic substances on fish acute toxicity/ *Daphnia spp* acute immobilization	TGAI	1 trial (conduct test by using rice fish (*Oryzias latipes*) or *Daphnia magna.*)	Test facilities conforming to GLP standards for agricultural chemicals	2-7-3
	(7) Freshwater shrimp acute toxicity test	TGAI	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-7-4
	(8) Amphipoda acute toxicity test	TGAI	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-7-5
	(9) *Chironomus* sp., acute immobilization test	TGAI	1 trial	Test facilities conforming to GLP standards for agricultural chemicals	2-7-6
	(10) Algae growth inhibition test	TGAI and formulation	1 trial for each test substance (In regard to TGAI, conduct test by using *Pseudokirchneriella subcapitata* (old scientific name: *Selenastrum capricornu*).)	Test facilities conforming to GLP standards for agricultural chemicals	2-7-7
Test results regarding toxicity on beneficial organisms other than aquatic animals and plants	(1) Bee toxicity test	TGAI or formulation	1 trial	No particular provisions	2-8-1
	(2) Silkworm toxicity test	TGAI or formulation	1 trial	No particular provisions	2-8-2
	(3) Natural enemy insect, etc. toxicity test	TGAI or formulation	Select 3 species among 2 orders from among the following orders: Diptera, Hymenoptera, Hemiptera, Coleoptera, Neuroptera, Acari, and Araneae	No particular provisions	2-8-3

	(4) Avian toxicity test				
	(i) Avian acute oral toxicity test	TGAI	1 trial	No particular provisions	2-8-4-1
	(ii) Avian dietary toxicity test	TGAI	1 trial	No particular provisions	2-8-4-2
Test results regarding the properties, stability, degradability, etc. of active ingredients	Color test, test on physical state of substance, odor test, spectrum test, melting point test, boiling point test, vapor pressure test, test on solubility in water, test on solubility in organic solvent, soil adsorption test, n-octanol/water partition coefficient test, density test, hydrolysis test, dissociation constant test, thermal stability test, test on photolysis in water, bioconcentration test	Active ingredients, etc. in their pure state (Note 5) (or if it is difficult to use active ingredients in their pure state; TGAI: if active ingredients are composed of multiple substances and those substances can be separated; each separated substance)	1 trial for each test substance	Test facilities conforming to GLP standards for agricultural chemicals (excluding color test, test on physical state of substance and odor test)	2-9-1~17
Test results regarding derivation of predicted environmental concentration	(1)Test on water polluting properties	Formulation	2 trials	Test facilities capable of adequate conduct of test on derivation of predicted environmental concentration	2-10-1
	(2) Test on agricultural chemical concentration measurement in paddy water of model paddy	Formulation	2 trials	Test facilities capable of adequate conduct of test on derivation of predicted environmental concentration	2-10-2
	(3) Test on agricultural chemical concentration measurement in paddy water of actual paddy	Formulation	2 trials	Test facilities capable of adequate conduct of test on derivation of predicted environmental concentration	2-10-3
	(4) Test on surface soil runoff in model field	Formulation	1 trial	Test facilities capable of adequate conduct of test on derivation of predicted environmental concentration	2-10-4
	(5) Drift test	Formulation	3 trials	Test facilities capable of adequate conduct of test on derivation of predicted environmental concentration	2-10-5
	(6) Monitoring test on agricultural chemical concentration in the rivers	Formulation	2 trials	Test facilities capable of adequate conduct of test on derivation of predicted environmental concentration	2-10-6
Test results regarding	Test on residues in crops	Formulation (Note6)	The number of trials for each applicable crop	Test facilities shall conform to	3-1-1

residues in crops.			shall follow the rules mentioned below. (In cases where the applicable crop is a crop group, the type of test crop to be tested shall follow the rules set separately by the Director of Plant Products Safety Division.). However, in the case of a crop whose production volume is particularly low, and when there is an another crop whose agricultural chemical residue level is considered higher than the relevant crop on the basis of the results of the initial adhesion study, the test results regarding residue in the another crop whose agricultural chemical residue level is supposed to be higher than the relevant crop may be submitted instead of the test results regarding residue of the relevant crop. ① For a crop whose production volume is particularly high, six or more trials on the crop are required. ② For a crop whose production volume is high, three or more trials on the crop are required. ③ For a crop whose production volume is low, two or more trials on the crop are required. ④ Regardless of ① and ②, for a crop whose production volume is particular high or high, when the relevant agricultural chemical is used only as warehouse fumigation or when it is clear that no residue of the relevant agricultural chemical remains in the crop in light of its application timing or application method etc., two or more trials on the crop are required.	the GLP standards for agricultural chemicals. However, for a crop whose production volume is low, GLP compliance is not required. Field trials shall be conducted according to the following standards: ① In cases where a crop whose production volume is particularly high is the applicable crop, trials shall be conducted in two or more years and in two or more prefectures that are major cultivation areas of the relevant crop in Japan. In cases where trials are conducted indoors (greenhouse/glasshouse), the requirement regarding years shall not apply. ② In cases where a crop whose production volume is high is the applicable crop, trials shall be conducted in two or more prefectures that are major cultivation areas of the relevant crop in Japan. ③ However, in cases where a crop whose cultivation area is limited to one prefecture is the applicable crop, trials shall be conducted at two or more test facilities or at the same test facility in two or more years. ④ In cases where a crop whose production volume is low is the applicable crop, trials shall be conducted at two or more test facilities or at the same test facility in two or more years. ⑤ In the case of ① to ③, two or more trials shall be	

				conducted in Japan for decline studies for an appropriate period which covers back and forth the application timing of the relevant agricultural chemical. However, in cases where it is not clear that the residue of the relevant agricultural chemical significantly declines with time, all trials shall be conducted for decline studies. ⑥ In the case of ① and ②, if the crop is cultivated two or more times per year, trials shall include a cropping season in which the residue of the relevant agricultural chemical in the crop will be higher. ⑦ Trials other than decline studies may be conducted outside of Japan. In this case, environmental conditions, sampling portion or other factors shall be equivalent to those in Japan. ⑧ In cases where the relevant agricultural chemical is a registered agricultural chemical whose application method is ground application, and when aerial application or unmanned helicopter application is added to the application method, the number of trials pertaining to the said aerial application or the said unmanned helicopter application shall be half or more of the required number. (In cases where the required number of trials is three or less, two or more trials shall be conducted.)	

Test results regarding residues in livestock	Test on residues in livestock	Active ingredients, etc.	2 types of test animals (1type of ruminant and 1 type of poultry)	Test facilities conforming to GLP standards for agricultural chemicals	3-2-1
Test results regarding persistence in soil	(1) Test on residues in soil	Formulation	2 trials	Test facilities capable of adequate conduct of test on persistence in soil	3-3-1
	(2) Test on residues in succeeding crops	Formulation	(1) For agricultural chemicals used in paddies, select 1 type of crop from among root vegetables, and 1 type from among grains, soybeans or other crops. (2) For agricultural chemicals used in upland fields, select 1 type of crop from among root vegetables, and 1 type from among plant categories that crops which are assumed as succeeding crops belong to.	Test facilities capable of adequate conduct of test on persistence in soil	3-3-2

Note 1: Efficacy test shall be conducted concurrently with phytotoxicity test.

Note 2: Phytotoxicity test shall be conducted concurrently with efficacy test.

Note 3: 1-year repeated dose oral toxicity test may be conducted concurrently with carcinogenicity test.

Note 4: Carcinogenicity test may be conducted concurrently with 1-year repeated dose oral toxicity test.

Note 5: “Pure state” shall mean at least 98% pure, in principle.

Note 6: In the case of spreader, the term “formulation” here shall mean a combination of the spreader and its applicable agricultural chemicals.

(Appended Table 1)

Test Items	Number of trials
Efficacy test and phytotoxicity test (efficacy / phytotoxicity combined test)	Efficacy test and phytotoxicity test shall be conducted for at least 2 years for each combination of applicable crops, applicable diseases, insect pests or weeds etc., and application method of the agricultural chemical pertaining to an application. (In cases where the applicable crop is a crop group, the crops included in the relevant crop group shall be tested (excluding the case of herbicides and plant growth regulators, in principle; hereinafter the same shall apply in this table.).) The test conducted each year shall be conducted in 3 or more facilities located in different prefectures, in principle. However, in the following cases, the number of trials implemented may be as suggested below. (1) If the combination of applicable crops and applicable diseases, insect pests or weeds pertaining to an application is the same as that of a registered agricultural chemical, and if it satisfies any of the following conditions, test shall be conducted in 3 or more facilities located in different prefectures for each combination of applicable crops and applicable diseases, insect pests or weeds pertaining to the application, in principle. (i) When the agricultural chemical pertaining to an application has the same active ingredients as a registered agricultural chemical, but is a different formulation type; (ii) When the agricultural chemical pertaining to an application has the same active ingredients and formulation type as a registered agricultural chemical, but the amount of the active ingredients applied is smaller than that of the registered agricultural chemical; (iii) When the agricultural chemical pertaining to an application is a mixed formulation including active ingredients from two or more registered agricultural chemicals, but the content of each active ingredient in the relevant agricultural chemical is different from the content of the active ingredients in the respective registered agricultural chemicals; (iv) When the agricultural chemical pertaining to an application is a registered agricultural chemical, but either its application concentration or its application dosage (the amount of the active ingredients applied) is reduced; (v) When the agricultural chemical pertaining to an application is a registered agricultural chemical, but its application method is changed; (2) If the combination of applicable crops and applicable diseases, insect pests or weeds pertaining to an application is the same as that of a registered agricultural chemical, and if it satisfies any of the following conditions, test shall be conducted in 2 or more facilities located in different prefectures for each combination of applicable crops and applicable diseases, insect pests or weeds pertaining to the application, in principle. (i) When the agricultural chemical pertaining to an application has the same active ingredients and formulation type as a registered agricultural chemical, but the amount of the active ingredients applied is either the same as or greater than that of the registered agricultural chemical; (ii) When the agricultural chemical pertaining to an application is a mixed formulation including active ingredients from two or more registered agricultural chemicals, and the content of each active ingredient in the relevant agricultural chemical is the same as the content of the active ingredients in the respective registered agricultural chemicals; (iii) When the agricultural chemical pertaining to an application is a registered agricultural chemical, but either its application concentration or its application dosage (the amount of the active ingredients applied) is increased; (3) If an application for registration satisfies any of the following conditions, test shall be conducted in 2 or more facilities located in different prefectures for each combination of applicable crops and applicable diseases, insect pests or weeds pertaining to the application, in principle. (i) When the agricultural chemical pertaining to an application is a registered agricultural chemical and secondary diseases, insect pests or weeds are added to the scope of applicable diseases, insect pests or weeds without adding any crops to the scope of the applicable crops; (ii) When the agricultural chemical pertaining to an application is a registered agricultural chemical and similar crops are added to the scope

	of the target crops which applicable diseases, insect pests or weeds of the registered agricultural chemical damage; (iii) When the agricultural chemical pertaining to an application is applied to crops that are only grown in limited areas, or that are produced in low volumes; (iv) When the agricultural chemical pertaining to an application is used against diseases, insect pests or weeds that only occur in limited areas; (v) When the agricultural chemical pertaining to an application is a registered agricultural chemical and there is an urgent need to expand the scope of the applicable diseases, pests or weeds for plant protection; (vi) When the agricultural chemical pertaining to an application is a spreading agent; (4) If an application for registration satisfies any of the following conditions, test shall be conducted in 3 or more facilities located in different prefectures for each combination of applicable crops and applicable diseases, pests or weeds pertaining to the application, in principle. (i) When the agricultural chemical pertaining to an application is a mixed formulation including new active ingredients as well as the active ingredients of a registered agricultural chemical, and when the combination of applicable crops and applicable diseases, insect pests or weeds relevant to the active ingredients of the registered agricultural chemical among all combinations pertaining to the application is identical with that of the registered agricultural chemical, and when test is conducted only for the combination of applicable crops and applicable diseases, pests or weeds relevant to the active ingredients of the registered agricultural chemical among all combinations pertaining to the application; (ii) When the agricultural chemical pertaining to an application is a registered agricultural chemical and new applicable crops are added for common diseases or pests which are difficult to control in many different crops and which are among applicable diseases or insect pests of the registered agricultural chemical; (iii) When the agricultural chemical pertaining to an application is a registered agricultural chemical which is used without crops or in a state of not having contact with crops, and new applicable crops are added for the applicable diseases or pests of the registered agricultural chemical; (5) Test shall be conducted in 3 or more facilities for each combination of applicable crops and applicable diseases or insect pests pertaining to the application, with regard to agricultural chemicals that are used in warehouses, silos, etc.
Test on metabolism in plants	For each plant category referred to in the left column of Appended Table 2 which the applicable crops pertaining to an application belong to, the test shall be conducted on 1 or more types of crops selected from among the main crops listed in the corresponding right column of the same table. Where crops to be used for food are included in the applicable crops, 1 or more types of crops to be used for food under the plant category to which that crops belong shall be selected and subject to the test. Where the applicant wishes to make an application to add crops to be used for food to the applicable crops of existing registered agricultural chemical, and where the test results already submitted pertaining to the plant category to which that crops belong were conducted only for forage crops, the applicant shall submit the test results conducted on 1 or more types of crops to be used for food included in that plant category. However, in cases where there are 3 or more types of plant categories relevant to the applicable crops pertaining to an application, and when it is considered that there is not a large difference of metabolism among crops involved in each plant category, the plant categories of the test may be 3 types. In cases where the applicable crops pertaining to an application are limited to only one plant category, and when the test plant is different from the applicable crops pertaining to the application, the plant subject to the test shall be 2 or more types. In cases where rice is included in the applicable crops, the test plant shall always include paddy rice. In cases where a genetically modified crop is included in the applicable crops, the test plant shall include the genetically modified crop in addition to that crop selected by the above description.

(Appended Table 2)

○ **Classification of plants as subjects for test on metabolism in plants**

Plant categories	Main crops
Rice	Paddy rice
Grains and sugar cane	Wheat, barley, rye, corn, buckwheat, sugar cane, oats, sorghum
Fruits (excluding citrus fruits and gourds)	Peach, loquat, kiwifruit, apple, pear, Japanese persimmon, nectarine, apricot, cherry, Japanese apricot (*Prunus mume*), strawberry, grape, gingko, Japanese chestnut, walnut
Citrus fruits	Satsuma mandarin(*Citrus unshiu*), large citrus fruits, small citrus fruits
Fruit vegetables (including gourds)	Bell pepper, okra, *shishito* pepper, squash, cucumber, tomato, eggplant, watermelon, melon
Plants with edible leaves or flowers	Cabbage, napa cabbage(*Brassica rapa* var. *pekinensis*), Japanese radish(*daikon*)(leaf), broccoli, *komatsuna*(*Brassica rapa var. perviridis*), *edamame*(immature soybeans in the pod), podded pea, green bean, onion, garlic, *rakkyo*, hop
Plants with edible roots or stalks	Japanese radish(*daikon*)(root), carrot, ginger, potato, sweet potato, taro(*Colocasia esculenta*), sugar beet
Pulses and oil-producing plants	Soybean, adzuki bean, pea, fava bean, rapeseed, sesame, safflower
Mushrooms	*Shiitake*, *enokitake*
Tea trees	Tea and leaves of crops classified under fruits (excluding citrus fruits and gourds) and citrus fruits

(Appendix Table 2)

The phrase "In the cases listed in Appendix Table 2" referred to in Section IV shall refer to cases described in the right column for each of test results listed in the left column of the following table.

Test results	Cases where the test results need not be submitted
Test results regarding phytotoxicity to the applicable crops	
(1) Test results regarding residual odor in tea	When the applicable crops do not include tea;
(2) Test results regarding tobacco taste	When the applicable crops do not include tobacco;
(3) Test results regarding limit for phytotoxicity	When it is found that there is no risk that the applicable crops will be exposed to the relevant agricultural chemical beyond its applicable range (application dosage, concentration), in light of its application method etc.;
Test results regarding phytotoxicity to adjacent crops	
(1) Test results regarding phytotoxicity due to drift and scattering	When it is found that there is no risk that the relevant agricultural chemical will affect adjacent crops (i.e. phytotoxicity) through its drifting and scattering, in light of the type of its active ingredients, its formulation type, its application method etc.;
(2) Test results regarding phytotoxicity due to runoff from paddy water	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is not used in paddies; (ii) When it is found that there is no risk that the relevant agricultural chemical will affect adjacent crops (i.e. phytotoxicity) through its runoff from paddy water into water systems such as rivers, in light of its application method etc. ;
(3) Test results regarding phytotoxicity due to volatilization	When it is found that there is no risk that the relevant agricultural chemical will affect adjacent crops (i.e. phytotoxicity) through its volatilization, in light of the properties of its active ingredients, its formulation type, its application method etc. ;
Test results regarding phytotoxicity to succeeding crops	When it is found that there is no risk that the relevant agricultural chemical will affect crops (i.e. phytotoxicity) that are to be cultivated after the applicable crops, in light of its application method, the degree of its persistence in soil etc.;
Test results regarding acute dermal toxicity	When it is found that the relevant agricultural chemical is corrosive (as are, for example, strong acid (in general, those of

	pH ≦2) or strong alkaline (in general, those of pH ≧11.5);
Test results regarding acute inhalation toxicity	Regarding tests using formulation, when it is found that there is no risk that users will be exposed to the relevant agricultural chemical through inhalation, in light of its formulation type, its application method etc.;
Test results regarding skin irritation	When it is found that the relevant agricultural chemical is corrosive (as are, for example, strong acid (in general, those of pH ≦2) or strong alkaline (in general, those of pH ≧11.5);
Test results regarding eye irritation	Cases that fall under either of the following items: (i) When it is found that the relevant agricultural chemical is corrosive (as are, for example, strong acid (in general, those of pH ≦2) or strong alkaline (in general, those of pH ≧11.5); (ii) When it is suspected that the relevant agricultural chemical is corrosive on the basis of the test results regarding skin irritation;
Test results regarding acute neurotoxicity	When it is found that there is no risk of neurotoxicity on the basis of the test results regarding acute toxicity etc.;
Test results regarding acute delayed neurotoxicity	Cases that fall under either of the following items: (i) When it is found that there is no risk of delayed neurotoxicity on the basis of the test results regarding acute toxicity etc.; (ii) When it is found that there is no risk of delayed neurotoxicity, in light of correlations with the chemical structures of chemicals that are known to have delayed neurotoxic effects;
Test results regarding 90-day repeated dose oral toxicity	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is found to be safe because the amount of exposure to its components (including substances that are produced through chemical changes in these substances; hereinafter referred to as "components etc.") is extremely very small when it is used, in light of its formulation type, its application method etc. ; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
Test results regarding 21-day repeated dermal toxicity	Cases that fall under either of the following items: (i) When it is found that there is no risk of long-term dermal

	exposure to the relevant agricultural chemical on persons who are applying it; (ii) When it is found that there is no risk that the relevant agricultural chemical has a high dermal toxicity on the basis of the test results regarding acute dermal toxicity;
Test results regarding 90-day repeated inhalation toxicity	Cases that fall under either of the following items: (i) When it is found that there is no risk of long-term inhalation exposure to the relevant agricultural chemical on persons who are applying it; (ii) When it is found that there is no risk that the relevant agricultural chemical has a high inhalation toxicity on the basis of the test results regarding acute inhalation toxicity;
Test results regarding repeated dose oral neurotoxicity	When it is found that there is no risk of neurotoxicity on the basis of the test results regarding 90-day repeated dose oral toxicity etc.;
Test results regarding 28-day repeated administration delayed neurotoxicity	When it is found that there is no risk of delayed neurotoxicity on the basis of the test results regarding acute delayed neurotoxicity test etc.;
Test results regarding 1-year repeated dose oral toxicity	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is found to be safe because there is no risk that humans will ingest its components etc. for a long period, or because the amount of ingestion of them would be extremely very small, in light of its formulation type, its application method etc.; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
Test results regarding carcinogenicity	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is found to be safe because there is no risk that humans will ingest its components etc. for a long period, or because the amount of ingestion of them would be extremely very small, in light of its formulation type, its application method etc. and; when it is clearly found that there is no mutagenicity; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
Test results regarding reproductive toxicity	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is found to be

	safe because there is no risk that humans will ingest its components etc. for a long period, or because the amount of ingestion of them would be extremely very small, in light of its formulation type, its application method etc.; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
Test results regarding teratogenicity	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is found to be safe because the amount of exposure to and ingestion of its components etc. is extremely very small when it is used, in light of its formulation type, its application method etc. ; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
Test results regarding mutagenicity	The same as in the case of test results regarding teratogenicity.
Test results regarding pharmacology	The same as in the case of test results regarding teratogenicity.
Test results regarding metabolism in animals	The same as in the case of test results regarding teratogenicity.
Test results regarding metabolism in plants	Cases that fall under any of the following items: (i) When the relevant agricultural chemical is used for crops other than those that are to be used for food (including industrial crops and crops to be used for animal feed); (ii) When the relevant agricultural chemical is found to be safe because there is no risk that humans will ingest its components etc. for a long period, or because the amount of ingestion of them would be extremely very small, in light of its formulation type, its application method etc.; (iii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.; (iv) When the relevant agricultural chemical is a registered agricultural chemical which is applied to other food crops, and a crop whose production volume is low is added to the applicable crops of that registered agricultural chemical;
Test results regarding metabolism in livestock	Cases that fall under any of the following items: (i) When the test results regarding residues in crops need not be submitted;

	(ii) When the relevant agricultural chemical is used for crops other than crops that are to be used for animal feed and crops whose by-products (rice straw, etc.) are to be used for animal feed; (iii) When the residual levels of test substance and its major metabolites are less than the limit of quantification in the tests on residues in the crops that are to be used for animal feed and crops whose by-products (rice straw, etc.) are to be used for animal feed; The limit of quantification shall be set aiming at around 0.01 to 0.05 mg/kg in principle (In the case of forage crops to which the residual standard value for pasture grass applies, the limit of quantification shall be set aiming at the concentration which becomes equivalent to 0.01 to 0.05 mg/kg if the moisture content of the crop is converted to 10%.).
Test results regarding behavior in soil	Cases that fall under either of the following items, or in any of the cases listed in the right column corresponding to the test results (1)-(3) listed in the left column below: (i) When it is found that there is no risk that the components etc. of the relevant agricultural chemical will contaminate farmland soil where it is used, in light of its formulation type, its application method etc.; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
(1) Test results regarding behavior in flooded aerobic soil	When the relevant agricultural chemical is not used in paddies;
(2) Test results regarding behavior in aerobic soil	When the relevant agricultural chemical is used only in paddies; however, this shall exclude cases where the test results are considered necessary, in light of the dissipation rate of its components etc. in flooded aerobic soil;
(3) Test results regarding behavior in anaerobic soil	Cases that fall under any of the following items: (i) When the relevant agricultural chemical is only used in paddies; (ii) When it is found that the components etc. of the relevant agricultural chemical dissipate rapidly in aerobic soil on the basis of the test results regarding behavior in aerobic soil; (iii) When the relevant agricultural chemical is found to be safe because its mobility in soil is low, in light of the physical and chemical properties of its components etc.;
Test results regarding behavior in water	

(1) Test results regarding hydrolytic behavior	Cases that fall under either of the following items: (i) When it is found that there is no risk that the components etc. of the relevant agricultural chemical will run off into water systems such as rivers, in light of its formulation type, its application method etc.; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
(2) Test results regarding photolytic behavior in water	The same as in the case of test results regarding hydrolytic behavior.
Test results regarding toxicity on aquatic animals and plants	
(1) Test results regarding fish acute toxicity	Cases that fall under either of the following items: (i) Regarding tests using TGAI, cases that fall under either of the following items: (a) When it is found that there is no risk that the components etc. of the relevant agricultural chemical will run off into water systems such as rivers, in light of its formulation type, its application method etc.; (b) When the relevant agricultural chemical is found not to be harmful because its components etc. are of very low toxicity, in light of their type etc.; (ii) Regarding tests using formulation, when it is found that there is no risk that the components etc. of the relevant agricultural chemical will run off into water systems such as rivers, in light of its formulation type, its application method etc.;
	When it is found that it is not necessary to conduct additional fish acute toxicity test on the basis of the test results regarding fish acute toxicity, *Daphnia spp* acute immobilization and algae growth inhibition etc. concerning the relevant agricultural chemical;
(2) Test results regarding fish(larvae) acute toxicity	When it is found that it is not necessary to conduct further tests regarding toxicity on aquatic animals and plants in conditions closer to actual environment on the basis of the test results regarding fish acute toxicity, *Daphnia spp* acute immobilization and algae growth inhibition, etc. concerning the relevant agricultural chemical;
(3) Test results regarding *Daphnia spp* acute immobilization	The same as in the case of test results regarding fish acute toxicity.

(4) Test results regarding *Daphnia spp* (adult daphnids) acute immobilization	The same as in the case of test results regarding fish (larvae) acute toxicity.
(5) Test results regarding *Daphnia spp* reproduction	Cases that fall under either of the following items: (i) When it is found that there is no risk that the components etc. of the relevant agricultural chemical will run off into water systems such as rivers, in light of its formulation type, its application method etc.; (ii) When there is no risk that the relevant agricultural chemical will affect the reproduction of crustaceans because its components etc. are of very low toxicity, in light of their type etc.;
(6) Test results regarding effects of coexistent organic substances on fish acute toxicity/ *Daphnia spp* acute immobilization	The same as in the case of test results regarding fish (larvae) acute toxicity.
(7) Test results regarding freshwater shrimp acute toxicity	The same as in the case of test results regarding fish (larvae) acute toxicity.
(8) Test results regarding amphipoda acute toxicity	The same as in the case of test results regarding fish (larvae) acute toxicity.
(9) Test results regarding *Chironomus* sp., acute immobilization	The same as in the case of test results regarding fish (larvae) acute toxicity.
(10) Test results regarding algae growth inhibition	The same as in the case of test results regarding fish acute toxicity.
Test results regarding toxicity on beneficial organisms other than aquatic animals and plants	When the relevant agricultural chemical is found not to be harmful because its components etc. are of very low toxicity, in light of their type etc., or in any of the cases listed in the right column corresponding to the test results (1)-(4) listed in the left column below:
(1) Test results regarding bee toxicity	When it is found that there is no risk that bees will be exposed to the relevant agricultural chemical, in light of its formulation type, its application method etc.;
(2) Test results regarding silkworm toxicity	When it is found that there is no risk that silkworms will be exposed to the relevant agricultural chemical through ingesting mulberry leaves etc., in light of its formulation type, its application method etc.;

(3) Test results regarding natural enemy insect, etc. toxicity	When it is found that there is no risk that natural enemy insects etc. will be exposed to the relevant agricultural chemical, in light of its formulation type, its application method etc.;
(4) Test results regarding avian toxicity Test results regarding avian acute oral toxicity Test results regarding avian dietary toxicity	(i) When it is found that there is no risk that birds will be exposed to the relevant agricultural chemical, in light of its formulation type, its application method etc.; (ii) As regards avian dietary toxicity test, when it is not confirmed that the substance is highly toxic on the basis of the test results regarding avian acute oral toxicity;
Test results regarding the properties, stability, degradability, etc. of active ingredients	Cases that fall under any of the following items: (i) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.; (ii) As regards soil adsorption test, hydrolysis test, photolysis in water test and bioconcentration test, when it is found that there is no risk that the components etc. of the relevant agricultural chemical will contaminate farmland soil where it is used or run off into water systems such as rivers, in light of its formulation type, its application method etc.; (iii) As regards hydrolysis test and photolysis in water test, when it is found that the results aimed at by the test are obtained from the test results regarding behavior in water; (iv) As regards bioconcentration test, when the value of n-octanol/water partition coefficient is less than 3.5;
Test results regarding derivation of predicted environmental concentration	Cases that fall under either of the following items, or in any of the cases listed in the right column corresponding to the test results (1)-(6) listed in the left column below: (i) When it is found that there is no risk that the components etc. of the relevant agricultural chemical will contaminate farmland soil where it is used or run off into water systems such as rivers, in light of its formulation type, its application method etc.; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
(1) Test results regarding water polluting properties	Cases that fall under any of the following items: (i) When the relevant agricultural chemical is not used in paddies; (ii) When the test results are not used in the calculation of the predicted environmental concentration pertaining to water pollution; (iii) When it is found that the results aimed at by the test are obtained from the test on agricultural chemical concentration measurement in paddy water of model paddy;

(2)Test results regarding agricultural chemical concentration measurement in paddy water of model paddy	Cases that fall under any of the following items: (i) When the relevant agricultural chemical is not used in paddies; (ii) When the test results are not used in the calculation of the predicted environmental concentration pertaining to damage to aquatic animals and plants; (iii) When it is found that the results aimed at by the test are obtained from the test on water polluting properties;
(3)Test results regarding agricultural chemical concentration measurement in paddy water of actual paddy	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is not used in paddies; (ii) When the test results are not used in the calculation of the predicted environmental concentration (meaning both the predicted environmental concentration pertaining to water pollution and the predicted environmental concentration pertaining to damage to aquatic animals and plants; the same shall apply hereinafter);
(4)Test results regarding surface soil runoff in model field	Cases that fall under either of the following items: (i) When the relevant agricultural chemical is used only in paddies; (ii) When the test results are not used in the calculation of the predicted environmental concentration;
(5)Test results regarding drift	Cases that fall under either of the following items: (i) When it is found that there is no risk that the relevant agricultural chemical will drift and run off into water systems such as rivers, in light of its formulation type, its application method etc.; (ii) When the test results are not used in the calculation of the predicted environmental concentration;
(6) Test results regarding monitoring agricultural chemical concentration in the rivers	When the test results are not used as a substitute for the predicted environmental concentration;
Test results regarding residues in crops, etc.	
Test results regarding residues in crops	Cases that fall under either of the following items: (i) Cases that fall under any of the following sub-items: (a) When the relevant agricultural chemical is used for crops other than those that are to be used for food (including industrial crops and crops to be used for animal feed; the same shall apply hereinafter); (b) When the relevant agricultural chemical is found to be safe because there is no risk that humans will ingest its components etc. for a long period, or

		because the amount of ingestion of them would be extremely very small even when ingested, in light of its formulation type, its application method etc.; (c) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.; (ii) As regards spreader, notwithstanding cases of preceding item (i), cases that fall under any of the following items: (a) When the relevant spreader is used for crops other than those that are to be used for food; (b) When it is found that there is no risk that the relevant spreader will affect the residue of the applicable agricultural chemicals in crops, and when it is found that the relevant spreader is safe because there is no risk that humans will ingest its components etc. for a long period, or because the amount of ingestion of them would be extremely very small even when ingested; (c) When it is found that there is no risk that the relevant spreader will affect the residue of the applicable agricultural chemicals in the applicable crops, and when the relevant spreader is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;

Test results regarding residues in livestock	Cases that fall under either of the following items: (i) When the test results regarding metabolism in livestock need not be submitted; (ii) When the residual levels of both test substance and its major metabolites in livestock products are less than 0.01 mg/kg on the basis of the test results regarding metabolism in livestock; (iii) When any residues of test substance or its major metabolites are found in livestock products on the basis of the test results regarding metabolism in livestock, cases fall under all of the following items; (a) When the concentration of residues of the test substance or its major metabolites found in livestock products in the test on metabolism in livestock is extremely near the limit of quantification; (b) When the dose of test substance to the livestock in the test on metabolism in livestock is extremely higher than the estimated maximum dietary burdens of livestock based on the residual level obtained by the test on residues in crops; and (c) When the residual level in livestock products derived from livestock fed with the estimated maximum dietary burdens is estimated scientifically less than 0.01 mg/kg, taking into account the ratio of the estimated maximum dietary burdens to the dose of test substance to the livestock in the test on metabolism in livestock;
Test results regarding persistence in soil	
Test results regarding residues in soil	Cases that fall under either of the following items: (i) When it is found that there is no risk that the components etc. of the relevant agricultural chemical will contaminate farmland soil where it is used, in light of its formulation type, its application method etc.; (ii) When the relevant agricultural chemical is found to be safe because its components etc. are of very low toxicity, in light of their type etc.;
Test results regarding residues in succeeding crops	When the relevant agricultural chemical is found to be safe because there is no risk that crops cultivated after the applicable crops will be contaminated with its components etc., in light of the degree of its persistence in soil etc.;

(Annex)

Guidelines on Preparation of Test Results Submitted When Applying for Registration of Agricultural Chemicals

Test Items **Identification number**

Section 1 Efficacy Test

Section 2 Phytotoxicity Test

Section 3 Toxicity Test

Basic Matters

1. Basic concept

(1) These guidelines are to be used as a standard when preparing test results regarding the efficacy, phytotoxicity, toxicity and persistence of the relevant agricultural chemical that are to be submitted with an application for registration of an agricultural chemical.

(2) Persons conducting tests are not required to strictly follow these guidelines. Moreover, these guidelines shall not preclude persons conducting tests from making changes in test methods for the purpose of more accurately achieving study objectives in accordance with the characteristics of the test substances.

2. Regarding test substances

(1) When technical grade active ingredients (hereinafter referred to as "TGAI") are used as test substances, they shall be equivalent to TGAI used as raw materials of the relevant agricultural chemical samples.

(2) When formulations are used as test substances, they shall be equivalent to the relevant agricultural chemical samples.

(3) Test substances from the same lot shall be used during the test period. If test substances from another lot are used for unavoidable reasons, it shall be sufficiently similar in composition to the previous lot. The lot number of the lot used shall be clearly indicated in the test results.

(4) The composition of each TGAI used in acute toxicity, repeated dose toxicity, carcinogenicity, reproductive toxicity, teratogenicity and mutagenicity shall be clearly indicated in each test results.

3. Regarding test organisms

In order to accurately conduct safety evaluations of agricultural chemicals, it is desirable to use test organisms of the same species and strain for all the relevant test items.

4. Regarding handling of experimental animals

When conducting experiments using animals, sufficient attention shall be paid to rearing management, experimental operations, methods of disposal, etc. from the perspective of protection of animals, in accordance with the Law on Welfare and Management of Animals (1973, Law No. 105), Standards relating to Breeding, Care and Reduction of Pain with regard to Experimental Animals (Ministry of the Environment Notification No. 88, April 28, 2006), international regulations and trends regarding animal welfare, etc.

<Efficacy Test>

Test on efficacy against the applicable diseases or pests

Efficacy and phytotoxicity test (1-1-1)

1. Objective

   The objective of these tests is to obtain scientific information concerning the effectiveness of agricultural chemicals (hereinafter referred to as "efficacy") in controlling disease, insect pests and in weeds, and concerning crop phytotoxicity.

2. Test crops

   Representative varieties of the target crops.

3. Test methods

   (1) Studies are to be carried out in fields (or in greenhouses, etc. when applicable). In order to achieve the test objectives, establish test product, untreated control of sufficient area, as well as, in principle, reference product.

   Conduct application of treatment according to the methods of use and the dosage (concentration) relevant to the registration application.

   (2) As regards test product, select appropriate time period for evaluation of efficacy and phytotoxicity under appropriate conditions as regards occurrence of disease, insect pests, and weeds, as well as the growth stage of crops.

   (3) In conducting tests, select appropriate methods based on consideration of the respective properties of the test chemicals, the target diseases, insect pests, and weeds, as well as the target crops.

4. Report items

   (1) Efficacy in the test product as compared to the untreated control and the reference product

   (2) Whether or not there is phytotoxicity; if so, their nature, degree (measurements of plant height, etc.), extent of recovery, etc.

   (3) Other items

   (i) Information regarding crops (growth details, growth stage at the time of treatment, etc.)

   (ii) Conditions under which the target diseases, insect pests, and weeds occur

   (iii) Weather conditions during the study period (temperature, precipitation, etc.)

<Phytotoxicity Test>

Test on phytotoxicity to the applicable crops (1-1-1～4)

Efficacy and phytotoxicity test (1-1-1)

Same as the above.

Limit Test for phytotoxicity (1-1-2)

1. Objective

   The objective of these tests is to clarify the maximum dosage and maximum concentration at which phytotoxicity do not occur, and to obtain scientific information concerning target crop phytotoxicity.

2. Test crops

   Use a representative variety of crops that are relevant to the registration application, and, in principle, crops that are healthy and at the stage of growth of maximum susceptibility.

3. Test methods

   (1) The tests are conducted with the objective of clarifying the maximum dosage or maximum concentration at which phytotoxicity does not occur; however, this does not need prevent the tests from being conducted double the maximum dosage that would actually be used. In such cases, conduct the test with the double dose that would actually be used in the method of use relevant to the application for registration, including an untreated control.

   (2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of phytotoxicity.

4. Reporting

   (1) Whether or not there is phytotoxicity; if so, their nature, degree (measurements of plant height, etc.), extent of recovery, etc.

   (2) Other items

   Information regarding crops (growth details, growth stage at the time of treatment, etc.)

Test on residual odor in tea (1-1-3)

1. Objective

The objective of these tests is to obtain scientific information as to whether or not there is an odor brought about by the agricultural chemical remains as a side effect of using it on tea plants.

2. Test crops

Use healthy tea plants cultivated according to the usual methods. Use the *Yabukita* variety of tea.

3. Test methods

(1) Conduct the test with the methods and dosage (or concentration) relevant to the application for registration, and establish an untreated section.

(2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of phytotoxicity.

4. Reporting

(1) Whether or not there is a residual odor

(2) Other items

(i) Information regarding crops (growth details, growth stage at the time of treatment, etc.)

(ii) Information regarding tea processing, storage, etc.

Tobacco taste test (1-1-4)

1. Objective

The objective of these tests is to obtain scientific information as to whether or not there is an effect on flavor brought about by the agricultural chemical remains as a side effect of using it on tobacco.

2. Test crops

Use healthy tobacco plants cultivated according to the usual methods. Select a representative variety.

3. Test methods

(1) Conduct the test with the methods and dosage (or concentration) relevant to the application for registration, and establish an untreated section.

(2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of tobacco flavor.

4. Reporting

(1) Whether or not there is an effect on flavor

(2) Other items

(i) Information regarding crops (growth details, growth stage at the time of treatment, etc.)

(ii) Information regarding tobacco processing, storage, etc.

Test on phytotoxicity to adjacent crops (1-2-1～3)

Test on phytotoxicity due to drift and scattering (1-2-1)

1. Objective

   The objective of these tests is to obtain scientific information regarding surrounding crop phytotoxicity due to scattering of the agricultural chemical.

2. Test crops

   (1) As regards varieties of test crops, care should be taken to select at least 1 representative variety each from among such crops as Solanaceae, Cucurbit, Brassicaceae, Legume, and Poaceae, etc. according to the type of crop and area to which the chemical is being applied.

   (2) Use representative crop varieties, at the stage of growth of maximum susceptibility.

3. Test methods

   (1) Conduct the test with the methods and dosage (or concentration) relevant to the application for registration, and establish an untreated control.

   (2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of phytotoxicity.

4. Reporting

   (1) Whether or not there is phytotoxicity; if so, their nature, degree (measurements of plant height, etc.), extent of recovery, etc.

   (2) Other items

   Information regarding crops (growth details, growth stage at the time of treatment, etc.)

Test on phytotoxicity due to runoff from paddy water (1-2-2)

1. Objective

   The objective of these tests is to obtain scientific information regarding side crop phytotoxicity, etc. that grow in water systems, with reference to agricultural chemicals, among those that are applied to rice paddies, that run off into water systems via paddy water.

2. Test crops

   Use representative varieties of such crops as rush, lotus root, *Kuwai* (*Sagittaria trifolia* var. *edulis*), etc. Use representative crop varieties, at the stage of growth of maximum susceptibility.

3. Test methods

   (1) Conduct the test with the methods and dosage (or concentration) relevant to the application for registration, and establish an untreated control.

(2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of phytotoxicity.

4. Reporting
   (1) Whether or not there are phytotoxicity; if so, their nature, degree (measurements of plant height, etc.), extent of recovery, etc.

   (2) Other items
   Information regarding crops (growth details, growth stage at the time of treatment, etc.)

Test on phytotoxicity due to volatilization (1-2-3)

1. Objective
   The objective of these tests is to obtain scientific information regarding surrounding crop phytotoxicity due to volatilization from water or soil of agricultural chemicals (herbicides only) especially with highly active in trace amount among chemicals which the active ingredient is a substance that has a high vapor pressure and low aqueous solubility.

2. Test crops
   Use representative crops that are likely to be highly susceptible. Use representative crop varieties, at the stage of growth of maximum susceptibility.

3. Test methods
   (1) Conduct the test with the methods and dosage (or concentration) relevant to the application for registration, and establish an untreated section.

   (2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of phytotoxicity.

4. Reporting
   (1) Whether or not there is phytotoxicity; if so, their nature, degree (measurements of plant height, etc.), extent of recovery, etc.

   (2) Other items
   Information regarding crops (growth details, growth stage at the time of treatment, etc.)

Test on phytotoxicity to succeeding crops

Succeeding crops phytotoxicity test (1-3)

1. Objective

   The objective of these tests is to obtain scientific information regarding phytotoxicity on succeeding crops of agricultural chemicals that are persist in soil for a long period and are deemed necessary due to the cultivation period of the target crop, etc., among soil treatment formulation or agricultural chemicals which have concern to be mixed with soil.

2. Test crops

   Select crops that are considered to be highly susceptible from crops which have a possibility to be cultivated as succeeding crops of the target crop. Use representative crop varieties at the stage of growth of maximum susceptibility.

3. Test methods

   (1) Conduct the test with the methods and dosage (or concentration) relevant to the application for registration, and establish an untreated section.

   (2) Conduct the test according to methods by which sufficient information will be obtained for evaluation of phytotoxicity.

4. Reporting

   (1) Whether or not there is phytotoxicity; if so, their nature, degree (measurements of plant height, etc.), extent of recovery, etc.

   (2) Other items

   Information regarding crops (growth details, growth stage at the time of treatment, etc.)

<Toxicity test>

Acute oral toxicity test (2-1-1)

1. Objectives

These studies are the first step in evaluating the toxicity of agricultural chemicals. Their objective is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific knowledge as to injuries to health that may result from a single oral exposure. The studies are also useful for obtaining initial scientific knowledge of the test substance's properties as regards toxic effects that will be of use in setting dosages for repeated dose toxicity studies and other studies.

2. Test method

Acute oral toxicity test methods include the fixed dose method and the toxic class method, among others.

I. Fixed dose method

1. Test animals

(1) Use young adult rodents (usually rats).

(2) In principle, use females. However, males should be used when there is information indicating that males are more sensitive to the test substance.

(3) Use females that are nulliparous and non-pregnant.

2. Administration method

Administer the test substance in a single dose by gavage, and when necessary, dissolve or suspend it in water or a suitable vehicle. However, the relevant vehicle should be of known toxicity and such as will not seriously affect the test results.

The species of animal used should be considered as regards the degree of fasting prior to test substance administration.

3. Observation period

Observations should be conducted for at least 14 days.

4. Setting the number of animals

(1) Sighting study

Use 1 animal for each dose level.

(1) Main study

Use 5 animals for each dose level. However, for dose levels that have been implemented in the sighting study, 4 animals are added to the 1 animal used in the sighting study, for a total of 5 animals.

5. Test procedure

(1) Sighting study

To select the starting dose levels for the main study, conduct the study in accordance with Annex 2-1-1-① using the doses of 5, 50, 300, and 2,000 mg/kg of body weight. As the initial dose level, select a dose at which evident toxicity is expected to be manifested. When no information is available regarding the acute toxicity of

the test substance, it is desirable to start with a dose of 300 mg/kg. A period of at least 24 hours should be allowed between the dosing of each level.

When death occurs at a dose level of 5 mg/kg, regard LD_{50} as ≦ 5 mg/kg of body weight, and terminate the study without conducting the main study. However, if further confirmation of LD_{50} is needed, additional procedures may be conducted.

(2) Main study

Conduct the study in accordance with Annex 2-1-1-②. However, regard the dose level at which a death is observed in the sighting study as one in which 2 deaths occur in the main study, without actually conducting the main study. Determine the interval between dosing at each level according to the duration and severity of toxic symptoms. Do not proceed to the next dose until survival or death can be confirmed for the previous dosed animals.

(3) Limit test

If there are no deaths in the sighting study at a dose level of 2,000 mg/kg, and 1 death or none in the main study at a dose level of 2,000 mg/kg, there is no need to administer doses exceeding 2,000 mg/kg.

6. Observation and examination

Conduct the items in (1) and (2) below.

(1) Observation as to general condition

(i) Carefully observe the general condition of animals at least once within 30 minutes after dosing, frequently during the following day, and thereafter at least once daily.

(ii) Keep a record of all types of symptoms of poisoning noted by gross observation in each animal, as well the time of occurrence, and the time of recovery or death.

(iii) Weigh the test animals immediately prior to and 1 week after test substance administration. If a test animal dies, weigh it at the time of death.

(iv) In order to minimize the loss of test animals that are useful for evaluating toxicity, institute appropriate measures (necropsy with gross observation, quarantining, etc.) promptly upon discovering dead, weakened, or moribund animals.

(2) Pathological examination

Conduct necropsies of all test animals, and record gross pathological findings. It is desirable to conduct histopathological examinations with reference to macroscopic gross observations of the organs of test animals that survived 24 hours or more after test substance administration.

II. Toxic class method

1. Test animals

In conformity with fixed dose method.

2. Administration method

In conformity with fixed dose method.

3. Observation period

In conformity with fixed dose method.

4. Setting the number of animals

Use 3 animals for each dose step.

5. Test procedure

(1) Select the starting test dose levels from among dosages of 5, 50, 300, and 2,000 mg/kg of body weight, and conduct tests in accordance with Annex 2-1-1-③. As the initial dose level, select a dose which is most likely to produce mortality in some of the dosed animals. When no information is available regarding the acute toxicity of the test substance, it is desirable to start with a dose of 300 mg/kg.

(2) Determine the interval between dosing at each step according to the duration and severity of toxic symptoms. Do not proceed to the next dosing until survival or death can be confirmed for the previous dosed animals.

(3) If there is 1 death or none at a dose level of 2,000 mg/kg administered to 3 animals, 2,000 mg/kg should be administered to an additional 3 animals. If the test substance causes 1 or no deaths after the second administration as well, there is no need to administer doses exceeding 2,000 mg/kg.

6. Observation and examination

Conduct the items in (1) and (2) below.

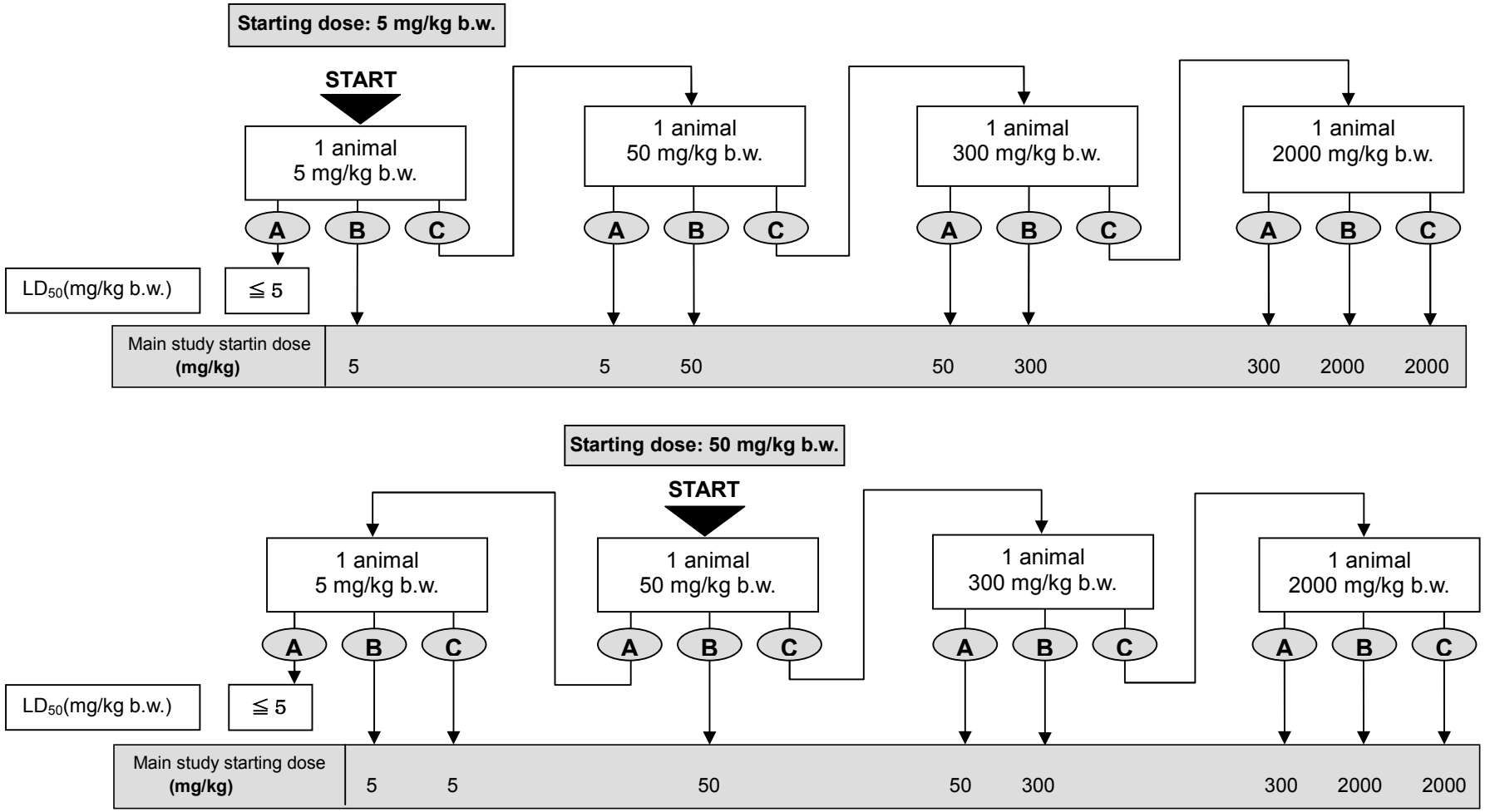
(1) Observation as to general condition

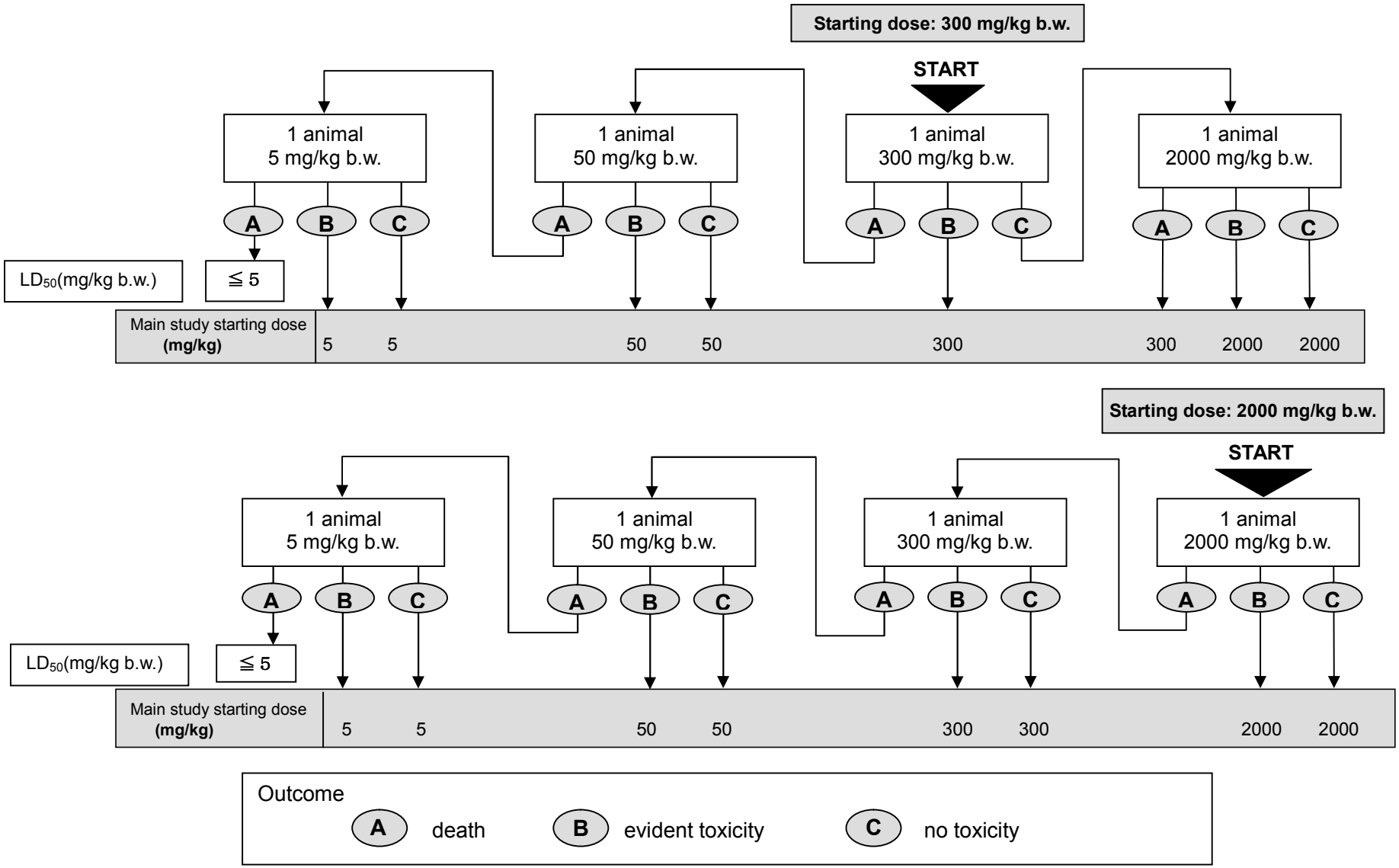
In conformity with fixed dose method.

(2) Pathological examination

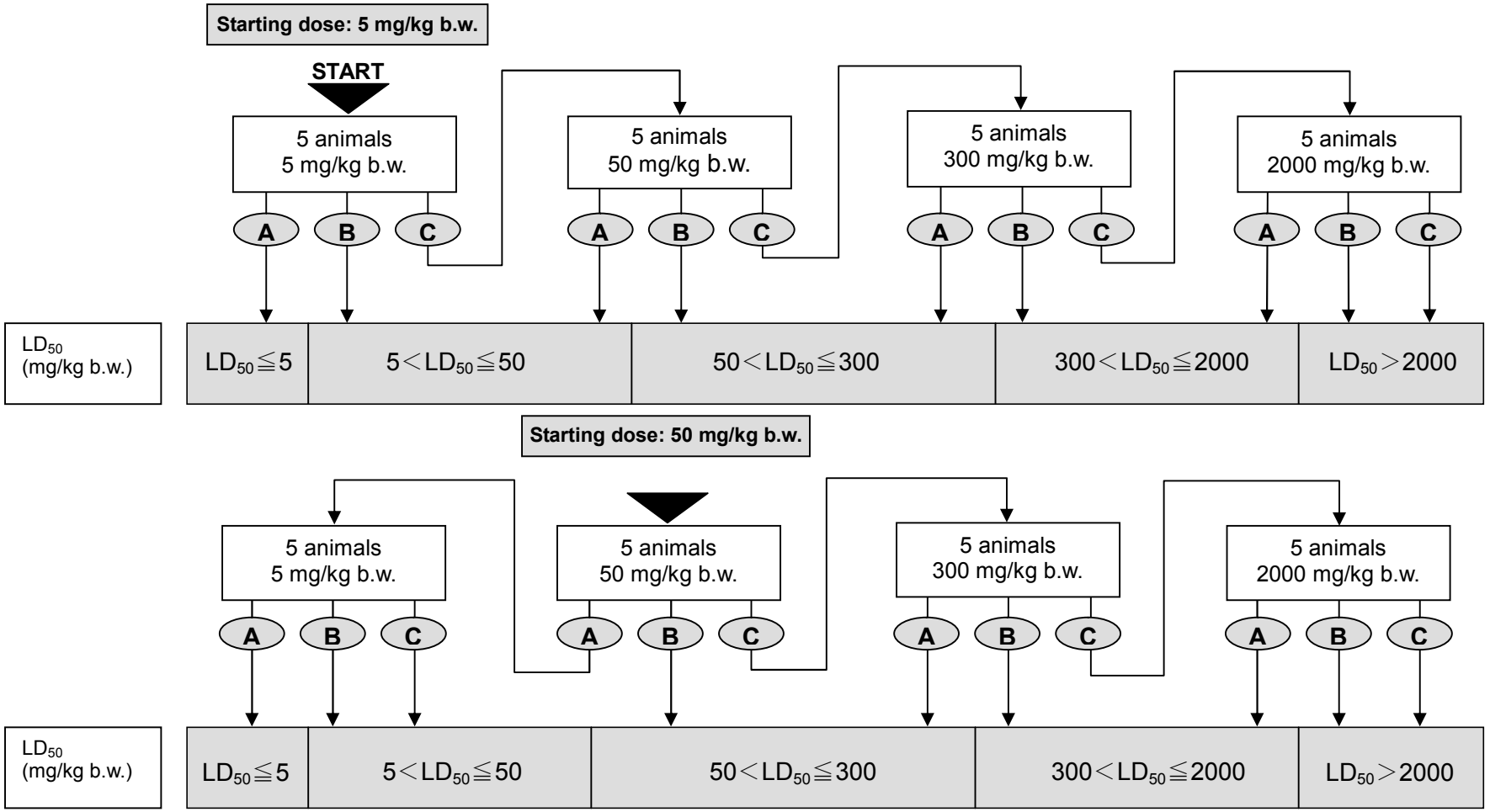
In conformity with fixed dose method.

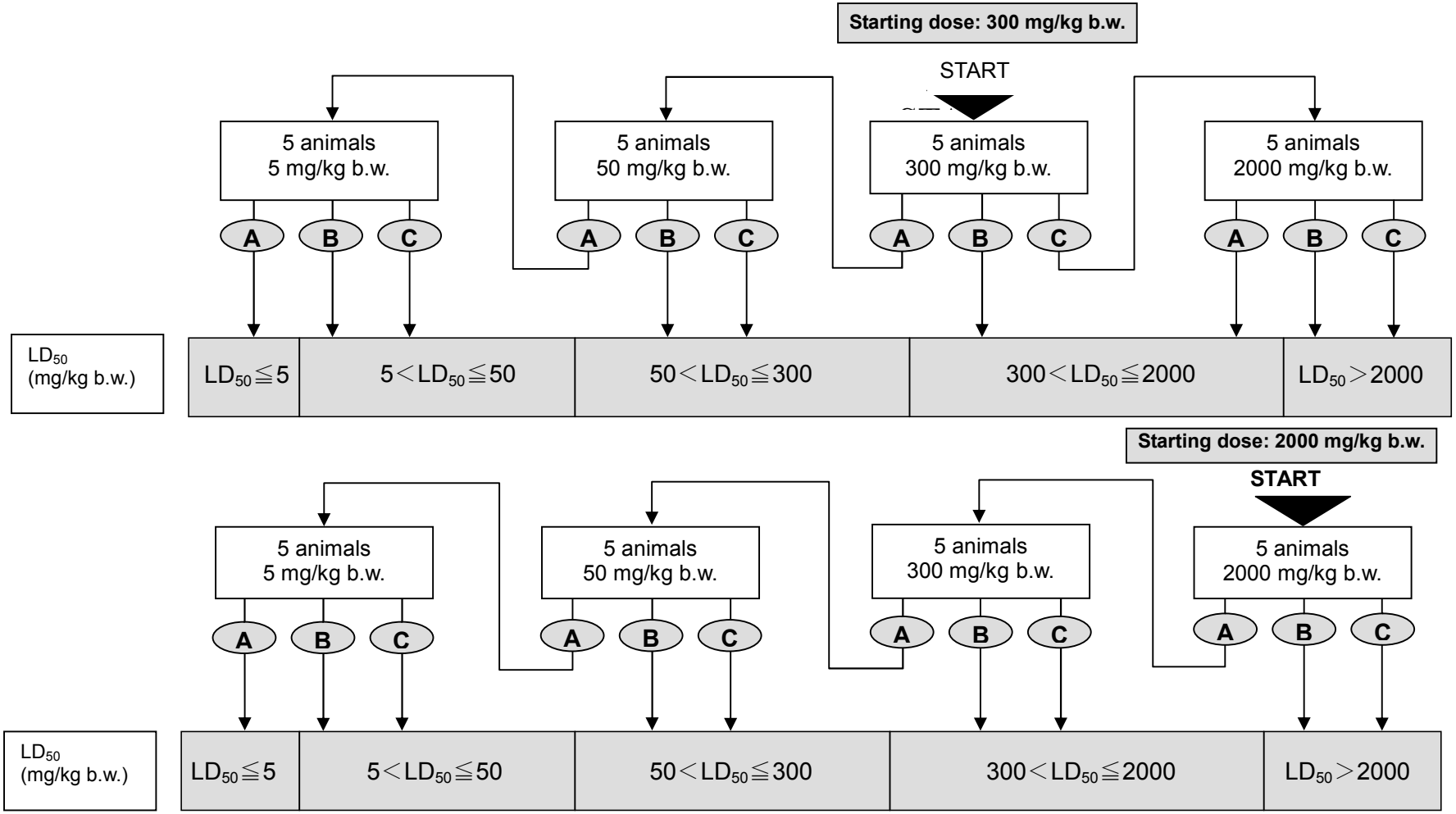
Annex 2－1－1－①：Fixed dose method／Flow chart for the sighting study




Starting dose: 300 mg/kg b.w.
START
1 animal
5 mg/kg b.w.
1 animal
50 mg/kg b.w.
1 animal
300 mg/kg b.w.
1 animal
2000 mg/kg b.w.
A
B
C
LD50(mg/kg b.w.)
≦ 5
Main study starting dose
(mg/kg)
5 5 50 50 300 300 2000 2000
Starting dose: 2000 mg/kg b.w.
START
1 animal
5 mg/kg b.w.
1 animal
50 mg/kg b.w.
1 animal
300 mg/kg b.w.
1 animal
2000 mg/kg b.w.
LD50(mg/kg b.w.)
≦ 5
Main study starting dose
(mg/kg)
5 5 50 50 300 300 2000 2000
Outcome
A death
B evident toxicity
C no toxicity

Annex 2－1－1－②：Fixed dose method／Flow chart for the main study



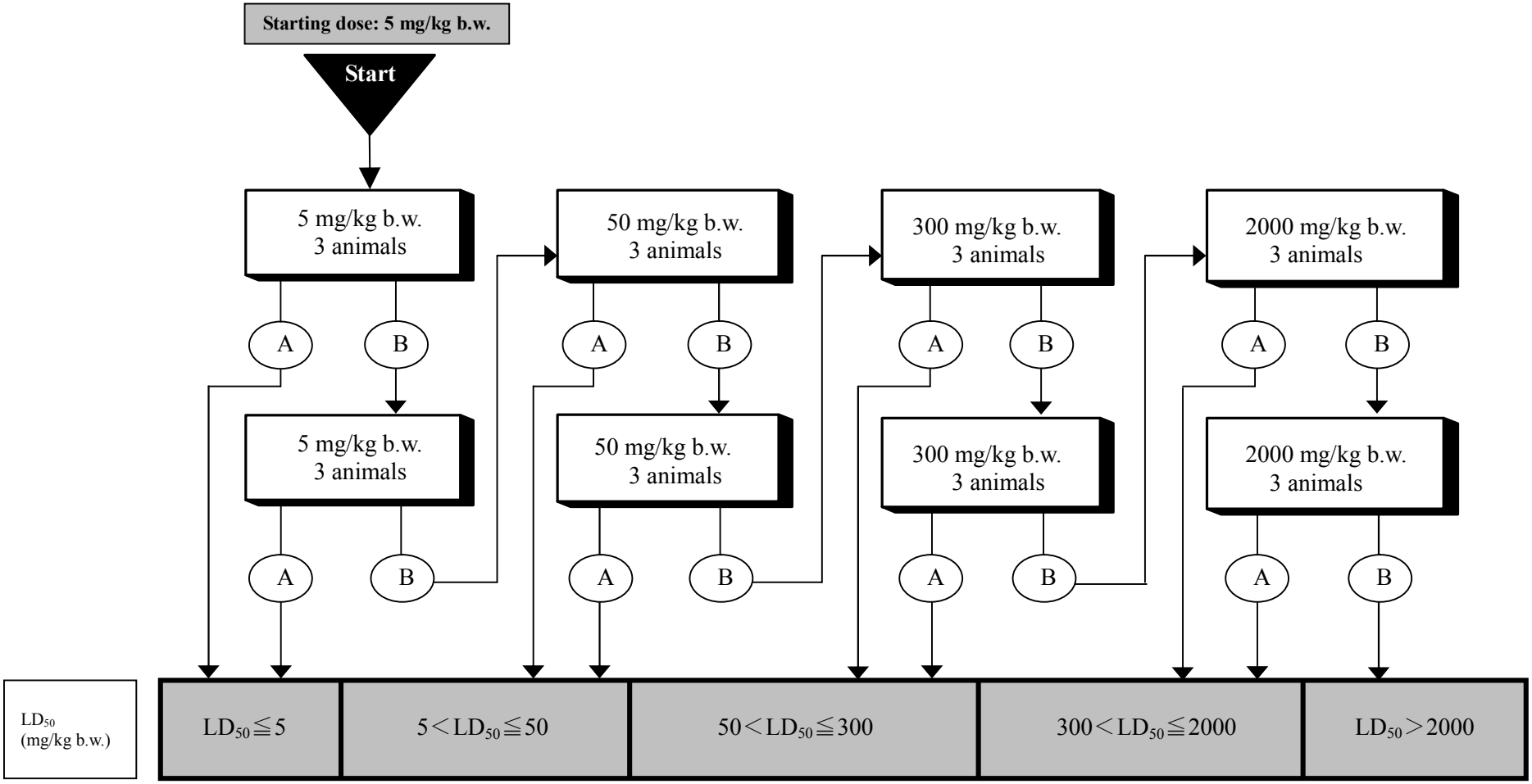


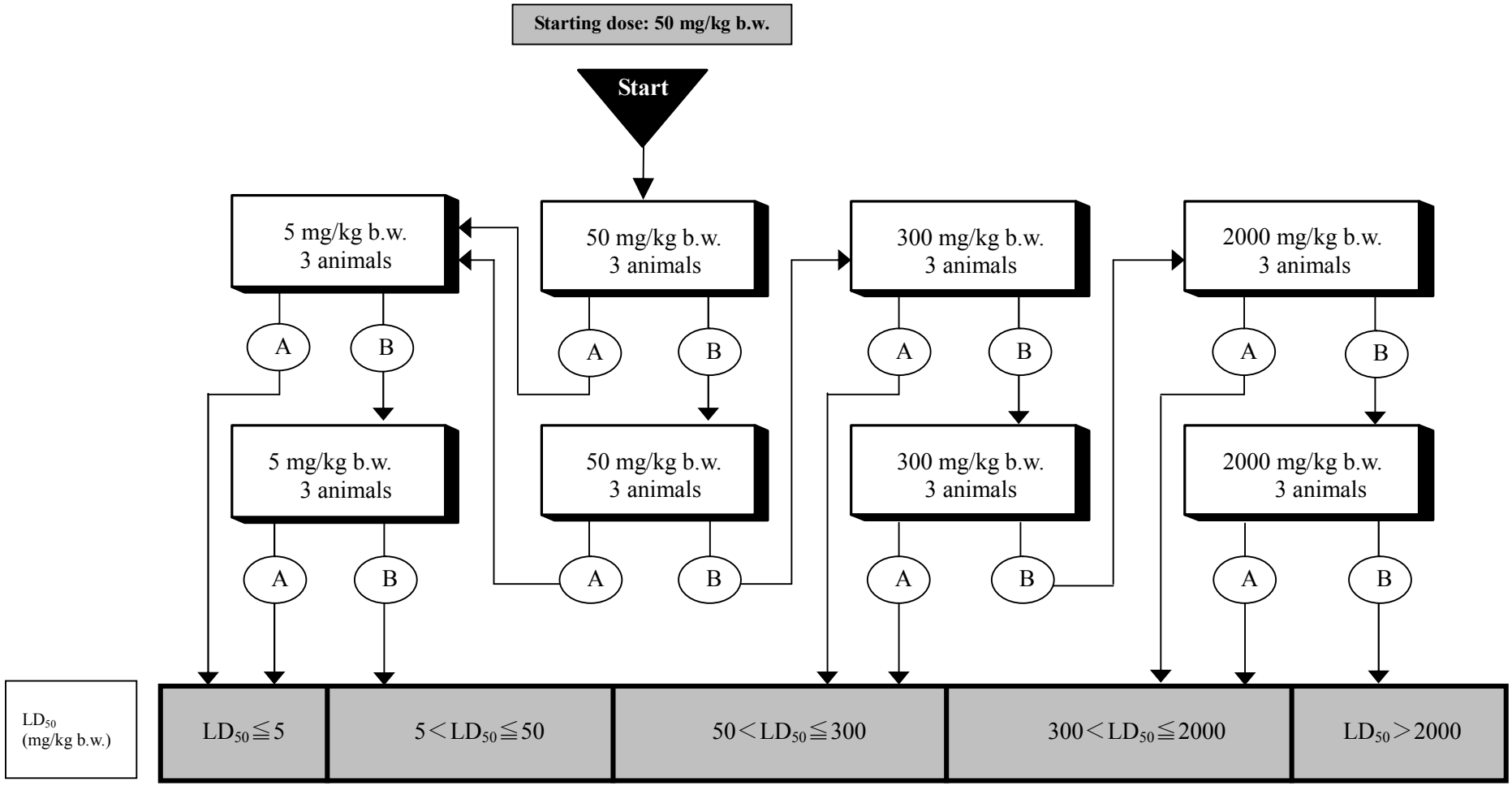
Outcome

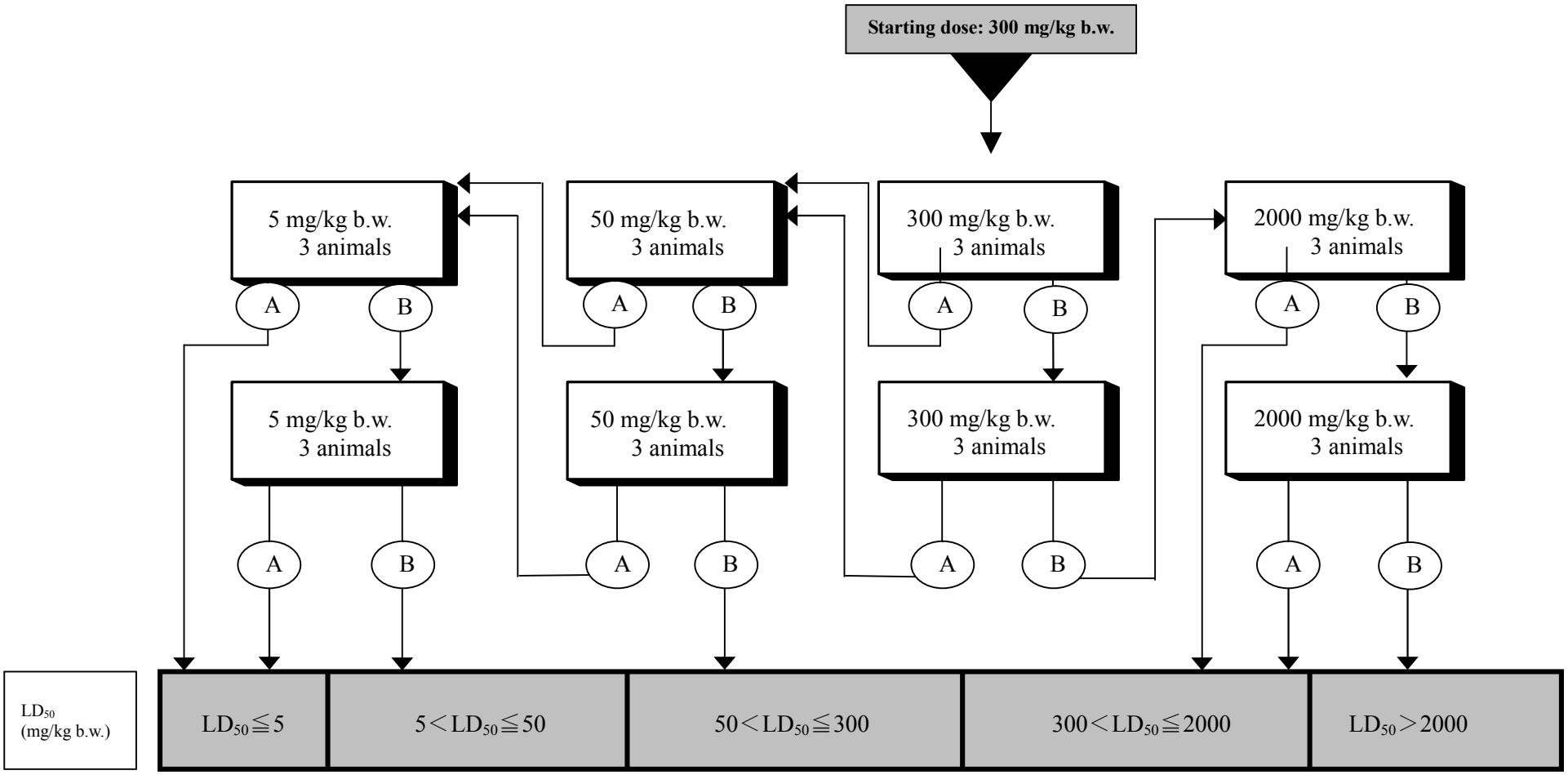
A ≧2 deaths　　B 1 death and/or ≧1 with evident toxicity　　C no toxicity

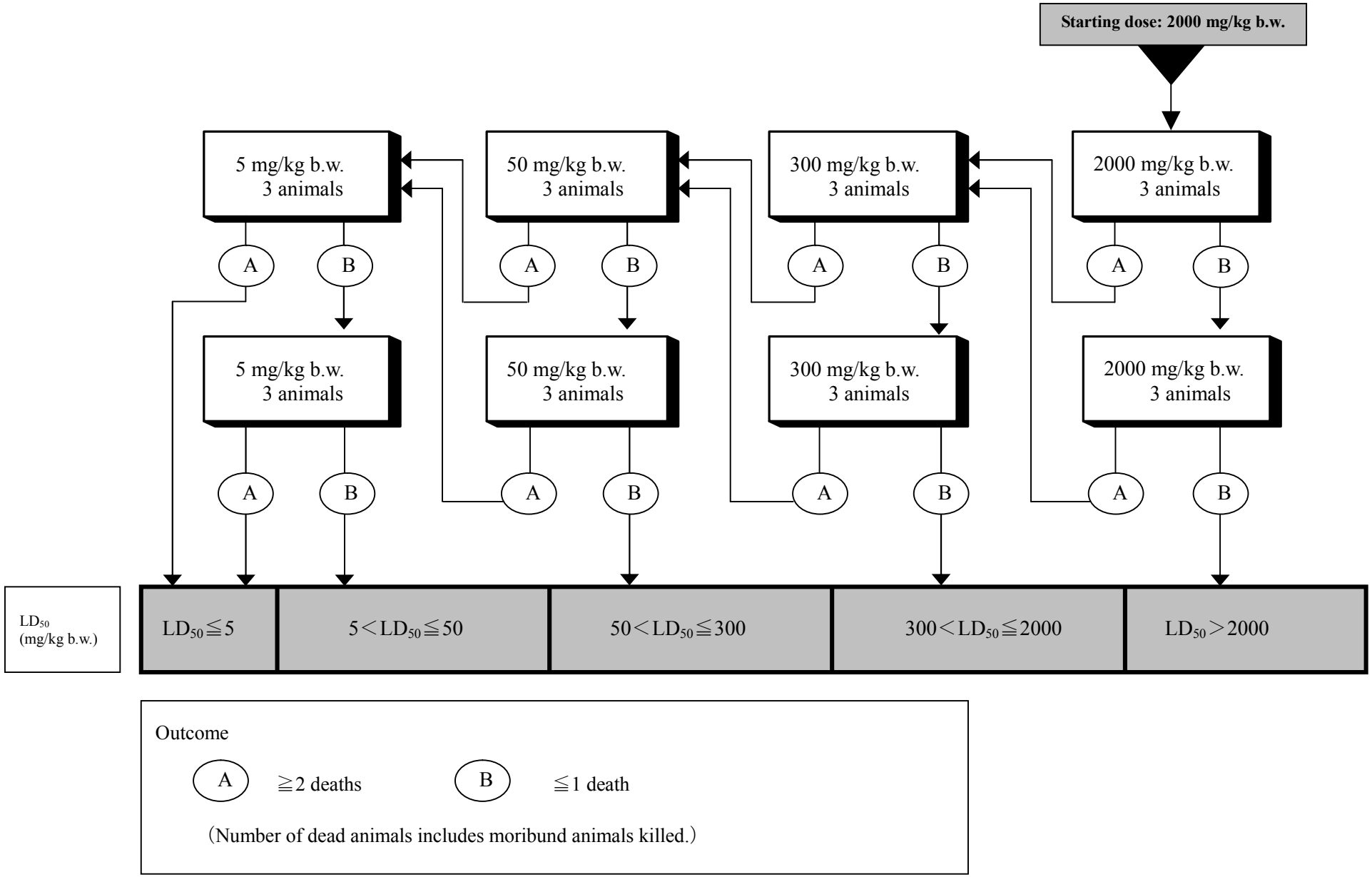
- For dose levels that have been implemented in the sighting study, 4 animals are added to the 1 animal used in the sighting study, for a total of 5 animals.
- Regard the dose level at which a death is observed in the sighting study as one in which 2 deaths occur in the main study, without actually conducting the main study.

Annex 2－1－1－③：Toxic class method／Flow chart




Starting dose: 50 mg/kg b.w.
Start
5 mg/kg b.w.
3 animals
50 mg/kg b.w.
3 animals
300 mg/kg b.w.
3 animals
2000 mg/kg b.w.
3 animals
A
B
5 mg/kg b.w.
3 animals
50 mg/kg b.w.
3 animals
300 mg/kg b.w.
3 animals
2000 mg/kg b.w.
3 animals
LD50
(mg/kg b.w.)
LD50≦5
5＜LD50≦50
50＜LD50≦300
300＜LD50≦2000
LD50＞2000


Starting dose: 300 mg/kg b.w.
5 mg/kg b.w. 3 animals
50 mg/kg b.w. 3 animals
300 mg/kg b.w. 3 animals
2000 mg/kg b.w. 3 animals
A
B
5 mg/kg b.w. 3 animals
50 mg/kg b.w. 3 animals
300 mg/kg b.w. 3 animals
2000 mg/kg b.w. 3 animals
LD50 (mg/kg b.w.)
LD50≦5
5＜LD50≦50
50＜LD50≦300
300＜LD50≦2000
LD50＞2000


Starting dose: 2000 mg/kg b.w.
5 mg/kg b.w. 3 animals
50 mg/kg b.w. 3 animals
300 mg/kg b.w. 3 animals
2000 mg/kg b.w. 3 animals
A
B
5 mg/kg b.w. 3 animals
50 mg/kg b.w. 3 animals
300 mg/kg b.w. 3 animals
2000 mg/kg b.w. 3 animals
A
B
LD50 (mg/kg b.w.)
LD50≦5
5＜LD50≦50
50＜LD50≦300
300＜LD50≦2000
LD50＞2000
Outcome
A ≧2 deaths
B ≦1 death
（Number of dead animals includes moribund animals killed.）

Acute dermal toxicity Test (2-1-2)

1. Objective

   The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding health hazards that may result from a single dermal exposure to the agricultural chemical.

2. Test animals

   (1) Use 1 or more species of mammals such as rats, rabbits, or guinea pigs. (Generally speaking, the weight ranges of test animals should be as follows: Rats, 200–300 g; rabbits, 2.0–3.0 kg; guinea pigs 350–450 g.)

   (2) Use young adults.

   (2) Nulliparous, non-pregnant females should be used.

3. Administration method

   (1) Remove the hair from the back trunks of the test animals by clipping or shaving 24 hours prior to test substance administration. At this time, be careful to avoid injuring the skin, since this will affect its permeability to the test substance.

   (2) Shave completely at least 10% of the body surface area (rats, 4 cm x 5 cm; rabbits, 12 cm x 14 cm; guinea pigs, 7 cm x 10 cm) for application of the test substance. Take the animals' body weights into account when determining the area to be shaved.

   (3) Apply the test substance within a range that is approximately 10% of the body surface area. There are cases in which test substances of high toxicity may be applied to a smaller area, but insofar as possible, apply the substance thinly and uniformly to the entire application site.

   (4) When the test substance is a solid, grind it as appropriate, and moisten it with water or other vehicle so that it makes good contact with the skin. If using a vehicle, use one that will not irritate the skin, and be careful that the vehicle does not affect the skin's permeability to the test substance.

   (5) The test substance is to be applied to the skin for a period of 24 hours. During that time, cover the application site with porous gauze, and secure it with non-irritating tape so as to preserve contact with the skin. An additional appropriate method of covering must be used to preserve the test substance and gauze, so that the test animals are not able to ingest the test substance.

   (6) Use water or an appropriate vehicle to remove test substance that is adhering to the skin at the end of the application period.

4. Observation period

   Conduct observations for at least 14 days.

5. Determining the number of animals, and establishing test groups

   (1) Determining the number of animals

   Assign 5 animals, all of the same sex, to each group.

(2) Establishing test groups

(i) Establish test substance dosage groups according to at least 3 dosage levels.

(ii) In addition to studies on one sex, administer the substance to at least 1 group of the other sex, to confirm that the other sex of the animal does not have a notably high susceptibility to the test substance. If sufficient information has been obtained to indicate that one sex is more susceptible to the test substance than the other, studies with the other sex may be omitted.

(iii) Establish groups with dosage levels at appropriate intervals for symptoms of poisoning and death among test animals. The groups must be established so as to be sufficient for determination of the dose-response curve and LD_{50}.

(3) Limit tests

If death is not confirmed as a result of a single administration of 2,000 mg/kg of body weight or more of the test substance, it is not necessary to conduct studies with groups that receive a higher dosage than that. However, 1 group of the opposite sex should also be administered 2,000 mg/kg of body weight, in order to check susceptibility.

6. Observation and examination

Conduct the items in (1) and (2) below.

(1) Observation as to general condition

(i) Carefully observe the general condition of animals, frequently during the day on which the test substance is administered, and thereafter at least once daily.

(ii) Keep a record of all types of symptoms of poisoning noted by gross observation in each animal, as well the time of occurrence, and the time of recovery or death.

(iii) Weigh the test animals immediately prior to and 1 week after test substance administration. If a test animal dies, weigh it at the time of death.

(iv) In order to minimize the loss of test animals that are useful for evaluating toxicity, institute appropriate measures (necropsy with gross observation, quarantining, etc.) promptly upon discovering dead, weakened, or moribund animals.

(2) Pathological examination

In conformity with acute oral toxicity studies.

Acute inhalation toxicity Test (2-1-3)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding health hazards that may result from a single exposure to the agricultural chemical via inhalation.

2. Test animals

(1) Use young adult mammals (usually rats) of 1 or more species.

(2) Nulliparous, non-pregnant females should be used.

3. Exposure method

(1) Expose animals to the set concentration of the substance for at least 4 hours, using inhalation equipment. The exposure method may be whole body or pernasal exposure. Do not provide food or water during exposure.

(2) Monitor flow rate, actual concentration of the test substance, particle size distribution, temperature, and humidity during exposure. Maintain the uniformity of these conditions.

(3) Particle size (aerodynamic mass median size) of 1-4 μm is preferable. Or, use the minimum particle size with which it is possible to conduct the test.

(4) If the test substance is volatile, ensure that it is not of a concentration that would cause an explosion.

4. Observation period

Conduct observations for at least 14 days.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

Assign 5 animals, all of the same sex, to each group.

(2) Establishing test groups

(i) Establish test substance dosage groups according to at least 3 dosage levels.

(ii) In addition to studies on one sex, administer the test substance to at least 1 group of the other sex, to confirm that the other sex of the animal does not have a notably high susceptibility to the test substance. If sufficient information has been obtained to indicate that one sex is more susceptible to the test substance than the other, studies with the other sex may be omitted.

(iii) Establish groups with exposure levels at appropriate intervals for symptoms of poisoning and death among test animals.

(iv) The groups must be established so as to be sufficient for determination of the concentration/response relationship, and the approximate median lethal dose (LC_{50}).

(v) When a vehicle is used for maintaining the proper concentration of the test substance in the exposure environment, it is desirable not to use a vehicle that is known to be toxic, or that will greatly affect the test results.

(vi) When necessary, conduct studies with a vehicle control group.

(3) Limit test

(i) If death ascribable to the test substance does not result in studies with an exposure concentration of 5 mg/L

for 4 hours, it is not necessary to conduct studies with higher concentrations than that. However, 1 group of the opposite sex should also be exposed to a concentration of 5 mg/l, in order to check susceptibility.

(ii) When an exposure concentration of 5 mg/L is impossible due to the physico-chemical properties of the test substance, and no death that is ascribable to the test substance occurs at the maximum concentration that can be obtained by means of the operational procedures in this test method, it is not necessary to conduct studies with higher concentrations than that. However, 1 group of the opposite sex should also be exposed to a high concentration, in order to check susceptibility.

6. Observation and examination

Conduct the items in (1) and (2) below.

(1) Observation as to general condition

In conformity with dermal toxicity tests.

(2) Pathological examination

(i) Pay attention to changes in the respiratory system, and take observed instances of poisoning into account. Conduct necropsies of all test animals, and record macroscopically pathological findings.

(ii) It is desirable to conduct histopathological examinations with reference to macroscopical gross observations of the organs of test animals that survived 24 hours or more.

Skin irritation test (2-1-4)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding skin irritant properties and corrosiveness of the test substance.

2. Test animals

Use 3 or more young adult albino rabbits.

3. Administration method

(1) Crip the hair on the back trunks of the animals 24 hours prior to the study. Be careful not to injure the skin, and only use healthy animals with undamaged skin.

(2) When the test substance is a solid, grind it, as appropriate, and moisten it with water or other vehicle, so that it makes good contact with the skin. If using a vehicle, use one that will not irritate the skin, and be careful that the vehicle does not affect its permeability by the test substance. In general use liquid test substance undiluted.

(3) Apply locally 0.5 ml of liquid test substance, or 0.5 g of test substance in the form of a solid or paste.

(4) Apply the test substance to a small area of the skin (approximately 6 cm^3). During the administration (application) period, cover the site with a gauze patch, secured with non-irritating tape. Procedures whereby the test substance, in the form of a liquid or paste, is applied to the gauze patch, which is then applied to the skin, are also acceptable. Using a semi-occlusive dressing, situate the patch in such a way that it will maintain contact with the skin throughout the exposure period (in some cases, an occlusive dressing may be used instead). Compare to the untreated areas of the animal's body.

(5) Normally, the exposure period should be 4 hours in duration. Use water or an appropriate vehicle to remove test substance that is adhering to the skin at the end of the exposure period.

4. Points to keep in mind regarding application

(1) When severe skin irritant properties or corrosiveness are suspected:

(i) Conduct the test with only 1 animal when it is suspected that the test substance has severe skin irritant properties or corrosiveness.

(ii) When corrosiveness is suspected, apply 3 test patches simultaneously to 1 animal. Remove the first patch after an exposure of 3 minutes. If no severe skin reaction is observed, remove the second patch after an exposure of 1 hour. If it is found at this stage, from the standpoint of prevention of cruelty to animals, that the study period can be extended to 4 hours, remove the third patch after 4 hours of exposure. If severe skin irritation is observed after 3 minutes or 1 hour of exposure, remove the remaining patch(es) and terminate the study.

Instead of the above, the 3 patches may be applied successively, and observations made accordingly.

(iii) When it is suspected that the test substance is a severe skin irritant, apply 1 patch to 1 animal for 4 hours.

(iv) If no severe skin irritation is observed after a 4-hour exposure, 2 other animals may be tested with the patch for 4 hours each.

(2) When the test substance is not expected to cause severe skin irritation or corrosion:

Commence studies using 3 animals, applying to each 1 patch for 4 hours of exposure.

5. Observation and rating of general condition

(1) Examine symptoms of erythema and edema when patches have been removed from the animals after 30 (or 60) minutes, 24 hours, 48 hours, and 72 hours. Rate the skin reactions.

(2) Rate and record skin irritation properties and corrosiveness according to the criteria in the table below. Continue observations thereafter when it is necessary to clarify whether the symptoms are reversible. In general, it is not necessary for observations to extend beyond 14 days.

(3) Keep proper records of severe injuries and other signs of poisoning, in addition to skin irritation and corrosion.

(Table) Skin irritation and corrosion criteria

1. Erythema and eschar formation

(1) No erythema 0
(2) Very slight erythema (barely perceptible)..... 1
(3) Well-defined clear erythema 2
(4) Moderate to severe erythema 3
(5) Severe erythema (deep redness) or eschar formation (erythema is not scorable)..... 4

Maximum score: 4

2. Edema formation

(1) No edema.....0
(2) Very slight edema (barely perceptible) 1
(3) Slight edema (edges of area well defined by definite raising) 2
(4) Moderate edema (swelling of approximately 1 mm) 3
(5) Severe edema (swelling > 1 mm, and extending beyond the exposed area).....4

Maximum score: 4

Eye irritation test (2-1-5)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding irritation and corrosion of eyes and ocular mucous membranes by the test substance.

2. Test animals

Use 3 or more young adult albino rabbits.

3. Administration method

(1) Examine both eyes of test animals within 24 hours prior to study commencement. Do not use animals with ocular abnormalities.

(2) Apply no more than 0.1 ml of undiluted liquid test substance, or 0.1 ml by volume or 0.1 g by weight of solid or paste. (Always record volume or weight.) If the test substance is solid or granular, grind it to a fine powder.

(3) Gently separate the lower eyelid of one eye from the eyeball, and apply the test substance to the conjunctival sac. Gently bring upper and lower eyelids together for approximately 1 second, in order to prevent loss of the test substance. Compare with the other, untreated eye.

Apply test substance that comes in pressurized aerosol containers by spraying a single jet for 1 second from 10 cm in front of the open eye.

(4) Local anesthetic may be used if it is thought that the test substance will cause severe pain. However, sufficient care should be taken that use of the local anesthetic does not lead to a significant difference in the organism's reactivity to the test substance.

(5) Do not wash the eye to which the test substance was applied for 24 hours following administration of the test substance eye drops. The eye may be washed 24 hours after administration if this is deemed appropriate.

(6) If severe ocular irritation occurs as a result of the test substance, conduct studies on at least 3 animals as to the effectiveness of washing their eyes. In such cases, wash the treated eye 30 seconds after administration for 30 seconds in a volume and at a flow velocity that will not injure the eye.

4. Points to keep in mind regarding application

Conduct the study with only 1 animal when it is suspected that the test substance is a severe eye irritant. If severe eye irritation or corrosion are observed as a result, it is not necessary to conduct studies with additional test animals.

5. Observation of general condition and scoring

(1) Observe the general condition of the eye 1 hour, 24 hours, 48 hours, and 72 hours after administration. Record observations, and also record reactivity (irritation or corrosion) of the eye to the test substance, based on the criteria in the table below. In addition, examination used fluorescein may be conducted in some or all of the test animals' eyes following observation 24 hours after administration.

(2) If no eye irritation is observed by 72 hours after administration, the studies can be regarded as finished.

(3) If persistent corneal injury or other ocular irritation is noted, continue to observe its progress (as to reversibility, irreversibility, etc.) for no more than 21 days following administration of the test substance.

(4) In addition to observation of the cornea, iris, and conjunctiva, keep records of all injuries, etc. that are observed.

(Table) Eye irritation and corrosion criteria

Cornea *

Opacity: Degree of opacity (determined according to the most opaque area)

(1) No ulcers or opacity observed 0
(2) Sporadic or diffused opacity (different from the degree of cloudiness having ordinary luster); the details of the iris are clearly translucent 1
(3) There are some clear areas left, but nearly all of the iris is obscured 2
(4) Nacreous areas, no details of iris visible, size of pupil barely discernible 3
(5) Corneal opacity; iris not discernible through opaque areas 4

Maximum score: 4

* Record the area of corneal opacity

Iris

(1) Normal 0
(2) Clear and deep rugae, congestion, swelling, moderate hyperemia in the corneal periphery; any of these singly or in combination; the iris still reacts to light (reaction is slow and dull) 1
(3) No reaction to light; hemorrhage; gross destruction (any or all of these) 2

Maximum score: 2

Conjunctiva

Redness (eyelid, bulbar conjunctiva, cornea, and/or iris)

(1) Blood vessels normal 0
(2) Clear hyperemia in some blood vessels 1
(3) Diffuse crimson; individual blood vessels cannot be readily discerned 2
(4) Diffuse beefy red 3

Maximum score: 3

Conjunctival edema (palpebral conjunctiva and/or nictitating membrane)

(1) No swelling 0
(2) Greater than normal swelling (including nictitating membranes) 1
(3) Obvious swelling accompanying ectropion of the eyelid 2
(4) Swelling such that the eyelid is less than half 3
(5) Swelling such that the eyelid is half or more 4

Maximum score: 4

Skin sensitization test (2-1-6)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding skin sensitization by the test substance.

2. Test animal species, age, and sex

(1) Use young adult guinea pigs.

(2) Nulliparous, non-pregnant females should be used.

3. Test methods

The Guinea Pig Maximization Test (hereinafter referred to as the "GPM method") and the Buehler Test (hereinafter referred to as the "Buehler method") are test methods that are conducted relatively frequently. However, other test methods may be substituted if information regarding sensitization can be obtained thereby.

4. Study Procedures

(1) GPM method

(i) Establishing test groups

Establish a test substance treatment group, a negative control group, and a positive control group. Conduct studies with the positive control group, using substances known to sensitize. Recent background data may be used if available.

(ii) Establishing the number of animals

a. There should be at least 10 animals in each test substance treatment group, and at least 5 in each control group.

b. If there are less than 20 animals in each test substance treatment group, and less than 10 animals in each control group, and it cannot be concluded whether the test substance causes sensitization, it would be desirable to conduct additional studies in which there are at least 20 animals in each test substance treatment group, and at least 10 animals in each control group.

(iii) Establishing dosages

a. The concentration of the test substance used for induction exposure should be such as the test animal is, in general, sufficiently able to tolerate, and should be the maximum concentration at which slight to moderate skin irritation occurs.

b. The concentration of the test substance used for challenge exposure should be the maximum at which irritation does not occur.

c. Use 2 or 3 animals, and determine the appropriate concentration of the test substance.

(iv) Initial induction (intracutaneous injection)

Use the following method.

a. Test substance treatment group

Administer the following set of 3 injections (0.1 ml) on both sides of the median line of the area of the back from which the hair has been removed.

Injection 1: Freund's complete adjuvant (hereinafter referred to as "FCA") and water (or saline) in 1 : 1 (v/v) mixture

Injection 2: The designated concentration of the test substance in an appropriate vehicle

Injection 3: The designated concentration of the test substance in a 1 : 1 (v/v) mixture of FCA and water(or saline)

b. Negative control group

Administer the following set of 3 injections (0.1 ml) to the same site as in the test substance

treatment group.

Injection 1: FCA and water (or saline) in a 1 : 1 (v/v) mixture

Injection 2: Only the vehicle used in the treatment group

Injection 3: FCA and water (or saline) in a 1 : 1 (v/v) mixture

(v) Re-induction (application by affixing to skin)

a. Re-induction 5-7 days after initial induction

If the test substance is not a skin irritant, apply 0.5 ml of petrolatum containing 10% sodium lauryl sulfate to the test site, on which the hair has been trimmed short or shaved approximately 24 hours prior to re-induction, in order to promote re-induction.

b. Re-induction 6-8 hours after initial induction.

Use the following method.

(a) Test substance treatment group

Remove the hair once again from the test site. Apply the test substance, which has been prepared with an appropriate vehicle, to the test site with filter paper or gauze sufficiently soaked in the preparation, and affix an occlusive dressing for 48 hours. It is necessary to have a reason for the vehicle selected. Grind solid test substance and mix it with an appropriate vehicle. Apply liquid test substance undiluted, when appropriate.

(b) Negative control group

Apply vehicle only, but in the same manner as with the treatment group, and affix an occlusive dressing for 48 hours.

(vi) Initial challenge (application by affixing to skin)

a. Conduct 14 days after re-induction (apply by affixing to skin).

b. Remove the hair from the ventral areas of animals in the teat substance administration and control groups. Affix a patch or chamber, to which the test substance has been applied, to one side of the animals' ventral areas, and, if necessary, affix another patch or chamber, to which only vehicle has been applied, to the other side in the same way.

c. Apply an occlusive dressing to the patch for 24 hours.

(vii) Observations

a. If necessary, remove the hair from the challenge area 21 hours after removing the pouch.

b. Note skin reactions 3 hours later (approximately 48 hours from the commencement of induction patch application), and record observations according to the rating standard shown below.

c. Observe skin reaction again 24 hours after the first observation, and record findings.

< Induction patch test reaction evaluation criteria >

No visible change 0

Diffuse or patchy erythema 1

Moderate dispersed erythema 2

Severe erythema and edema 3

(viii) Re-challenge

If it is necessary to further confirm results obtained from the initial challenge, re-challenge may be conducted 1 week after the initial challenge, after establishing a new control group, as appropriate. The control group used for the initial challenge may be used again.

(ix) Observations of general condition

Record all skin reactions and abnormal findings resulting from induction and challenge.

(2) Buehler method

(i) Establishing test groups

Establish a test substance treatment group, a negative control group, and a positive control group. Conduct studies with the positive control group, using substances known to sensitize. Recent background data may be used if available.

(ii) Establishing the number of animals

Use 20 animals in the test substance treatment group, and 10 in the control group.

(iii) Establishing dosages

Same as with the GPM method.

(iv) Initial induction (application by affixing to skin)

Use the following method.

a. Test substance treatment group

Remove the hair from one shoulder or side. Apply a test patch containing the test substance, which has been prepared with an appropriate vehicle, to the site, and secure it with an occlusive dressing for 6 hours.

b. Negative control group

Apply vehicle only, but otherwise use the same method as with the test substance treatment group.

(v) Re-induction (application by affixing to skin)

a. Conduct 6-8 days and 13-15 days after the initial induction

b. Treat in the same way the same shoulder or side used in the initial induction.

(vi) Challenge

a. Conduct 14 days after re-induction.

b. Remove the hair from the ventral areas of animals in the teat substance administration and control groups. Affix a patch or chamber, to which the test substance has been applied, to one side of the animals' ventral areas, and, if necessary, affix another patch or chamber, to which only vehicle has been applied, to the other side in the same way. Affix the patch with an occlusive dressing for 6 hours.

(vii) Observations

a. If necessary, remove the hair from the challenge area 21 hours after removing the patch.

b. Note skin reactions 3 hours later (approximately 30 hours from the commencement of induction patch application), and record observations according to the scoring system shown in the GPM method.

c. Observe skin reaction again 24 hours after the first observation, and record findings.

(viii) Re-challenge

Same as in the GPM method.

(ix) Observation of general condition.

Same as in the GPM method.

Acute neurotoxicity test (2-1-7)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by clarifying the neurotoxic properties of the relevant agricultural chemical following a single exposure, and by obtaining scientific information regarding the maximum dosage at which no toxic changes are observed (no observed adverse effect level: NOAEL).

2. Test animals

(1) Use rodents (rats, usually).

(2) Normally, use, as soon as possible after weaning and following acclimatization period, animals that are the same age, 5-6 weeks old.

(3) In general, use the same number of male and female animals. Nulliparous and non -pregnant females should be used.

3. Administration method

(1) Select the method of administration (oral, dermal, or inhalation) as necessary.

(2) Use the same methods as in the acute oral toxicity studies, the acute dermal toxicity studies, or the acute inhalation toxicity studies.

4. Observation period

Observe animals for 14 days following single exposure to the test substance.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) Use 10 or more each of male and female animals in each group for the studies, as necessary for detailed observations of symptoms and examination of functions. From among these, select 5 or more each of male and female animals in each group as necessary for neurohistopathological examination.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups.

(iii) It is essential, ultimately, to ensure that there is a sufficient number of animals for evaluation of test results.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. Establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in death and the minimum dose at which no toxic effects can be determined, and so that the relationship between dosages and reactions can be discerned.

c. Refer to the results of previously conducted toxicity studies when establishing dosages. In addition, indicate the grounds for the dosages established.

d. If no toxic effects are confirmed as a result of a single administration of the maximum dose that is technically possible, or 2,000 mg/kg of body weight of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.
b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct items (1)-(4) below.

(1) Observations as to general condition

(i) Carefully observe the general condition of the animals every day.

(ii) Observe overall condition, and whether or not any abnormal behavior or deaths occur.

(iii) Weigh all animals prior to the commencement of administration, and at least 1 week after the commencement of administration.

(2) Detailed observation of condition

(i) Conduct these observations in regard to 10 or more each of male and female animals in each relevant group prior to the commencement of administration, and within 8 hours after administration, when the effect is expected to be most pronounced, as well as 7 and 14 days after administration. Observe the animals both in their cages and on the observation stand.

(ii) Use rating methods for which the criteria for judgment and measurement have been clearly stipulated, and follow standardized procedures.

(iii) In general, conduct examinations in regard to the following items:

Appearance (skin; fur; changes in eyes, eyeballs, and mucous membranes), body position and posture (hunchback posture, etc.), autonomic nervous system function (lacrimation, piloerection, pupil diameter, respiration, excretion, etc.), motor coordination, ambulatory abnormalities, animal's reactions to being handled and to environmental stimulation, nervous system (tremor, convulsion, muscular contractions, etc.), changes in exploratory behavior, ordinary behavior (changes in grooming, headshaking, gyration, etc.), abnormal behavior (autophagia, backward motion, abnormal vocalization, etc.), aggression, etc.

(3) Functional examination

(i) Conduct these observations in regard to 10 or more each of male and female animals in each relevant group prior to the commencement of administration, and within 8 hours after administration, when the effect is expected to be most pronounced, as well as 7 and 14 days after administration.

(ii) In general, conduct examinations in regard to the following items:

Sensorimotor reactions to various stimuli (including auditory, visual, and proprioceptor stimuli), grip strength, and amount of spontaneous motor activity (using an automatic recording apparetus).

(iii) If it is suspected, on the basis of other toxicity studies, that the test substance is a neurotoxin, conduct studies and careful examinations regarding the appropriate sensory mechanisms, motor function, and learning/memory relating to the suspected neurotoxicity.

(4) Physiological examination

(i) Conduct histopathological examinations in regard to 5 or more each of male and female animals in each relevant group.

(ii) If abnormalities are noted in specific animals during symptomatic observations and functional examinations, examine these animals.

(iii) Fix tissues by means of perfusion fixation or other appropriate method. Record all gross observations of physiological changes.

(iv) In general, embed tissue sample in paraffin, and use a staining agent, such as hematoxylin and eosin stain.

However, if nerve damage in the peripheral nervous system is observed or suspected, adjust and examine peripheral nerve tissues embedded in resin.

(v) Conduct additional examinations and special staining, as appropriate, based on symptomatic observations.

(vi) Evaluate neurohistopathological findings by relating them to other toxicity test results and behavioral effects.

(vii) Conduct phased examinations of representative sections from the central and peripheral nervous systems.

(viii) First of all, compare sections from the maximum dosage group and the control group. If no neurohistopathological changes are observed in the maximum dosage group, no further examinations are necessary. If changes are observed in the maximum dosage group, examine sections from the median and minimum dosage groups, in that order.

(ix) In general, examine the following tissues:

The central cerebrum, including the prosencephalon and the hippocampus; the mesencephalon, cerebellum, pons, and medulla oblongata; the eyeball, including the optic nerve and retina; the cervical and lumbar enlargements of the spinal cord; the spinal ganglion, the ventral and dorsal roots of nerve fibers, the proximal sciatic nerve, the proximal tibial nerve (in the knee area), the gastrocnemius bifurcation of the tibial nerve, and the skeletal muscles (especially the gastrocnemius).

(x) The sections from the spinal cord and peripheral nerves should include both horizontal and vertical sections.

Acute delayed neurotoxicity test (2-1-8)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific findings regarding agricultural chemicals that may be expected to have delayed neurotoxic properties based on test results in regard to acute toxicity and other toxicity, or on the correlation of their chemical structures with those of other substances known to exhibit delayed neurotoxicity.

2. Test animals

(1) Use hens of ordinary varieties and strains.

(2) It is desirable to use young adult hens of standard size, 8-12 months old.

(3) Use healthy animals that have not had viral diseases or drug treatments that would affect actual test results, nor any ambulatory abnormalities.

3. Administration method

(1) Administer once, forcibly and orally.

(2) If the test substance is a liquid, administer it as liquid concentrate, or after dissolving in an appropriate vehicle.

(3) If the test substance is a solid, administer it in solution, insofar as possible.

4. Observation period

Conduct observations for 21 days following administration.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) Use a number of animals in both the administration and control groups such that the 6 birds needed for biochemical studies, as well as 6 birds that can survive until the end of the studies and can be used in histopathological studies, will be available.

(ii) Use a number of animals the positive control group such that the 3 birds needed for biochemical studies, as well as 3 birds that can survive until the end of the studies and can be used in histopathological studies, will be available.

(2) Establishing test groups

(i) Test substance administration group

Set the highest dosage possible (maximum non-lethal dose), at which fatalities were not observed in preliminary studies.

Administer the maximum non-lethal dose of the test substance, with 2,000 mg/kg/day as the upper limit.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration group, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which

sufficient information on toxicity cannot be obtained.

(iii) Positive control group

Conduct studies with substances known to have delayed neurotoxic properties (for example, TOCP). Recent background data may be used if available.

6. Note regarding care

Keep the animals in a cage or pen that is large enough to allow the animals to walk around freely, and readily permit observation of their ambulatory status.

7. Observation and examination

Conduct the following items (1)-(3).

(1) Observation of general condition

(i) Commence observation of all animals immediately after administration. Carefully observe them several times per day for the next 2 days, and then at least once per day for the following 21 days, or until planned sacrifice.

(ii) Keep records of all symptoms of toxicity, including their type, time of occurrence, degree, and duration. Evaluate ataxia on the basis of criteria comprising at least 4 levels.

(iii) Animals used in pathological examinations should be brought out of their cages at least twice per week. Conduct forced movement at fixed times, in order to be able to observe even slight toxic effects.

(iv) Conduct gross pathological examinations of moribund animals after removing and sacrificing them.

(v) Weigh all animals prior to administration and at least once a week thereafter.

(2) Biochemical examination

(i) Sacrifice 6 birds each chosen at random from the administration and control groups, and 3 birds from positive control group, within 72 hours after administration of the test substance. Harvest their brains and lumbar spinal cords, and measure the neuropathy target esterase (NTE, also called neurotoxic esterase) activity.

Normally, sacrifice 3 birds each from the administration and control groups 24 hours and 48 hours after administration, and sacrifice 3 birds from the positive control group 24 hours after administration. Harvest the brains and lumbar spinal cords from each of these birds. If it is determined that excretion of the test substance is extremely slow, based on moderate symptoms of toxicity, it would be desirable to sacrifice 3 birds each two times between the 24th and, at the latest, the 72nd hour after administration, taking into account the optimal interval for detecting delayed neurotoxicity induction, and harvest their brains and lumbar spinal cords.

(ii) Measuring the acetylcholine esterase activity in the organs of the same animals in which NTE activity was measured would be helpful in evaluation.

(3) Pathological examination

(i) Conduct gross pathological examinations of all animals sacrificed, either as planned or as circumstances required, including observation of the appearance of the brain and spinal cord.

(ii) Conduct studies of nerve tissue harvested from at least 6 surviving birds from each group when testing is complete.

(iii) Fix the tissue by means of perfusion fixation, or other appropriate method.

a. Include sections from the cerebellum (central longitudinal plane), medulla oblongata, spinal cord, and peripheral nerves.

b. Collect spinal cord sections from the upper cervical, mesothorax, and lumbo-sacral areas.

c. Collect proximal, distal, and bifurcating sections of the tibial nerve, and also harvest the sciatic nerve.

(iv) Stain sections of the myelin sheaths and the axons according to an appropriate procedure.

90-day repeated dose oral toxicity test (2-1-9)

1. Objective

The objective of these studies is to obtain scientific information regarding toxic changes that occur following oral administration of the test substance, repeated for at least 90 days, as well as the maximum dosage at which toxic changes are not observed (NOAEL). These studies are also useful for obtaining information concerning establishment of dosages for carcinogenicity studies and 1-year repeated oral toxicity studies.

2. Test animals

(1) Conduct studies on 1 species of rodent (usually rats) and 1 species of non-rodent (dogs, usually).

(2) As regards rodents, use, as soon as possible after weaning and following acclimatization period (usually 5-6 weeks old), animals that are same age; as regards non-rodents (dogs), use animals that are 4-6 months old.

(3) In principle, use the same number of male and female animals. Use nulliparous, non-pregnant females.

3. Administration method

Carry out repeated oral administration, usually mixed with the animals' feed or water. However, gavage administration may be conducted if these methods of administration prove difficult.

4. Administration period

At least 90 consecutive days.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) As regards rodents, use at least 10 males and 10 females per group; as regards non-rodents, use at least 4 males and 4 females per group.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups. If interim sacrifice is anticipated, establish the additional number of animals required for this reason.

(iii) It is essential to ensure that there is a sufficient number of animals for evaluation of test results.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in large numbers of fatalities and the minimum dose at which no toxic effects can be determined, and so that the relationship between dosages and response can be discerned. (Indicate the grounds for establishment of dosages.)

c. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount

of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(5).

(1) Observation of general condition

(i) Observe all animals daily to determine their general condition (times at which symptoms of toxicity occur, and their degree), and mortality. In addition, weigh them regularly, and measure the food consumption. (When the test substance is administered in drinking water, also measure water consumption. Same below.)

(ii) In general, weigh the animals and measure the food consumption they ingest once prior to commencement of administration, and at least once per week following the commencement of administration. Also compute the amount of the test substance ingested.

(2) Detailed observation of condition

(i) Conduct observations once prior to commencement of administration, and at least once per week following the commencement of administration.

(ii) Observe the animals both in their cages and on the observation stand. Observe the animals carefully, in accordance with an observation procedure in which observation items, as well as the definitions of these items, and the rating criteria and order of observation, have been established sufficiently for ascertaining changes in general condition. Be careful to ensure that the handling of animals at observation time does not affect test results.

(iii) In general, conduct examinations in regard to the following items:

External appearance (skin; fur; changes in eyes, eyeballs, and mucous membranes. etc.), body position and posture (hunchback posture, etc.), autonomic nervous system function (lacrimation, piloerection, pupil diameter, respiration, excretion, etc.), motor coordination, abnormal gait, animal's reactions to being handled and to environmental stimulation, nervous system (tremor, convulsion, muscular contractions, etc.), changes in exploratory behavior, ordinary behavior (changes in grooming, headshaking, pivoting, etc.), abnormal behavior (autophagia, backward motion, abnormal vocalization, etc.), aggression, etc.

(3) Functional examination

(i) Conduct examination of rodents near the end of administration.

(ii) In general, conduct examinations in regard to the following items:

Sensorimotor reactions to stimuli (including auditory, visual, and proprioceptor stimuli), grip strength, and amount of motor activity (measure using an automatic recording apparatus).

(4) Blood tests

(i) It would be desirable, in principle, to conduct blood tests of all animals, at least once at the end of the study, in the case of rodents, and both prior to and at the end of the study, in the case of non-rodents. However, in the case of rodents, the examinations may be limited a part of each group (at least 5 animals), for practical reasons.

(ii) Except in the case of mice, it would be desirable for animals to fast for 1 night prior to examination.

(iii) Usually, conduct examinations with regard to the following points. In addition to these, other items could be selected and added as appropriate. Make selections with regard to examination items and methods that are widely accepted internationally.

a. Hematological tests

Red blood cells, leukocyte cells, hemogram (percentage by leukocyte group), platelet count, hemoglobin, hematocrit; in addition to these, reticulocyte count, clotting capability, (prothrombin time, activated partial thromboplastin time) etc.

b. Blood biochemical tests

Serum (plasma) protein, albumin, A/G ratio, glucose, cholesterol, triglyceride, bilirubin, urinary nitrogen, creatinine, transaminase (AST (GOT), ALT (GPT)), γ-GTP, alkaline phosphatase, electrolytes (sodium, potassium, chlorine, calcium, inorganic phosphorus, etc.), etc.

(5) Urinalysis

(i) As regards rodents, conduct urinalyses of a fixed number (5 or more) of male and female animals in each group; as regards non-rodents, conduct urinalyses of all animals. Conduct urinalyses at the same time as blood tests.

(ii) Usually, conduct urinalyses with regard to the following points.

Urine volume, pH, protein, glucose, keton bodies, bilirubin, urobilinogen, occult blood, sediment, specific gravity, etc.

(6) Ophthalmological examination

Conduct ophthalmological examinations of all animals among non-rodents, and among at least the high dosage and control groups among rodents, prior to administration and at the end of the study. If abnormality is observed that are ascribable to the test substance, conduct examinations of all animals.

(7) Pathological examination, etc.

(i) Promptly conduct necropsies, as well as gross observations, of organs and tissues, and histopathological examinations, in cases of death during the administration period. Determine the cause of death, and the degree of toxic changes at the time of death.

(ii) Promptly sacrifice and dissect animals that become moribund during the test period; conduct observations and examinations as in (i) above. Determine the cause of moribundity, and the degree of toxic changes at the time the animal became moribund.

(iii) Sacrifice and dissect all surviving animals at the end of the administration period, after having collected blood and urine for the various tests. Conduct gross examinations of organs and tissues, and weigh each organ.

(iv) Usually, weigh the following organs. Except in the case of mice, it would be desirable for animals to fast for 1 night prior to examination.

Liver, kidneys, adrenal glands, testes, ovaries, thymus gland, spleen, heart, brain, prostate gland(NOTE), thyroid gland, parathyroid gland(NOTE), and pituitary gland(NOTE)

NOTE: Examine the prostate, thyroid, parathyroid, and pituitary glands of non-rodents only.

(iv) Conduct histopathological examinations of all animals among non-rodents, and at least of those in the high dosage and control groups among rodents.

(v) Usually, conduct pathological examinations of the following organs and tissues, and add others as appropriate upon gross examination.

Skin, mammary glands, lymph nodes, (cervical and mesenteric lymph nodes, etc.), aorta, salivary glands, bones and marrow (sternum and femur), thymus gland, trachea, lungs and bronchia, heart, thyroid and parathyroid glands, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), liver (and gallbladder), pancreas, spleen, kidneys, adrenal glands, bladder, seminal vesicle and coagulating gland, prostate, testes(NOTE), epididymis, ovaries, uterus, vagina, brain, pituitary gland, sciatic nerve, skeletal muscle, spinal cord (cervical, thoracic, and lumbar areas), eyeballs and appendages, and other organs and tissues in which changes can be confirmed with the gross.

NOTE: Examine the testes using a fixative, such as Bouin fixative, that is appropriate for retaining the structure of seminiferous tubules.

(vi) If, in dosage groups of animals other than rodents, there are organs in which changes due to administration of the test substance are observed with the gross examination, or if it is deemed necessary on the basis of findings in high dosage groups, conduct histopathological examinations of all animals in those groups. Among rodents, histopathological examination of all animals would be helpful in evaluations.

(vii) Even after the completion of studies, preserve organs and tissues so that histopathological examinations can be conducted if necessary.

7. Other

It would be desirable to conduct the following studies in cases in which effects on the nervous system, immune system, or endocrine system are confirmed on the basis of above-mentioned test results.

(1) Nervous system: repeated oral dose neurotoxicity test

(2) Immune system: immunohistochemical staining of fresh frozen samples, measurement of splenic lymphocyte composition, measurement of natural killer (NK) cell activity, measurement of immunoglobulin (IgG, IgM, IgE, etc.) etc.

(3) Endocrine system: Measurement of steroid and thyroid hormones, etc. in the blood.

21-day repeated dermal toxicity test (2-1-10)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding toxic changes that occur following repeated dermal administration of the test substance, repeated for 21 days, as well as the maximum dosage at which toxic changes are not observed (NOAEL).

2. Test animals

(1) Use 1 or more species of mammals such as rats, rabbits, or guinea pigs.

(2) Use adult animals. In order to conduct the studies readily, it is desirable to stay within the following weight ranges:

Rats, 200-300 g; rabbits, 2.0-3.0 kg; guinea pigs 350-450 g

(3) In general, use the same number of male and female animals. Use nulliparous and non-pregnant females.

3. Administration method

(1) Clip the hair of an appropriate site on the trunk of the animals immediately prior to the study. The hair may be shaved, but in this case do it 24 hours prior to the study.

In general the animals are shaved at 1-week intervals. Be careful in shaving not to injure the skin, since this may affect its permeability by the test substance.

(2) Clip the hair from approximately 10% of the total body surface area. Take body weight into account when determining the application area and area to be shaved.

(3) When the test substance is a solid, grind it to powder, as appropriate, and moisten it with water or other vehicle, so that it makes good contact with the skin. If using a vehicle, use one that will not irritate the skin, and be careful about the effect of the vehicle on the permeability of the test substance. In general, use liquid test substance undiluted.

(4) Apply the test substance uniformly within a range that is approximately 10% of the body surface area (for example, with animals within the above-mentioned weight ranges: rats, 4 cm x 5 cm; rabbits, 12 cm x 14 cm; guinea pigs, 7 cm x 10 cm). There are cases in which test substances of high toxicity may be applied to a smaller area, but insofar as possible, apply the substance thinly and uniformly to the entire application site.

(5) During the test substance application period, cover the application site with porous gauze, and secure it with non-irritating tape so as to preserve contact with the skin. An additional appropriate method of covering must be used to preserve the test substance and gauze, so that the test animals are not able to ingest the test substance. Braces may also be used to prevent animals from ingesting test substance, but complete fixation is not desirable.

4. Administration period

It is desirable to administer the substance for 21 consecutive days, with 6 hours of exposure per day, 7 days per week.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) Use at least 5 male and 5 female animals per group in the studies.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups. It is essential to ensure that there will ultimately be a sufficient number of animals for evaluation of test results.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in large numbers of fatalities and the minimum dose at which no toxic effects can be determined, and so that the relationship between dosages and response can be discerned. Also indicate the grounds for the dosages established.

c. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(5).

(1) Observation of general condition

(i) Observe all animals daily to determine their general condition (times at which symptoms of toxicity occur, and their degree), and mortality. In addition, weigh them regularly, and measure the food consumption.

(ii) In general, weigh the animals and measure the food consumption once prior to commencement of administration, and at least once per week following the commencement of administration.

(2) Blood tests

(i) If would be preferable, in principle, to conduct blood tests of all animals, at least once at the end of the study, in the case of rodents, and both prior to and at the end of the study, in the case of non-rodents.

(ii) It would be desirable for animals to fast for 1 night prior to examination.

(iii) Usually, conduct examinations with regard to the following points. In addition to these, other items could be selected and added as appropriate. Make selections with regard to examination items and methods that are widely accepted internationally.

a. Hematological tests

Red blood cells, white blood cells, hemogram (percentage by leukocyte group), platelet count, hemoglobin, hematocrit; in addition to these, reticulocyte count, clotting capability, (prothrombin time, activated partial thromboplastin time) etc.

b. Blood biochemical tests

Serum (plasma) protein, albumin, glucose, bilirubin, urinary nitrogen, creatinine, transaminase (AST (GOT), ALT (GPT)), γ-GTP, alkaline phosphatase, electrolytes (sodium, potassium, chlorine, calcium, inorganic phosphorus, etc.), etc.

(3) Urinalysis

(i) As regards rodents, conduct urinalyses of a fixed number of male and female animals in each group; as regards non-rodents, conduct urinalyses of all animals. Conduct urinalyses at the same time as blood tests.

(ii) Usually, conduct urinalyses with regard to the following points.

Urine volume, pH, protein, glucose, keton bodies, bilirubin, urobilinogen, occult blood, sediment, specific gravity, etc.

(4) Pathological examinations, etc.

(i) Necropsies

a. Conduct gross examinations of all animals, including those that have died during the study, or those that have been sacrificed due to moribundity.

b. Examine the surface of the body.

c. Weigh the major organs of all animals, including the following organs:
Liver, kidneys, adrenal glands, and testes

(ii) Preservation of organs and tissues

Preserve organs and tissues as stipulated below.

a. Organs and tissues in which lesions visible to the gross or changes in size are noted.

b. Untreated and treated skin.

c. Liver

d. Kidneys

(iii) Histopathological examinations

Among non-rodents, carry out histopathological examinations of all animals. Among rodents, carry out histopathological examinations of the following, for example.

a. All animals in control and maximum dosage groups.

b. All animals that have died or have been sacrificed during the study period.

c. Parts of any animal in which lesions visible to the gross have occurred.

d. The target tissues of all animals.

e. The livers and kidneys of all animals.

In addition to the above, sites in which effects have occurred in maximum dosage groups must be histologically examined.

90-day repeated inhalation toxicity test (2-1-11)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals when they are used, by obtaining scientific information regarding toxic changes that occur following repeated exposure to inhalation of the test substance, repeated for 90 days, as well as the maximum dosage at which toxic changes are not observed (NOAEL).

2. Test animals

(1) Use young adult mammals (usually rats) of 1 or more species.

(2) Use young adults.

(3) In general, use the same number of male and female animals. Use nulliparous, non-pregnant females.

3. Exposure method

(1) Use inhalation equipment of appropriate capacity. Do not provide food or water during exposure.

(2) Monitor flow rate, actual concentration of the test substance, particle size distribution, temperature, and humidity during exposure. Maintain the uniformity of these conditions.

(3) Particle size (mass median aerodynamic size) of 1-4 μm is preferable, or use the minimum particle diameter that it is possible to conduct.

(4) If the test substance is volatile, be careful that it is not of a concentration that would cause an explosion.

4. Exposure period

The exposure time should be at least 6 hours per day, at least 5 days per week, for a period of at least 90 days.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) Use at least 10 males and 10 females per group.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups. Ensure that there is a sufficient number of animals for evaluation of test results.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in large numbers of fatalities and the minimum dose at which no toxic effects can be determined, and so that the relationship between dosages and reactions can be discerned. Indicate the grounds for establishment of dosages.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control

group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(5).

(1) Observation of general condition

(i) Observe all animals daily to determine their general condition (times at which symptoms of toxicity occur, and their degree), and mortality. In addition, weigh them regularly, and measure the food consumption.

(ii) In general, weigh the animals and measure the food consumption once prior to commencement of administration, and at least once per week following the commencement of administration.

(2) Blood tests

(i) If would be desirable, in principle, to conduct blood tests of all animals, at least once at the end of the study. However, based on operational considerations, this may be limited to some of the animals (at least 5) in each group.

(ii) It would be desirable for animals to fast for 1 night prior to examination.

(iii) Usually, conduct examinations with regard to the following points. In addition to these, other items could be selected and added as appropriate. Make selections with regard to examination items and methods that are widely accepted internationally.

a. Hematological tests

Red blood cells, white blood cells, hemogram (percentage by leukocyte group), platelet count, hemoglobin, hematocrit; in addition to these, reticulocyte count, clotting capability (prothrombin time, activated partial thromboplastin time), etc.

b. Blood biochemical tests

Serum (plasma) protein, albumin, glucose, bilirubin, urinary nitrogen, creatinine, transaminase (AST (GOT), ALT (GPT)), γ-GTP, alkaline phosphatase, electrolytes (sodium, potassium, chlorine, calcium, inorganic phosphorus, etc.), etc.

(3) Urinalysis

(i) Conduct urinalyses of a fixed number of male and female animals (at least 5 of each) in each group at the same time as blood tests.

(ii) Usually, conduct urinalyses with regard to the following points.

Urine volume, pH, protein, glucose, keton bodies, bilirubin, urobilinogen, occult blood, sediment, specific gravity, etc.

(4) Ophthalmological examination

Conduct ophthalmological examinations of all animals, insofar as possible (at least those in the high dosage and control groups), prior to administration and at the end of the study. If abnormalities occur, conduct examinations of all animals.

(5) Pathological examinations, etc.

(i) Necropsies

a. After necropsies have been performed, examine the surface of the body, orifices, cranium, pleural cavity, peritoneal cavity, and internal organs.

b. Weigh the major organs of all animals, including the following organs:
Liver, kidneys, adrenal glands, and testes

(ii) Preservation of organs and tissues

Preserve organs and tissues as stipulated below, so that it will be possible to conduct histopathological

examinations as necessary after the study has been completed.

Organs and tissues in which lesions visible to the gross are noted, skin, brain, pituitary gland, thyroid gland (including the parathyroid gland), thymus gland, lungs (including bronchia), nasopharynx, heart, sternum, salivary gland, liver (including gallbladder), spleen, kidneys, adrenal glands, pancreas, gonads, uterus, appendages to genital organs, mammary gland, muscle, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), bladder, lymph nodes, peripheral nerves, spinal cord, eyes, aorta

(iii) Histopathological examinations

Carry out the following histopathological examinations in regard to all of the following animals, etc.

a. All animals in control and maximum dosage groups.
b. All animals that have died or have been sacrificed during the study period.
c. Parts of any animal in which lesions visible to the gross have occurred.
d. The target tissues of all animals.
e. The respiratory tract, liver, and kidneys of all animals.

In addition to the above, sites in which effects have occurred in maximum dosage groups must be histologically examined.

Repeated dose oral neurotoxicity test (2-1-12)

1. Objective

The objective of these studies is to obtain scientific information regarding neurotoxic changes that occur following repeated oral administration of the test substance, as well as the maximum dosage at which toxic changes are not observed (NOAEL).

These studies may be conducted in conjunction with repeated dose toxicity studies, in order to evaluate the relationship between neurotoxicity and general toxicity.

2. Test animals

(1) Use rodents (rats, usually).

(2) Usually, use, as soon as possible after weaning and following acclimatization period, animals that are the same age, 5-6 weeks old.

(3) In general, use the same number of male and female animals. Use nulliparous, non-pregnant females.

3. Administration method

Carry out repeated orally administration, usually mixed with the animals' feed or water. However, gavage administration may be conducted if these methods of administration prove difficult.

4. Administration period

Conduct for 90 days or 1 year, as necessary.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) At least use 10 male and 10 female animals in each group for the studies, as necessary for detailed observations of condition and examination of functions. From among these, select at least 5 male and 5 female animals in each group as necessary for neurohistopathological examination.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups.

(iii) When conducting these studies in conjunction with others, adjust the number of animals as appropriate, based on the objectives of each study. If interim sacrifice or recovery group is considered, establish the additional number of animals required for this reason. It is essential, ultimately, to ensure that there is a sufficient number of animals for evaluation of test results.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. Establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in large numbers of fatalities and the minimum dosage at which no toxic effects can be determined, and so that the relationship between dosages and reactions can be discerned.

c. Refer to the results of previously conducted toxicity studies when establishing dosages. In addition, indicate the grounds for the dosages established.

d. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies

with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle.

c. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(6).

(1) Observation of general condition

(i) Observe the general condition of all animals carefully every day.

(ii) Observe general condition, and whether or not abnormal behavior or deaths occur.

(iii) Body weight and food consumption (When the test substance is administered in drinking water, also measure the water consumption. Same below.)

a. For 90-day studies, weigh the animals and measure the amount of feed they ingest once prior to commencement of administration, and at least once per week following the commencement of administration.

b. For 1-year studies, weigh the animals and measure the food consumption once prior to commencement of administration, and at least once per week for 3 months following the commencement of administration, and at least once every 4 weeks thereafter.

c. Compute the amount of the test substance ingested.

(2) Detailed observation of condition

(i) Observe at least 10 males and 10 females from each group studied.

(ii) The frequency of observations is indicated in the table below.

(iii) If recovery groups are used, examine animals upon completion of the recovery period.

(iv) Observe the animals both in their cages and on the observation stand. Observe the animals in accordance with a standards observation procedure in which observation items, as well as the definitions of these items, and the scoring criteria have been established.

(v) In general, conduct examinations in regard to the following items:

Appearance (skin; fur; changes in eyes, eyeballs, and mucous membranes; presence or absence of discharges; etc.), body position and posture (hunchback posture, etc.), autonomic nervous system function (lacrimation, piloerection, pupil diameter, respiration, excretion, etc.), abnormal gait, ambulatory abnormalities, animal's reactions to being handled and to environmental stimulation, nervous system (tremor, convulsion, muscular contractions, etc.), changes in exploratory behavior, ordinary behavior (changes in grooming, headshaking, pivoting, etc.), abnormal behavior (autophagia, backward motion, abnormal vocalization, etc.), aggression, etc.

(3) Functional examination

(i) Observe 10 males and 10 females from each group studied.

(ii) The frequency of examinations is indicated in the table below.

(iii) If recovery groups are used, examine animals near the end of test, insofar as possible.

(iv) In general, conduct examinations in regard to the following items:

Sensorimotor reactions to stimuli (including auditory, visual, and proprioceptor stimuli), grip strength, and amount of spontaneous motor activity(measure using an automatic recording apparatus).

(v) If it is suspected, on the basis of other toxicity studies, that the test substance is a neurotoxin, conduct studies

and careful examinations regarding the appropriate sensory mechanisms, motor function, and learning/memory relating to the suspected neurotoxicity.

(4) Ophthalmological examination

Conduct ophthalmological examinations of, at least, animals in the high dosage and control groups, prior to administration and at the end of the study. If abnormality is observed, ascribable to the test substance occur, conduct examinations of all animals.

(5) Histopathological examinations

(i) Examine at least 5 males and 5 females from each group studied.

(ii) Examine specific animals in which abnormalities were observed in observations of symptoms and in functional examinations.

(iii) Fix the tissue by means of perfusion fixation, or other appropriate method. Record all gross observations of pathological changes.

(iv) In general, embed tissue sample in paraffin, and use a staining agent, such as hematoxylin and eosin stain. However, if nerve damage in the peripheral nervous system is observed or suspected, adjust and examine peripheral nerve tissues embedded in resin.

(v) Conduct additional examinations and special staining, as appropriate, based on symptomatic observations.

(vi) Evaluate neurohistopathological findings by relating them to other toxicity test results and behavioral effects.

(vii) Conduct phased examinations of representative sections from the central and peripheral nervous systems.

(viii) First of all, compare sections from the maximum dosage group and the control group. If no neurohistopathological changes are observed in the maximum dosage group, no further examinations are necessary. If changes are observed in the maximum dosage group, examine sections from the median and minimum dosage groups, in that order.

(ix) In general, examine the following tissues:

The central cerebrum, including the prosencephalon and the hippocampus; the mesencephalon, cerebellum, pons, and medulla oblongata; the eyeball, including the optic nerve and retina; the cervical and lumbar enlargements of the spinal cord; the spinal ganglion, the ventral and dorsal roots of nerve fibers, the proximal sciatic nerve, the proximal tibial nerve (in the knee area), the gastrocnemius bifurcation of the tibial nerve, and the skeletal muscles (especially the gastrocnemius).

(x) The sections from the spinal cord and peripheral nerves should include both horizontal and vertical sections.

(xi) Preserve organs and tissues, so that it will be possible to conduct histopathological examinations as necessary after the study has been completed.

Table: Frequency of detailed observation of symptoms and functional studies

Type of Study	Relevant animals	90-day studies	1-year studies
Observation of general condition	All animals	Every day	Every day
Detailed observation of condition	Animals selected for detailed observation	(1) Prior to commencement of administration (2) Once during the 1st or 2nd week of administration (3) Each month after commencement of administration	(1) Prior to commencement of administration (2) Once, 1 month after commencement of administration (3) Every 3 months after commencement of administration
Functional examination	Animals selected for functional examination	(1) Prior to commencement of administration (2) Once during the 1st or 2nd week of administration (3) Each month after commencement of administration	(1) Prior to commencement of administration (2) Once, 1 month after commencement of administration (3) Every 3 months after commencement of administration

28-day repeated administration delayed neurotoxicity test (2-1-13)

1. Objective

The objective of these studies is to obtain information regarding the details of toxic changes that occur following repeated dose of the test substance over a 28-day period, and the maximum dosage at which toxic changes are not observed (NOAEL), in order to provide additional information regarding delayed neurotoxicity that has been confirmed or is suspected on the basis of acute delayed neurotoxicity studies.

2. Test animals

(1) Use hens of ordinary varieties and strains.

(2) It is desirable to use young adult hens of standard size, 8-12 months old.

(3) Use healthy animals that have not had viral diseases or drug treatments that would affect actual test results, nor any ambulatory abnormalities.

3. Administration method

(1) Carry out successive forcible oral administration, by means of stomach tube, gelatin capsule, or an equivalent method.

(2) If the test substance is a liquid, administer it as liquid concentrate, or after dissolving in an appropriate vehicle. If the test substance is a solid, administer it in solution, insofar as possible.

4. Administration and observation periods

(i) The administration period is 28 days.

(ii) Conduct observations for 14 days following the end of administration.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

Use a number of animals in both the administration and control groups such that the 6 hens needed for biochemical tests, as well as 6 hens that can survive until the end of the studies and can be used in histopathological studies, will be available.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. Establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that the toxic effect of the test substance, and insofar as possible its delayed neurotoxicity, will be clear, and so that the animals do not die or show conspicuous signs of suffering. Set the dosage at which no toxic effects can be determined as the minimum dosage. Set other dosage levels such that the relationship between dosages and reactions can be discerned.

c. Refer to the results of acute delayed neurotoxicity studies, and other toxicity studies, when establishing dosages. In addition, indicate the grounds for the dosages established.

d. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Note regarding care

Keep the animals in a cage or pen that is large enough to allow the animals to walk around freely, and readily permit observation of their ambulatory status.

7. Observation and examination

Conduct the following items (1)-(3).

(1) Observation of general condition

(i) Commence observation of all animals immediately after administration. Carefully observe them at least once per day during the administration period, and for 14 days after the end of the administration period, or until planned sacrifice.

(ii) Keep records of all symptoms of toxicity, including their type, time of occurrence, degree, and duration. Evaluate ataxia on the basis of criteria comprising at least 4 levels.

(iii) Animals used in pathological examinations should be brought out of their cages at least twice per week. Conduct forced movement at fixed times, in order to be able to observe even slight toxic effects.

(iv) Conduct gross pathological examinations of moribund animals after removing and sacrificing them.

(v) Weigh all animals once prior to administration and once 1 week after administration.

(2) Biochemical examinations

(i) Sacrifice 6 hens each, chosen at random, from the administration and control groups, within 72 hours after last administration of the test substance. Harvest their brains and lumbar spinal cords, and measure the neuropathy target esterase (NTE, also called neurotoxic esterase) activity.

Usually, sacrifice 3 hens each from the administration and control groups 24 hours and 48 hours after administration, and harvest their brains and lumbar spinal cords.

If, based on the results of acute delayed neurotoxicity studies or other test results, another interval is considered more appropriate for detecting the capability of inducing delayed neurotoxicity, it would be desirable to sacrifice 3 hens for a second time, and harvest their brains and lumbar spinal cords.

(ii) Measuring the acetylcholine esterase activity in the relevant organs (brain and cerebrospinal system) of the same animals in which NTE activity was measured would be helpful in evaluation.

(3) Pathological examinations

(i) Conduct gross pathological examinations of all animals sacrificed, either as planned or as circumstances require, including observation of the appearance of the brain and spinal cord.

(ii) Conduct studies of nerve tissue harvested from at least 6 surviving hens from each group when the study is complete.

(iii) Fix the tissue by means of perfusion fixation, or other appropriate method.

a. Include sections from the cerebellum (central longitudinal plane), medulla oblongata, spinal cord, and peripheral nerves.

b. Collect spinal cord sections from the upper cervical, mesothorax, and lumbo-sacral areas.

c. Collect proximal, distal, and bifurcating sections of the tibial nerve, and also harvest the sciatic nerve.

(iv) Stain sections of the myelin sheaths and the axons according to an appropriate procedure.

1-year repeated dose oral toxicity test (2-1-14)

1. Objective

The objective of these studies is to obtain scientific information regarding toxic changes that occur following long-term oral administration of the test substance, as well as the maximum dosage at which clear signs of toxic changes are not observed (NOAEL: NOAEL, no observed adverse effect level).

2. Test animals

(1) Conduct studies with 1 species of rodent (usually, rats) and 1 species of non-rodent (usually, dogs).

(2) As regards rodents, use as soon as possible after weaning and following acclimatization period (usually, 5-6 weeks old); as regards non-rodents (dogs), use animals that are 4-6 months old.

(3) In principle, use the same number of male and female animals. Use nulliparous and non-pregnant females.

3. Administration method

Carry out repeated oral administration, usually mixed with the animals' feed or water. However, gavage administration may be conducted if these methods of administration prove difficult.

4. Administration period

Repeated for at least 1 year.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) As regards rodents, use at least 20 males and 20 females per group; as regards non-rodents, use at least 4 males and 4 females per group.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups. If interim sacrifice is anticipated, establish the additional number of animals required for this reason.

(iii) It is essential to ensure that there is a sufficient number of animals for evaluation of test results.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in death and the minimum dose at which no toxic effects can be determined, and so that the relationship between dosages and response can be discerned.

c. Refer to the results of 90-day repeated dose oral toxicity studies in establishing dosages. Indicate the grounds for establishment of dosages.

d. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount

of vehicle.

c. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(5).

(1) Observation of general condition

(i) Observe daily the general condition of all animals.

(ii) Weigh the animals regularly, and measure the food consumption. (When the test substance is administered in drinking water, also measure the water consumption.; the same shall apply hereinafter.)

(iii) In general, weigh the animals and measure the food consumption, once prior to commencement of administration, and at least once per week until 3 months after the commencement of administration. Thereafter, weigh them at least once every 4 weeks. Also compute the amount of the test substance ingested.

(2) Blood tests

(i) Conduct tests 6 months following commencement of administration and at the end of the study, in the case of rodents, and prior to commencement of administration, 6 months following commencement of administration and at the end of the study, in the case of non-rodents. In principle, test all animals, but in the case of rodents, the examinations may be limited to a part of each group (at least 10 males and 10 females), for operational reasons.

(ii) Except in the case of mice, it would be desirable for animals to fast for 1 night prior to examination.

(iii) Usually, conduct examinations with regard to the following points. In addition to these, other items should be selected and added as appropriate. Make selections with regard to examination items and methods that are widely accepted internationally.

a. Hematological tests

Red blood cells, white blood cells, hemogram (percentage by leukocyte group), platelet count, hemoglobin, hematocrit; in addition to these, reticulocyte count, clotting capability (prothrombin time, activated partial thromboplastin time), etc.

b. Blood biochemical tests

Serum (plasma) protein, albumin, A/G ratio, glucose, cholesterol, triglyceride, bilirubin, urinary nitrogen, creatinine, transaminase (AST (GOT), ALT (GPT)), γ-GTP, alkaline phosphatase, electrolytes (sodium, potassium, chlorine, calcium, inorganic phosphorus, etc.), etc.

(3) Urinalysis

(i) As regards rodents, conduct urinalyses of a fixed number (10 or more) of male and female animals in each group; as regards non-rodents, conduct urinalyses of all animals. Conduct urinalyses at the same time as blood tests.

(ii) It is best to conduct urinalysis of the same animals used for blood tests.

(iii) Usually, conduct urinalyses with regard to the following points.

Urine volume, pH, protein, sugar, keton bodies, bilirubin, urobilinogen, occult blood, sediment, specific gravity, etc.

(4) Ophthalmological examination

Conduct ophthalmological examinations of all animals among non-rodents, and among at least the high dosage and control groups among rodents, prior to administration and at the end of the study. If abnormality is observed ,that are ascribable to the test substance, conduct examinations of all animals.

(5) Pathological examination, etc.

(i) Promptly conduct necropsies, as well as gross observations, of organs and tissues, and histopathological examinations, in cases of deaths during the administration period. Strive to determine the cause of death, and the degree of toxic changes at the time of death.

(ii) Promptly sacrifice and dissect animals that become moribund during the study period; conduct observations and examinations as in (i) above. Determine the cause of moribundity, and the degree of toxic changes at the time the animals became moribund.

(iii) Sacrifice and dissect all surviving animals at the end of the administration period, after having collected blood and urine for the various tests. Conduct gross observations of organs and tissues. Usually, weigh the following organs of all animals among non-rodents, and of 10 or more male and female animals in each group among rodents. Except in the case of mice, it would be desirable for animals to fast for 1 night prior to examination.

Liver, kidneys, adrenal glands, testes, ovaries, spleen, heart, brain, prostate gland(NOTE), thyroid gland, parathyroid gland(NOTE), and pituitary gland(NOTE)

NOTE: Examine the prostate, parathyroid, and pituitary glands of non-rodents only.

(iv) Conduct histopathological examinations of all animals among non-rodents, and at least of those in the high dosage and control groups among rodents.

(v) Usually, conduct pathological examinations of the following organs and tissues, and add others as appropriate upon gross examination.

Skin, mammary glands, lymph nodes, (cervical and mesenteric lymph nodes, etc.), aorta, salivary glands, bones and marrow (sternum and femur), thymus gland, trachea, lungs and bronchia, heart, thyroid and parathyroid glands, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), liver (and gallbladder), pancreas, spleen, kidneys, adrenal glands, bladder, seminal vesicle and coagulating gland, prostate, testes, epididymis, ovaries, uterus, vagina, brain, pituitary gland, sciatic nerve, skeletal muscle, spinal cord (cervical, thoracic, and lumbar areas), eyeballs and appendages, and other organs and tissues in which changes can be confirmed with the gross.

(vi) If, in dosage groups of animals other than rodents, changes due to administration of the test substance are observed in the relevant organs and tissues with the gross, during 90-day repeated dose oral toxicity studies or if it is deemed necessary on the basis of findings in high dosage groups, conduct histopathological examinations of all animals in those groups.

(vii) Among rodents, histopathological examination of all animals would be helpful in evaluations.

(viii) Even after the completion of studies, preserve organs and tissues so that histopathological examinations can be conducted if necessary.

Carcinogenicity test (2-1-15)

1. Objective

The objective of these studies is to obtain scientific findings regarding whether or not repeated oral administration of the test substance is carcinogenic.

2. Test animals

(1) Use at least 2 species of rodent (usually, rats and mice).

(2) Usually, use, as soon as possible after weaning and following acclimatization period, animals that are the same age, 5-6 weeks old. As regards selection of varieties and strains, those that are known to have such characteristics as resistance to infectious diseases, long life, and sensitivity to known carcinogens are widely used as test animals. In particular, select strains regarding which there is accumulated evidence regarding incidence of spontaneous tumors.

(3) In general, use the same number of male and female animals. Use nulliparous, non-pregnant females.

3. Administration method

Carry out repeated oral administration, usually mixed with the animals' feed or water. However, gavage administration may be conducted if these methods of administration prove difficult.

4. Administration period

(1) Establish the test substance administration period necessary for achievement of study objectives, taking into account the average life expectancy of the varieties and strains of animals used.

(2) Usually, the administration period should be 24-30 months for rats, and 18-24 months for mice.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) Use at least 50 male and 50 female animals in each group.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups.

(iii) It is unacceptable to lose 10% or more of any group, due to cannibalism or other causes related to animal care.

(iv) In principle, it is unacceptable for the survival rate of rats (at 24 months after the commencement of administration) or mice (at 18 months after the commencement of administration) to fall below 25%. If interim sacrifice is anticipated, establish the additional number of animals required for this reason.

(2) Establishing test groups

(i) Test substance administration groups

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish groups according to the dosage levels listed below, such that the relationship between dosage and reaction will be clear. Refer to the results of 90-day repeated oral toxicity studies in establishing dosages. Indicate the grounds for establishment of dosages.

(ii) Establishing dosages

a. Maximum dosage

Select as the maximum a dosage at which several toxic affects are confirmed, but at which the death

rate from causes other than tumors is not significantly higher than that of the control group.

If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

b. Minimum dosage

Usually, the minimum dose must not be less than 10% of the maximum dose.

c. Intermediate dosage

It is desirable to establish the geometrical mean of the maximum and minimum dosages as the intermediate dosage. Usually, the common ratio between groups is a value from 2 to 3.

d. Other

It is not necessary to determine NOAEL of the test substance on the basis of these studies, but if carcinogenicity, etc., is noted, determine its mechanism through additional studies, etc., and determine the NOAEL of the test substance with respect to carcinogenicity, according to appropriate parameters.

(iii) Control group

a. Establish the same conditions for the control group as for the test substance administration group, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(3).

(1) Observation of general condition

(i) In general, observe daily the general condition of all animals. In addition, weigh them regularly, and measure the food consumption. (When the test substance is administered in drinking water, also measure the water consumption; the same shall apply hereinafter.)

(ii) In general, weigh the animals and measure the food consumption once prior to commencement of administration, and at least once per week for 3 months following the commencement of administration, and at least once every 4 weeks thereafter. Also, compute the amount of the test substance ingested.

(2) Blood tests

Adjust blood samples of animals that have died or become moribund during the study period, or when sacrificing survivors at the end of the study period. Adjust smears of examples in which hematopoietic tumors are anticipated, due to tumefaction, etc. of the thymus gland, lymph nodes, liver, or spleen.

(3) Pathological examination, etc.

(i) Promptly conduct necropsies, as well as gross observations, of organs and tissues, and histopathological examinations, in cases of fatality during the study period. It is necessary to add findings on all kinds of changes (hyperplasia, precancerous lesion, etc.), up to and including tumors, in records of tumorous lesions (same as regards (ii) and (iii) below).

(ii) Usually, conduct pathological examinations of the following organs and tissues, and add others as appropriate upon gross examination.

Skin, mammary glands, lymph nodes, (cervical and mesenteric lymph nodes, etc.), aorta, salivary glands, bones and marrow (sternum and femur), thymus gland, trachea, lungs and bronchia, heart, thyroid and parathyroid glands, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), liver (and gallbladder), pancreas, spleen, kidneys, adrenal glands, bladder, seminal vesicle and coagulating gland, prostate, testes, epididymis, ovaries, uterus, vagina, brain, pituitary gland, sciatic nerve, skeletal muscle, spinal cord (cervical, thoracic, and lumbar areas), eyeballs and

appendages, nasal cavity, and other organs and tissues in which changes can be confirmed with the gross.

(iii) Promptly sacrifice and dissect animals that become moribund during the study period; conduct observations and examinations as in (i) above.

(iv) At the end of the study period, promptly sacrifice and dissect all surviving animals, and examine organs and tissues with the gross. Conduct histopathological examinations, as in (i) above, of all animals in the control and maximum dosage groups. However, if there are organs or tissues regarding which there is a difference in rate of tumorigenesis between the maximum dosage and control groups, conduct histopathological examinations of the relevant organs and tissues of all other animals in the other dosage groups.

It would be helpful in evaluations to conduct histopathological examinations of all animals.

(v) Even after the completion of studies, preserve organs and tissues so that histopathological examinations can be conducted if necessary.

1-year repeated dose oral toxicity / carcinogenicity combined test (2-1-16)

1. Objective

These studies are conducted in order to detect adverse effects that occur following long-term repeated dose of the test substance, and their objective is to obtain scientific information regarding the 1-year repeated dose oral toxicity and carcinogenicity of the test substance simultaneously.

2. Test animals

(1) Conduct the studies using 1 species of rodent (usually, rats). Use, as soon as possible weaning and following acclimatization period, animals that are same age (5-6 weeks old, usually).

(2) As regards selection of varieties and strains, those that are known to have such characteristics as resistance to infectious diseases, long life, and sensitivity to known carcinogens are widely used as test animals. In particular, select strains regarding which there is accumulated evidence regarding incidence of spontaneous tumors.

(3) In general, use the same number of male and female animals. Use females that have never given birth and are not pregnant.

3. Administration method

Carry out repeated oral administration, usually mixed with the animals' feed or water. However, forcible oral administration may be conducted if these methods of administration prove difficult.

4. Administration period

(1) Establish the test substance administration period necessary for achievement of study objectives, taking into account the average life expectancy of the varieties and strains of animals used.

(2) Usually, the administration period should be 24-30 months for rats, and 18-24 months for mice.

(3) For the satellite group and its control group, the period for detecting 1-year repeated oral toxicity should be at least 1 year, in general.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

(i) Use at least 50 male and 50 female animals in each group.

(ii) Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups.

(iii) It is unacceptable to lose 10% or more of any group, due to cannibalism or other causes related to animal care.

(iv) In principle, it is unacceptable for the survival rate of rats (at 24 months after the commencement of administration) or mice (at 18 months after the commencement of administration) to fall below 25%. If interim sacrifice is anticipated, establish the additional number of animals required for this reason.

(v) Regarding the satellite groups

a. The number of animals established for detecting 1-year repeated dose oral toxicity should be at least 10 males and 10 females per group. However, 20 of each should be used in the maximum dosage group.

b. Use an appropriate random sampling method, by body weight differential, etc., for allocating animals to groups.

c. If interim sacrifice is anticipated, establish the additional number of animals required for this reason.

d. It is essential to be able to ensure that the number of animals is sufficient for evaluating test results.

(2) Establishing test groups

(i) Dosage levels for detecting carcinogenicity

In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels. In addition to these, establish satellite groups and a control group for 1-year repeated oral toxicity studies.

Establish groups according to the dosage levels listed below, such that the relationship between dosage and reaction will be clear.

Refer to the results of 90-day repeated dose oral toxicity studies in establishing dosages. Indicate the grounds for establishment of dosages.

a. Maximum dosage

Select as the maximum a dosage at which several toxic affects are confirmed, but at which the death rate from causes other than tumors is not significantly higher than that of the control group.

If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

b. Minimum dosage

Usually, the minimum dose must not be less than 10% of the maximum dose.

c. Intermediate dosage

It is best to establish the geometrical mean of the maximum and minimum dosages as the intermediate dosage. Usually, the common ratio between groups is 2 to 3.

d. Other

It is not necessary to determine NOAEL of the test substance on the basis of these studies, but if carcinogenicity, etc., is noted, determine its mechanism through additional studies, etc., and determine the NOAEL of the test substance with respect to carcinogenicity, according to appropriate parameters.

(ii) Dosage levels for detecting 1-year repeated dose oral toxicity

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. As regards maximum dosages, set each dose level such that the dosage at which toxic effects not resulting in death and the minimum dose at which no toxic effects can be determined, and so that the relationship between dosages and reactions can be discerned.

c. Refer to the results of 90-day repeated dose oral toxicity studies in establishing dosages. Indicate the grounds for establishment of dosages.

d. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(iii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle.

c. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct the following items (1)-(5).

(1) Observation of general condition

(i) Observe daily the general condition of all animals. Weigh them regularly, and measure the food

consumption. (When the test substance is administered in drinking water, also measure the water consumption; the same shall apply hereinafter.)

(ii) In general, weigh the animals and measure the food consumption once prior to commencement of administration, and at least once per week until 3 months after the commencement of administration. Thereafter, weigh them at least once every 4 weeks. Also compute the amount of the test substance ingested.

(2) Blood tests

(i) In the dosage groups for detection of carcinogenicity, adjust blood samples of animals that have died or become moribund during the study period, or when sacrificing survivors at the end of the study period. Adjust smears of examples in which hematopoietic tumors are anticipated, due to tumefaction, etc. of the thymus gland, lymph nodes, liver, or spleen.

(ii) In the satellite groups for detection of 1-year repeated oral toxicity, collect blood samples from at least 10 males and 10 females in each group 6 months after the commencement of administration and at the end of the study (at 12 months). Conduct hematological and blood biochemical tests.

(iii) Except in the case of mice, it would be desirable for animals to fast for 1 night prior to examination.

(iv) In the satellite groups, usually conduct examinations with regard to the following points. In addition to these, other items should be selected and added as appropriate. Make selections with regard to examination items and methods that are widely accepted internationally.

a. Hematological tests

Red blood cells, white blood cells, hemogram (percentage by leukocyte group), platelet count, hemoglobin, hematocrit; in addition to these, reticulocyte count, clotting capability (prothrombin time, activated partial thromboplastin time), etc.

b. Blood biochemical tests

Serum (plasma) protein, albumin, A/G ratio, glucose, cholesterol, triglyceride, bilirubin, urinary nitrogen, creatinine, transaminase (AST (GOT), ALT (GPT)), γ-GTP, alkaline phosphatase, electrolytes (sodium, potassium, chlorine, calcium, inorganic phosphorus, etc.), etc.

(3) Urinalysis

(i) Conduct urinalyses of a fixed number (10 or more) of male and female animals in each satellite group for detection of 1-year repeated oral toxicity. Conduct urinalyses at the same time as blood tests.

(ii) It is desirable to conduct urinalysis of the same animals used for blood tests.

(iii) Usually, conduct urinalyses with regard to the following points.

Urine volume, pH, protein, sugar, keton bodies, bilirubin, urobilinogen, occult blood, sediment, specific gravity, etc.

(4) Ophthalmological examination

Conduct ophthalmological examinations of at least the high dosage and control groups among satellite groups for detection of 1-year repeated oral toxicity, prior to administration and at the end of the study. If abnormality is observed that are ascribable to the test substance, conduct examinations of all animals.

(5) Pathological examination, etc.

(i) Promptly conduct necropsies, as well as gross observations, of organs and tissues, and histopathological examinations, in cases of deaths during the administration period. Strive to determine the cause of death, and the degree of toxic changes at the time of death.

It is necessary to add findings on all kinds of changes (hyperplasia, precancerous lesion, etc.), up to and including tumors, in records of tumorous lesions (same as regards (ii) and (iii) below).

(ii) Usually, conduct pathological examinations of the following organs and tissues, and add others as appropriate upon gross examination.

Skin, mammary glands, lymph nodes, (cervical and mesenteric lymph nodes, etc.), aorta, salivary glands, bones and marrow (sternum and femur), thymus gland, trachea, lungs and bronchia, heart, thyroid and parathyroid glands, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), liver (and gallbladder), pancreas, spleen, kidneys, adrenal glands, bladder, seminal vesicle and coagulating gland, prostate, testes, epididymis, ovaries, uterus, vagina, brain, pituitary gland, sciatic nerve, skeletal muscle, spinal cord (cervical, thoracic, and lumbar areas), eyeballs and appendages, nasal cavity, and other organs and tissues in which changes can be confirmed with the gross.

(iii) Promptly sacrifice and dissect animals that become moribund during the study period; conduct observations and examinations as in (i) above. Strive to clarify the cause of moribundity, and the degree of toxic changes at the time the animals became moribund.

(iv) Sacrifice and dissect all surviving animals in the dosage groups for detection of carcinogenicity and satellite groups for detection of 1-year repeated oral toxicity at the end of the administration period, and conduct gross examinations of organs and tissues. Weigh the organs of all animals in the satellite groups. Usually, weigh the following organs. Except in the case of mice, it would be desirable for animals to fast for 1 night prior to examination.

Liver, kidneys, adrenal glands, testes, ovaries, spleen, heart, brain

(v) Conduct histopathological examinations, as in (i) above, of all animals in the control and maximum dosage groups. However, conduct histopathological examinations of organs or tissues of all other animals regarding which a difference in rate of tumorigenesis between the maximum dosage and control groups is noted, of the relevant organs and tissues in 90-day repeated oral toxicity studies, of sites in which lesions are noted with the gross in the course of these studies, and organs and tissues regarding which such examinations are deemed necessary on the basis of findings in the high dosage groups.

It would also be helpful in evaluation to conduct histopathological examinations of all animals in circumstances other than those noted above.

(vi) Even after the completion of studies, preserve organs and tissues so that histopathological examinations can be conducted in the future if necessary.

Reproductive toxicity test (2-1-17)

1. Objective

The objective of these studies is to obtain scientific information regarding the effect of administering the test substance to two generations (first generation (P) and second generation (F1)) of animals on their reproductive functions, such as estrous cycle, coitus, conception, parturition, and lactation, and on their offspring.

2. Test animals

(1) Use at least 1 species of rodent (usually, rats).

(2) When selecting strains of test animals, avoid those with a low fertility. Select strains that were bred for use in general toxicity and reproductive toxicity.

3. Administration method

(1) Carry out repeated dose oral administration, usually mixed with the animals' feed or water. However, gavage administration may be conducted if these methods of administration prove difficult.

(2) Computes dosages weekly based on the body weight of each individual. Dosages for pregnant females may be computed on the basis of body weight on gestation days 0 and 6.

4. Administration period

The test substance administration period should be as follows.

(1) First generation (P)

Commence administration from the time animals are 5-9 weeks old, after acclimating for 5 days, and continue for at least 10 weeks, until mating. Thereafter, continue administering to males at least until they have stopped mating, and to females until the weaning of F1 offspring.

(2) Second generation (F1)

Commence administration at the time of weaning until mating. Thereafter, continue administering to males at least until they have stopped mating, and to females until the weaning of F2 offspring.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

Use an equal number of male and female animals in the studies sufficient for obtaining at least 20 gravid animals per group.

(2) Establishing test groups

(i) Establishing test substance administration groups

a. Establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated. Set the maximum dosage such that the dosage at which toxic effects not resulting in death, such as suppression of weight gain, are observed in parents or offspring, and the minimum dose at which no toxic effects can be determined in either parents or offspring, and set other dosage levels so that the dose –response relationship can be discerned. Indicate the grounds for establishment of dosages.

c. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Mating, adjusting number of animals per litter, and second generation (F1) selection

(1) First generation (P)

(i) Allow male/female pairs from the same dosage groups to cohabit and mate until copulation is confirmed. Limit the cohabitation period to 2 weeks.

(ii) Check daily whether coitus has occurred, according to the presence or absence of spermatozoa in vaginal smears, or the presence or absence of a vaginal plug. Establish the day on which spermatozoa or vaginal plug are confirmed as gestation day 0.

(iii) Obtaining second litter from the first generation may be considered, as necessary.

(iv) Allow litters to nurse until weaning. In adjusting the number per litter, exclude excess neonates at random, 4 days after birth, until 4 males and 4 females remain in each. If it is impossible to make adjustments to obtain 4 male and 4 female animals per litter, there is no objection to making adjustments such that there are a total of 8 animals (for example, 5 males and 3 females) per litter. Do not adjust litters of less than 8 offspring.

(v) When weaning F1, make selections so that males and females that each mate with 1 or 2 partners are, within any given group, from as many mothers as possible.

(2) Second generation (F1)

Allow mating and adjust the number of animals per litter in the same way as in the first generation (P). Prevent mating between members of the same litter.

7. Observation and examination

Conduct the following items (1)-(3).

(1) Parent animals

(i) General condition

a. Observe daily the general condition of P animals, as well as F1 animals used for breeding. During reproductive period, examine females in regard to pregnancy and parturition.

b. Observation of general condition should include signs of excitation, seizure, sedation, and ambulatory irregularity, in addition to survival/fatality and outward appearance. Observations in regard to pregnancy and parturition should include miscarriage, premature birth, and delayed birth.

(ii) Body weight and food consumption

a. Weigh regularly all P animals, as well as F1 animals used for breeding and measure the food consumption. (When the test substance is administered in drinking water, also measure the water consumption; the same shall apply hereinafter.) In general, the animals should be weighed, and the food consumption measured, on the day administration commences, and at least once per week thereafter.

b. Weigh females during the reproductive period, on gestation days 0, 7, 14, and 21, and on nursing days 0, 7, 14, and 21.

c. Also compute the amount of the test substance ingested.

(iii) Observations as to sexual maturation

Observe the development of the external sexual organs of the F1 animals used for mating.

(iv) Estrous cycle

Observe the estrous cycles of P females, as well as F1 females used for mating, at least 2 weeks prior to mating. If necessary, also observe the estrous cycles of 3rd generation (F2) females.

(v) Gestation, parturition, and nursing

a. Compute the following values, based on the number of females engaging in copulation, the number of pregnant females (and the number of males that fertilize females), the number of delivered dams, and the number of offspring weaned.

Copulation rate < (Number of animals which copulated / number of animals used for mating) x 100 >

Conception rate < (Number of animals pregnant / number of females which copulated) x 100 >

Birth rate < (Number of females delivering live offspring / number of pregnant females) x 100 >

Weaning rate < (Number of surviving offspring at weaning / number of offspring, adjusted 4 days after birth) x 100 >

b. Study causal factors as regards females that fail to copulate successfully.

(vi) Sperm tests

At the time of sacrifice, determine the number, motility, and morphology of spermatids inside the testes and epididymis of P males and F1 males used for mating.

(2) Offspring

(i) Immediately after birth, note the number of offspring of each female, as well as their weight, the number of stillborn offspring, the number of live offspring, and whether or not any of them exhibit external abnormalities. If necessary, measure anogenital distance (AGD).

(ii) Note the number of live offspring on the 4th, 7th, 14th, and 21st days after birth. Compute survival rates and weigh each individual.

(iii) In addition observe the general condition of the offspring.

(3) Pathological examinations

(i) Promptly sacrifice the P animals and the F1 animals used for mating after the offspring of them are weaned, and the F2 young and the F1 animals not used for mating after weaning. Dissect them, and conduct gross examinations, with special reference to the organs in the reproductive systems.

(ii) Determine the number of implantations in the uteruses of females used for mating. Dissect individuals that die during the study period promptly, to determine the cause. Also promptly sacrifice and dissect animals that become moribund during the study period, and investigate the cause of moribundity.

(iii) Usually, weigh the following organs.

a. Parent animals: Ovaries, uterus, testes, epididymis, seminal vesicle and coagulating gland, prostate, brain, liver, kidneys, adrenal glands, spleen, pituitary gland, thyroid gland, and other target organs

b. Offspring: Brain, spleen, thymus gland, and uterus

(iv) Conduct histopathological examinations of the reproductive organs and other target organs of P animals, as well as F1 animals used for breeding in the high dosage and control groups. If abnormalities are observed that are thought to be effects of the test substance, examine the moderate and low dosage groups in the same way. In addition, conduct histopathological examinations of organs and tissues of offspring in which abnormalities thought to be the effects of the test substance are noted. Refer to the results of repeated dose toxicity studies.

(v) Even after the completion of studies, preserve organs of parent animals and offspring, particularly those of the reproductive system, so that histopathological examinations can be conducted as needed.

Teratogenicity test (2-1-18)

1. Objective

The objective of these studies is to obtain scientific information regarding the effects of exposing pregnant mother animals to the test substance on the growth and development of fetuses, with special reference to teratogenicity.

2. Test animals

Use at least 2 species of animals, including 1 or more species of rodent (usually, rats) and non-rodents (usually, rabbits).

3. Administration method

(1) In general, conduct gavage continuous oral administration.

(2) Compute dosages based on weight as close as possible to the day of administration. The test substance may also be administered in feed or water if it is possible to ensure uniform dosage on the basis of blood concentration level, food consumption, etc.

4. Administration period

(1) Administer the test substance on consecutive days during a period extending at least from implantation to the last day but one prior to the expected delivery date.

(2) Establish the day on which spermatozoa are confirmed in vaginal plug or vaginal smear as gestation day 0.

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

Use a sufficient number of pregnant animals to allow for interpretation of data.

(2) Establishing test groups

(i) Test substance administration groups

a. In addition to the control group, establish test substance dosage groups according to at least 3 dosage levels.

b. Establish dosage levels such that symptoms of the test substance toxicity will be clear, and so that the NOAEL can be estimated.

c. In order to discern clearly the dose-response relationship, set the maximum dosage such that toxic effects on mother animals and fetuses, such as suppression of weight gain, can be observed, and set the minimum dosage such that no toxic effects on parent animals or fetuses are observed. Indicate the grounds for establishment of dosages.

d. If no toxic effects are confirmed as a result of administration of the maximum dose that is technically possible, or 1,000 mg/kg of body weight/day of the test substance, it is not necessary to conduct studies with higher dosages than that.

(ii) Control group

a. Establish the same conditions for the control group as for the test substance administration groups, except that the test substance is not administered.

b. When a vehicle, etc. is being used for administration of the test substance, administer to the control group the same amount of vehicle as to the dosage group that is being administered the largest amount of vehicle. Establish an additional untreated control group when using a vehicle, etc. regarding which sufficient information on toxicity cannot be obtained.

6. Observation and examination

Conduct items (1)-(2) below.

(1) Parent animals

(i) General condition

a. Observe daily the general condition and gestational status of all dam.

b. Regularly weigh the animals, and determine the food consumption. (When the test substance is administered in drinking water, also measure the water consumption; the same shall apply hereinafter.) In general, weigh the animals and measure food intake on gestation day 0, on the day of dissection, and at least once every 3 days during the administration period.

c. Observation of general condition should include signs of excitation, seizure, sedation, and ambulatory irregularity, in addition to survival/fatality and outward appearance. Observations in regard to pregnancy and parturition should include abortion and premature birth.

(ii) Necropsy

a. Promptly sacrifice animals that display symptoms of abortion or premature birth, and conduct gross observations of their organs and tissues. Do the same in regard to animals that die or become moribund.

b. Sacrifice all animals on the day prior to the expected delivery date, and conduct gross examination of all organs and tissues, after removing the uterus. Weigh the extracted uteruses, and conduct examinations per (2) below.

c. Determine the number of corpora luteum. If conceptus is not observed in the uterus, conduct further examinations to determine whether implantation has occurred.

(2) Fetuses

(i) Dissect the uteruses extracted from dam. Determine the number of embryonic deaths and fetal deaths, as well as the number of surviving fetuses.

Insofar as possible, record the grounds for estimated time of death of embryos and fetuses. Determine the sex of fetuses, weigh them individually, and record these data items. Compute the mean weights of male and female fetuses in each group.

(ii) After examining all extracted fetuses for external abnormalities, examine half of the fetuses from each rat litter for skeletal abnormalities, and examine the remainder for visceral abnormalities. Examine all rabbit fetuses for visceral abnormalities (dissect the heads of 1/2 of them, and make detailed examinations), and then examine them for skeletal abnormalities.

Mutagenicity test (2-1-19-1~3)

1. Objective

The objective of these studies is to determine whether the test substance induces gene mutation, chromosome configuration abnormalities, or numerical abnormalities.

2. Selection of test method

Conduct reverse mutation studies using bacteria, chromosomal aberration studies using cultured mammalian cells, and micronucleus studies using rodents as studies of mutagenicity. If any of these studies are inapplicable, for technical or scientific reasons, it is acceptable to substitute test systems that provide similar indications. Conduct additional mutagenicity studies if further investigation is deemed necessary, on the basis of these test results.

Reverse mutation test (2-1-19-1)

1. Bacterial strains to be used

Select 1 strain from each of the following types of bacteria, and use a total of 5 strains of bacteria in the studies.

(i) *Salmonella typhimurium* TA98
(ii) *Salmonella typhimurium* TA100
(iii) *Salmonella typhimurium* TA1535
(iv) *Salmonella typhimurium* TA1537, TA97, or TA97a
(v) *Escherichia coli* WP2 *uvrA*, *Escherichia coli* WP2 *uvrA*/pKM101, or *Salmonella typhimurium* TA102

Other bacteria strains may be added, if necessitated by the properties of the test substance.

2. Dosage levels

(1) Using concentrations that will permit analysis, set at least 5 levels, at appropriate intervals.

(2) It would be preferable to conduct range findings studies for establishing the maximum concentration, using bacteria strains used in main studies. Keep growth inhibition and solubility in mind when establishing concentrations.

(3) In principle, set the concentration at which growth is inhibited as the maximum concentration; use 5 mg/plate of substances that do not appear to inhibit growth, as the highest concentration. The concentration at which precipitation occurs may be set as the maximum dose for substances that display no growth inhibition and that are difficult to dissolve.

3. Control

Set a negative control, for which vehicle is used, and a positive control, for which a known mutagen is used, for each study.

4. Number of plates used

Use 2 or more plates for each test substance dosage level and each control.

5. Test method

(1) Conduct studies either according to the preincubation method or the plate method. A different method may be used, if there is a scientifically valid reason to do so.

(2) Whichever method is used, conduct experiments with and without metabolic activation. Use S9 mix in which coenzymes have been added to the supernatant fraction (S9) of homogenate of livers from animals that have been appropriately treated so as to induce development of a drug metabolic enzyme system.

6. Observation

After culturing all plates for 48-72 hours at 37° C, count the number of revertant colonies per plate. At the same time, note growth inhibition and precipitation of the test substance.

7. Evaluation of results

(1) If the range finding studies have been conducted regarding which sufficient information has been provided as to bacterial strains used, dosage levels, controls, and plate number, they may be used to confirm reproducibility.

(2) Make a positive judgement if there is clearly a greater number of revertant colonies as compared to the negative control group, and if reproducibility and dose dependency are confirmed. If reproducibility is not confirmed, conduct confirmation studies. If neither a positive nor a negative judgement can be clearly made, conduct confirmation studies under different experimental conditions.

7. Displaying results

Display the actual measured value for number of revertant colonies per plate and the mean for the group.

Chromosomal aberration test (2-1-19-2)

1. Cells used

(1) Use primary, culture and subculture cells, or established cell lines, including human cells. Examples are Chinese hamster fibroblast cell line and human peripheral blood lymphocyte.

(2) Examine the cells used in studies as to chromosome number (modal number), whether the mycoplasma is contaminated, cell cycle, etc.

2. Dosage levels

(1) Use at least 3 levels of dosage, with appropriate intervals between them (with a common ratio of 2, in general), so that they chromosome analysis can be performed.

(2) It would be desirable to conduct range findings studies in advance.

(3) The maximum dose should be the concentration at which cell proliferation is suppressed by at least 50%, regardless of the test substance solubility in the culture solution. If suppression of cell proliferation by at least 50% is not observed, make 5 mg/ml or 10 mM (whichever is lower) the maximum dosage. If absolutely no suppression of proliferation is noted, and test substance precipitation is noted at the end of the treatment, the dosage at which precipitation occurs make be used as the maximum dosage.

3. Control

Set a negative control, for which vehicle is used, and a positive control, for which a known clastogen is used, for each study.

4. Number of plates used

Use 2 or more plates each for each test substance dosage level and each control.

5. Test method
   (1) Use cells in proliferation phase. At first, use the short treatment method, with and without metabolic activation. Treat the cells with the test substance for 3-6 hours, and adjust chromosome specimens after approximately 1.5 cell cycles from the commencement of treatment.

   (2) If negative results are obtained using the short treatment method, conduct successive treatment for 1.5 cell cycles without metabolic activation. The cell cycle may be noticeably delayed by the test substance. If successive treatment for longer than 1.5 cell cycles without metabolic activation is necessary, or if a later period than 1.5 cell cycles is required for specimen preparation with metabolic activation, conduct confirmation studies as necessary. Use S9 mix in which coenzymes have been added to the supernatant fraction (S9) of homogenate of livers from animals that have been appropriately treated so as to induce drug metabolic enzymes.

6. Observation
   (1) All the slide specimens, including positive and negative controls, are coded so that the treatment conditions are unknown for the examination.

   (2) Observe at least 200 well-spread metaphases per dosage (100 per plate); record the number and frequency of cells with structural chromosome aberrations. Also record the number and frequency of each type of structural aberration.

   (3) Record gaps separately from other aberrations, and do not include them in total aberration incidence. Define gaps as achromatic chromosomal positions that are narrower than the chromatid width. If polyploidy or endoreduplication are observed, note the number of instances, and their incidence.

8. Evaluation of results
   (1) Make a positive determination if there is clearly a greater incidence of cells with chromosomal aberration as compared to the negative control group, and if reproducibility and dose relation are confirmed.

   (2) If neither a positive nor a negative determination can be clearly made, conduct confirmation studies under different experimental conditions.

9. Displaying results

   Display all observation data on each plate, together with mean group values. Display data concerning cell proliferation in the dosage groups and negative control group. Clearly record the dosage at which precipitation of the test substance is observed.

Micronucleus test (2-1-19-3)

1. Types of animals

   In general, use either mice or rats when using red blood cells from bone marrow; use mice when using red blood cells from red blood cells from peripheral blood. However, other appropriate types of animals may be used. The studies may be conducted with males only if no clear sex difference is observed as to toxicity.

2. Number of animals

   Use 5 or more animals per group.

3. Administration route

   Use gavage or intraperitoneal administration.

4. Number of administrations

Conduct either single or multiple administrations.

5. Dosage levels

(1) Establish at least 3 levels of dosage, at appropriate intervals.

(2) Set the maximum dosage as the dosage at which cytotoxicity is confirmed by a decline in immature red blood cells, etc. in bone marrow, or at which symptoms of toxicity are observed, or beyond which it is expected that any dosage would be lethal.

(3) Set 2,000 mg/kg as the maximum dosage if toxicity is not confirmed.

6. Control

Set a negative control, for which vehicle is used, and a positive control, for which a known micronucleus inducer is used, for each study.

7. Methods

(1) Use red blood cells from bone marrow or peripheral blood.

(2) Adjust samples twice at appropriate times following administration of the test substance, in the case of single administration, and once at an appropriate time following administration of the test substance, in the case of multiple administration.

(3) If it is possible to confirm the time at which susceptibility is maximized, it is satisfactory to adjust samples only once, even following single administration.

8. Observations

(1) All the slide specimens, including positive and negative controls, are coded so that the treatment conditions are unknown for the examination.

(2) Observe at least 2,000 immature red blood cells per individual, and record the incidence of cells that have micronuclei.

(3) Observe at least 200 red blood cells per individual, in the case of bone marrow, and at least 1,000 per individual, in the case of peripheral blood, and record the ration of immature red blood cells to total red blood cells.

9. Evaluation of results

(1) Make a positive determination if dose relation is noted in the increase in the number of cells with micronuclei, or if there is a clear increase in the number of cells with micronuclei in any administration groups.

(2) If neither a positive nor a negative determination can be clearly made, conduct confirmation studies under different experimental conditions.

10. Displaying results

Display observational data for each individual, and the average values for each group.

Pharmacology test (2-2-1)

1. Objective

The objective of these studies is to pharmacologically analyze the acutely toxic effects of the test substance, to clarify the possibility of the occurrence of symptoms of acute intoxication, as well as their characteristics, and to obtain useful information on mechanisms of and treatment for acute intoxication. Study items and methods should be selected that are appropriate for the test substance's form of use, the characteristic ways in which its toxicity manifests, and its physical characteristics.

2. Test animals

Use young male and female animals, such as mice, rats, guinea pigs, rabbits, and dogs, as appropriate for the relevant study items.

3. Study plan basics

(1) Administration route

In general choose an administration route by which acute exposure can be anticipated.

(2) Number of administrations

A single dose is given in general in test using whole animals.

(3) Establishing dosages

Establish administration groups such that the no observed adverse effect level (NOAEL) and dose relation of toxic response will be clear in each study. Set the maximum dose as the dosage at which fatalities occur. With oral administration, it is not necessary to perform studies with dosages higher than 2,000 mg/kg of body weight.

(4) Control group

Establish a negative (vehicle) control group for each study.

(5) Observation (examination) period

Observations are performed at times which will demonstrate the onset of toxic response, maximum effects and their disappearance.

(6) When using anesthetized animals

Take changes in absorption of the test substance due to anesthetic into account when conducting studies.

(7) When using extracted organ (tissue) samples

Establish a negative (vehicle) control group, and also establish a group receiving a concentration such that NOAEL and dose relation will be clear. It would be desirable to set concentration according to estimated tissue concentrations in the active site. Employ appropriate organic vehicles and surfactants for test substances of low solubility, and apply them to liquid nutrients in a dissolved or emulsified state. Also examine the reversibility of effects.

(8) Number of examples

The number of animals per group should be such that appropriate statistics can be obtained. However, use at least 3 animals per group for items regarding which time-lapse tracking is important, to confirm reproducibility.

4. Examination items

Conduct examinations in regard to the following items, in order to ascertain toxic response on the basis of acute

pharmacological effects. Not all the items are required for fulfilling the principles of these studies. When selecting examination items, refer to the items listed below, and decide based on information obtained from results of other toxicity studies.

Select examination methods that facilitate scientifically correct evaluation of toxic effects, based on the pharmacological effects of the test substance.

(1) Items that should usually be conducted

(i) Observation of symptoms

Observe systems such that they are ascertained objectively, quantitatively, and temporally.

(ii) Central nervous system

Effects on spontaneous movement, convulsions, etc.

(iii) Respiratory and circulatory systems

Effects on respiration, blood pressure, heart rate, and electrocardiogram

(iv) Renal functions

Urine volume, electrolyte concentration in urine, urine osmotic pressure (specific gravity), etc.

(2) Items to be added and conducted as deemed necessary on the basis of results obtained in (1) above, and information obtained from other toxicity study results

(i) Autonomic nervous system

Effects on pupil diameter, nictitating membrane, extracted ductus deferens, etc.

(ii) Skeletal muscle

Effects on grip strength, extracted skeletal muscle, etc.

(iii) Blood system

Hemolytic effects, effects on clotting functions, etc.

(iv) Digestive system

Effects on peristalsis, extracted intestines, agonist contraction, etc.

(v) Other

Other items deemed necessary on the basis of observation of symptoms and other toxicity studies.

Test on metabolism in animals (2-3-1)

1. Objective

The objective of these studies is to contribute to evaluation, etc. of test results regarding toxicity of agricultural chemicals, by administering test substances to animals and obtaining scientific findings concerning their pharmacokinetics (absorption, distribution, excretion, metabolism, etc.).

2. Test substance

Use compounds, of a high degree of purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled or unlabeled with radioactive isotopes. Verify their source, purity and stability. Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the isotope labels.

3. Test animals

(1) Use young adult animals of a single species (usually, rats).

(2) It would be preferable to use animals of the same strain as those used in the 1-year repeated dose oral toxicity and carcinogenicity studies.

(3) In principle, use both male and female animals.

(4) If there are conspicuous differences between rodents and non-rodents as regards the target organs or degree of toxic symptoms, it would be desirable to use non-rodents in addition. Pregnant animals may also be used, if necessary.

4. Administration method

(1) Administration route

Use oral administration, in general. If necessary, supplement with studies using intravenous administration, etc.

(2) Number of administrations and administration period

Conduct single administration, in general. Consider conducting repeated dose if accumulation is anticipated. In case of repeated dose, estimate the test substance's *in vivo* steady state and accumulation, and allow for sufficient intervals (usually once per day) and administration period (in general, 14 days).

5. Determining the number of animals, and establishing test groups

(1) Determining the number of animals

Use an appropriate number of animals (in general, at least 4 in each dosage group) for achieving test objectives, taking into account individual differences, and the number of specimens required for observations and measurements.

(2) Establishing test groups

(i) Use at least 2 different dosages for single administration. When establishing the 2 dosage levels, use guidelines whereby the high dose is the dosage at which some toxic effects are observed, while the low dose is the dosage at which no toxic effects are observed.

(ii) For repeated dose, use only the low dose in general, and use the high dose if necessary.

6. Items to consider

Items to consider in regard to adsorption, distribution, excretion, and metabolism are usually as follows.

(1) Absorption

Obtain information regarding the amount of the test substance absorbed, and the absorption speed. These are obtained based on analyses of estimates, etc. of amounts excreted and blood concentrations (serum concentration, plasma concentration, and total blood concentration).

(2) Distribution

Measure the distribution (concentration and distribution rate) of the test substance and metabolites in major organs and tissues (including organs and tissues in which toxic effects have been noted) at multiple points in time, as appropriate, including Tmax and at the end of the examination of excretion. And obtain information concerning the distribution, temporal change, and accumulation of them.

(3) Excretion

Measure the amount of the test substance and metabolites excreted in excrement and exhalation, and obtain from these the total amount excreted. Also obtain information concerning the excretion route, as well as the degree and speed of excretion. Measurements of amount excreted should be measured over time, until 7 days after administration, or until 90% of the amount administered has been excreted, whichever occurs sooner. If necessary, measure the amount excreted in bile and milk, as well.

(4) Metabolism

Use appropriate methods to identify and quantify the test substance. Also obtain information concerning the metabolic pathway, as well as the degree and speed of metabolism.

(5) Other

It would be desirable to investigate, insofar as possible, in order to elucidate, for example, various items suggesting relationships to toxicity such as biopolymer bonding, organs and tissues that contribute to metabolism, etc.

Test on metabolism in plants (2-4-1)

1. Objective

The objective of these studies is to obtain scientific information regarding the absorption and translocation of the test substance inside plant bodies, as well as concerning the major metabolic routes including photochemical reactions on the surface of plants and the amounts of metabolites. Together with results of studies of fate in animals, the studies should also contribute to confirmation of dissimilarities between metabolites found in animals and plants, and to determinations regarding substances analyzed in studies of residue in crops.

2. Test substance

Use compounds, of a high degree of purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled or unlabeled with radioactive isotopes. Verify their source, purity and stability.

Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the isotope labels.

3. Test plants

It would be desirable to conduct studies in which plants are cultivated under conditions close to those under which the plants are usually cultivated.

4. Treatment method

(1) Use a test substance that is representative of the formulation for which registration is intended, or has been adjusted so as to be similar in form or composition.

(2) In general, carry out treatment according to methods of use, usage period, and amount used that are relevant to the registration application.

(3) If multiple differing application methods are anticipated, it would be desirable, in general, to conduct multiple studies.

5. Collecting samples

(1) In general, gather samples when the relevant crops are harvested. However, if the period from application to harvest is lengthy, and it would be difficult to elucidate metabolic route merely upon observation at harvest time, data may be collected multiple times from the first to last period of application.

(2) It is desirable to gather samples at various times during the harvest period, in the case of crops that have long harvest periods.

(3) In general, distinguish between samples gathered from various parts of plants, such as roots, leaves, stems, or fruit, and between treated and untreated parts, if relevant.

6. Analysis

(1) Determine in each of the gathered and separated samples the amount of metabolites (includes unchanged test substance; same below) present, etc.

(2) As regards the treated parts of plants, analyze metabolites that are left on the surface separately, insofar as possible, from metabolites that permeate the plant body.

(3) In general, analyze samples promptly. Adopt appropriate methods for minimizing decomposition of metabolites when storing plant samples and liquid extracts. It is also necessary to ensure that changes in metabolites can be

ascertained during the storage period.

7. Metabolite identification, etc.

It is desirable, in general, to identify metabolites as follows:

(1) Identify or characterize the metabolites that account for 10% or more of the total residual in the sample parts, equivalent to, if converted into test substance, represent a concentration of 0.01 mg/kg or more of all metabolites and similar substances.

(2) Insofar as technically possible, identify metabolites that account for 10% or more of the total residual in the sample parts, equivalent to test substance, represent a concentration of 0.05 mg/kg or more of all metabolites and similar substances.

(3) Insofar as technically possible, endeavor to identify metabolites of plant bodies (in the case of conjugates, their aglycon portions), even when the quantity available is insufficient for this purpose.

(4) It would be desirable to identify metabolites with residuals of 50% or more of extractable residues in sample parts, and to characterize those that represent 70% or more of the total residual, when the radioactive residual concentration is 0.01 mg/kg or more equivalent to test substance.

(5) Characterize the metabolites that represent 10% or more of the total residual, and of extracted residual components that are generated in concentrations of 0.05 mg/kg or more, by treating them with surfactants, enzymes, acids, bases, etc.

(6) It would be desirable to characterize the metabolites by converting them to common molecular components, insofar as possible, when the radioactive residual concentration is 0.01 mg/kg or more equivalent to test substance, and multiple metabolites are present that each metabolite accounts for 10% or less of total residues.

Test on metabolism in livestock (2-4-2)

1. Scope

(1) This test guideline specifies the livestock metabolism studies using radiolabelled test substance (hereinafter referred to as "labelled test substance") for the purpose of qualitatively and quantitatively clarifying the metabolism of an agricultural chemical in livestock (including poultry; the same shall apply hereinafter) when crops to be used for feed and agricultural byproducts such as rice straw, etc. (hereinafter referred to as "feed commodities") in which the agricultural chemical (including plant metabolites, etc. of the agricultural chemical) remains are fed to livestock.

(2) The guidelines are based on OECD Test Guidelines 503 "Metabolism in Livestock" (adopted in January 8, 2007). However, of the uses of agricultural chemicals specified in the OECD Guidelines, the provisions regarding the "direct application to livestock" and "animal premise treatment" are not included.

2. Objective

(1) Predict the properties of the components containing the radioisotope derived from a labelled test substance (hereinafter referred to as "radioactive residue") in tissues, organs, milk, and eggs (limited to those edible; hereinafter referred to as "edible tissues") and excreta of livestock, and estimate the total concentration of the radioactive residue (total radioactive residue, hereinafter referred to as "TRR").

(2) Identify the major components of the residue in edible tissues and indicate the components to analyse in livestock residue studies (i.e. the residue definition for both enforcement and exposure assessment).

(3) Elucidate the metabolic pathway for the agricultural chemical in ruminants and poultry.

(4) Provide evidence of whether or not the residue is fat-soluble (a property of being distributed in fat tissue, milk fat, or egg yolk; the same applies hereinafter).

(5) Provide information to explain the absorption, distribution, metabolism and excretion of the agricultural chemical in the body of livestock if there is a possibility of human consumption of agricultural chemical components remaining in livestock commodities.

3. General considerations

(1) Animal metabolism study (2-3-1) data do not substitute for livestock metabolism study data. However, animal metabolism study data may be used to supplement the livestock metabolism studies if they are used as a reference in designing the livestock metabolism studies or if sufficient characterization or identification of the residue components was not achieved in livestock metabolism studies.

(2) If the livestock metabolism study data are used as part of livestock residue study (3-2-1) data, additional data are required from a study that uses a second animal (or group of animals in the case of poultry) treated with a dose comparable to dietary levels expected in the actual feeding practice. In this case, the dosing period need to be extended if it is suspected that the residue concentration in edible tissues is not likely to reach a plateau. The use of livestock metabolism data as part of livestock residue data (3-2-1) would require fully adequate scientific reasoning, especially if a plateau has not been reached in milk and eggs.

4. Conditions under which the studies are required

Livestock metabolism studies are required when the level of the active ingredient or major metabolite is not less than the limit of quantification (LOQ) in a crop residue study (3-1-1) on feed commodities. The LOQ should be set

using 0.01-0.05 mg/kg as a general standard (levels that become 0.01-0.05 mg/kg when the water content is converted to 10% in the case of feed commodities to which the residual standard value for pasture grass applies.).

5. Study method

(1) Test substance

(i) Test substance

In principle, the test substance should be the active ingredient, etc. of the relevant agricultural chemical (hereinafter referred to as the "parent compound") and should not be a mixture of the parent compound and plant metabolites, etc. If major metabolites in plants are also found to be animal metabolites, then additional livestock metabolism studies that involve dosing with the plant metabolites will not generally be needed. However, if the major plant metabolite accounts for the predominant part of the total residue in feed commodities, a livestock metabolism study using the relevant metabolite may be required.

(ii) Labelling position

a. A stable position should be chosen so that residue components can be tracked.

b. If multiple ring structures or significant side chains are present and if it is anticipated that cleavage between these moieties may occur, separate studies should be conducted for each ring or side chain labelled. A scientifically based rationale may be stated in lieu of conducting the relevant studies if no cleavage is anticipated. However, if cleavage of the molecule is evident, the application may be required to conduct an additional study with radiolabel that tracks the portion of the molecule that is cleaved.

(iii) Radioisotopes used for labelling

a. Radioisotope used for labelling is generally ^{14}C.

b. ^{32}P, ^{35}S, or other radioisotopes may be used if no carbons or only labile carbon side chains exist in the molecule. The use of ^{3}H is generally discouraged due to the possibility of hydrogen exchange with natural constituents. If a potentially labile side chain or ^{3}H labelling is chosen, the study data can be used only if all radioactive residues are identified and found to be associated with the agricultural chemical tested.

c. Stable isotopes such as ^{13}C, ^{15}N, ^{2}D (nonexchangeable) may be used in combination to aid in identification of metabolites by mass spectrometry (hereinafter referred to as "MS") or nuclear magnetic resonance method (hereinafter referred to as "NMR").

(iv) Specific activity

The specific activity of the test substance should be adequate to allow the quantification of 0.01 mg/kg as the amount of the total residue in each edible tissue.

(2) Application

(i) Application method

The test substance should be orally given by gavage to ensure complete administration of the test substance.

(ii) Dose levels

a. The dose level used in livestock metabolism studies should approximate the level of exposure expected from the feeding of crops treated with the highest residue level (hereinafter referred to as "expected dietary maximum burden"). However, livestock should be dosed at least at a level of 10 mg/kg in the diet to obtain sufficient residue in the tissues for characterization and/or identification.

b. All dosage estimates should be based on a dry feed weight. The calculation method for expected dietary maximum burden is according to the Tests on residues in livestock (3-2-1).

c. If dosages are insufficient to provide adequate radioactive substance concentrations for characterization and/or identification of residues, additional studies will be needed. The additional studies should be designed to provide sufficient radioactive substance concentrations by appropriate means, e.g., increased specific radioactivity, suitable time of sacrifice, or exaggerated dose.

(iii) Duration of application

Ruminants and non-ruminants should be administered daily for not less than 5 days and poultry for not less than 7 days.

(3) Test animals and sampling

(i) Test animals

a. The species of choice are lactating goats for ruminants and laying hens for poultry.

b. The number of test animals per labelled test substance is given below. However, the number of animals may be increased when scientifically considered necessary. When non-ruminants are used, the number of animals should be in accordance with the case of non-ruminants.

(a) Lactating goats: 1 animal

(b) Laying hens: 10 animals (10 animals per dose group if more than one dose level is tested in the study)

c. It is not necessary to include control animals.

d. Non-ruminant (swine) metabolism studies may be necessary if metabolism from the animal metabolism study is significantly different from ruminant or poultry metabolism from the livestock metabolism study (e.g., the following cases).

(a) Differences in metabolic pathway

(b) Difference in major residues

(c) The appearance of metabolites with sub-structures, which are of known potential toxicological concern

e. The acclimatization period should be such to ensure that the livestock maintain good levels of milk and egg production prior to dosing in the study.

f. Test animals should not be pre-dosed with the test substance for the following reasons, which may result in low levels of radioactive residue concentration in tissues, thus precluding the detection of their distribution to tissues or making it difficult to compare relative amounts of parent and metabolites:

(a) Predosing may result in the induction of metabolic enzymes, and

(b) Residue of the predosed test substance can alter the distribution in the animal body and lead to changes in the specific activity of the parent compound and metabolites present in each tissue.

(ii) Sampling of animal parts

a. The following shall be considered in choosing the time of slaughter. A rationale for the time of slaughter should be described in the study report.

(a) Typical time of slaughter (e.g., 1, 3, 6, 9, 12 hours after the last administration) and kinetic information

(b) In principle, the time of slaughter should be 6 to 12 hours after the last administration. However, under no circumstances should the time of sacrifice be later than 24 hours after the last administration. Animals should not be slaughtered before Tmax (time taken by the concentration of the test substance, etc. to reach Cmax (the maximum concentration of the test substance, etc.)) to avoid over-emphasising the contribution of the unchanged parent compound.

(c) Sufficient radioactive residue concentration should be available for metabolite identification/characterization.

(d) If at the time of sacrifice chosen, an insufficient concentration of radioactive residue is anticipated, the maximum concentration in tissues should be estimated from kinetic information to validate the time of slaughter. The maximum concentration in tissues occurs close to Tmax assuming a rapid distribution of radioactivity among tissues, etc. Tmax can be derived by analysing blood samples from test animals. Also, experience has shown that in many cases the biokinetic behaviour of the orally administered pesticide is similar in, rats, ruminants, and, in large part, poultry, and therefore kinetic information, whole-body autoradiography results, etc. in laboratory animals may be used as a reference.

b. Excreta, milk and eggs should be collected twice daily, if applicable.

c. At least the following tissues should be sampled at the time of slaughter.
   (a) Muscle (loin and flank muscles for ruminants and leg and breast muscles for poultry)
   (b) Liver (whole organ for the goat and poultry and representative parts of the different lobes of the liver for cattle and swine)
   (c) Kidney (ruminants only)
   (d) Fat (perirenal, omental and subcutaneous fat for ruminants and pigs and visceral and subcutaneous fat for poultry)
d. Pathological examination of the collected organs should be performed. Abnormalities should be recorded and reported.

(4) Sample analysis
(i) Identification and characterisation of radioactive residue
a. A livestock metabolism study should identify and characterize at least 90 % of the TRR in each edible tissue according to Appendix 1. However, if identification is difficult for the following reasons, clarify the concentration of each residue component and only attempt to characterise them, and report the reason why identification is difficult and the results of characterisation.
   (a) Amount of each radioactive residue is very low
   (b) Radioactive residue is incorporated into natural constituents
   (c) The labelled test substance is easily metabolised to numerous low level components
b. Where the structure of a metabolite is identical to that of an active ingredient or metabolite of another registered agricultural chemical, available information on the compound should be collected.
(ii) Tissues to be analysed
a. The radioactive residue concentration should be quantified for all tissue/organ, etc. and excreta samples. For poultry, samples from ten birds can be pooled if individual sample analysis is difficult. For milk, too, samples collected from the same animal on the same day can be pooled if individual sample analysis is difficult. For milk, the fat fraction should be separated from the aqueous fraction, and the radioactive residue concentration in each fraction quantified.
b. Where the residue cannot be sufficiently characterized because of the low levels of residue present in tissues, etc., characterisation and identification of the residue in excreta may be required.
c. The concentration of the residual radioactive substance in muscle and fat shall be determined for each sampling part. If the residual concentration is similar between different parts (e.g., abdominal muscle and flank muscle), samples from the different parts of the same tissue may be pooled before metabolite analysis. In this case, however, the relative proportion of these different parts in the pooled sample shall be comparable to the relative proportion of the corresponding parts in the test animal. If the residue concentration clearly differs between different parts, the characterization and identification of the radioactive residue should be performed separately for each part.
(iii) Analytical method
a. Samples for analysis are homogenised, the radioactivity measured, and the TRR calculated. The entire whereabouts of the administered radioactive substance shall be clearly determined.
b. Samples are extracted from tissues with a series of solvent systems (including aqueous) with various polarities and other characteristics depending on the nature of the expected residues. The residues contained in the extracted liquids obtained are defined as extractable residue, and the residues contained in the remaining unextracted portion as unextractable residues.
c. The extractable and unextractable residues are identified and characterized according to Appendix 1.

(5) Storage of samples
(i) In principle, samples for metabolism studies should be stored at –18°C or lower. Storage under any other conditions need to be recorded and justified.
(ii) The stability of the residue in samples during tissue collection analytical sample preparation, and storage

must be confirmed.

(iii) Storage stability data are not normally necessary where tissues collected and analytical samples (including extracts) prepared during the study period were stored under appropriate conditions and the analytical samples were analysed within six months of collection.

(iv) In cases where analysis cannot be completed within six months of sample collection, evidence should be provided for the storage stability during the period between collection and final analysis. Tissues, etc. used for the verification of storage stability should be those actually stored as samples; e.g., where extracts from tissues are stored as samples, the stability should be verified in the extracts.

(v) If instability of the active ingredient is suspected based on other information, the stability during the study period should be confirmed even within six months of sampling.

(vi) If the extraction procedures in the analytical methods of livestock residue studies (3-2-1) etc. differ from those used in metabolism studies, radiovalidation data may be required. Therefore, if specific metabolites accumulate in specific organs, samples of these organs, together with liver and milk, should also be retained.

6. Items to be reported in the study report

(1) Summary/introduction

(i) The purpose of the study, testing strategies employed, and rationale for the selection of these strategies.

(ii) Guidelines observed for the conduct of this study and information on the study conduct system; and unexpected experimental problems and resulting deviations from the study protocol and the effects of those deviations on the results of the study.

(iii) Summary of the results (detected metabolites and expected metabolic pathways (including a description of the identity and quantity (free and non-extractable) of all components of the residue and their distribution within edible tissues))

(iv) Analysis of the results (a conclusion concerning the nature of the residue in the tissues, milk, and eggs analysed)

(v) Experimental problems and their validity evaluated in light of the purpose of the study.

(2) Materials and methods

(i) Test substance

a. Identification of the test substance, including chemical name (IUPAC name), common name (ISO name, etc.), company developmental name, CAS name and No., Lot No., purity and structural formula (analysis certificate should be attached)

b. Chemical structures for the parent compound and metabolites constituting the residue and their developmental or experimental names (With respect to standard substance used for identification, an analysis certificate that describes information on its purity and structure such as NMR or MS analysis results, etc., should be provided if available)

c. Information on the dosing formulation (e.g., solvent used in the administration of the labelled test substance, carrier, supplementary components, etc.)

d. Chemical purity of the labelled test substance, radiochemical purity, specific activity (MBq/mg), type of the radioisotope and its origin. Information on the chemical structures of impurities, if any, that are radiolabeled with the radioisotope contained in the labeled test substance. The site(s) of labelling in the molecule for the radiolabelled test substance should be provided. Where a radioisotope other than ^{14}C is selected, its validity and the rationale for the decision of the site of the molecule to be labelled should be provided.

e. Specific radioactivity (MBq/mg) of the dosing formulation. Sample calculation for converting experimental data into the residual radioactive substance concentration (mg/kg). Sufficient information to verify the concentration in tissues and organs, and various chromatographic fractions.

f. All additional information considered relevant to the radiolabelled test substance (e.g., physical chemical

properties)

(ii) test conditions and animal health
   a. A detailed description of test environment (e.g., animal housing conditions)
   c. Information on the species, strain, age, body weight, milk and egg production, and developmental stage of the test animals
   d. Description of animal health throughout the study; e.g., observations of changes in animal health status during the study, changes or observations in the liver and kidney (when such observations were reported in studies with laboratory animals)
   e. If the reported test conditions have deviated from the animal handling procedures specified in the study protocol, a description of the deviation and rationale for the method of handling employed should be provided.

(iii) Administration
   a. A description of the vehicle and administration method in which the radiolabelled test substance was administered to the test animals.
   b. Relationship between the dose level (mg/kg body weight) and the expected maximum dietary burden (mg/kg)
   c. The number of administrations per day and dosing period
   d. A discussion or rationale if the dose levels deviated from those specified in the study protocol

(iv) Sampling of tissues and organs
   a. Where animals other than lactating goats, laying hen, or swine were selected for use in the study, the reason for the selection and a statement of its validity should be provided. If a metabolism study was not conducted in either ruminants or poultry, its rationale or explanation should be provided.
   b. Tissues and organs subjected to the analysis of radioactive residues
   c. Collecting procedures for edible tissues and excreta and their amounts
   d. All additional information considered relevant.

(v) Time of slaughter
   a. Duration from the last administration to slaughter
   b. Information that justifies the time of slaughter. In the case of using information directly obtained from the test animals or kinetic information from laboratory animals, such information should be summarised or relevant data attached.

(vi) Handling of samples and storage stability (See 7. (3))
   a. Handling of collected samples, storage conditions and storage period before shipping and shipping procedures
   b. Conditions and period of storage for samples in laboratories
   c. Conditions and period of storage for analytical samples until identification, etc. of residues

(vii) Analytical method used for the analysis of radioactive residues
   a. The capacity of the analytical methods utilized to determine the components of the residue, whether free, conjugated, or non-extractable.
   b. Analytical method for residues in the collected tissues
   c. Oxidative combustion or liquid scintillation analysis
   d. Quantitative results of major components of the residue detected from the test animals
   e. Analytical conditions including detailed description of equipment used for determining the amount of residue in each sample
   f. Counting times, disintegration per minute (dpm), the equivalent concentration to the parent compound of detected compounds , sensitivity, limit of detection, and representative calculations
   g. In the case of radioassay using quenching correction, the method of quenching correction and the method used to reduce quenching effects should be described.

(viii) Extraction and fractionation of radioactive residues
   a. A discussion of and rationale for the selection of extraction procedures (including fractionation)

employed

b. Hydrolysis conditions used to release radioactive residues from extracts or conjugates of unextractable residues and rationale for the selection.
c. The ratio and amounts of free or conjugated parent compound or metabolites in each extracted fraction.
d. A quantitative estimate of the radioactive residue (percentage to the TRR (%TRR) and concentration (parent compound equivalent (mg/kg))) remaining in the extracted sample matrix following solvent extraction and hydrolytic treatments.
e. Radiochemical extraction efficiencies for each tissue and organ collected.
f. Discussion on the loss of the radioactive substance in the fractionation and isolation procedures and on the measures taken to minimize the loss.
g. Whether or not the major part of the unextractable radioactive residue has been incorporated into the endogenous components.
h. The percentage (%TRR) and concentration (parent compound equivalent (mg/kg)) of each radioactive residue in each sample fraction (water soluble, organosoluble, released by hydrolysis, etc.).

(xii) Characterisation/identification of radioactive residues

a. A detailed list of the parent compound, all known metabolites, and expected intermediates used for characterization and identification of radioactive residues(their structure and the purity of their reference substances)
b. Sample and reference Rf values on TLC radioautograms and relative retention times on GC and HPLC columns. Deviations or variances from expected values and steps taken to correct these problems should be discussed.
c. Images of TCL plates or autoradiograms used for the identification and HPLC/GC chromatograms including mass spectral scans, etc. (including chromatograms for analytical standards).
d. Details of complementary analytical procedures used to separate and characterise/identify metabolites (high-voltage electrophoresis, ion-exchange, exclusion chromatography, derivatisation, etc.) and instrumental analytical methods (MS, NMR, etc.) for ultimate identification of metabolites.
e. Percentage (%TRR) and concentration (parent compound equivalent (mg/kg)) of the radioactive substance detected from tissues or organs, or the extraction fraction analysed.
f. Procedures used to characterise or identify the unextractable radioactive residue
g. Residue components should be reported either as:
   (a) Free residues
   Metabolites normally extractable by organic solvents that do not require chemical treatment to be released
   (b) Conjugated residues
   Metabolites made up of two parts, one derived from the agricultural chemical and one from the animal, such as glucuronic acid, sulphuric acid, amino acid, and glutathione. The pesticide-derived components is normally identified after cleavage of the conjugated bond by acid, base, or enzymatic hydrolysis.
   (c) Unextractable residues
   Metabolites binding covalently with endogenous components that cannot be extracted by extraction with polar or non-polar solvent. Those that can be released from the matrix by acid, base, or enzymatic hydrolysis should be categorised as unextractable residues.
   (d) Natural constituent
   This category applies to the case where the agricultural chemical is degraded, finds its way into anabolic cycles, and is incorporated into cell constituents. If the natural constituents are unextractable, they are difficult to distinguish from unextractable residues under (c).
h. Additional information relevant to the conduct of the livestock metabolism study and the determination of the TRRs.

(3) Results and discussion

(i) Test strategies

A discussion of deviations made from the study protocol as a result of unexpected experimental problems encountered. These include difficulties in extraction, fractionation, and characterization of residues and specific extraction and characterization strategies employed for unextractable or bound residues. It should include a discussion of the effects, if any, of those deviations on the results of the study.

(ii) Metabolic pathways

If possible, a detailed discussion, accompanied by a flowsheet format, of the pathways of metabolism observed in test animals. The results obtained in the livestock metabolism study should be compared to the results obtained in the animal metabolism studies as well as to the results observed in plant metabolism studies. Based on the results of the characterization/identification studies, the chemical definition of the metabolic pathway (chemical reactions involved, etc.) should be proposed, including a table with associated chemical structures and names (IUPAC and CAS, including CAS numbers). Any postulated intermediates/metabolites should also be clearly indicated in the pathway.

(iii) Characterisation, identification, and distribution of TRR

a. Use a tabular or graphic format. Identify all major components of TRR (free, conjugated, and non-extractable), including name, structure, and quantity (expressed as %TRR and concentration (as parent compound equivalents (mg/kg)), and report their distribution within each fraction. They should be reported as free, conjugated, or non-extractable metabolites or natural constituents.

b. Provide information on the total amount of major unidentifiable or uncharacterisable components of the terminal residues and their distribution within each fraction.

c. Statistical treatments. (Include representative examples of any statistical tests applied to the raw data obtained during sampling/analyses in the course of the livestock metabolism study.) Provide the LOQ for radioactivity determination and chromatography.

(iv) Provide all additional information considered relevant to provide a detailed description of the livestock metabolism study including quality control measures/precautions taken to ensure validity of the study.

(4) Conclusion

(i) The pathways and mechanisms of metabolism observed in test animals.

(ii) The total amount, components, and distribution of the residue in the sampled tissues, eggs and milk

(iii) Extraction and analytical methods for the analytical substance; results of validation studies conducted on animal tissues, organs, milk, or eggs to demonstrate the capability of the relevant analytical methodology.

(iv) Conclusion to provide a complete and thorough understanding of the kinetic and metabolic processes occurring in livestock

(5) Appendices

(i) Representative chromatograms, spectra, etc. (where applicable)

(ii) A list of study reports, etc. used as a reference in the conduct of the study

7. Literature

(1) OECD Guidelines for the testing of chemicals: Metabolism in Livestock (2007)

(2) OECD Guidelines for testing of chemicals: Residues in Livestock (2007)

(3) OECD Guidance Document: Overview for Residue Chemistry Studies (2006)

(4) OECD Guidance Document on the Definition of Residue (2006)

(5) Commission of the European Communities (1997). Document 7030/VI/95-Rev.3 (22/7/1997); Appendix F: Metabolism and Distribution in Domestic Animals.

(6) Food and Agriculture Organisation of the United Nations (2009) FAO Manual on the Submission and Evaluation of Pesticide Residues Data.

(7) Food and Agriculture Organisation of the United Nations (2002) Submission and Evaluation of Pesticide Residues Data for the Estimation of Maximum Residue Levels in Food and Feed. Rome.

(8) Food and Agriculture Organisation of the United Nations (1996). Guidelines on Pesticide Residue Trials to

Provide Data for the Registration of Pesticides and the Establishment of Maximum Residue Limits, Section 2.1 Radiolabelled Studies (Metabolism Studies). Rome.

Appendix 1 Identification and characterisation of radioactive residue

1. Identification and characterisation of extractable residues
   (1) Identification
      (i) "Identification" refers to structural determination of the radioactive residue. Identification is accomplished by any of the following:
         a. Identification by co-chromatography with a reference substance of known structure
         b. Structural identification by such techniques as MS, NMR, etc.
         c. Co-chromatography should be performed with two independent systems, such as reverse and normal phases. If the separation of reverse phase and normal phase thin layer chromatography (TLC) or high performance layer chromatography (HPLC) is of suitable quality, then additional confirmation by MS, etc. is not necessary.
      (iii) For metabolites with a radioactivity of less than 0.05 mg/kg or 10% of the TRR, identification by a co-chromatography technique using putative metabolites as reference standards is acceptable.

   (2) Characterisation
      (i) "Characterisation" refers to the elucidation of the characteristics of the radioactive residue in a stage before identification. Organosoluble, aqueous soluble, neutral, acidic, basic, polar, nonpolar, unextractable, etc.
      (ii) Characterisation also involves descriptions of chemical moieties present in the molecule based on conversion to a common structure or due to reactivity with particular reagents.
      (iii) When identification is not accomplished in all radioactive residues, the degree of characterization required for the unidentified radioactive residues will depend on the following factors:
         a. Amount of the radioactive residues
         b. Proportion of the TRR already identified
         c. Importance as a food
         d. Toxicological concern
         e. capacity of analytical methods to detect radioactive residues

   (3) Others
      (i) In the metabolism studies in which highly exaggerated feeding levels are employed and low radioactivity is detected in edible tissues, characterization and/or identification are less necessary.

      In the following cases, for example, minimal characterization and/or identification of residues should be adequate, unless any special toxicological concerns are raised at this residue level

      (Example) The normally anticipated maximum dietary burden is not greater than 0.01 mg/kg, a dietary concentration of not less than 10 mg/kg is administered in a livestock metabolism study, and the resulting TRR in edible tissues is not greater than 0.1 mg/kg.
      (ii) The stereochemistry of metabolites does not necessarily need to be determined. If there are toxicological concerns, however, the ratio of the stereoisomers needs to be taken into consideration.
      (iii) New techniques such as supercritical fluid extraction (SFE), microwave extraction, and accelerated solvent extraction method (ASE) are used as far as possible to fully elucidate the metabolic pathway.

   (4) Situations in which identification of metabolites is required
      (i) The threshold values of radioactive residue concentration at which characterization and/or identification is required for each tissue or organ are shown in Table 1.

      It should be noted that the threshold values based on concentration are not absolute standards, but approximate guides as to how much characterization is adequate. If there are toxicological concerns at lower levels, identification and characterisation is required.
      (ii) Attention should be paid to the possibility that a single metabolite can be distributed among multiple extracted fractions.

(iii) In cases where a particular radioactive residue partitions into multiple extracted fractions, each fraction should be analysed by chromatography for the total amount of the component distributed among these fractions to be calculated to determine whether identification and/or characterization is required.

Table 1. Criteria for the identification and characterisation of radioactive residue

%TRR	Concentration (mg/kg)	Required characterisation and identification
<10	<0.01	No need for identification, etc. if no toxicological concern.
<10	0.01-0.05	Characterise. Only attempt to confirm identity if straightforward, e.g., a reference compound is available or the identification is known from a previous study.
<10	>0.05	Characterization/identification needs to be decided on a case-by-case basis taking into account how much of the TRR has been identified.
>10	<0.01	Characterise. Only attempt to identify if straightforward.
>10	0.01-0.05	Attempt to identify if needed to establish a metabolic pathway. At least characterisation is required.
>10	>0.05	Identify wherever technically possible.
>10	>0.05 Unextractable radioactive substance	Release, characterisation, etc. of radioactive residues are required according to "2. Release of unextractable residues" and Figure 1.

2. Release of unextractable residues

(1) The following situations are considered in which radioactive residues are detected as unextractable in livestock:

(i) Incorporation into biomolecules (i.e., amino acids, sugars, etc.). This occurs when the labelled test substance is degraded into such small (usually one or two) carbon units as to be used in the biosynthesis of endogenous components.

(ii) Tight binding with specific moieties in biomolecules to form bound residues, which can be released via chemical reactions (e.g., acidic, basic, or enzymatic hydrolysis).

(iii) Physical encapsulation or integration of radioactive residues into tissues. Release of residues in this situation may require solubilisation of the tissue, usually by influx treatment with strong base, but use of surfactants may allow the radioactive residue to be released under less severe conditions.

(2) General procedures for the release of unextractable residues are shown below (See Figure 1).

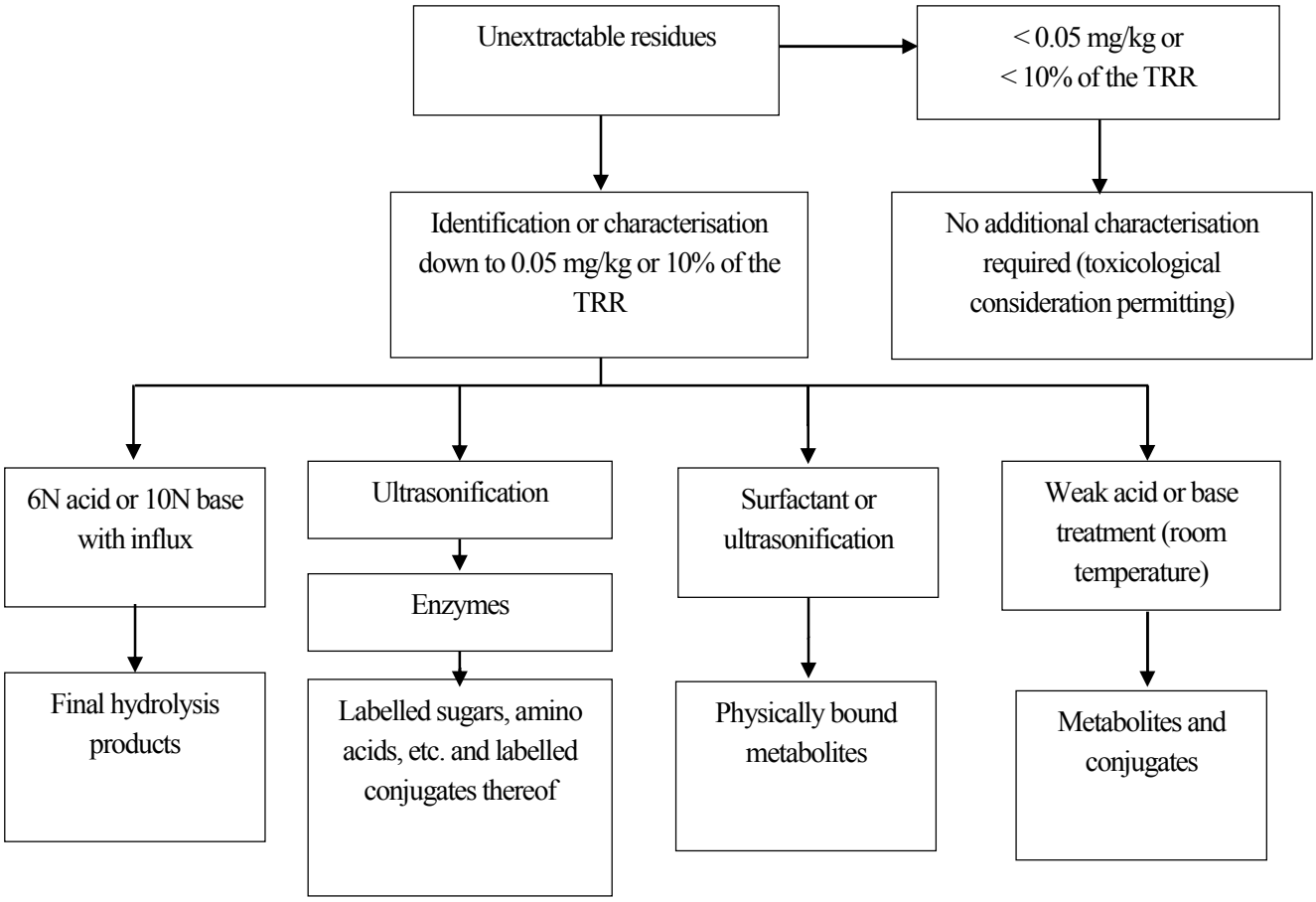
(i) After extraction, if the radioactive residue concentration in the matrix is not less than 0.05 mg/kg or 10% of the TRR, release of the radioactive residues should be attempted (See Table 1). After release treatment, if the unextractable radioactive substance concentration in the matrix is less than 0.05 mg/kg or less than 10% of the TRR, further release is not necessary.

(ii) The released radioactive residue should be quantified, and identified and/or characterized according to the criteria in Table 1. With respect to characterisation, the chromatographic behaviour of the released radioactive residues should be compared to that of the parent compound and likely metabolites, which are close in structure to the parent compound.

(iii) Release treatments are performed sequentially or on sub-samples. The types of treatments includes the following (See Figure 1). The milder the treatment, the more greater the integrity of the metabolic structures released.

a. Addition of weak acid or weak base at 37°C
b. Use of surfactants
c. Use of enzymes
d. Use of 6N acid or 10N base with reflux

Figure 1 Characterisation and identification of unextractable residues



(3) If an unextractable residue is considered to comprise the major portion of the total residue, it is desirable to determine whether or not the residue is endogenous components. Evidence of the radioisotope being incorporated into endogenous compounds like amino acids, sugars, phenol compounds, nucleotides, etc., may alleviate the need for further characterisation and identification. This usually means that the agricultural chemical has been degraded into such small carbon units as to be used in the biosynthesis of endogenous components. In this case, identification and characterization may not be required even if the unextractable residue level exceeds the criteria provided in Table 1.

This condition does not, however, apply to 3H labelled compounds or to agricultural chemicals in which the ^{14}C label is incorporated at a labile site in the molecule, or in cases where a single released metabolite, which comprises a significant portion of the TRR (greater than 0.05 mg/kg or greater than 10 % of the TRR), has not been identified.

Test on behavior in soil (2-5-1~3)

Test on behavior in flooded aerobic soil (2-5-1)

1. Objective

The objective of these studies is to obtain scientific information regarding the main metabolic pathway of the test substance in flooded soil under aerobic conditions, the types of metabolites, and the mass balance, etc. of the test substance. These studies contribute to the analysis of the results of animal and plant metabolism studies, and to the selection of substances to be analyzed in tests on residues in soil, etc.

2. Test substance

Use compounds, of a high purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled with radioactive isotopes or unlabeled. Verify their source, purity and stability.

Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the labeled substance.

3. Test soil

(1) Use fresh surface soil collected from rice paddies.

(2) Use soil that is fresh soil, or soil that has been subjected to minimal air-drying at room temperature, if necessary; pass it through a 2 mm sieve (in general) prior to use. Do not air dry the soil completely.

(3) It would be desirable to use sterilized soil additionally, in order to observe the effects of microorganisms.

4. Test conditions

(1) The soil depth should be at least 5 cm. Flood the soil in water depth of at least 1 cm.

(2) The tests should be conducted under dark conditions, and in general at 25° C (± 2°).

(3) The tests should be conducted, in general, under conditions such that gases are exchanged freely, and that test substance etc. released in the gas phase during the study period can be recovered.

(4) After preliminary incubation of soil (preincubation) for at least 2 weeks under the same conditions as those maintained during the test period and after confirming reduction zone formation, treat with test substance.

5. Test procedures

(1) Treatment

(i) Dissolve the test substance in water or a small amount of organic solvent (acetone, etc.); after the test system has been treated once, stir and shake, etc. so that there is a uniform distribution of the test substance in the flooded water.

(ii) Set a level of the treatment concentration, using as a standard the concentration when the maximum conventional application of the test substance (or active ingredient) is applied once and distributed uniformly, in soil with a depth of 10 cm. However, if the conventional application is so low as to interfere with analyses, etc., use a concentration within the range enabling analysis.

(2) Test period

Set a period sufficient for ascertaining decline of the test substance, as well as formation and decline of its

main metabolites. Set 6 months as the maximum period.

(3) Collecting samples for analysis

Collect samples of test system soil, water, and gas at least 6 times, including immediately after treatment and at the end of the study period, so as to enable appropriate analysis of the formation and decline of metabolites, etc. (including unchanged test substance; same below). By means of decantation method, separate water from soil.

(4) Analysis

In general, analyze samples promptly. Adopt appropriate methods for minimizing decomposition of metabolites when storing soil samples, extract solution, etc. It is also necessary to ensure that changes of metabolites can be ascertained during the storage period.

6. Items to consider

Items to consider in regard to all steps of distribution and metabolism are as follows.

(1) Distribution

Obtain information regarding time-dependent changes in the distribution of the test substance, etc. in soil, water, and gas phases of test system, and clarify the mass balance of the substance. If bound residues have formed after soil extraction, obtain information on the ratio at which they form.

(2) Metabolism

Use appropriate methods to identify and/or characterize and quantify test substance and major transformation products present in test system soil, water, and gas phase; characterize and quantify them. Obtain information regarding metabolic pathway. Also obtain information regarding decline of the test substance, as well as, when possible, decline of the main metabolites. Characterize the properties of bound residues that form after soil extraction.

Test on behavior in aerobic soil (2-5-2)

1. Objective

The objective of these studies is to obtain scientific information regarding the main metabolic pathways of the test substance in soil under aerobic conditions, the types of metabolites, and the mass balance, etc. of the test substance. These studies contribute to the analysis of the results of animal and plant metabolism studies, and to the selection of substances to be analyzed in tests on residues in soil, etc.

2. Test substance

Use compounds, of a high purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled with radioactive isotopes or unlabeled. Verify their source, purity and stability. Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the labeled substance.

3. Test soil

(1) Use fresh surface soil collected from fields.

(2) Use soil that is fresh soil, or soil that has been subjected to minimal air-drying at room temperature, if necessary; in general pass it through a 2 mm sieve prior to use. Do not air dry the soil completely.

(3) It would be desirable to use sterilized soil additionally, in order to observe the effects of microorganisms.

4. Test conditions

(1) The soil depth should be 1-5 cm.

(2) Maintain the water content of the soil at 40-60% of its maximum water holding capacity.

(3) The tests should be conducted under dark conditions, and in general at 25° C (± 2°).

(4) The tests should be conducted, in general, under conditions such that gases are exchanged freely, and such that test substance etc. that is released in the gas phase during the study period can be recovered.

(5) After preliminary incubation of soil for at least 2 weeks under the same conditions as those maintained during the study period, treat with test substance.

5. Test procedure

(1) Treatment

(i) Dissolve the test substance in water or a small amount of organic solvent (e.g., acetone, etc.); after the test system has been treated once, stir and shake, etc. so that there is uniform distribution of the test substance in the soil.

(ii) Set a level of the treatment concentration, using as a standard the concentration when the maximum conventional application of the test substance (or active ingredient) is applied once and distributed uniformly, in soil with a depth of 10 cm. However, if the conventional application is so low as to interfere with analyses, etc., use a concentration within the range enabling analysis.

(2) Test period

Set a period sufficient for ascertaining decline of the test substance, as well as formation and decline of its main metabolites. Set 6 months as the maximum period.

(3) Collecting samples for analysis

Collect samples of test system soil and gas at least 6 times, including immediately after treatment and at the end of the study period, so as to enable appropriate analysis of the formation and decline of metabolites, etc. (includes unchanged test substance; same below).

(4) Analysis

In general, analyze samples promptly. Adopt appropriate methods for minimizing decomposition of metabolites when storing soil samples, extract solution, etc. It is also necessary to ensure that changes of metabolites can be ascertained during the storage period.

6. Items to consider

Points to investigate in regard to all steps of distribution and metabolism are as follows.

(1) Distribution

Obtain information regarding time-dependent changes in the distribution of the test substance and its metabolites, etc. in soil and gas phase of test system, and clarify the mass balance of the substance. If bound residues have formed after soil extraction, obtain information on the ratio at which they form.

(2) Metabolism

Use appropriate methods to identify and/or characterize and quantify test substance and major transformation products present in test system soil and gas phase. Obtain information regarding metabolic pathways. Also obtain information regarding decline of the test substance, as well as, when possible, decline of the main metabolites. Characterize the properties of bound residues after soil extraction.

Test on behavior in anaerobic soil (2-5-3)

1. Objective

The objective of these studies is to obtain scientific information regarding the main metabolic pathways of the test substance in soil under anaerobic conditions, the types of metabolites, and the mass balance, etc. of the test substance. These studies contribute to the analysis of the results of animal and plant metabolism studies, and to the selection of substances to be analyzed in tests on residues in soil, etc.

2. Test substance

Use compounds, of a high degree of purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled with radioactive isotopes or unlabeled. Verify their source, purity and stability. Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the labeled substance.

3. Test soil

(1) Use fresh surface soil collected from fields.

(2) Use soil that is fresh, or soil that has been subjected to minimal air-drying at room temperature, if necessary; in general pass it through a 2 mm sieve prior to use. Do not air dry the soil completely.

4. Test conditions

(1) The soil depth should be at least 5 cm.

(2) Flood the soil so that the water is at least 1 cm deep to formulate anaerobic condition.

(3) The tests should be conducted under dark conditions, and in general at 25° C (± 2°).

(4) The tests should be conducted, in general, under anaerobic conditions such that anaerobic condition is maintained by free exchange of inert gases, and such that test substance etc. that is released in the gas phase during the study period can be recovered.

(5) After preliminary incubation for at least 2 weeks under the same conditions as those maintained during the test period and after confirming reduction zone formulation, treat with test substance.

5. Test procedures

(1) Treatment

(i) Dissolve the test substance in water or a small amount of organic vehicle (acetone, etc.); after the test system has been treated once, stir and shake, etc. so that there is uniform distribution of the test substance in the flooded water.

(ii) Set a level of the treatment concentration, using as a standard the concentration when the maximum conventional application of the test substance (or active ingredient) is applied once and distributed uniformly, in soil with a depth of 10 cm. However, if the conventional application is so low as to interfere with analyses, etc., use a concentration within the range enabling analysis.

(2) Test period

Set a period sufficient for ascertaining decline of the test substance, as well as formation and decline of its main metabolites. Set 6 months as the maximum period.

(3) Collecting samples for analysis

Collect samples of test system soil, water, and gas at least 6 times, including immediately after treatment and at the end of the study period, so as to enable appropriate analysis of the formation and decline of metabolites, etc. (includes unchanged test substance; same below). By means of decantation method, separate water from soil.

(4) Analysis

In general, analyze samples promptly. Adopt appropriate methods for minimizing decomposition of metabolites when storing soil samples, extract solution, etc. It is also necessary to ensure that changes of metabolites can be ascertained during the storage period.

6. Items to consider

Investigate to consider in regard to all steps of distribution and metabolism are as follows.

(1) Distribution

Obtain information regarding time-dependent changes in the distribution of the test substance, etc. present in test system soil, water, and gas phases, and clarify the mass balance of the substance. If bound residues have formed after soil extraction, obtain information on the ratio at which they form.

(2) Metabolism

Use appropriate methods of identify and/or characterize and quantify test substance and major transformation products present in test system soil, water, and gas phases. Obtain information regarding metabolic pathways. Also obtain information regarding decline of the test substance, as well as, when possible, decline of the main metabolites. Characterize the properties of bound residues that form after soil extraction.

Test on behavior in water (2-6-1, 2)

Test on hydrolytic behavior (2-6-1)

1. Objective

The objective of these tests is to obtain information regarding the hydrolytic behavior of the test substance in water, their main transformation pathways, transformation product, and the mass balance, etc. of the test substance. These studies contribute to the analysis of the results of animal and plant metabolism studies and to the selection of substances to be analyzed in tests on water polluting properties, etc.

2. Test substance

Use compounds, of a high degree of purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled with radioactive isotopes or unlabeled. Verify their source, purity and stability. Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the labeled substance.

3. Test water

Use buffer solutions of pH 4.0, 7.0, and 9.0.

4. Test conditions

(1) Conduct studies at 25 ± 1° C.

(2) Eliminate causes of transformation other than hydrolysis (light, oxygen, etc.).

(3) Sterilize test water and test containers.

5. Test procedures

(1) Treatment

Use a single test concentration of the test substance. In general, select 0.01 M or less than 1/2 of the water solubility, whichever is lower.

(2) Test period

Set a period sufficient for ascertaining decline of the test substance, as well as formation and decline of its major transformation products. Set 30 days as the maximum period. At the end of the study period, it would be desirable to check whether sterile conditions have been maintained in the test system.

(3) Collecting samples for analysis

Collect samples (water and gas) at least 7 times, including immediately after treatment and at the end of the study period, so as to enable appropriate analysis of the decline of the test substance and the formation and decline of major transformation products.

(4) Analysis

(i) Analyze samples promptly after collecting them.

(ii) Adopt appropriate methods for minimizing degradation of transformation products, etc. when storing samples, extract solution, etc. It is also necessary to ensure that changes of transformation, etc. products can be ascertained during the storage period.

6. Items to consider

Items to consider are usually as follows.

(1) Mass Balance

Clarify the mass balance.

(2) Transformation products, etc.

Use appropriate methods to identify and/or characterize and quantify test substance and major transformation products present in water and quantify them. If volatile substances are formed, identify and/or characterize and quantify them. Obtain information regarding hydrolytic pathways, as well as formation and decline of the major transformation products.

(3) Hydrolysis rate

Obtain information regarding decline of the test substance, as well as, when possible, formation and decline of the major transformation products.

Test on photolytic behavior in water (2-6-2)

1. Objective

The objective of these tests is to obtain information regarding the photolytic behavior in water of the test substance under light, as well as their main transformation pathways, transformation products, and the mass balance of the test substance. These studies contribute to the analysis of the results of animal and plant metabolism studies and to the selection of substances to be analyzed in tests on water polluting properties, etc.

2. Test substance

Use compounds, of a high degree of purity, that are the active ingredients, etc. of agricultural chemicals, and that are either labeled with radioactive isotopes or unlabeled. Verify their source, purity and stability. Clarify the method of synthesis, labeled nuclide, labeled position, and specific radioactivity of the labeled substance.

3. Test water

Use natural water and distilled water (or buffer solution).

4. Test conditions

(1) Use, as a test light source, artificial lighting that is similar to sunlight that would reach the ground, in terms of wavelength distribution. Conduct continuous irradiation. Measure the wavelength distribution and light intensity of on the sample stage.

(2) The surfaces of the test containers that receive incident light must be made of materials that will not absorb the light described above.

(3) Sterilize test water and test containers. When using natural water, sterilize in such a way as not to change its components.

(4) Conduct studies at 25 ± 2° C.

5. Test procedures

(1) Treatment

Use a single test concentration of the test substance. Use a concentration that is less than 1/2 of water solubility, and which is sufficient for analyses of the test substance decline rate and formation and decline of transformation products.

(2) Test period

Set the study period such that decline of the test substance and the formation and decline of the major transformation products can be ascertained. The test period need not to exceed 30- day equivalent to sunlight at 35° north latitude (Tokyo); spring (from April to June). Following completion of the study, it is desirable to confirm that sterile conditions have been maintained in the test system.

(3) Collecting samples for analysis

Collect samples (water and gas) at least 7 times, including immediately after treatment and at the end of the study period, so as to enable appropriate analysis of the decline of the test substance, as well as the formation and decline of the major transformation products.

(4) When conducting studies, establishing a sample under dark area as a control.

(5) Analysis

(i) Analyze samples promptly after collecting them.

(ii) Adopt appropriate methods for minimizing degradation of transformation products, etc. when storing samples, extract solution, etc. It is also necessary to ensure that changes of transformation products can be ascertained during the storage period.

6. Items to consider

Points to investigate are usually as follows.

(1) Mass Balance

Clarify the mass balance.

(2) Transformation products, etc.

Use appropriate methods to identify and/or characterize and quantify test substance and major transformation products present in water. If volatile substances are formed, identify and/or characterize and quantify them.

Obtain information regarding transformation pathways, as well as formation and decline of the major transformation products.

(3) Photolysis rate

Obtain information regarding decline of the test substance, as well as, when possible, formation and decline of the transformation products.

Test on impact on aquatic animals and plants

Fish acute toxicity test (2-7-1-1)

1. Objective

The objective of these studies is to establish safe use methods of using agricultural chemicals by obtaining scientific information regarding the short-term effects of the test substance on fish.

2. Definitions

(1) Death: A fish is considered dead if there is no observable movement (of the gill covers, etc.), and there is there is no response when the tail is touched.

(2) Median lethal concentration (LC50): The test substance concentration at which 50% of the test animals die during the exposure period.

(3) Test substance: TGAI or formulation of the agricultural chemical to be studied.

(4) Standard substance: Substance used for confirming the reproducibility of test conditions.

(5) Test chemical: Test substance and standard substance used in the studies.

(6) Static test: A test that is conducted according to a system in which the test solution is not exchanged during the exposure period.

(7) Semi-static test: A test that is conducted according to a system in which the test solution is exchanged in each container in each fixed period.

(8) Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.

3. Test organisms

(1) Organism species

(i) Select the test fish from the table at the end of this section.

(ii) The LC50 of the standard substance should be recommended to confirm.

(2) Acclimatization

(i) The test fish must be acquired by the 12th day prior to their use in studies, and maintained from that time.

(ii) Dip the fish in a medicated bath when received, if necessary.

(iii) The fish must be acclimated to the same environmental conditions (water quality, etc.) under which studies will be conducted for at least 9 days prior to use in studies.

(iv) The fish should be fed at least 5 times per week, but must not be fed for 24 hours prior to use for the study.

(v) Acclimatization should be conducted under the following conditions, and record the mortality rate.

a. If during the 7-day period following the stabilization period (the 2 days following the commencement of acclimatization) the mortality rate exceeds 10% of the individuals in a group, that group should be excluded.

b. If the group mortality rate is 5-10%, and is still 5% or more after acclimatization for 7 days, exclude the group, or continue acclimatization until the mortality rate falls below 5%.

c. If the group mortality rate is less than 5%, the fish in that group may be used in the studies.

4. Exposure method.

Conduct the studies under static, semi-static, and flow-through conditions.

5. Exposure period

Conduct the studies for 96 hours.

6. Determining the number of test fish, and establishing test groups

(1) Number of test fish

Use at least 7 fish in each experimental group.

(2) Establishing test groups

(i) Test concentration groups

a. Set groups of at least 5 different concentrations in a geometrical series.

b. Determine test concentrations and a factor for concentration on the basis of preliminary test results. As for a common ratio, it is desirable not to exceed 2.2.

c. It would be desirable to include within the concentration range the concentrations at which all test fishes die, and that at which there are no fatalities, as 1 level each, and at least 2 more levels at which some of the fishes die.

(ii) Control groups

a. Establish as a control a group that is not treated with the test substance.

b. When using a solubilizing agent to adjust the test liquid concentrate, establish a solubilizing agent control group, treated with the same concentration of solubilizing agent to be used in the study.

7. Preparation of the test solution

The test solution is prepared as described below. It would be desirable to prepare the test solution and test concentrate immediately prior to their use in the study.

(1) When using TGAI as the test substance

(i) When using a water soluble TGAI, dissolve it in the water that is to be used to dilute the test substance, and prepare the test solution or test solution concentrate.

(ii) When using a TGAI that is not water soluble, use mechanical means to disperse the test substance, and prepare the test solution or test solution concentrate, or prepare the test solution concentrate with a solubilizing agent, such as organic vehicle, emulsifier, or dispersant. Use a solubilizing agent that is of low toxicity to fish, which has not been noted to have adverse effects on fish at the concentration used in the study, and which does not change the properties of the test substance.

(iii) The solubilizing agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

(2) When using a formulation as the test substance

(3) Add the formulation to the dilution water, and stir. Prepare the test solution or test solution concentrate. Do not use a solubilizing agent to prepare the formulation.

8. Environmental conditions

(1) Population of test fish

(i) In static and semi-static studies, it is necessary to use at least 1 L of test solution per gram of test fish body weight.

(ii) A still higher population may be used in flow-through studies.

(2) Water temperature

Set the temperature according to the species of test fish, as shown in the table below, within a variance range of ± 2°C.

(3) Light

Light for 12-16 hours.

(4) Feeding

Do not feed during the exposure period.

(5) Dilution water

(i) Do not use water that contains hazardous substances for the study. Use water after its quality has been demonstrated to be favorable to the survival and development of fish, from the same source as the water in which they were bred.

(ii) Use dechlorinated tap water, natural water supplies, or reconstituted water.

(iii) Aerate the water to air sufficiently prior to use, and prepare the temperature.

(6) Dissolved oxygen concentration

Maintain the dissolved oxygen concentration as at least 60% of the saturate concentration. As far as there is no significant loss of the test substance, gentle aeration may be applied as required.

(7) pH

Do not adjust the pH of the test solution.

9. Observation and measurement

(1) Observation of the general condition of test fish

At the very least, observe the general condition of test fish at the 24, 48, 72, and 96 hours after the commencement of exposure, and keep records. Promptly remove dead fish from the test system. Also record any abnormalities observed.

(2) Measuring test substance concentration.

(i) When using TGAI as the test substance, measure the concentration of the test substance in each test concentration group at least at the commencement and end of exposure, at the 48 hours after the commencement of exposure, and prior to and following water changes.

(ii) During the exposure period, the test substance concentration should be 80% or higher than the nominal concentration.

(3) Measurement of environmental conditions

(i) Confirm the quality of the dilution water prior to the study.

(ii) Measure water temperature, dissolved oxygen concentration, and pH of the test solution in each test group at least at the commencement and end of exposure, and prior to and following water changes.

10. Method of processing results

(1) Use an established method for computing LC_{50}, based on the mortality rate results for each concentration.

(2) If the measured values for test substance concentration fluctuate ± 20% or more above the nominal concentration, compute LC_{50} on the basis of the mean measured concentration.

11. Reporting

(1) The test substance

(2) The test fish

Species name, water source, breeding method, acclimatization, number of test fish, length and weight of fish, LC_{50} with standard substance, etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, etc.

(4) Results

(i) LC50 (when TGAI is the test substance, LC_{50} is based on the active constituent concentration) and the 95% confidence limit (at the time of each observation, if possible)
(ii) Method of calculating LC_{50}
(iii) The cumulative mortality rate in each test group at the time of each observation
(iv) A graph of the concentration/mortality curve at the end of the exposure period
(v) Abnormal symptoms and responses of test fish
(vi) Measured values of the test substance concentration (only when using TGAI as the test substance)
(vii) Results of measurement of environmental conditions

Water quality, dissolved oxygen concentration, pH, etc.

(viii) Other items

Test solution conditions, and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

12. Study validity

(1) The mortality rate must not exceed 10% in control groups at the end of the exposure period. If less than 10 fish are used, it is unacceptable for more than 1 of them to die.

(2) The dissolved oxygen concentration must be maintained as at least 60% of the saturate concentrate.

Table: Conditions and temperatures to be set according to test organism species

Fish Species	Set Temperature (°C)	Test Fish Length (cm)
Cyprinus carpio	20-24	4.0 ± 2.0
Oryzias latipes	21-25	2.3 ± 1.2
Lepomis macrochirus	21-25	2.0 ± 1.0
Oncorhynchus mykiss	13-17	5.0 ± 1.0
Poecilia reticulata	21-25	2.0 ± 1.0
Danio rerio	21-25	2.0 ± 1.0
Pimephales promelas	21-25	2.0 ± 1.0

Fish (larvae) acute toxicity test (2-7-1-2)

1. Objective

As per fish acute toxicity studies.

2. Definitions

As per fish acute toxicity studies.

3. Test organisms

(1) Organism species

(i) Use larvae (no more than 24 hours old) of ricefish (*Oryzias latipes*).
(ii) The LC50 of the standard substance should be recommended to confirm.

(2) Acclimatization
(i) Breed male and female parent ricefish under conditions adopted to laying eggs (water temperature about 25℃, photoperiod more than 13 hours illumination, sufficient food). If fish are obtained from another organization, acclimatize in the same way as in acute fish toxicity studies. If fish are maintained continuously, an acclimatization period is not necessary.
(ii) Collect the fertilized eggs that were laid, remove the filaments from them, and incubate them in clear water (with the same water quality as the dilution water to be used for the study).
(iii) After the eggs hatch, collect the fry swimming in the water by using a glass tube and so on.

4. Exposure method.
As per fish acute toxicity studies.

5. Exposure period
As per fish acute toxicity studies.

6. Determining the number of test fish, and establishing test groups
As per fish acute toxicity studies.

7. Preparation of the test solution
As per fish acute toxicity studies.

8. Environmental conditions
As per fish acute toxicity studies.

9. Observation and measurement
As per fish acute toxicity studies.

10. Method of processing results
As per fish acute toxicity studies.

11. Reporting
As per fish acute toxicity studies.

12. Study validity
As per fish acute toxicity studies.

Daphnia spp **acute immobilization test (2-7-2-1)**

1. Objective
The objective of these studies is to establish safe use methods of using agricultural chemicals by obtaining scientific information regarding the short-term effects of the test substances on crustaceans.

2. Definitions
(1) Immobilization: If there is no swimming movement at all in a test solution for 15 seconds after the test container is shaken lightly, immobilization is considered to have occurred.

(2) Median effect concentration (EC_{50}): The test substance concentration at which 50% of the test organisms are immobilized during the exposure period.

(3) Test substance: TGAI or formulation of the agricultural chemical to be tested.

(4) Standard substance: Substance used for confirming the reproducibility of study conditions.

(5) Test chemical: Test substance and standard substance used in the studies.

(6) Static test: A test that is conducted according to a system in which the test solution is not changed during the exposure period.

(7) Semi-static test: A test that is conducted according to a system in which the test solution is changed in each container during each fixed period.

(8) Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.

3. Test organisms

(1) Organism species

(i) Use *Daphnia magna*. However, other species of daphnia may be used if equivalent test results can be obtained thereby.

(ii) Use test organisms of known history (the supplier, breeding methods, etc.), offspring of the same strain parent daphnia.

(iii) It would be desirable to confirm the EC_{50} of a standard substance. The first offspring of the parent daphnia shall not be used in order to reduce variability.

(2) Life stage

Use individuals that are no more than 24 hours old (hereinafter referred to as "young daphnia").

(3) Breeding of parent daphnia

The parent daphnia used to obtain young daphnia should be bred for a fixed period under conditions as close to the test environmental conditions as possible (same water quality as that of the dilution water used in the studies, water temperature, etc.). Daphnia should be healthy and in their reproductive prime (usually 2-4 weeks old).

4. Exposure method.

Conduct the studies under static, semi-static, and flow-through conditions.

5. Exposure period

Conduct the studies for 48 hours.

6. Determining the number of test organisms, and establishing test groups

(1) Number of test organisms

Use at least 20 test organisms in each test group. In this case, it is desirable to divide the test organisms into 4 groups of 5.

(2) Establishing test groups

(i) Test concentration groups

a. Establish at least 5 different concentrations in a geometrical series. As for a common ratio, it is desirable not to exceed 2.2.
b. Determine test concentrations and a factor for concentration on the basis of preliminary test results.
c. It would be desirable to include within the concentration range the concentrations at which all test organisms are immobilized, and that at which there is no immobilization, as 1 level each, and at least 2 more levels at which some of the organisms are immobilized.

(ii) Control groups
a. Design as a control a group that is not treated with the test substance.
b. When using a solubilizing agent to adjust the test solution concentrate, design a solubilizing agent control group, treated with the same concentration of solubilizing agent to be used in the study.

7. Preparation of the test solution

The test solution is prepared as described below. It would be desirable to prepare the test solution and test solution concentrate immediately prior to their use in the study.

(1) When using TGAI as the test substance
(i) When using a readily water soluble TGAI, dissolve it in the water that is to be used to dilute the test substance, and prepare the test solution or test solution concentrate.
(ii) When using a TGAI that is not water soluble, use mechanical means to disperse the test substance, and prepare the test solution or test solution concentrate, or prepare the test solution concentrate with a solubilizing agent, such as organic solvent, emulsifier, or dispersing agent. Use a solubilizing agent that is of low toxicity to the organisms, which has not been noted to have adverse effects on the organisms at the concentration used in the study, and which does not change the properties of the test substance.
(iii) The solubilizing agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

(2) When using a formulation as the test substance

Add the formulation to the dilution water, and stir. Prepare the test solution or test solution concentrate. Do not use a solubilizing agent to prepare the formulation.

8. Environmental conditions

(1) Test solution volume

Use at least 5 ml of test solution per individual daphnia.

(2) Water temperature

Set the temperature at 20°C, with the range of ± 1°C during the study period.

(3) Light

It is desirable to light for 16 hours.

(4) Feeding

Do not feed during the exposure period.

(5) Dilution water
(i) Do not use water that contains hazardous substances in the study. Use water after its quality has been demonstrated to be favorable to the survival and reproduction of daphnia, from the same source as the water in which they were bred.
(ii) Use dechlorinated tap water, natural water supplies, or reconstituted water.
(iii) Aerate sufficiently prior to use, and prepare the temperature.
(iv) Water containing chelating agent, such as Elendt M4 or M7 culture medium, shall not be used in the test of

substances containing a metal.

(6) Dissolved oxygen concentration

Maintain the dissolved oxygen concentration as at least 3 mg/L. During exposure, aeration should not be applied as a rule.

(7) pH

Do not adjust the pH of the test solution.

9. Observation and measurement

(1) Observation of the general condition of test organisms

Observe and record whether immobilization has occurred 24 and 48 hours after the initiation of exposure. Record any abnormal behavior or appearance when it is observed, in addition to immobilization.

(2) Measuring of the test substance concentration

(i) When using TGAI as the test substance, measure the concentration of the test substance in each test group at least at the initiation and end of exposure, and prior to and following water changes. When setting the number of containers in each test group, take equal volumes of test solution from all containers, mix them, and use the mixture to measure of the test sample. However, a test solution from a different container may be used for analysis when necessary. In this case, processing should be applied under the identical conditions of the test solution.

(ii) During the exposure period, the test substance concentration should be 80% or more than the setting concentration.

(3) Measurement of environmental conditions

(i) Confirm the quality of the dilution water prior to the study.

(ii) Measure water temperature, dissolved oxygen concentration, and pH of the test solution in each test group at least at the initiation and end of exposure, and prior to and following water changes. During exposure, the pH shall not fluctuate by 1.5 or more in ordinary cases.

10. Method of processing results

(1) Use an established method for calculating EC_{50}, based on the immobilization rates results for each concentration.

(2) If the measured values for test substance concentration range ± 20% or more above the setting concentration, compute EC_{50} on the basis of the mean value of the measured concentrations.

11. Reporting

(1) The test substance

(2) The test organisms

Species name, history (source from which they were obtained, breeding method, etc.), EC_{50} of positive standard substance, etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, etc.

(4) Results

(i) EC_{50} (when TGAI is used as the test substance, LC_{50} is based on the active constituent concentration),

and its 95% confidence limit (at the time of each observation, if possible)

(ii) Method of calculating EC_{50}

(iii) The cumulative immobilization rate in each test group at the time of each observation

(iv) A graph of the concentration/immobilization rates curve at the end of the exposure period

(v) Observed effects

(vi) Measured values of the test substance concentration (only when using TGAI as the test substance)

(vii) Results of measurement of environmental conditions

Water quality, dissolved oxygen concentration, pH, etc.

(viii) Other items

Test solution conditions and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

12. Study validity

(1) The immobilization rate must not exceed 10% in control groups during exposure period.

(2) During exposure, daphnia in test groups exceeding 10% of all control groups shall not indicate any abnormal symptom or behavior such as decoloration or drifting on the water surface.

(3) The dissolved oxygen concentration must be maintained as at least 3 mg/L at the end of exposure period.

Daphnia spp (adult daphnids) acute immobilization test (2-7-2-2)

1. Objective

As per *Daphnia* acute immobilization studies.

2. Definitions

As per *Daphnia* acute immobilization studies.

3. Test organisms

(1) Organism species

As per *Daphnia* acute immobilization studies.

(2) Life stage

Use individuals that are 7 days old after breeding and feeding.

(3) Breeding of parent daphnia

As per *Daphnia* acute immobilization studies.

4. Exposure method.

As per *Daphnia* acute immobilization studies.

5. Exposure period

As per *Daphnia* acute immobilization studies.

6. Determining the number of test organisms, and establishing test groups

As per *Daphnia* acute immobilization studies.

7. Preparation of the test solution
   As per *Daphnia* acute immobilization studies.

8. Environmental conditions
   As per *Daphnia* acute immobilization studies.

9. Observation and measurement
   As per *Daphnia* acute immobilization studies.

10. Method of processing results
    As per *Daphnia* acute immobilization studies.

11. Reporting
    As per *Daphnia* acute immobilization studies.

12. Study validity
    As per *Daphnia* acute immobilization studies.

Daphnia spp reproduction test (2-7-2-3)

1. Objective
   The objective of these studies is to establish safe use methods of using agricultural chemicals by obtaining scientific information regarding effects of the test substances for the reproducibility on crustaceans.

2. Definitions
   (1) Reproduction rate: This refers to the mean cumulative number of living offspring produced (surviving young daphnia) per parent.

   (2) Median effect concentration (EC_{50}): The test substance concentration at which the reproduction rate of the test organisms is inhibited by 50% during the exposure period as compared to the control.

   (3) Lowest observed effect concentration (LOEC): The lowest test concentration at which effects are observed, in terms of reproductivity, the mortality rate among parent daphnia, etc. as compared to the control.

   (4) No observed effect concentration (NOEC): The highest test concentration at which no effects are observed, in terms of reproductivity, the mortality rate among parent daphnia, etc. as compared to the control.

   (5) Test substance: TGAI used in studies.

   (6) Semi-static test: A test that is conducted according to a system in which the test solution is changed in each container during each fixed period.

   (7) Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.

3. Test organisms
   (1) Organism species

(i) Use *Daphnia magna*. However, another species of *daphnia* may be used if equivalent test results can be obtained thereby.

(ii) Use test organisms of known history (the source from which they were obtained, methods of breeding, etc.), offspring of the same strain parent daphnia.

(2) Life stage

Use individuals that are no more than 24 hours old (hereinafter referred to as "young daphnia"). The first offspring of the parent daphnia shall not be used in order to reduce variability.

(3) Breeding of parent daphnia

The parent daphnia used to obtain young daphnia should be kept for a fixed period under conditions as close to the study environmental conditions as possible (same water quality as that of the dilution water used in the studies, water temperature, etc.). Use daphnia that are healthy and in their reproductive prime (usually 2-4 weeks old).

4. Exposure method.

Conduct the studies under semi-static and flow-through conditions.

5. Exposure period

Conduct the studies for 21 days.

6. Determining the number of test organisms, and establishing test groups

(1) Number of test organisms

Use at least 10 test organisms in each test group. Divide the organisms into numbers of individuals as necessary for observation.

(2) Establishing test groups

(i) Test concentration groups

a. Set groups of at least 5 different concentrations in a geometrical series. As for a common ratio, it is desirable not to exceed 3.2.

b. Establish test concentrations and a factor for concentration on the basis of preliminary study or acute immobilization test results.

c. It would be desirable to include within the concentration range the concentrations at which all test organisms are affected, and that at which there are no effects, as 1 level each, and at least 2 more levels at which some of the fish die.

(ii) Control groups

a. Establish as a control a group that is not treated with the test substance.

b. When using a solubilizing agent to prepare the test solution concentrate, establish a control group for a solubilizing agent, treated with the same concentration of solubilizing agent to be used in the study.

7. Preparation of the test solution

The method of preparing the test solution is as described below.

(1) Usually, dilute test solution concentrate of a high concentration to obtain test solution. Use a solubilizing agent, such as organic vehicle, emulsifier, or dispersing agent, if necessary for precise preparation of the test solution concentrate, but it is desirable to avoid using auxiliary agents insofar as possible.

(2) Use a solubilizing agent that is of low toxicity to the organisms, which has not been noted to have adverse effects on the organisms at the concentration used in the study, and which does not change the properties of the test substance.

(3) The auxiliary agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

8. Environmental conditions

(1) Amount of test solution

Use at least 50 ml of test solution per individual daphnia.

(2) Water temperature

It is desirable to set the temperature in the range of 18°C –22°C, and allow it to vary within a range of ± 1°C.

(3) Light

It is desirable to light for 16 hours.

(4) Feeding

Feed the daphnia unicellular green algae, such as chlorella.

(5) Dilution water

(i) Do not use water that contains hazardous substances in the study. Use water after its quality has been demonstrated to be favorable to the survival and reproduction of daphnia, from the same source as the water in which they were raised.

(ii) Use dechlorinated tap water, natural water supplies, or reconstituted water.

(iii) Expose to air sufficiently prior to use, and prepare the temperature.

(iv) Water containing chelating agent, such as Elendt M4 or M7 culture medium, shall not be used in the test of substances containing a metal.

(6) Dissolved oxygen concentration

Maintain a dissolved oxygen concentration of at least 3 mg/L during the exposure period. During exposure, aeration should not be applied as a rule.

(7) pH

Do not adjust the pH of the test solution.

9. Observation and measurement

(1) Observation of the general condition of test organisms

Count the number of living and dead parent daphnia, as well as the number of surviving offspring. Regularly observe and record the condition of parent daphnia, as well as whether or not dead young daphnia, aborted eggs, or resting eggs are present.

(2) Measuring test substance concentration

(i) Measure the test substance concentration in each test concentration group.

(ii) During the exposure period, the test substance concentration should be 80% or more than the nominal concentration.

(3) Measurement of environmental conditions

(i) Confirm the quality of the dilution water prior to the study.

(ii) Measure water temperature, dissolved oxygen concentration, hardness, and pH of the test solution in each test group. During exposure, pH shall not fluctuate by 1.5 or more in ordinary cases.

10. Method of processing results

(1) EC_{50} and 95% confidence limit

Calculate EC_{50} according to the common method, in so far as possible, using the mean total number of living offspring produced per parent (surviving young *daphnia*) in each control group (or auxiliary agent control group) and each test concentration group.

(2) LOEC and NOEC

Calculate the total cumulative number of living offspring produced per parent (surviving young *daphnia*) in each test container, and determine by statistical means whether there are significant differences between the control group (or solubilizing agent control group) and each test concentration group. Determine the lowest concentration (LOEC) at which significant differences are noted and the maximum concentration (NOEC) at which significant differences are not observed, in comparison with the control group (or solubilizing agent control group).

(3) If the measured values for test substance concentration vary ± 20% or more from the nominal concentration, compute EC_{50} on the basis of the mean measured concentration.

11. Reporting

(1) The test substance

(2) The test organisms

Species name, history (source from which they were obtained, method of care, etc.), etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, etc.

(4) Results

(i) Mortality rate of parent daphnia
(ii) Reproductive rate
(iii) EC_{50}, and its 95% confidence limit on the basis active ingredient concentration (if possible)
(iv) Calculating method of EC_{50}
(v) LOEC and NOEC on the basis active ingredient concentration (If these have not been obtained, record the reason.)
(vi) Calculating method of LOEC and NOEC
(vii) The mortality rate and reproductive rate among parent daphnia in each test group at the time of each observation
(viii) Graph of the concentration/parent daphnia mortality rate curve at the end of the exposure period
(ix) Observed effects
(x) Days until the first offspring appear in each test concentration group
(xi) Measured values of the test substance
(xii) Results of measurement of environmental conditions
(xiii) Water quality, dissolved oxygen concentration, hardness, pH, etc.
(xiii) Other items

Test solution conditions, and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

12. Study validity

(1) The mortality rate of parent daphnia must not exceed 20% in control groups at the end of the exposure period.

(2) The mean total cumulative number of living offspring produced per parent in the control group must be at least

60 individuals.

Test on effects of coexistent organic substances on fish acute toxicity / Daphnia spp acute immobilization (2-7-3)

1. Objective

The objective of these studies is to establish safe methods for using agricultural chemicals by obtaining scientific information regarding the short-term effects of the test substances on fish or crustaceans.

2. Definitions

(1) Death (Fish): Test organism makes no response to light incentive.

(2) Immobilization (*Daphnia spp)*: If there is no swimming movement at all in a test solution for 15 seconds after the test container is shaken lightly, immobilization is considered to have occurred.

(3) Median lethal concentration (LC50): The test substance concentration at which 50% of the test animals die during the exposure period.

(4) Median effect concentration (EC_{50}): The test substance concentration at which 50% of the test organisms are immobilized during the exposure period.

(5) L(E)C_{50}: LC_{50} or EC_{50}

(6) Test substance: TGAI of the agricultural chemical to be studied.

(7) Solubilizing agent: solvent used to dissolve test substance.

(8) Static test: A test that is conducted according to a system in which the test solution is not exchanged during the exposure period.

(9) Semi-static test: A test that is conducted according to a system in which the test solution is exchanged in each container in each fixed period.

(10) Dissolved Organic Carbon (DOC): various organic molecules occurring in lotic and lentic ecosystem, which in this test are restricted to a heterogeneous group of humic substances.

(11) Total Organic Carbon (TOC): The sum of all organic carbon molecules, which are dissolved, and suspended, occurring in test dilution waters.

(12) Humic substance: Humic acids (HAs), fulvic acids, humine fractions, and their various salts, resulting from chemical fractionation of this heterogeneous naturally occurring organic substances. For the purposes of this test, HA, sodium salt may be used as the sources of DOC.

3. Test organisms

(1) Organism species

<Fish>

Ricefish (*Oryzias latipes*)

<Daphnia>

Daphnia magna.

(2) Life stage

<Fish>

Use juvenile fish. Fish used in a particular test should be the same age and be of normal size and appearance for their age. The longest fish should not be more than twice the length of the shortest fish.

<Daphnia>

Use individuals that are no more than 24 hours old. The first offspring of the parent daphnia shall not be used in order to reduce variability.

Use test organisms of known history (the supplier, breeding methods, etc.).

(3) Acclimatization and Breeding of parent daphnia

As per *Daphnia* acute immobilization studies or fish acute toxicity studies.

4. Exposure method

Conduct the studies under static and semi-static conditions.

5. Exposure period

96 hours for ricefish, 48 hours for daphnid

6. Determining the number of test organisms, and establishing test groups

(1) Preliminary study

(i) If the toxicity of the test substances in HA is not already known, a preliminary study should be performed to determine the range of concentrations to be used in the main study.

(ii) Initially, about 5 fish or 10 Daphnids is performed at 10 mg./L of HA. In some cases, the 10 mg HA/L concentration may be so high that no toxicity is present due to the formation of viscous, colloidal, complexes. If this occurs, the 10 mg HA/L concentration should be decreased to less than 10 mg/L, or an appropriately lower concentration.

(iii) If the acute toxicity value in the preliminary study performed at 10 mg/L HA is lower than those of test under non-HA.

(2) Main study

(i) Number of test organisms

Use at least 7 fish or 20 Daphnids in each test group, divide the organisms into the number of individuals as necessary for observation. In this case, an equal number of test organisms should be placed in each test chamber.

(ii) Establishing test groups

a. Test concentration groups

(a) HA concentration should be set at 2.5, 5, 10mg/L. Establish at least 5 different concentrations in a geometrical series and each HA concentrations.

(b) Determine test concentrations and a factor for concentration on the basis of preliminary test results.

(c) It is desirable to include within the concentration range the concentrations at which all test fish (or test Daphnids) die (or are immobilized), and that at which there are no fatalities (or immobilizations), as 1 level each, and at least 2 more levels at which some of the fish (or test Daphnids) die (or are immobilized).

b. Control groups

(a) Every test should be include a control consisting of the same dilution water, conditions, procedures,

and test organisms from the same group used in the test, except that none of the test substance is added.

(b) Every test should also include negative controls consisting of dilution water with HA alone.

7. Preparation of the test solution

Use only dilution water for preparation of the test solution. Do not use organic aids for this study in principle. However, in case the study is difficult to complete without the use of organic aids for agricultural chemicals of low water-solubility, the minimal amount required may be used.

8. Environmental conditions

(1) Test material

(i) Constituent material

Constituent material and equipment that contact the stock solution, the test solution, or the dilution water shall not include material that may leach out or dissolve into the solution to a degree that might affect the test results. Select material and equipment that contact the stock solution or the test solution, so that the amount of the test substance absorption is minimized. If possible, use glass, stainless steel, or fluorine plastics for the material.

(ii) Cleaning test system

Wash the test tank sufficiently before each study.

(iii) Dilution water

a. Do not use water that contains hazardous substances that might affect the study, and use water after its quality has been demonstrated to be favorable to the survival and growth of fish and to the survival and reproduction of daphnia, from the same source as the water in which they were bred.

b. Use dechlorinated tap water, natural water, or reconstituted water.

c. Expose to air well prior to use, and adjust the temperature.

(2) Population

(i) Fish

The number of fish in the test tank must not be so large as to affect the test results. Test fish body weight per 1L of test solution in the test tank must not exceed 0.5g. This population target shall be set so that the dissolved oxygen concentration cited in (3) can be maintained.

(ii) Daphnids

Use at least 10 mL of test solution per 1 daphnid.

(3) Dissolved oxygen concentration

Maintain the dissolved oxygen concentration as at least 60% of the saturate concentration. Gentle aeration applied, as necessary.

(4) Water temperature

Set the temperature to 23°C for ricefish, and 20°C for daphnids. The variance range must not exceed ± 2°C for ricefish, and ± 1°C for daphnids.

(5) Light

Light for 12-16 hours.

9. Observation and measurement

(1) Observation of the general condition of test organisms

At the very least, observe any abnormalities such as mortality, immobilization, or others, every 24 hours after the commencement of exposure, and keep records. Promptly remove dead fish from the test system.

(2) Measuring test substance concentration.

Test substance concentration of test solution needs not be measured in principle. However, measure the test substance concentration of stock solution at preparation of the test solution as needed.

(3) Measurement of environmental conditions

(i) Dilution water and test solution

a. Confirm the quality of the dilution water prior to the study.

b. Measure water temperature, dissolved oxygen concentration, and pH of the test solution in each test group at least at the initiation and end of exposure, and prior to and following water changes.

(ii) Dissolved organic carbon (DOC)

HA shall be used in this study as naturally-derived DOC

(iii) Sampling for TOC measurement

Collect samples for TOC analysis from the upper and the bottom part, and the center part between the both sides of the test tank in each test group and the control group. Do not include surface scum (scum or thin film in the surface) or substances removed from the bottom or side of the test tank.

(iv) Measurement of TOC

Regarding static test, measure DOC (as TOC) for each HA concentration at the commencement of the study (prior to adding test substance and test organisms).

Regarding semi-static test, measure TOC of the prepared test solution as per static test, at water changes (prior to adding test substance).

10. Method of processing results

(1) Use nominal concentration of the test substance based on 100% active ingredients for all L(E) C_{50} calculation and for plotting a dose-response curve.

(2) Obtain a dose-response curve and L(E) C_{50} values for each HA concentration, based on the mortality and immobilization obtained from the study, by using a conventional method.

(3) Perform regression analysis as to relationship between the mean concentration of TOC measured for each HA concentration and L(E) C_{50} values, and calculate L(E) C_{50} values at the TOC concentration of 1.5 mg/L.

(4) Obtain the toxicity mitigation coefficient at TOC concentration of 1.5 mg/L by dividing L(E) C_{50} values at the TOC concentration of 1.5 mg/L by L(E) C_{50} values at HA concentration of 0 mg/L.

11. Reporting

(1) The test substance

(2) The test organisms

Species name, history (source from which they were obtained, method of care, etc.), etc.

(3) Test method

HA (chemical properties and source), chemical analysis method of TOC (including validating method and blank test solution), exposure conditions, environmental conditions (DO, pH, temperature and lighting method, etc), items for observation and measurement, etc. When organic aids were used, add the following information as a reference to discuss the current test results; (i) discussion on necessity of organic solvent use, (ii) discussion on validity of type and amount of used organic solvent, (iii) discussion on affects that the organic solvent might have exerted on test results, and (iv) outline of results of the study using substances similar to the test substance, and so on.

(4) Results

(i) L(E) C_{50}values at each HA concentration and at TOC of 1.5 mg/L, and their 95% confidence limits (at the time of each observation, if possible), and toxicity mitigation coefficient.

(ii) Method of calculating L(E)C_{50}

(iii) Result of regression analysis as to relationship between TOC and L(E)C_{50}, and the method used for the analysis.

(iv) The cumulative mortality rate or the cumulative immobilization rate in each test group at the time of each observation

(v) A graph of the concentration/ mortality (or immobilization) rates curve at the end of the exposure period

(vi) Observed effects

(vii) Measured values of TOC

(viii) Results from any preliminary studies performed at 10mg/L of HA.

(ix) Results of measurement of environmental conditions (water quality, dissolved oxygen concentration, pH, etc.)

(x) Other items

Test solution conditions, and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

12. Study validity

(1) The mortality rate (or the immobilization rate) must not exceed 10% in control groups at the end of the exposure period. If less than 10 fish are used, it is unacceptable for more than 1 of them to die.

(2) The dissolved oxygen concentration must be maintained as at least 60% of the saturate concentrate.

(3) It is unacceptable for daphnia to be floating on the surface of the water in the control group at the initiation of exposure.

Freshwater shrimp acute toxicity test (2-7-4)

1. Objective

The objective of these studies is to establish safe use methods of using agricultural chemicals by obtaining scientific information regarding the short-term effects of the test substances on crustaceans (shrimp).

2. Definitions

(1) Death: Test organism makes no response for light incentive.

(2) Median lethal concentration (LC50): The test substance concentration at which 50% of the test animals die during the exposure period.

(3) Test substance: TGAI of the agricultural chemical to be studied.

(4) Standard substance: Substance used for confirming the reproducibility of study conditions.

(5) Test chemical: Test substance and standard substance used in the studies.

(6) Static test: A test that is conducted according to a system in which the test solution is not changed during the exposure period.

(7) Semi-static test: A test that is conducted according to a system in which the test solution is changed in each container during each fixed period.

(8) Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.

3. Test organisms
   (1) Organism species
      (i) Use freshwater shrimp. It is desirable to use Green neon shrimp (*Neocaridina denticulate*) or *Paratya compressa improvisa.*
      (ii) Use test organisms of known history (the supplier, breeding methods, etc.).
      (iii) It would be desirable to confirm the EC_{50} of a standard substance.

   (2) Growth stage
      Use test organisms at the growth stage in which they are not morphologically different from adults, and those without eggs.

   (3) Acclimatization
      (i) Test organisms must be collected and held for at least 12 days prior to studying.
      (ii) Test organisms must be acclimatized under similar environmental conditions to those used in the study for at least 9days prior to studying.
      (iii) Feed appropriate amount at least 5 days .Test organisms should not be fed for 24 hours before exposure.
      (iv) Acclimatize following standard and record mortality.
         a. If mortality of 7 days following stable period after 2days exceed 10% of group numbers, remove the group.
         b. In case the mortality of group is 5-10%, after acclimatize for more 7 days continuously, If the mortality of group is more than 5%, remove the group or continue to acclimatize until mortality become under 5%..
         c. In case the mortality of group is less than 5%, the group can be used in the study.
      (v) If shrimp cultured continuously are used, acclimatization period is not necessary specially.

4. Exposure method
   Conduct the studies under static, semi-static, and flow-through conditions.

5. Exposure period
   Conduct the studies for 96 hours.

6. Determining the number of test organisms, and establishing test groups
   (1) Number of test organisms
      Use at least 10 test organisms in each test group.

   (2) Establishing test groups
      (i) Test concentration groups
         a. Establish at least 5 different concentrations in a geometrical series.
         b. Determine test concentrations and a factor for concentration on the basis of preliminary test results.
         c. It would be desirable to include within the concentration range the concentrations at which all test organisms die, and that at which there are no fatalities, as 1 level each, and at least 2 more levels at which some of the organisms die.
      (ii) Control groups

a. Establish as a control a group that is not treated with the test substance.
b. When using a solubilizing agent to adjust the test solution concentrate, design a solubilizing agent control group, treated with the same concentration of solubilizing agent to be used in the study.

7. Preparation of the test solution

The test solution is prepared as described below. It would be desirable to prepare the test solution and test solution concentrate immediately prior to their use in the study.

(1) When using a readily water soluble TGAI, dissolve it in the water that is to be used to dilute the test substance, and prepare the test solution or test solution concentrate.

(2) When using a TGAI that is not water soluble, use mechanical means to disperse the test substance, and prepare the test solution or test solution concentrate, or prepare the test solution concentrate with a solubilizing agent, such as organic solvent, emulsifier, or dispersing agent. Use a solubilizing agent that is of low toxicity to the organisms, which has not been noted to have adverse effects on the organisms at the concentration used in the study, and which does not change the properties of the test substance.

(3) The solubilizing agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

8. Environmental conditions

(1) Population

Sufficient amount of test solution is needed depending on the size and the number of test organisms. Population density shall be set so that the dissolved oxygen concentration is maintained at 60% or more of the saturate concentration.

(2) Water temperature

Set the temperature at 20-24℃ and standard temperature is 22℃, with the range of ± 1℃ during the study period.

(3) Light

It is desirable to light for 12-16 hours.

(4) Feeding

Do not feed during the exposure period.

(5) Dilution water

(i) Do not use water that contains hazardous substances for the study. Use water after its quality has been demonstrated to be favorable to the survival and development of test organisms, from the same source as the water in which they were bred.
(ii) Use dechlorinated tap water, natural water supplies, or reconstituted water.
(iii) Aerate sufficiently prior to use, and prepare the temperature.

(6) Dissolved oxygen concentration

Maintain the dissolved oxygen concentration as at least 60% of the saturate concentration. Gentle aeration applied , as necessary.

(7) pH

Do not adjust the pH of the test solution.

9. Observation and measurement

(1) Observation of the general condition of test organisms

At the very least, observe the general condition of test organisms at the 24, 48, 72, and 96 hours after the commencement of exposure, and keep records. Promptly remove dead test organisms from the test system.

(2) Measuring of the test substance concentration

(i) Measure the concentration of the test substance in each test group at least at the initiation and end of exposure, at the 48 hours after the commencement of exposure, and prior to and following water changes.

(ii) During the exposure period, the test substance concentration should be 80% or more than the setting concentration.

(3) Measurement of environmental conditions

(i) Confirm the quality of the dilution water prior to the study.

(ii) Measure water temperature, dissolved oxygen concentration, and pH of the test solution in each test group at least at the initiation and end of exposure, and prior to and following water changes.

10. Method of processing results

(1) Use an established method for computing LC_{50}, based on the mortality rate results for each concentration.

(2) Generally compute LC_{50} on the basis of average values for measured active ingredient concentration, if the fluctuations of measured values are less than ± 20% the nominal concentration compute LC_{50} on the basis of the nominal concentration.

11. Reporting

(1) Test substance

(2) The test organisms

Species name, water source, breeding method, acclimatization, number of test organisms, length and weight of organisms, LC_{50} with standard substance, etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, test substance concentration etc.

(4) Results

(i) LC_{50} on the basis active ingredient concentration, and its 95% confidence limit (at the time of each observation, if possible)

(ii) Method of calculating LC_{50}

(iii) The cumulative mortality rate in each test group at the time of each observation

(iv) A graph of the concentration/mortality curve at the end of the exposure period

(v) Abnormal symptoms and responses of test organisms

(vi) Measured values of the test substance concentration

(vii) Results of measurement of environmental conditions

Water quality, dissolved oxygen concentration, pH, etc.

(viii) Other items

Test solution conditions, and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

12. Study validity

(1) The mortality rate must not exceed 10% in control groups at the end of the exposure period.

(2) The dissolved oxygen concentration must be maintained as at least 60% of the saturate concentrate.

Amphipoda acute toxicity test (2-7-5)

1. Objective

The objective of these studies is to establish safe use methods of using agricultural chemicals by obtaining scientific information regarding the short-term effects of the test substances on crustaceans (Amphipoda).

2. Definitions

(1) Death: Test organism makes no response for light incentive.

(2) Median lethal concentration (LC50): The test substance concentration at which 50% of the test animals die during the exposure period.

(3) Test substance: TGAI of the agricultural chemical to be studied.

(4) Standard substance: Substance used for confirming the reproducibility of study conditions.

(5) Test chemical: Test substance and standard substance used in the studies.

(6) Static test: A test that is conducted according to a system in which the test solution is not changed during the exposure period.

(7) Semi-static test: A test that is conducted according to a system in which the test solution is changed in each container during each fixed period.

(8) Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.

3. Test organisms

(1) Organism species

(i) Use freshwater shrimp (Amphipoda). It is desirable to use *Gammarus fasciatus*, *G. pseudolimnaeus*, and *Hyalella azteca*. But other Amphipoda shrimp can be used.

(ii) Use test organisms of known history (the supplier, breeding methods, etc.).

(iii) Gammarids can be cultured in the laboratory or collected from natural resources.

(iv) Gammarids used in the study should be of similar age and/or size and from the same source or culture population.

(v) It would be desirable to confirm the EC_{50} of a standard substance.

(2) Growth stage

Use test organisms at the growth stage in which they are not morphologically different from adults, and those without eggs.

(3) Acclimatization

(i) Test organisms must be collected and held for at least 12 days prior to studying.

(ii) Test organisms must be acclimatized under similar environmental conditions to those used in the study for at least 9days prior to studying.

(iii) Feed appropriate amount at least 5 days .Test organisms should not be fed for 24 hours before exposure.

(iv) Acclimatize following standard and record mortality.

a. If mortality of 7 days following stable period after 2days exceed 10% of group numbers, remove the group.

b. In case the mortality of group is 5-10%, after acclimatize for more 7 days continuously, If the mortality of group is more than 5%, remove the group or continue to acclimatize until mortality become under 5%.

c. In case the mortality of group is less than 5%, the group can be used in the study.

4. Exposure method

Conduct the studies under static, semi-static, and flow-through conditions.

5. Exposure period

Conduct the studies for 96 hours.

6. Determining the number of test organisms, and establishing test groups

(1) Number of test organisms

Use at least 20 test organisms in each test group.

(2) Establishing test groups

(i) Test concentration groups

a. Establish at least 5 different concentrations in a geometrical series.

b. Determine test concentrations and a factor for concentration on the basis of preliminary test results.

c. It would be desirable to include within the concentration range the concentrations at which all test organisms die, and that at which there are no fatalities, as 1 level each, and at least 2 more levels at which some of the organisms die.

(ii) Control groups

a. Establish as a control a group that is not treated with the test substance.

b. When using a solubilizing agent to adjust the test solution concentrate, design a solubilizing agent control group, treated with the same concentration of solubilizing agent to be used in the study.

7. Preparation of the test solution

The test solution is prepared as described below. It would be desirable to prepare the test solution and test solution concentrate immediately prior to their use in the study.

(1) When using a readily water soluble TGAI, dissolve it in the water that is to be used to dilute the test substance, and prepare the test solution or test solution concentrate.

(2) When using a TGAI that is not water soluble, use mechanical means to disperse the test substance, and prepare the test solution or test solution concentrate, or prepare the test solution concentrate with a solubilizing agent, such as organic solvent, emulsifier, or dispersing agent. Use a solubilizing agent that is of low toxicity to the organisms, which has not been noted to have adverse effects on the organisms at the concentration used in the study, and which does not change the properties of the test substance.

(3) The solubilizing agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

8. Environmental conditions

(1) Population

Sufficient amount of test solution is needed depending on the size of test organisms.

For static test, more than 1 L of test solution per 10 test organisms is needed. On the other hand, for flow-through

test, denser population is acceptable to conduct the study.

(2) Water temperature

Set standard temperature to 18 - 23℃, yet the temperature may be set according to the species of test organism as optimal breeding temperature. It is desirable that a variance range during the study is within ± 1°C of the set temperature.

(3) Light

It is desirable to light for 16 hours.

(4) Feeding

Do not feed during the exposure period.

(5) Dilution water

(i) Do not use water that contains hazardous substances for the study. Use water after its quality has been demonstrated to be favorable to the survival and development of test organisms, from the same source as the water in which they were bred.

(ii) Use dechlorinated tap water, natural water supplies, or reconstituted water.

(iii) Aerate sufficiently prior to use, and prepare the temperature.

(6) Dissolved oxygen concentration

Maintain the dissolved oxygen concentration as at least 60% of the saturate concentration. Gentle aeration applied , as necessary.

(7) pH

Do not adjust the pH of the test solution.

9. Observation and measurement

(1) Observation of the general condition of test organisms

At the very least, observe the general condition of test organisms at the 24, 48, 72, and 96 hours after the commencement of exposure, and keep records. Promptly remove dead test organisms from the test system.

(2) Measuring of the test substance concentration

(i) Measure the concentration of the test substance in each test group at least at the initiation and end of exposure, at the 48 hours after the commencement of exposure, and prior to and following water changes.

(ii) During the exposure period, the test substance concentration should be 80% or more than the setting concentration.

(3) Measurement of environmental conditions

(i) Confirm the quality of the dilution water prior to the study.

(ii) Measure water temperature, dissolved oxygen concentration, and pH of the test solution in each test group at least at the initiation and end of exposure, and prior to and following water changes.

10. Method of processing results

(1) Use an established method for computing LC_{50}, based on the mortality rate results for each concentration.

(2) Generally compute LC_{50} on the basis of average values for measured active ingredient concentration, if the fluctuations of measured values are less than ± 20% the nominal concentration compute LC_{50} on the basis of the nominal concentration.

11. Reporting

(1) Test substance

(2) The test organisms

Species name, water source, breeding method, acclimatization, number of test organisms, length and weight of organisms, LC_{50} with standard substance, etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, test substance concentration etc.

(4) Results

(i) LC50 on the basis active ingredient concentration, and its 95% confidence limit (at the time of each observation, if possible)
(ii) Method of calculating LC_{50}
(iii) The cumulative mortality rate in each test group at the time of each observation
(iv) A graph of the concentration/mortality curve at the end of the exposure period
(v) Abnormal symptoms and responses of test organisms
(vi) Measured values of the test substance concentration
(vii) Results of measurement of environmental conditions
Water quality, dissolved oxygen concentration, pH, etc.
(viii) Other items
Test solution conditions, and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

12. Study validity

(1) The mortality rate must not exceed 10% in control groups at the end of the exposure period.

(2) The dissolved oxygen concentration must be maintained as at least 60% of the saturate concentrate.

***Chironomus* sp., acute immobilization test (2-7-6)**

1. Objective

The objective of this test is to establish safe use methods of using agricultural chemicals by obtaining scientific information regarding the short-term effects of the test substances on *Chironomus* sp. Larvae.

2. Definitions

(1) Immobilization: Those animals that are not able to change their position (by crawling or swimming movements) within 15 seconds after mechanical stimulation, e.g. by subjecting the larvae to a gentle stream of water from a Pasteur pipette or agitation of the test vessel, are considered to be immobilized.

(2) Median effect concentration (EC_{50}): The concentration of the test substance that immobilizes 50% of the test organisms during the exposure period.

(3) Test substance: TGAI of the agricultural chemical to be studied.

(4) Standard substance: Substance used for confirming the reproducibility of test conditions.

(5) Test chemical: Test substance and standard substance used in the studies.

(6) Static test: A test that is conducted according to a system in which the test solution is not changed during the exposure period.

(7) Semi-static test: A test that is conducted according to a system in which the test solution is changed in each container during each fixed period.

(8) Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.

3. Test organisms
   (1) Organism species
      (i) *Chironomus riparius, C. yoshimatsui or C. dilutes* are used.
      (ii) The larvae should be derived with known history (the supplier, breeding methods, etc.).
      (iii) It is recommended to confirm the EC_{50} of a standard substance.

   (2) Growth stage
      First instar larvae of *Chironomus* sp. are used in this test. First instar larvae can be obtained by collecting fresh egg masses 4 or 5 days before starting the test and allowing them to hatch.

   (3) Breeding of *Chironomus* sp.
      Chironomus sp. for obtaining first instar larvae are kept for a specified period under conditions as close as possible to the environmental conditions of the test (dilution water of the same quality, the same water temperature, etc. as that used in the test), and healthy ones are used in the test.

4. Exposure method
   Conduct the studies under static, semi-static, and flow-through conditions.

5. Exposure period
   Conduct the studies for 48 hours.

6. Determining the number of test organisms, and establishing test groups
   (1) Number of test organisms
      It is recommended that at least 20 larvae are used for each test concentration and controls. They preferably are divided into four groups of five larvae each.

   (2) Establishing test groups
      (i) Test concentration groups
         a. At least five concentrations were arranged in a geometrical series with a spacing factor preferably not exceeding 2.2.
         b. Determine test concentrations and a factor for concentration on the basis of preliminary test results.
         c. It is recommended to include at least one test concentration resulted in 100 % immobilization, another concentration resulted in no immobilization and two test concentrations in which some larvae are immobilized in a series of the test concentrations.
      (ii) Control groups
         a. Establish as a control a group that is not treated with the test substance.
         b. When using a solubilizing agent to adjust the test solution concentrate, design a solubilizing agent

control group, treated with the same concentration of solubilizing agent to be used in the test.

7. Preparation of the test solution

The test solution is prepared as described below. It would be desirable to prepare the test solution and test solution concentrate immediately prior to their use in the test.

(1) When using a readily water soluble TGAI, dissolve it in the water that is to be used to dilute the test substance, and prepare the test solution or test solution concentrate.

(2) When using a TGAI that is not water soluble, use mechanical means to disperse the test substance, and prepare the test solution or test solution concentrate, or prepare the test solution concentrate with a solubilizing agent, such as organic solvent, emulsifier, or dispersing agent. Use a solubilizing agent that is of low toxicity to the organisms, which has not been noted to have adverse effects on the organisms at the concentration used in the test, and which does not change the properties of the test substance.

(3) The solubilizing agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

8. Environmental conditions

(1) Test solution volume

Use at least 2 mL of test solution per individual larva.

(2) Water temperature

The water temperature should be within the range of 18 to 22°C for *C. riparius*, and 23 to 25°C for *C. yoshimatsui* and *C. dilutes*, which should be constant within ±1°C during the test period.

(3) Light

It is recommended to light for 12-16 hours.

(4) Feeding

Do not feed during the exposure period.

(5) Dilution water

(i) Do not use the water that contains hazardous substances for the test. Use the water after its quality has been demonstrated to be favorable to the survival and development of test organisms, from the same source as the water in which they were bred.

(ii) Use dechlorinated tap water, natural water supplies, or reconstituted water.

(iii) Aerate sufficiently prior to use, and prepare the temperature.

(6) Dissolved oxygen concentration

The dissolved oxygen concentration should be 3 mg/L or more throughout the exposure period.

(7) pH

Do not adjust the pH of the test solution.

9. Observation and measurement

(1) Observation of the general condition of test organisms

Check for immobilized larvae at 24 and 48 hours after start of exposure.

In addition to immobility, any abnormal behavior or unusual appearance should be reported.

(2) Measuring of the test substance concentration

(i) Measure the concentration of the test substance in each test group at least at the initiation and end of exposure, and prior to and following water changes.

(ii) During the exposure period, the test substance concentration should be 80% or more than the setting concentration.

(3) Measurement of environmental conditions

(i) Confirm the quality of the dilution water prior to the test.

(ii) Measure water temperature, dissolved oxygen concentration, and pH of the test solution in each test group at least at the initiation and end of exposure, and prior to and following water changes. The pH should normally not vary by more than 1.5 units during the exposure period.

10. Method of processing results

(1) EC_{50} should be calculated using a standard method based on the immobility rate results for each concentration.

(2) EC_{50} should be calculated based on the means of the measured concentrations if they vary from the nominal concentrations by ±20% or more.

11. Reporting

(1) Test substance

(2) Test organisms

Species name, historical background (source, method of breeding, etc.), EC_{50} of the standard substance, etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, etc.

(4) Results

(i) EC_{50} based on active ingredient concentrations, and its 95% confidence limit (at each observation time, if possible)

(ii) Method of calculating EC_{50}

(iii) Cumulative immobilization rate for each test group at each observation time

(iv) A graph of the concentration-immobility curve at the end of exposure

(v) Observed adverse effects

(vi) Measured test substance concentrations

(vii) Results of measurement of environmental conditions

Water quality, dissolved oxygen concentration, pH, etc.

(viii) Other items

Conditions of the test solution, events that might have affected the results of the test (details of the deviation from this test method and possible effect on the test results), etc.

12. Test validity

(1) The immobility rate must not exceed 15% in control groups at the end of the exposure period.

(2) The dissolved oxygen concentration must be maintained at 3 mg/L or more.

Algae growth inhibition test (2-7-7)

1. Objective

The objective of these studies is to establish safe use methods of handling agricultural chemicals by obtaining scientific information regarding the effects of the test chemical on algae growth.

2. Definitions

(1) Biomass: Dry mass of the organism per unit volume. However, it sometimes refers to the parameter, such as cell density, that is used as an alternative to biomass.

(2) Growth rate: Increment of biomass per unit time (day) in natural logarithm

(3) Median effect concentration (EC_{50}): Concentration of the test substance in which growth of the test organism is inhibited by 50% compared with the control group.

(4) No observed effect concentration (NOEC): The maximum concentration at which no effects are observed as compared to the control group.

(5) Test substance: TGAI or formulation of the agricultural chemical to be tested.

(6) Standard substance: Substance used for confirming the reproducibility of test conditions.

(7) Test chemical: Test substance and standard substance used in the studies.

3. Test organisms

(1) Organism species

(i) It is desirable to use *Pseudokirchneriella subcapitata* (previous scientific name: *Selenastrum capricornu*). However, the species and strains listed below, as well as others, may be used if they are more convenient for culturing and testing, and validity of the test is satisfied.

a. *Pseudokirchneriella subcaptitata* (ATCC 22662 strain)

b. *Desmodesmus subspicatus* (previous scientific name *: Scenedesmus subspicatus*) (86.81 SAG strain)

(ii) It would be desirable to confirm EC_{50} with the standard substance.

(2) Culturing method

Algae should be cultivated under conditions similar to the study conditions. Use algae in its logarithmic growth phase. In principle, culture under sterile conditions.

(3) Initial biomass

The initial biomass shall not exceed 0.5 mg/L, and when the recommended strain of the *Pseudokirchneriella subcapitata* is used, 5×10^{3} - 1×10^{4} cells/ml will be appropriate as the initial cell concentration of the test culture medium.

4. Exposure method

Use a method whereby algae are exposed in the culture medium with test substance. Use shaken culturing.

5. Exposure period

In principle, set a 72-hour period.

6. Determining the number of animals, and establishing test groups

(1) Test concentration groups

(i) Establish at least 5 different concentration groups in geometric series.

(ii) Determine test concentrations and their factor on the basis of preliminary studies. As for a common ratio, it is desirable not to exceed 3.2.

(iii) It would be desirable to include within the concentration range the concentration at which test algae growth is inhibited by more than 75%, and that at which there is no inhibition, as 1 level each, and at least 2 more levels at which algae growth is partially inhibited.

(2) Control groups

(i) Establish control groups that are not treated with the test substance.

(ii) When using a solubilizing agent to prepare the test culture medium, establish a solubilizing agent control group, treated with the same concentration of solubilizing agent to be used in the study.

(3) Replication of test group groups

Each test concentration group shall consist of 3 groups or more, and as to the control groups (when solubilizing agent is used, control group of the solubilizing agent), twice the number of the test concentration group is recommended.

7. Preparation method of test culture medium

The test culture medium is prepared by a method as described below. Note that it is desirable to prepare test culture medium immediately prior to use in the study.

(1) When using TGAI as the test substance

(i) When using a water soluble TGAI, dissolve the test substance in culture medium that has been properly sterilized, and prepare it as the test stock solution. After the test stock solution has been diluted with sterilized culture medium, prepare test culture medium by adding the algae suspension, and prepare.

(ii) When using a TGAI that is not water soluble, prepare the test culture medium according to either of the following methods.

a. Prepare the test culture medium, using test stock solution consisting of the test substance dissolved in a solubilizing agent, such as organic vehicle. Use a solubilizing agent that is of low toxicity to test organisms, which has not been noted to have adverse effects on test organisms at the concentration used in the study, and which does not change the properties of the test substance.

The solubilizing agent concentration in the test solution should be the same in all test concentration groups as a rule and should not exceed 100 mg/l (or 0.1 ml/l).

b. Add the amount of the test substance necessary for each concentration to the sterilized culture medium, by sterilized conditions. After stirring or ultrasonic treatment etc., prepare the test culture medium by adding algae suspension.

(2) When using formulation as a test substance

Prepare test stock solution by adding formulation to sterilized culture medium and stirring. After the test stock solution has been diluted with culture medium, prepare test culture medium by adding the algae suspension, and prepare. Do not use a solubilizing agent with the formulation.

8. Environmental conditions

(1) Culturing method

(i) Culturing under sterilizing conditions.

(ii) Maintain the test culture medium as a suspension throughout the test period; shake or stir the test containers, in order to promote ventilation.

(2) Culture temperature

Set the temperature at 21-24°C, and allow it to vary within a range of ± 2 °C during the exposure period.

(3) Light

Lighting shall be provided continuously and uniformly, and when the recommended strain of *Pseudokirchneriella subcapitata* is used, illuminance of 60 – 120 μE/m^2/s (4440–8880 lux) in the measurement range of the wavelength, 400– 700 nm is recommended near the surface of the fluid.

(4) Culture medium

(i) Types of media

It is desirable to use either OECD medium (OECD Test Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006)) or AAP (AGP) medium (US EPA: Alga Assay Procedure: Bottle Test, National Environmental Research Center, Corvallis, Oregon (1971)).

(ii) Amount of culture medium

Recommended volume of culture medium per container is 100 ml, although it depends on the method of measuring the biomass and the method of measuring the concentration of the test substance.

9. Observation and measurement

(1) Measurement of biomass

Measure biomass in each test containers every 24 hours following the initiation of exposure, until the end of exposure. Record abnormality in appearance, when it is observed.

(2) Measurement of the test substance concentration

(i) If stock solution has been used as the test substance, measure the concentration of the test substance in each test concentration group at least at the beginning and end of exposure.

(ii) Collect test solution from each container in each test substance concentration group, combine the solution thus collected, and use it as a sample for measurement. When no difference is expected after several repetitions, samples may be collected from one container. Where the algae concentration (inhibitory rate) is almost the same at the completion of the study, it is considered a case where no difference is expected after repeated trials if the distribution is made from the same preparation at the start of the study. Where the algae concentration (inhibitory rate) is significantly different between repetitions, a measurement should be made for each container. When required for analysis, the test solution from a different container may be used. In this case, processing should be made under the same conditions for the test solution.

(3) Measurement of environmental conditions

(i) Measure test water temperature and pH readings of test culture medium in each test group (test concentration and control groups).

(ii) Measure at least at the beginning and end of exposure.

10. Processing methods of results

(1) Methods of calculating concentration-percentage inhibition rate of cell growth

Tabulate the biomass in each test concentration and control group, together with the measurement time, and the test substance concentration. Draw a growth curve by plotting the mean number of cells in each test concentration group and control group versus time. Calculate the growth inhibition rate for each concentration by comparing growth rates.

(2) Calculating EC_{50}

Use an established method for calculating EC_{50}, based on the growth inhibition rate results for each concentration.

(3) Derive EC_{50} based on the average of the measured concentrations when the measured value of the test substance concentration fluctuates ±20% or more from the set concentration.

11. Reporting

(1) The test substance

(2) The test organisms

Species name, strain name, EC_{50} of standard substance, etc.

(3) Test method

Exposure conditions, environmental conditions, items for observation and measurement, etc.

(4) Results

(i) EC_{50} (When TGAI is used as the test substance, EC_{50} is based on the active ingredient) and its 95% confidence limit (at the time of each observation, if possible)
(ii) Method of calculating EC_{50}
(iii) NOEC (When TGAI is used as the test substance, NOEC is based on the active constituent concentration. This is only when NOEC is obtained from the test concentration group established in 6. (1) 2).)
(iv) Biomass and the average in each test group at the time of each observation
(v) Method of measuring biomass
(vi) Growth curve
(vii) A graph showing the concentration/growth inhibition rate relationship
(viii) Observed effects
(ix) Measured values of the test substance concentration (only when using TGAI as the test substance)
(x) Measurement results of environmental conditions
Water quality, pH, etc.
(xi) Other items
Test solution conditions, and other items that might affect the test results (details of the deviation from this test method and the possible effect on the test results).

13. Study validity

(1) The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period.

(2) The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3) in the control cultures should be calculated. These mean coefficient must not exceed 35%.

(3) The coefficient of variation of average specific growth rates during the test period (0-3 days) in replicate control cultures must not exceed 7%.

Test on toxicity on beneficial organisms other than aquatic animals and plants (2-8-1～4)

Bee toxicity test (2-8-1)

1. Objective

The objective of these studies is to establish safe use methods of handling agricultural chemicals by obtaining scientific information regarding the influence of the test substance on bees.

2. Test method

(1) Conduct acute toxicity studies (acute oral toxicity studies or contact toxicity studies). However, there is no objection to conduct of studies according to other methods, if they are scientifically valid.

(2) If severe toxicity is noted in acute toxicity test results, field toxicity studies should be conducted.

Silkworm toxicity test (2-8-2)

1. Objective

The objective of these studies is to establish safe use methods of handling agricultural chemicals by obtaining scientific information regarding the influence of the test substance on silkworms.

2. Test method

(1) Conduct acute oral toxicity studies. However, there is no objection to conduct of studies according to other methods, if they are scientifically valid.

(2) If severe toxicity is noted in acute oral toxicity test results, residual toxicity studies should be conducted.

Natural enemy insect, etc. toxicity test (2-8-3)

1. Objective

The objective of these studies is to establish safe use methods of handling agricultural chemicals by obtaining scientific information regarding the influence of the test substance on non-target insect, such as natural enemy insect.

2. Test method

(1) Conduct acute toxicity studies. However, there is no objection to performance of studies according to other methods, if they are scientifically valid.

(2) If severe toxicity is noted in acute toxicity test results, field effect studies should be conducted.

Avian impact test (2-8-4-1, 2)

Avian acute oral toxicity test (2-8-4-1)

1. Objective

The objective of these studies is to establish safe use methods of handling agricultural chemicals by obtaining scientific information regarding the effects of a single oral administration of the test substance on birds.

2. Test method

(2) No particular guidelines. Conduct by a scientifically valid method.

(3) For example, the US EPA document, "Ecological Effects Test Guidelines OPPTS 850.2100 Avian Acute Oral Toxicity Test — Public Draft (712-C-96-139, April, 1996)" is available as a test method.

Avian dietary toxicity test (2-8-4-2)

1. Objective

The objective of these studies is to establish safe methods of handling agricultural chemicals by obtaining scientific information regarding the effects of the test chemical on birds when it is administered to them via their feed, as a more realistic exposure route.

2. Test method

(1) No particular rules. Conduct a scientifically valid method.

(2) For example, the OECD Test Guideline 205, entitled "Avian Dietary Toxicity Test (1984)" is available as a test method.

Test on the properties, stability, degradability, etc. of active ingredients (2-9-1~17)

1. Objective

The objective of these tests is to obtain basic scientific information such as their properties, stability and degradability that is indispensable for evaluating aspects of the safety of agricultural chemicals.

2. Specific test content, etc.

This set of tests is comprised of the following (2-9-1~16).

(1) Color tests (2-9-1)

(i) Test method

Color should be observed visually at room temperature and ordinary pressure.

(ii) Reporting

Color (Record as "white", "light yellow", "brown", etc.)

(2) Tests on physical state of the substance (2-9-2)

(i) Test method

Physical state should be observed visually at room temperature and ordinary pressure.

(ii) Reporting

Physical state (Record as "solid (crystal)", "solid (powder)", "liquid", "gas", etc.)

(3) Odor tests (2-9-3)

(i) Test method

Odor should be measured organoleptically at room temperature and ordinary pressure.

(ii) Reporting

Odor (Record as "pungent odor", "aromatic odor", etc.)

(4) Spectrum tests (2-9-4)

(i) Test method

a. Ultraviolet-visible absorption (UV/VIS) spectrum should be conducted in accordance with OECD Guideline 101 (adopted May 12, 1981).

b. Infrared (IR) spectrum, nuclear magnetic resonance (NMR) spectrum (^{1}H, ^{13}C, etc.), and mass spectrum (MS) should be measured, using appropriate measuring devices.

(ii) Reporting

Measurement conditions and chart. For UV/VIS, record absorption wavelength (nm) and molar absorption coefficient; for IR, record absorption wavenumber (cm^{-1}); for NMR, record ppm; for MS, record measured values as m/z. Insofar as possible, clarify the assignment of each NMR and MS peak with relevant structural formula.

(5) Melting point tests (2-9-5)

(i) Test Method

The test should be conducted in accordance with OECD Test Guideline 102 (adopted July 27, 1995).

(ii) Reporting

Melting point (°C)

(6) Boiling point tests (2-9-6)

(i) Test Method

The test should be conducted in accordance with OECD Test Guideline 103 (adopted July 27, 1995). When boiling does not occur at normal pressure, take readings under reduced pressure conditions.

(ii) Reporting

Boiling point (°C). Record observed decomposition, if any.

(7) Vapor pressure tests (2-9-7)

(i) Test Method

The test should be conducted in accordance with OECD Test Guideline 104 (adopted July 27, 1995).

(ii) Reporting

a. Vapor pressure (Pa)
b. Test temperature (°C)

(8) Tests on solubility in water (2-9-8)

(i) Test Method

The test should be conducted in accordance with OECD Test Guideline 105 (adopted July 27, 1995).

(ii) Reporting

a. Solubility in water (mg/l or g/l)
b. Test temperature (°C)

(9) Tests on solubility in organic solvent (2-9-9)

(i) Test Method

a. The test should be conducted in accordance with OECD Test Guideline 105 (adopted July 27, 1995).
b. As an organic solvent, use a nonpolar hydrocarbon (such as hexane or heptane), an aromatic hydrocarbon (such as xylene or toluene), a halogenated hydrocarbon (such as dichloromethane), a ketone (such as acetone), an alcohol (such as methanol or ethanol), or an ester (such as ethyl acetate).

(ii) Reporting

a. Solubility in organic solvent (mg/l or g/l)
b. Test temperature (°C)

(10) Soil adsorption tests (2-9-10)

(i) Test Method

The tests should be conducted in accordance with OECD Test Guideline 106 (adopted January 21, 2000). However, in general more than one soil should be selected among each Types 2, 3, 4 and 5 of the 7 soil types indicated in said guideline and at least one must be volcanic ash soil. Conduct studies at 25°C .

(ii) Reporting

a. Soil adsorption coefficient (in units of K^{ads}_{F} and $K^{ads}_{F\,oc}$)
b. Test temperature (°C)

(11) n-octanol/water partition coefficient tests (2-9-11)

(i) Test Method

The test should be conducted in accordance with OECD Test Guideline 107 (adopted July 27, 1995) or 117 (adopted March 30, 1989) at a temperature of 25°C.

(ii) Reporting

a. n-octanol/water partition coefficient ($\log_{10}$ values)
b. Test temperature (°C)

(12) Density tests (2-9-12)

(i) Test Method

The test should be conducted in accordance with OECD Test Guideline 109 (adopted July 27, 1995).

(ii) Reporting
a. Density (g/cm^3)
b. Test temperature (°C)

(13) Hydrolysis tests (2-9-13)
(i) Test Method
The test should be conducted in accordance with OECD Test Guideline 111 (adopted May 12, 1981).
(ii) Reporting
a. Estimated half-life for each pH level
b. Test temperature (°C)

(14) Dissociation constant tests (2-9-14)
(i) Test Method
The test should be conducted in accordance with OECD Test Guideline 112 (adopted May 12, 1981).
(ii) Reporting
a. Dissociation constant (pKa)
b. Test temperature (°C)

(15) Thermal stability tests (2-9-15)
(i) Test Method
The test should be conducted in accordance with OECD Test Guideline 113 (adopted May 12, 1981; excluding accelerated storage studies).
(ii) Reporting
Whether or not quality is changed due to heat, and the temperature of beginning significant transformation (°C)

(16) Tests on photolysis in water (2-9-16)
(i) Test method
Prepare a dilute solution, at a concentration of aqueous solubility or lower, and within a range enabling analysis, by adding the test substance to sterile distilled water. Determine the test substance's photolysis in water, using an artificial light source (one that has a wavelength distribution and light intensity similar to those of natural light). In such cases, conduct the studies at a water temperature of 25°C. Use quartz glass containers. Conduct control studies without light.
(ii) Reporting
a. Estimated half-life
b. Test temperature (°C), light intensity (w/m^2), and measured wavelength range (nm)

(17) Bioconcentration tests(fish bioconcentration tests)(2-9-17)
(i) Definitions
a. Bioconcentration: Increase of test substance concentration in an organism or on its body surface or in a specific organ compared with the concentration in the environment or in the medium.
b. Bioconcentration factor: Ratio of agricultural chemical concentration in the fish to the concentration in water at any time during the uptake period.
c. Steady state: Condition where fluctuation of the bioconcentration factor is within 20% after consecutive three measurements at 48-hour intervals.
d. Uptake phase: Time period when exposure of the test fish in the test substance takes place.
e. Depuration phase: Period to investigate the degree of test substance discharge from the fish or from the specific organs after the test fish is transferred from the test solution containing the test substance to a solution without it.

f. Test substance: TGAI of the agricultural chemical to be studied.
g. Test chemical: Test substance and standard substance used in the studies.
h. Flow-through test: A test that is conducted according to a system in which test solution is supplied continuously.
i. Semi-static test: A test that is conducted according to a system in which the test solution is exchanged in each container in each fixed period.

(ii) Test organisms

a. Organism species

Select the test fish from the table at the end of this section.

b. Acclimatization

a) The test fish must be acquired by the 14th day prior to their use in studies, and maintained from that time.
b) Dip the fish in a medicated bath when received, if necessary.
c) The fish must be acclimated to the same environmental conditions (water quality, etc.) under which studies will be conducted for at least 14 days prior to use in studies.
d) The fish should be fed at least 5 times per week,.
e) Acclimatization should be conducted under the following conditions, and record the mortality rate.
[a] If during the 7-day period following the stabilization period (the 2 days following the commencement of acclimatization) the mortality rate exceeds 10% of the individuals in a group, that group should be excluded.
[b] If the group mortality rate is 5-10%, and is still 5% or more after acclimatization for 7 days, exclude the group, or continue acclimatization until the mortality rate falls below 5%.
[c] If the group mortality rate is less than 5%, the fish in that group may be used in the studies.

(iii) Exposure method.

As a rule, tests should be conducted in the flow-through condition. However, when the flow-through test is impossible (detrimental to the test organisms), a semi-static test may be conducted.

(iv) Test Period

a. The uptake phase and depuration phase should be established during the test period (when the bioconcentration factor is obtained in BCFk).
b. The uptake phase should be 28 days or until steady state is established. If steady state cannot be reached within 28 days, the period should be extended until steady state is established or up to 60 days, whichever comes sooner.
c. The depuration phase should be the time where 95% or more of the test substance concentration in steady state of the fish body is discharged.

(v) Establishing test groups

a. Test concentration groups

a) Set groups of at least two different concentrations.
b) 1/100 (or less) of LC_{50} by acute toxicity studies should be used as a guideline for the maximum concentration of the test concentration, and two concentration groups should be set with the concentration as low as possible to allow analysis using the analytical method.
c) As a rule, the lower concentration group should have a concentration 1/10 of the concentration in the high concentration group. However, if the concentration in the low concentration group is not measurable due to the detection limit of the concentration, the ratio may be increased to greater than 1/10.
d) When the concentration to be set is not measurable due to the detection limit, the test substance containing a radioisotope marker may be used.
e) The test concentration should not exceed the solubility in water of the test substance.

b. Control groups

a) Establish as a control a group that is not treated with the test substance.

b) When using a solubilizing agent to adjust the test liquid concentrate, establish a solubilizing agent control group, treated with the same concentration of solubilizing agent to be used in the study. When a solubilizing agent that does not affect the fish is used, either of the untreated control groups or the solubilizing agent control group should be established.

(vi) Preparation of the test solution

a. When using a water soluble TGAI, dissolve it in the water that is to be used to dilute the test substance, and prepare the test solution or test solution concentrate.

b. When using a TGAI that is not water soluble, prepare for the stock solution by mechanical means to disperse the test substance or with solubilizing agents, such as organic solvent, emulsifier, or dispersant. Use a solubilizing agent that is of low toxicity to fish, which has not been noted to have adverse effects on fish at the concentration used in the study, and which does not change the properties of the test substance. The solubilizing agent concentration in the test solution should not exceed 100 mg/l (or 0.1 ml/l).

(vii) Environmental conditions

a. Population of test fish

For the flow-through condition, 1–10 liters of test solution per 1g of fish weight should be replaced per day.

b. Water temperature

Set the temperature according to the species of test fish, as shown in the table below, within a variance range of ± 2°C.

c. Light

Light for 12-16 hours.

d. Feeding

Feed the fish every day. The fish should be fed 1%–2% of their weight per day.

Any remaining food and excrement should be removed after feeding.

e. Dilution water

Do not use water that contains hazardous substances for the study. Use water after its quality has been demonstrated to be favorable to the survival and development of fish, from the same source as the water in which they were bred. Use dechlorinated tap water, natural water supplies. Aerate the water to air sufficiently prior to use, and prepare the temperature.

f. Dissolved oxygen concentration

Maintain the dissolved oxygen concentration as at least 60% of the saturate concentration. Gentle aeration applied, as necessary.

g. pH

Do not adjust the pH of the test solution.

(viii) Observation and measurement

a. Life or death and symptoms in the fish

Observe and record every 24 hours after exposure is started.

Promptly remove dead fish from the test system. Also record any abnormalities observed.

b. Test substance concentration in the fish body

Measurements should be made at least 5 times during the uptake period and at least 4 times during the discharge period.

4 or more fish should be used for each analysis. Analysis should be made by individual fish.

c. Lipid content in the fish body

Preferably, measure the lipid content when measuring the test substance concentration in the fish body.

d. Test substance concentration in the test solution

Measurements should be made at least 5 times before the start of exposure, on the day exposure is started, and during the uptake period. During the uptake period, measurements should be made at the

same time the analysis of the test substance is made.

During the uptake period, fluctuation of the test substance concentration should be within ±20% of the average of the measured values.

e. Water quality

Prior to the study, the general water quality of the dilution water should be measured.

Water temperature, dissolved oxygen concentration, and pH of the test solution for all test groups should be measured.

(ix) Derive the bioconcentration factor

Derive the bioconcentration factor from the measured values of the test substance concentration in the fish body and test solution.

(x) Reporting

a. The test substance

Common name, chemical name, structural formula, purity, lot number, solubility in water, Pow (n-octanol/water partition coefficient) etc.

When a radioisotope is used, the position of the marker and the grounds for the position, radiochemical purity, specific radioactivity, etc.

b. The test fish

Species name, water source, breeding method, acclimatization, number of test fish, length and weight of test fish, etc.

c. Test method

Exposure conditions, environmental conditions, items for observation and measurement, etc.

d. Results

[a] Bioconcentration factor (BCFss or BCFk) and derivation method.

[b] Uptake rate constant and discharge rate constant.

[c] Death or abnormal symptoms or response of the test fish.

[d] Measured value of lipid content in the fish body.

[e] Measured value of the test substance concentration in the test water.

[f] Measured value of the test substance in the fish body.

[g] Measured value of the water quality.

[h] When the test substance labeled with a radioisotope is used, and when BCF of total radioactivity exceeds1000, indicate the concentration of metabolites and transformation products with 10% or more total radioactivity.

[i] Other items that might affect the test results, such as condition of the test solution (details of the deviation from this test method and the possible effect on the test results) etc.

(xi) Study validity

a. Fluctuation of water temperature shall be within the range of ± 2°C.

b. Incidence of death and disease in the control group or the test group shall not exceed 10% when the studies are complete. When the studies are extended for several weeks or months, death or abnormalities in the control group or in a test group shall be less than 5% for one month and less than 30% for the entire period.

c. The dissolved oxygen concentration must be maintained as at least 60% of the saturate concentrate.

d. Fluctuations in the test substance concentration in the tank shall be within ±20% of the average of the measured values.

Table: Conditions and temperatures to be set according to test organism species

Fish Species	Set Temperature (°C)	Test Fish Length (cm)
Cyprinus carpio	20～25	8.0 ± 4.0
Oryzias latipes	20～25	3.0 ± 2.0
Lepomis macrochirus	20～25	5.0 ± 2.0
Oncorhynchus mykiss	13～17	8.0 ± 4.0
Poecilia reticulata	20～25	3.0 ± 1.0
Danio rerio	20～25	3.0 ± 0.5
Pimephales promelas	20～25	5.0 ± 2.0

Test on derivation of predicted environmental concentration (2-10-1～6)

Test on water polluting properties (2-10-1)

1. Objective

The objective of these tests is to obtain scientific information regarding pollution of paddy water by agricultural chemicals used in paddies, to the PEC for human health (Predicted Environmental Concentration for long-term risk assessment on human health).

2. Design test plots (test paddies)

Design test plots treated and untreated with the test substance, as follows.

(1) Treatment of test plots (test paddies)

(i) In general, use concrete container, etc. of at least 1 m² (1 m x 1 m), enabling the amount of leaching water to be adjusted.

(ii) Use paddy soil, comprising gray lowland soil, gley soil, high-humidity andosol, or brown lowland soil, etc. for package. In general, use soil after pulverizing the soil without air drying, then selectively removing small stones and bulky organic matter, etc. and mixing well, pack it with water into a soil layer of approximately 50 cm deep, while sufficiently de-aerating it.

(iii) Establish the plots so as to sufficiently reflect atmospheric and other environmental conditions in fields.

(iv) It would be desirable to set up a roof so that rainwater cannot enter, in order to prevent sudden increases, etc. in the amount of paddy water due to rainfall. In such cases, be sure that air flow is not impeded; and use materials with good light transmittance for the roof.

(2) Test plot (test paddy) control

(i) Allow approximately 1-2 cm of leaching per day throughout the study period, and maintain the plots in a flooded state with a water depth of approximately 5 cm. Eliminate drainage and runoff.

(ii) The water used should not contain substances that are likely to affect decomposition or interrupt analysis, etc. of the test substance.

(3) Crops cultivated in test plots

The crops cultivated in test plots should be the same one in application for registration, and in general should be cultivated in the conventional way.

3. Handling and treatment of the test substance

(1) The test substance should be used soon after it is prepared.

(2) The test substance should be stored under appropriate conditions. If the substance is to be stored for a long period after opening, its stability during the storage period should be confirmed.

(3) Apply the test substance once properly, in a formulation and a method (in terms of time, quantity, etc.) that is relevant to the application for registration, and use the tools customarily used.

However, if it is difficult to use conventional tools in test paddies, other equivalent methods may be employed.

(4) Do not apply the test substance during rainy weather, or when rainfall is expected shortly after application. Unless rain will not affect results, e.g. the system is under a roof.

4. Collecting samples (paddy water)
   (1) Collecting samples
      (i) Collect samples in glass bottles, using a syringe, etc. and taking care not to mix in bulky organic matter and soil particles. Mix well.
      (ii) Collect samples from at least 4 different places at a time (with, in general, water depth 2-3 cm).

   (2) Time and number of sample collection
      (i) In general, collect samples immediately before and after application (1-3 hours afterwards), as well as 1 day, 3 days, 7 days, and 14 days after application.
      (ii) When there is a possibility that substances targeted for analysis may be detected in paddy water more than 14 days after application, continue collecting samples until the concentration detected is at least 1/10 of the maximum detected.
      (iii) If set water outlet closing period is established in the method of usage relevant to the application for registration, water samples should also be collected on the last day period.

5. Handling of samples
   (1) Transporting samples
      (i) Care should be taken in transporting samples not to let the samples deteriorate or become contaminated. They should be transported rapidly, at low temperatures, but not frozen.
      (ii) Handle samples properly, by affixing identifying labels, etc., in order to prevent confusion, etc. of samples during transport.

   (2) Handling samples after transportation

   Immediately upon receiving samples, verify their authenticity according to their identification labels, etc. Handle them properly, so as to avoid confusion among samples, and use them promptly for analyses.

6. Analysis of samples
   (1) Target substances for analysis

   The substances to be analyzed are the active ingredients of agricultural chemicals related to the test substance, as well as substances formed biologically and chemically. However, this does not apply to substances detected in extremely minor quantities in water, or which have been deemed non-harmful due to their extremely low toxicity, etc.

   (2) Analysis method
      (i) Adopt a method by which the target substance can be accurately analyzed.
      (ii) Express the amount of target substance residue as mg/l.
      (iii) Analyze each sample at least twice, and use the mean value from these analyses as the measured value.
      (iv) Confirm the precision of the analytical method by relative standard deviation(RSD) of recovery study within the range of concentrations in which detection of the target substances is anticipated.
      (v) The sensitivity of the analytical method is expressed as the limit of quantification, that is, the minimum concentration at which a sufficient recovery rate can be obtained in all operations for analysis of samples. Sensitivity should meet the objective of the study.
      (vi) Confirm the recovery rate of the analytical method, at the limit of quantification and at the range of concentrations in which detection of the target substances is anticipated, using samples that have been collected from untreated plots, and to which a known quantity of the target substance has been added.
      (vii) In general, analyze the samples for analysis promptly after the collection. If tentative storage of samples is unavoidable, store them under appropriate conditions, conduct storage stability examinations to determine the stability of target substances during the storage period.
      (viii) Conduct storage stability examinations of stored samples, using samples that have been collected from

untreated plots, and to which a known quantity of the target substance has been added and stored, under the same conditions, for at least the same period, and analyze according to the same methods by which test samples are analyzed.

7. Reporting
   (1) Institution that created study report (site for field test or analysis)

   (2) Test substance

   (3) Test conditions

   (4) Method of analysis (summary and details)

   (5) Limit of quantification and recovery rate for each target substance

   (6) Details regarding sample preparation

   (7) Results of analysis (analytical values at each time of sample collection)

   (8) Estimated half-life and method of derivation

Test on agricultural chemical concentration measurement in paddy water of model paddy (2-10-2)

1. Objective

   The objective of tests is to obtain scientific information regarding the formation and decline of agricultural chemical in paddy water of model paddy to derive the PEC for aquatic organisms (Predicted Environmental Concentration for short-term risk assessment on aquatic organisms).

2. Design test plots (test paddies)

   As per tests on water polluting properties.

3. Handling and application of the test substance

   As per tests on water polluting properties.

4. Collecting samples (paddy water)
   (1) Collecting method

   As per tests on water polluting properties.

   (2) Time and number of sample collection
      (i) Collect samples immediately before and after the application of the test substance (1 to 3 hours after application), and during the evaluation period (for 14 days), at as short intervals as possible.
      (ii) Collect samples at the same time of the day for each collection day.
      (iii) If set water outlet closing period is established in the method of usage relevant to the application for registration, water samples should also be collected on the last day period.

5. Handling of samples

As per tests on water polluting properties.

6. Analysis of samples
   (1) Substance to be analyzed
   The same substance as the one evaluated in toxicity studies concerning aquatic organisms shall be analyzed.

   (2) Analysis method
   As per tests on water polluting properties.

7. Reporting
   As per tests on water polluting properties

Test on agricultural chemical concentration measurement in paddy water of actual paddy (2-10-3)

1. Objective
   The objective of these tests is to obtain scientific information concerning the formation and decline of agricultural chemical in paddy water of actual paddy to derive the predicted environmental concentration.

2. Design test plots (test paddies)
   (1) Test paddies
   Tests shall be conducted in well-managed paddies with sufficient area (5 – 30 a) and little leak of water.

   (2) Management of test paddies
   (i) Water for test paddies shall not include substances that might affect decomposition or analysis, etc. of the test substance.
   (ii) Crops grown in test paddies shall be applicable crops that are relevant to the application for registration, and shall be grown by conventional farming method.
   (iii) Keep the test paddies in a static condition after agricultural chemical treatment, and pay attention to water management so that constant depth of paddy water shall be maintained (about 5 cm deep).
   (iv) In case agricultural chemical other than test agricultural chemical is to be used, choose one that does not affect the analysis.

3. Handling and application of the test substance
   (1) Apply the test substance promptly after preparation.

   (2) Store the test substance under appropriate control. If the substance is to be stored for a long period after opening, confirm the stability during the storage period.

   (3) Apply the test substance once by using the conventional apparatuses, based on the type and usage (time, dose, etc) specified in the application for registration. However, in case it is difficult to use the said apparatuses in test paddies, another equivalent method may be used.

   (4) Application shall not be performed when it is raining or likely to rain.

4. Collecting samples (paddy water)
   (1) Collecting method
      (i) Collect required amount of samples into glass bottles etc., using a stainless or glass dipper or syringe, etc. taking care not to mix in bulky organic matter or soil particles. Mix samples well.
      (ii) Collect samples from as many different places as possible (more than 10 places per 10 a per one collection) to prevent biased sampling. Take equal volume of samples from each collection point, and mix well.

   (2) Time and number of sample collection
      When a test is conducted to derive the PEC for human health (Predicted Environmental Concentration for long-term risk assessment on human health), it should follow the water pollution properties test, and when a test is performed to derive the PEC for aquatic organisms (Predicted Environmental Concentration for short-term risk assessment on aquatic organisms) it should follow the studies of agricultural chemical concentration measurements in the paddy water of the model paddy.

5. Handling of samples
   As per studies of water polluting properties.

6. Analysis of samples
   When a test is performed to derive the PEC for human health, it should follow the water pollution properties test, and when a test is performed to derive the PEC for aquatic organisms, it should follow the studies of agricultural chemical concentration measurements in the paddy water of the model paddy.

7. Other investigation items
   Investigate weather conditions (weather, temperature, precipitation, and if possible, evaporation), water temperature, pH, and water depth during the test period. In addition, observe whether or not there was overflow from the test paddy.

8. Reporting
   As per tests on water polluting properties.

Test on surface soil runoff in model field (2-10-4)

1. Objective
   The objective of these tests is to obtain scientific information concerning the surface runoff of agricultural chemicals in fields other than paddies, by using a model field, to derive the predicted environmental concentration.

2. Test equipments etc.
   Use an artificial rainfall generator, a bulk container for soil filling, and a surface flow collector that meet the following requirements, respectively.
   (1) Artificial rainfall generator
      The one that allows at least 2 m height from the soil surface, and generates artificial rainfall with arbitrary precipitation over the whole small field evenly and accurately.

   (2) Bulk container
      A large, square or rectangular container (area of at least 0.7 m^2) that allows 20 cm thick when soil was filled, with a structure that the soaked water can be drained from its bottom. The container shall be installed at inclines of 5 degrees under the artificial rainfall generator.

(3) Surface flow collector

A surface flow collector is to be installed on one side of a small field filled with soil. This shall have a structure that can collect surface flow without fail, avoiding the artificial rain from mixing in.

3. Test plot (model field)

(1) Test soil

Use agricultural soils equivalent to Andosols.

(2) Filling of soil

Fill the test soil up to about 5 cm higher than the top of the container wall, with appropriate density lest surface effect should occur. Expose the container to natural or artificial rainfall to soak the rain water to the bottom of the soil, and then dry it in air until the surface soil is dry. If the surface soil level at this point is lower than the top of the container, replenish the soil up to about 5 cm higher than the wall top. Then plow well the surface soil of 2 – 3 cm inside the wall (about 15 cm deep).

(3) Installation of soil-moisture meter

Install a soil-moisture meter in the soil container to measure moisture of soil of about 15 cm deep.

(4) Crop cultivation

Do not cultivate crops.

(5) Repetition

Repeat the study 3 times.

4. Confirmation of surface flow occurrence state

Apply artificial rainfall of 30 mm/hr to each test plot prior to agricultural chemical treatment several times. Confirm that the time it took to obtain a surface flow of 1.5 L per 1 m^2 of area (starting from the soil moisture of pF1.8 to 2.0) is within the range of 30 to 120 minutes, and that the time difference among test plots are within 30 %. Thus arbitrary obtained surface flow samples shall be used as samples for recovery examinations and stock stability studies (samples from untreated plot).

5. Handling and application of the test substance

(1) Apply the test substance promptly after preparation.

(2) Store the test substance under appropriate control. If the substance is to be stored for a long period after opening, confirm the stability during the storage period.

(3) Apply the test substance once by using the conventional apparatuses, based on the type and usage (time, dose, etc) specified in the application for registration. However, in case it is difficult to use the said apparatuses in test paddies, another equivalent method may be used.

(4) Treatment of agricultural chemical shall be conducted under the moisture range of pF 2.0 to 2.2. Immediately prior to treatment, plow the test plot well and prepare the surface soil. Apply treatment taking care not to contaminate the collection equipment with agricultural chemical.

6. Treatment of artificial rainfall and collection of samples (surface flow water)

(1) Apply artificial rainfall to each plot at Day 1, 3, 7, and 14 after the agricultural chemical treatment, to obtain 1.5 L of surface flow per 1m^2 of soil area.

(2) Target volume for collection of surface flow shall be classified into 5 or so varied levels, and record the collection time for each target level.

(3) Intensity of artificial rainfall on Day 1 after agricultural chemical treatment shall be 30mm/hr, and on Day 3 and after, the intensity of artificial rainfall shall be adjusted properly within the range of 10 to 30 mm /hr, so that its collection times for each target level shall be as equivalent as possible to those on Day 1.

(4) Collect surface flow into a glass or stainless container, let it sit for 10 minutes. Decant off to another glass or stainless container, taking care not to mix in deposit. Promptly subject it to analysis.

7. Management during test period

During the test period, expose each test lot to sufficient natural light, and keep it from influence of natural rainfall.

8. Analysis of samples

(1) Target substances for analysis

When a test is performed to derive the PEC for human health (Predicted Environmental Concentration for long-term risk assessment on human health), it should follow the water pollution properties test, and when a test is performed to derive the PEC for aquatic organisms (Predicted Environmental Concentration for short-term risk assessment on aquatic organisms), it should follow the studies of agricultural chemical concentration measurements in the paddy water of the model paddy.

(2) Analysis method

(i) Adopt appropriate method that enables analysis of the analyte with accuracy.
(ii) Stir samples well, take a batch of samples and subject it to analysis without removing the suspended solids particles in the water.
(iii) The amount of analyte shall be expressed in mg/l.
(iv) Conduct the analysis at least twice for each sample, and the mean value shall be used as measured value.
(v) Accuracy of analysis method shall be validated by coefficient of variance within the concentration range in which analyte is expected to be detected.
(vi) Sensitivity of analysis method shall be expressed as the limit of quantification, the minimum concentration at which sufficient recovery will be obtained when all the operations are conducted for the samples. Sensitivity should meet the study objective.
(vii) Recovery of the analysis method shall be confirmed either at the limit of quantification or within the concentration range in which the analyte is expected to be detected, using the samples prepared by adding a known quantity of analyte to the samples collected from the untreated plot.
(viii) Samples shall be subjected to analysis immediately after collection in principle. In case the samples have to be stored temporarily, store them under appropriate control, and conduct stock stability study to confirm the stability of analyte during the storage period.
(ix) To conduct stock stability study, add known quantity of analyte to the samples collected from the untreated plot, store the samples under the same conditions as and during the same or longer period as analyte storage, and analyze thus prepared samples.

9. Reporting

(1) Institution that created study report (site for field test or analysis)

(2) Test substance

(3) Test conditions (Soil properties and details of study procedures)

(4) Analysis method (Outline and details)

(5) Quantification limit and recovery rate for each analyte

(6) Details on sample preparation

(7) Analysis results (analytical values for each sample collection)

(8) Runoff rate (mean runoff rate during the study period)

Drift test (2-10-5)

1. Objective

The objective of this test is to investigate the drift rate in relation to the amount of ingredient included in applied agricultural chemical by distance, by applying ground-spray agricultural chemical to the field and measuring the amount of downwind agricultural chemical droppage by distance, to derive the predicted environmental concentration.

2. Test fields

Test fields shall measure at least 20 m in depth, and be located where sufficient space of investigation area is afforded on the downwind side.

3. Application of agricultural chemical

(1) Agricultural chemical shall be applied at mean wind speed of about 2.0 m/s or more.

(2) Apply the test substance promptly after preparation.

(3) Store the test substance under appropriate control. If the substance is to be stored for a long period after opening, confirm the stability during the storage period.

(4) Apply the test substance once by using the conventional apparatuses, based on the type an d usage (time, dose, etc.) specified in the application for registration.

(5) Observe weather conditions such as wind speed at the time of spraying test substance, and keep a record.

4. Investigation of droppage

(1) Traps

Use glass petri dishes for traps.

(2) Placing traps

Place traps on the investigation area, directed down-wind along the main wind direction at appropriate intervals. Place the traps horizontally, parallel to the soil surface, more than 3 traps in the equal distance at several meters intervals.

(3) Collection of traps

As soon as spraying is finished and all the sprayed particles have dropped completely, cover the traps, collect them, and subject them to analysis promptly.

5. Analysis

(1) Substance to be analyzed

Active ingredient of agricultural chemical is to be analyzed in principle.

(2) Analysis method

(iii) Adopt appropriate method that enables analysis of the analyte with accuracy.

(iv) Group a few traps within the equal distance and analyze them at least twice for each sample, and use the mean values of these as measured values.

(v) The accuracy of the analysis method shall be validated by the coefficient of variance within the concentration range in which analyte is expected to be detected.

(vi) The sensitivity of the analysis method shall meet the study objective, and be expressed as the limit of quantification.

(vii) Recovery of analysis method shall be confirmed by using the samples dissolved out with a certain volume of solvent used in the study, after adding known quantity of analyte to the trap.

(viii) Samples shall be subjected to analysis immediately in principle. In case the samples have to be stored temporarily, store them under appropriate control, and conduct stock stability study to confirm the stability of analyte during the storage period.

(ix) A stock stability study will be conducted by analyzing the samples stored under the same conditions as and during the same or longer period as analyte storage, as per (v).

6. Reporting

(1) Institution that created study report (site for field test or analysis)

(2) Test substance

(3) Test conditions (Test fields, investigation methods and details of study procedures)

(4) Analysis methods (Outline and details)

(5) Quantification limit and recovery rate for each analyte

(6) Details on sample preparation

(7) Analysis results

(8) Drift rate by distance to theoretical application dose

Monitoring test on agricultural chemical concentration in the rivers (2-10-6)

1. Objective

The objective of this monitoring test is to obtain information concerning the concentration of the agricultural chemicals (only for registered active ingredients), in water in public water area.

2. Investigation area

(1) Of the prefectures whose prevalence of agricultural chemical use rank in the top, based on recent shipping statistics, select 2 preferences or more that are expected to have the highest concentration of agricultural chemicals in river, concerning the relevant agricultural chemical and usage, considering the actual status of use.

(2) Select the investigation river for which it is confirmed that drainage water flows into the river from the agricultural chemical use area.

(3) Select at least the following points as investigation points.

(i) When used in assessment on water pollution properties

a. Assessment points

It should be a downstream area close to the intersection of main drains from the relevant area with the river to be investigated.

b. Behavior observation points

Preferably, set a behavior observation point in the main drains where agricultural chemical runoff from the relevant area can be monitored appropriately.

c. Upstream observation points

It should be upstream of the intersection of the drains from the relevant area with the river to be investigated.

(ii) When used in assessment on aquatic organisms

a. Assessment points

Regularly monitored points in public water areas that are located near the downstream site of the relevant area (water environment standard points or supplementary environmental reference points).

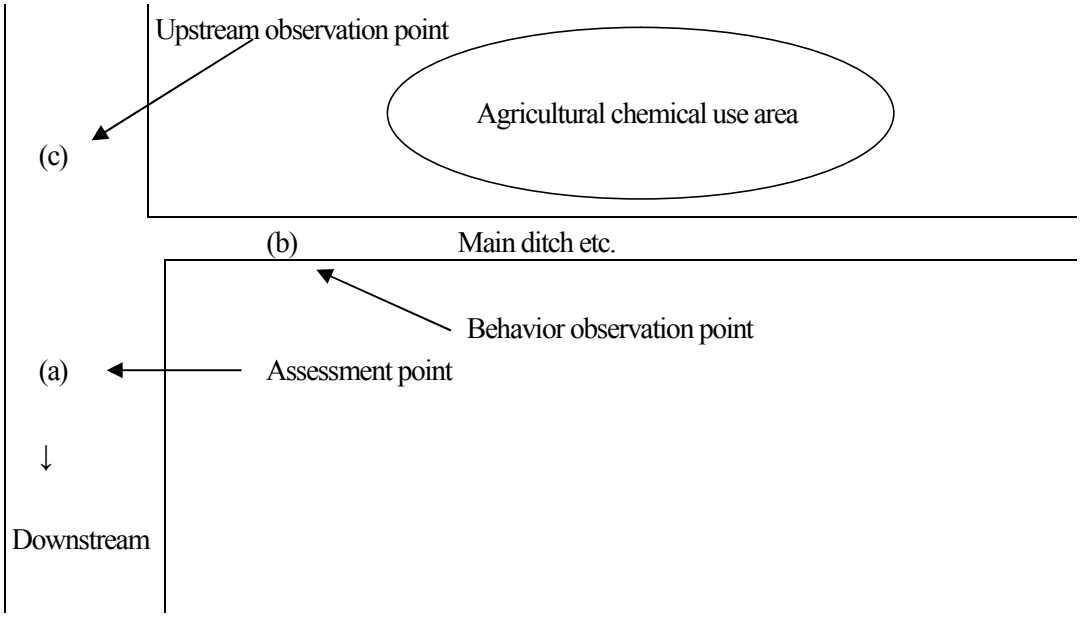
b. Behavior observation points

Main drains or so where behavior of agricultural chemical runoff from the relevant area can be kept up adequately.

However, in case there are 2 or more agricultural chemical use plots within one area, it is desirable to set behavior observation points in 2 or more areas.

c. Upstream observation points

Upstream of meeting where the drainage water from the relevant plot flows into the river to be investigated.



3. Measurement of flow rate and weather observation

Flow rate (m^3/s) should be measured at the assessment point once per quarter or more frequently when used in assessment on water pollution properties, and once per investigation period or more frequently when used in risk assessment on aquatic organisms, Keep a record of the weather during the test period.

4. Collecting samples (river water)

(1) Collecting method

(i) Use appropriate collection apparatuses made of stainless or glass, and collect the river water from the center line of the stream of each investigation point in principle.

(ii) Take care not to mix in the sediment, and remove coarse supernatant substances.

(2) Collection period and collection intervals

(i) For assessment on water pollution properties

a. Agricultural chemicals to be used in paddies

Collection of samples should start before application of the agricultural chemical and shall be made once a week during the application period and once a month after the application period until it is confirmed that the agricultural chemical concentration at the assessment point decreases to the limit of quantification or less,

b. Agricultural chemicals to be used in upland fields other than paddies

Collection of samples should be made every two weeks during the application period of the agricultural chemical and once a month after the application period until it is confirmed that the agricultural chemical concentration at the prediction point decreases to the limit of determination or less.

(ii) For risk assessment on aquatic organisms

1. Agricultural chemicals to be used in paddies

Start collecting samples prior to agricultural chemical application, and collect every day (if possible) at the peak of agricultural chemical use, and at a few days to 1 week intervals after that, until the agricultural chemical concentration at assessment point is confirmed to attenuate irreversibly.

2. Agricultural chemicals to be used in fields other than paddies

Collect samples about every 1 week during the agricultural chemical use period, until about 1 month after the use period.

5. Handling of samples

As per tests on water polluting properties.

6. Analysis of samples
   (1) Target substances for analysis

   When used for assessment on water pollution properties, it should follow the water pollution properties test, and when used for risk assessment on aquatic organisms, it should follow the studies of the agricultural chemical concentration measurement in the paddy water of the model paddy.

   (2) Analysis methods
      (i) Adopt appropriate method that enables analysis of the target substance with accuracy.
      (ii) Concentration of analyte shall be expressed in μg/l.
      (iii) Conduct the analysis at least twice for each sample, and the mean values shall be used as measured values.
      (iv) The accuracy of the analysis method shall be validated by the coefficient of variance within the concentration range in which the analyte is expected to be detected.
      (v) Sensitivity of the analysis method shall be expressed as the limit of quantification, the minimum concentration at which sufficient recovery will be obtained when all the analysis operations are conducted for the samples. Sensitivity should meet the study objective.
      (vi) Samples shall be subjected to analysis immediately after collection in principle. However, in case the samples have to be stored temporarily, store them under appropriate control, and conduct a stock stability study to confirm the stability of the analyte during the storage period.
      (vii) To conduct a stock stability study, add a known quantity of analyte to similar samples not including analytes, and analyze the samples stored under the same conditions as and during the same or longer period as the stored samples.
      (viii) Add a known quantity of analyte to similar samples (either river water collected from upstream in which no relevant agricultural chemical is mixed, or river water collected other than the agricultural chemical use period), either at the limit of quantification or within the concentration range in which the analyte is expected to be detected, and use thus prepared samples to confirm the recovery of the analysis methods.

7. Report items
   (1) Institution that created the study report (site for field test or analysis)

   (2) Test substance

   (3) Test conditions (investigated area, investigation methods, details of weather, sample collection operations during the investigation period, etc.)

   (4) Analysis method (Outline and details)

   (5) Limit of quantification and recovery rate for each analyte

   (6) Analysis results

   (7) Discussion on factors of agricultural chemical runoff

   (8) Average concentration throughout the year or during the maximum concentration period.

＜Persistence Test＞

Test on residues in crops

Test on residues in crops (3-1-1)

1. Objective

   The objective of these studies is to obtain scientific information regarding the degree of residue of agricultural chemicals in crops.

2. Test crops
   (1) Choose representative varieties and cropping types from among crops relevant to the application for registration.

   (2) Select a standard method of cultivation. Base the method on greenhouse cultivation or cultivation without bags, since cultivation conditions (greenhouse versus outdoor, with bags versus without bags) have an especially large effect on residue.

3. Establishing test plots (fields)
   (1) Establish plots treated and untreated with the test substance as test plots.

   (2) The test plots must be of sufficient area to ensure a sufficient quantity of crops for performance of analyses.

   (3) Measures must be devised for preventing contamination of the test plots with scattered agricultural chemicals from elsewhere.

   (4) For each field, establish the number of days during which observations of fate of residue of the test substance are made, in principle, in each test substance treatment plot. However, these limitations do not apply to test substances that are used in the initial growth stage of crops, or to those used on limited occasions, such as soil treatment chemicals.

4. Cultivation of test crops
   (1) Conduct cultivation management by the ordinary methods, such that the test crops will be in a marketable condition at harvest time.

   (2) If extermination of harmful insects, etc. is unavoidable, choose a method that will not affect the test results.

5. Handling and preparation of the test substance
   (1) The test substance should be employed soon after it is prepared.

   (2) The test substance should be stored under appropriate conditions. If the substance is to be stored for a long period, after being opened, its stability during the storage period should be confirmed.

   (3) Apply the test substance once properly, in a formulation and according to a method of usage (in terms of time, number of applications, quantity, etc.) that is relevant to the application for registration, and using the tools customarily employed. However, if it is difficult to use said tools in test paddies, other equivalent methods may be substituted.

(4) Do not apply the test substance during rainy weather, or when rainfall is expected shortly after application. However, when rain will not affect results, due to construction of a roof, this limitation is unnecessary.

6. Test samples

(1) Sampling portions and amounts collected should be in accordance with Appendix Table 1.

(2) Use a proper method of collecting samples, so that there is no bias in sampling.

(3) Samples should be in a marketable condition and should also be, insofar as possible, of uniform size (in terms of length and thickness). Do not collect defective crops (immature crops, as well as those injured by disease, insects, or chemicals).

(4) Use proper methods of collecting and packaging samples so as to avoid confusion among samples, as well as contamination.

7. Handling of samples

(1) Transporting samples

(i) Care should be taken in transporting samples so that they do not deteriorate or become contaminated. They should be transported rapidly, at low temperatures, but not frozen; this does not apply to sample that have been sun-dried or mechanically dried.

(ii) Handle samples properly, by affixing identifying labels, etc., in order to prevent confusion among samples during transport.

(2) Handling samples after transportation

Immediately upon receiving samples, verify their authenticity according to their identification labels, etc. Handle them properly, so as to avoid confusion among samples, and use them promptly for analyses.

8. Analysis of samples

(1) Target substances for analysis

The substances to be analyzed are the active ingredients of agricultural chemicals related to the test substance, as well as substances generated in the course of the biological and chemical changes they undergo (hereinafter referred to as "component substances, etc."). However, this does not apply to substances that leave extremely low residue concentration, and which have been deemed negligible risk to human health due to their extremely low toxicity, etc.

In general, analyze the component substances, etc. of spreading agent as well as the agricultural chemicals to which they are applied. However, spreader may be listed in Appendix Table 2 for any valid reason, based on such factors as their effect on residue of the substances to which they are applied.

(2) Analysis portions

Analysis portions for crops to be used for food should be in accordance with "Specifications and Standards for Food, Food Additives, etc."(Ministerial Notification No. 370 of the Ministry of Health and Welfare, dated December 28, 1959).

(3) Method of analysis

(i) Use all or uniform portions of samples from each analysis portions, after they have been homogenized.

(ii) Adopt a method by which the target substance can be scientifically analyzed. However, when an analytical method has been stipulated upon establishment of food regulations (standards for residual agricultural chemicals), use the method so stipulated.

(iii) Express the amount of target substance residue as ppm. (ppm in this case is the ratio by weight.)

(iv) Analyze each sample at least twice.

(v) Confirm or verify the validity of the analytical method in every crops by means of the following items.

a. Selectivity

Analyze samples which don't contain the target substance and confirm the absence of interference in the samples.

b. Recovery rate

Calculate the average of the ratio of the quantitative value to the spiked concentration ,within the limit of quantification and the range of concentrations in which detection of the target substances is anticipated, by using samples that have been collected from untreated plots and to which a known quantity of the target substance has been added.

c. Precision

Calculate the Relative Standard Deviation under repeatability conditions (RSDr) within the limit of quantification and the range of concentrations in which detection of the target substances is anticipated.

(vi) Ensure the sensitivity needed for study objectives, and express the limit of quantification with the minimum concentration at which a sufficient recovery rate and precision can be obtained if all operations involved in analysis of samples are conducted.

(vii) In general, use samples for analysis promptly after they have been in receipt. If temporary storage of samples is unavoidable, store them under appropriate conditions, conduct storage stability examinations to determine the stability of target substances during the storage period.

(viii) Conduct storage stability examinations of stored samples, using samples that have been collected from untreated plots and made the same form as samples of the crop plant residue tests, and to which a known quantity of the target substance has been spiked, under the same conditions, for at least the same period, and according to the same methods by which test samples are analyzed.

9. Reporting

(1) Test substance

(2) Methods of test crop cultivation, test substance use, etc.

(3) Weather conditions during the cultivation period (air temperature, rainfall, sunshine, etc.)

(4) Target substance for analysis

(5) Method of analysis (summary and details)

(6) Limit of quantification and recovery rate in each analysis

(7) Details regarding sample preparation

(8) Results of analysis

Appendix Table1

Crops	Sampling portions	Amount of sample collected
Rice	Hulled rice Unhulled rice Rice straw	1 kg 1 kg 5 bundles (2kg)
Wheat	Threshed seeds	1 kg
Barley	Threshed seeds	1 kg
Immature corn	Ear	1 kg
Corn(grain) and corn to be used for animal feed	Dried seeds Early harvested corn (limited to corn to be used for animal feed)	1kg 12 stubbles
Citrus unshiu	Fruit	2kg
Citrus natsudaidai	Fruit	2kg
Citrus iyo	Fruit	2kg
(*Citrus unshiu* × *C. sinensis*) × *C. reticulate*	Fruit	2kg
Citrus hassaku	Fruit	2kg
Citrus reticulate blanco	Fruit	2kg
Prunus persica (L.) Batsch	Fruit	2kg
Citrullus lanatus	Fruit	5kg
Cucumis melo L.	Fruit	5kg
Malus pumila	Fruit	2kg
Pyrus pyrifolia var. Culta, Pyrus communis L.	Fruit	2kg
Diospyros kaki	Fruit	2kg
Prunus mume	Fruit	1kg
Fragaria ananassa	Fruit	1kg
Vitis spp.	Fruit	1kg
Actinidia deliciosa	Fruit	1kg
Capsicum annuum var.	Fruit	1kg
Cucurbita	Fruit	2kg
Cucumis sativus	Fruit	2kg
Solanum lycopersicum	Fruit	2kg
Solanum lycopersicum (Mini tomato)	Fruit	1kg
Solanum melongena	Fruit	2kg
Glycine max Merr	Fruit	1kg
Phaseolus vulgaris	Pod	1kg
Brassica oleracea var. capitata	Head	5kg
Brassica rapa L. var. glabra Regel	Foliage	5kg
Brassica oleracea var. botrytis	Flower bud	2kg
Brassica oleracea var. italica	Flower bud	2kg
Brassica rapa var. peruviridis	Foliage	1kg
Chrysanthemum coronarium	Foliage	1kg
Apium graveolens L. var. *Dulce*	Foliage	1kg
Brassica rapa var. *chinensis*	Foliage	1kg
Brassica rapa L. var. *hakabura*	Foliage	1kg
Lactuca sativa	Foliage	3kg
Allium fistulosum	Foliage	1kg
Spinacia oleracea L.	Foliage	1kg
Allium tuberosum	Edible part	1kg
Brassica rapa	Root Leaf	2kg 1kg
Raphanus sativus	Root Leaf	5kg 1kg
Arctium lappa L.	Root	2kg

Daucus carota L.	Root	2kg
Nelumbo nucifera	Underground stem	2kg
Zingiber officinale	Root stock	1kg
Phyllostachys heterocycla f. pubescens	Bamboo shoots	2kg
Allium cepa	Bulb	2kg
Ipomoea batatas L.	Root tuber	2kg
Solanum tuberosum	Root tuber	2kg
Amorphophallus konjac	Corm	2kg
Colocasia esculenta	Root tuber	2kg
Dioscorea japonica	Tuber(rhizophore)	2kg
Glycine max	Dried grain	1kg
Vigna angularis	Dride grain	1kg
Beta vulgaris	Root	5kg
Saccharum officinarum	Stem	5kg
Camellia sinensis	Unprocessed tea	200g
Crops to be used for food other than the above	Edible part	The amount of sample collected should be determined appropriately considering fluctuations within the same sample to secure the accuracy of the analysis. At least 5 samples or more should be collected.
Crops to be used for animal feed other than the above	Part to be used for animal feed	1 kg (0.5 kg in the case of dried grass)

Note: For crops where the quantity to satisfy the column for the amount of the sample collected is less than 5 pieces, 5 pieces of the same size should be collected.

Appendix Table 2

	Effect of the relevant spreader on the residue of the agricultural chemical to which it is applied	
	When it is confirmed that there is no likelihood that it increases the residue of the agricultural chemical to which it is applied	When it is confirmed that there is a likelihood that it increases the residue of the agricultural chemical to which it is applied
1. Safety confirmed, for the reason that the toxicity of the component substances of the relevant spreader are of extremely low toxicity, etc.		Component substances, etc. of the agricultural chemical to which the spreader is to be applied
2. Safety confirmed for the reason that there is no likelihood that humans would ingest the component substances of the relevant spreader over the long term; or because, if they did ingest it, the amount consumed would be extremely minute.		Component substances, etc. of the agricultural chemical to which the spreader is to be applied
3. Cases to which neither 1 nor 2 above applies.	Component substances, etc. of the spreader	Component substances, etc. of the spreader and the agricultural chemical to which it is applied

Test on residues in livestock (3-2-1)

1. Scope of application
   (1) This guideline applies to livestock residue studies conducted to quantify levels of residues in meat, fat, liver, kidney, milk, eggs, etc. (hereinafter referred to as "tissues") derived from livestock (including poultry, the same applies hereinafter) that are fed with agricultural commodities for feed or their by-products, such as rice straw (hereinafter referred to as "feed commodities"), that contain residues of pesticides (including their metabolites in plants).

   (2) This guideline is compliant with OECD Test Guideline 505 "Residues in Livestock" (adopted on January 8, 2007), but does not include such modes of application as "direct application to livestock" and "livestock premise treatment" provided in the OECD Test Guideline.

2. Purpose
   The purpose of livestock residue studies is to provide data for determining the maximum residue limits (MRLs) in livestock commodities and for exposure assessment.

3. General considerations
   (1) Tests on residues in livestock allow quantitative estimation of transfer of residue to livestock commodities.

   (2) Application of agricultural chemical to livestock shall be based on the intake predicted on the premise that maximum residues are present in the crop to be fed (the estimated maximum dietary burden).

   (3) In principle, livestock residue studies are conducted in ruminants and poultry. In cases where a metabolic study was conducted in pigs and it showed a metabolic pathway different from that in ruminants, a pig residue study must be conducted.

   (4) Study results shall be extrapolated to other species according to the following principles:
      (i) Residue levels in meat, fat, liver, kidney and milk determined in residue studies using lactating dairy cattle may be extrapolated to the same tissues of any terrestrial mammal. In cases where a pig residue study is conducted, however, the results of the pig study serve as residue levels in pigs.
      (ii) Residue levels in meat, fat, liver, and eggs determined in residue studies using laying hens may be extrapolated to the same tissues derived from any poultry.

4. Conditions under which a study is required
   A livestock residue study is required when the results of Tests on metabolism in livestock (2-4-2) indicate that the residue level of the test substance or a major metabolite in a livestock commodity exceeds 0.01 mg/kg.
   However, even in the case where residues are present in livestock commodities, livestock residue studies are not necessary in the cases where the residue levels are infinitesimally close to the limit of quantification (LOQ), where the dose levels administered to animals in Tests on metabolism in livestock (2-4-2) is substantially greater than the estimated maximum dietary burden, and where the residue levels in livestock commodities resulting from administration of the estimated maximum dietary burden, estimated by taking into consideration the ratio of the expected dietary burden with the dose level administered to livestock in Tests on metabolism in livestock (2-4-2), are less than 0.01 mg/kg when considering.

5. Study method
   (1) Test substance
      (i) In principle, the test substance shall be an active ingredient of an agricultural chemical.

(ii) In cases where it is evident that the major metabolite in a plant is present as a metabolite in an animal, an additional study for this metabolite is not necessary. If a metabolite uniquely detected in a plant is the predominant residue in commodities, it is appropriate to administer this metabolite. In general, it is not recommended to administer more than one test substance. Any use of a mixture needs to be justified.

(2) Application

(i) Application method

The test substance shall be applied preferably by capsule to ensure consistent exposure over a specified period of time based on the residue level in commodities. If the substance is applied to the feed, it must be thoroughly mixed with the feed and regular analytical checks must be made to ensure the consistency and stability of the test substance in the feed over the study duration.

(ii) Dose levels

a. Determine the estimated maximum dietary burden for each type of livestock (cattle, pigs, or poultry) and conduct the study generally with three dose levels (1X, 3X, and 10X the estimated maximum dietary burden). The estimated maximum dietary burden shall be the highest of those calculated for the same kind of animals. For example, where it is estimated that the dietary burden is higher for beef cattle than lactating dairy cattle, 1X the maximum dietary burden estimated for beef cattle serves as the 1X the dose level, even if lactating dairy cattle are used in the livestock residue study.

b. Dose levels lower than one time the estimated maximum dietary burden may be added, on the assumption that the dietary burden may be lower than estimated in such a case that the diet is processed before fed to animals.

c. A specific calculation method for calculating the estimated maximum dietary burden is shown in Appendix 1.

d. Information obtained with multiple dose levels is used as follows:

(a) Revision of residue limits for livestock commodities and exposure assessment may be required if the mode of application is expanded and thus the estimated daily maximum burden becomes higher than those at the start of the study. In the case where the estimated maximum dietary burden is within the dose levels tested in the livestock residue study conducted, the residue levels in livestock commodities based on the new mode of application can be calculated by linear regression of dose levels and residue concentrations in livestock and by interpolation of the estimated maximum dietary burden.

(b) If there is no linear relationship between dose levels and residue levels, care should be taken in the estimation of residue levels from dose levels. Furthermore, it may not be appropriate to extrapolate from ruminants to other types of livestock animals fed with substantially different feed.

(iii) Duration of administration

The test substance shall be administered to test animals every day for at least 28 days. If the residue levels in milk or eggs do not reach a plateau during the 28-day administration period, the test substance shall be administered every day until reaching a plateau.

(3) Test animals and sampling

(i) Test animals

a. Species

In principle, the species of choice were lactating dairy cattle for ruminants and laying hens for poultry.

b. Number of test animals

(a) Lactating dairy cattle: 1 untreated (control) animal and 3 animals for each dose group (each dose level)

(b) Laying hens: 1 untreated (control) animal per dose group and 9-10 animals for each dose group (per dose level)

(c) The study conditions for a residue study for beef cattle or pigs shall be in accordance with that for lactating dairy cattle unless otherwise specified.

(ii) Use of control animals

Control animals are necessary to determine whether there are any effects other than those of the test substance on egg production, milk yield and general health of the animals in the residue study. Control animals also provide samples that can be used for validation of analytical methods.

(iii) Condition of animals

a. Record the information on the age, body weight, daily feed consumption (individual or group mean), milk yield or egg production as well as on the conditions of animals during acclimation and administration periods. It should be confirmed before use in the study that lactating dairy cattle are producing an average milk yield for commercial milk production, and that laying hens are in sufficient egg production. In cases where feed consumption is not recorded individually but on a group mean basis, attention should be paid to inter-individual difference.

b. Any health problem, abnormal behaviour, low feed consumption, or unusual treatment given to animals should preferably be recorded and discussed where relevant to determine whether it has any effect on study results.

c. Provide animals with an appropriate period of acclimation and make sure that their feed consumption, change in body weight, and milk yield or egg production during the acclimation period are normal.

(iv) Sampling of animal tissues, etc.

a. Lactating dairy cattle shall be slaughtered within 24 hours after the last administration. Laying hens shall be slaughtered with 6 hours after the last administration. When slaughtering, care should be taken not to contaminate tissues with blood, urine, feces, or other body fluids.

b. Detailed information on samples collected from lactating dairy cattle, laying hens, and pigs is shown in Tables 1, 2, and 3. Residue levels must be analysed individually. Residue levels in laying hens may be analysed correctively in groups of three. In cases where the analyte is considered fat-soluble, fat samples from different parts shall not be pooled but analysed separately. (See (10) (i).)

c. Record findings for the liver and kidney samples collected. Special care should be taken for findings reported in animal metabolism studies (2-3-1).

d. Milk and egg samples shall be collected in the following manner:

(i) Collect milk and egg samples from all test animals before starting administration to serve as control.

(ii) After administration, samples shall be collected on at least two days a week (e.g., every 3 or 4 days).

(iii) Milk shall be sampled individually once in the morning and once in the evening on each day of sampling. When the day of sampling coincides with the day of administration, collected the sample before administration.

(iv) After collecting samples from control animals, collect samples from the treatment groups

(v) Milk collected on the same day from the same animal may be mixed. Milk collected from different animals shall not be mixed.

(vi) Eggs shall be collected two times a day of sampling. Remove feces, etc. attached during sampling

(vii) Egg samples collected on the same day from the laying hens of the same dose group may be pooled if necessary to achieve the same sample weight suitable for analysis and stock. However, make sure to have three samples from each dose group at each time point.

Table 1. Samples from ruminants

Sample material	Sampling method	Sample preparation for analysis	Sample weight
Meat	Collect approx. equal amounts of loin, flank and hind-leg (round piece).	Chop into pieces and mix them homogeneously.	0.5 kg
Fat	Collect approx. equal amounts of subcutaneous, omental, perirenal fat.	Chop into pieces and mix them homogeneously.	0.5 kg
Liver	Collect the entire organ or representative parts thereof (e.g., a section of each lobe).	Chop into pieces and mix them homogeneously.	0.4 kg
Kidney	collected from the both kidneys		0.2 kg
Milk*[1]	Collect from each animal separately.		0.5 L*[2]

*1 For fat-soluble compounds, residue levels in milk need to be analysed at the time of and after reaching a plateau until the last administration. It is recommended to separate milk fat from milk by physical method for use in the analysis. This is because solvent extraction before separation results in residues being extracted from both the water and fat phases. Separate cream (containing 40-60% fat) from fat-free milk and record the fat content in cream.

*2 If samples need to be stored frozen until analysis, it is allowable to pool the individually collected samples and reduce the sample amounts at the time of analysis.

Table 2. Samples from poultry [*1]

Sample material	Sampling method	Sample preparation for analysis	Sample weight
Meat	Collect approx. equal amounts of leg and breast.	Chop the samples from three animals into pieces and mix them homogeneously.	0.5 kg
Fat	Collect abdominal fat and pool the fat from three animals.	Chop the samples from three animals into pieces and mix them homogeneously.	0.05 kg
Liver	Collect the entire organ.	Chop the samples from three animals into pieces and mix them homogeneously.	0.05 kg
Eggs	Collected for individual animals.	Clean shells, break eggs from three animals, combine the whites and yolks*[2]. In cases where residues are fat-soluble, whites and yolks need to be analysed separately to confirm distribution between whites and yolks*[3].	3 units

*1 At least 3 samples per group shall be available (i.e., at least 9 animals per group)

*2 Samples can be prepared either before or after transport to the analytical laboratory. However, the addition of solvent shall be performed at the start of analysis.

*3 In cases where the weights of the yolk and white are known, they shall be analysed separately so that the residue level can be calculated on a whole egg basis for the purpose of MRL setting. In this case, yolk and white need to be separated before storing the samples.

Table 3. Samples from pigs

Sample material	Sampling method	Sample preparation for analysis	Sample weight
Meat	Collect approx. equal amounts of loin, flank and hind-leg muscle.	Chop into pieces and mix homogeneously.	0.5 kg
Fat	Collect approx. equal amounts of subcutaneous, omental and perirenal fat.	Chop into pieces and mix homogeneously.	0.5 kg
Liver	Collect the entire organ or representative parts thereof (e.g., a section of each lobe).	Chop into pieces and mix homogeneously.	0.4 kg
Kidney	Collect from both kidneys.	Chop into pieces and mix homogeneously.	0.2 kg

(8) Sample analysis

(i) Analyte

Samples shall be analysed not only for active ingredients of the agricultural chemical of interest but also for major metabolites produced in Tests on metabolism in livestock (2-4-2), etc. Of these metabolites, those with no toxicological concern or possibility to remain as a residue need not be analysed.

(ii) Analytical method

a. The validity of the analytical method shall be confirmed or validated according to Test on residues in crops(3-1-1).

b. The LOQ, which varies with the toxicity of the compound shall, in principle, set to roughly less than 0.01-0.05 mg/kg.

(iii) Edible tissues and organs

Analysis preferably begins with samples from the highest dose group. If the residue level in the samples from the highest dose group is less than the LOQ, samples from the lower dose groups need not be analysed.

To make clear the interindividual variability in the residue level, tissues shall be analysed individually for lactating dairy cattle and pigs. For poultry, samples from three animals shall be pooled and used as one sample. Three animals shall be included in each treatment group.

(iv) Milk and eggs

All samples shall be analysed before starting administration and until residue levels plateau. After the plateau levels are reached, analysis may be performed once every week (e.g., on Days 14, 21 and 28). Three cases shall be analysed at each time point for each treatment group. However, if the samples of the highest dose group are less than the LOQ, analysis is not necessary for lower dose groups.

(9) Stock stability

Data to certify the Stock stability of representative tissues for the duration from sampling through analysis shall be submitted. However, this can be omitted in cases where the stock period is not more than 30 days and where the stability of the substance of interest can be explained from its physicochemical properties etc. The method for the Stock stability test shall be in accordance with the Test on residues in crops (3-1-1).

(10) Other considerations for the conduct of the study

(i) Considerations for fat-soluble compounds

a. Whether a compound should be classified as fat-soluble or not shall be evaluated based on the distribution ratio of the analyte between muscle and fat mainly observed in livestock metabolism/residue studies. Sampling procedures for livestock commodities vary depending on whether the compound is fat-soluble or not, and therefore requires careful consideration.

b. If the compound is considered fat-soluble, combining fat tissues of different types can lead to underestimation of residue levels, and therefore fat tissues must be analysed individually. Furthermore, record the following information for each type of tissue.

(a) Types of fat and regions to be sampled (e.g., subcutaneous, omental and perirenal fat)

(b) Fat content (purified or extracted fat is assumed to be 100% fat) or values in the literature

(ii) Considerations when conducting a depuration study

When conducting a depuration study, refer to descriptions relating to depuration studies specified in OECD Test Guideline 505.

(iii) If there is a possibility of exposure due to direct application of the pesticide to livestock as well as exposure due to contribution of feed commodities containing pesticide residue, consult with the Food and Agricultural Materials Inspection Center (FAMIC) in advance.

6. Items that should be reported in the study report

(1) Summary and introduction

(i) Purpose of the study, study design, and rationale for selecting the study design

(ii) Guidelines used for the conduct of the study, information on the implementation system of the study,

unexpected problems on the study, deviations from the study protocol due to such problems, and effects of such deviations on the study results.

(iii) Summary of results (after administration, residue transfer to tissues, whether residue accumulated in certain tissues, maximum residue level in each tissues, and whether the residue level plateaued in milk or eggs)

(iv) Results of analysis (conclusion on residue transfer through feed to tissues. and discussion on the degree of transfer)

(v) Problems on the study and its validity when the problems are evaluated in the light of the study purpose

(2) Materials and methods

(i) Test substance

a. The chemical name (IUPAC name), common name (ISO name, etc.), company's developmental name, CAS name and number, Lot No., purity, structural formula, etc. of the test substance. An analysis certificate shall be attached.

b. Chemical structures of analytes and their developmental or experimental names. An analysis certificate that describes information on their purities and structures, if any, shall be attached.

c. Information on the administered formulation (e.g., the solvent for the test substance, single substance or auxiliary component, etc.)

d. Rationale for using a compound other than active ingredients of the pesticide as the test substance, if applicable

(ii) Housing conditions and conditions of test animals

a. Housing methods and facilities (sizes of enclosures, unit of housing, containers for feed and drinking water, temperature, lighting, and handling of excreta)

b. Species, breed, age, body weight (including changes), and general conditions of test animals

c. Method of individual identification (e.g., ear tags)

d. Body weights and egg/milk production during the acclimation, administration, and withdrawal periods

e. Health problems, abnormal behaviour, or unexpected treatment of animals, and discussion on their effects on study results

f. Findings in the liver and kidney samples

(iii) Feed

a. Report the following concerning the feeds given to animals during the acclimation and administration periods.

(a) Types of feed and drinking water

(b) Amounts provided (e.g., in specified amounts or ad libitum)

b. Feed consumption (dry weight for ruminants) on an individual or dose group basis.

(iv) Administration

a. Dose levels and administration method

b. Administration methods, such as mixed in feed, in gelatine capsules, etc., dose levels (concentration of the test substance per dry weight feed (ppm(mg/kg feed)), and rationale for the selection of dose levels

c. Date of preparation of the test substance and stock conditions until administration

d. Analytical method used in the spike/recovery test of feed and analysis results; certificate to ensure that the test substance was stable from the preparation of the feed through the administration period.

e. The frequency of administration should be reported if the test substance was not mixed in feed.

f. Start date and end date of administration (or the administration period). Dose levels shall be reported in mg/kg feed, mg/animals/day, or mg/kg body weight/day.

g. Number of test animals per treatment group and control group

(v) Sampling of milk and eggs

In cases where milk or egg samples were collected by a method other than normal ones, the method used shall be described. In cases where multiple samples were pooled, its method shall be described.

(vi) Sampling after slaughter

a. The method of slaughter and the interval between the last administration and slaughter. In cases where the interval between the last administration and slaughter is longer than 24 hours, the reason shall be stated and the effect on residues discussed.
b. Tissues collected after slaughter and their type (e.g., loin, flank and hind-leg muscle, etc.) and weight; method of pooling samples from different animals if applicable.

(vii) Handling of samples and their Stock stability
a. Stock and handling of samples during the period from sampling to analysis shall be reported for the following items:
(a) Method of sample preparation before stock
(b) Containers for stock
(c) Time interval between sampling and stock
(d) Stock temperature
(e) Duration of stock (dates of collection, shipping, analysis, etc.)
(f) Method of transfer, if applicable
b. In cases where samples were not analysed within 30 days, state the rationale to ensure that the stock did not affect the study results (Stock stability, etc.).

(viii) Methods used for extraction, purification, measurement, and analysis
Details of the methods used for extraction, purification and measurement of samples; methods used for identification of residues in tissues, quantification and analysing the results

(ix) Analysis of samples
a. Detailed description of analytical method employed (including the results of method validation, recovery rate, and sensitivity of analysis); statement of the selection of the analyte. If the information on the analytical method has been submitted in a separate report, it is permissible to simply cite it as reference. This is also the case when analysing metabolites.
b. Data that support residue levels and recovery rates (sample weight , amount of the final extract, and peak height or peak area on the chromatogram, etc. for the control group, recovery sample (including samples for confirming Stock stability) and treatment group)
c. Analytical instruments used (including measuring conditions) and reagent; flow charts of extraction and purification procedures if they are complicated
d. The following shall be included when describing the results of a recovery study to validate the analytical method and establish its sensitivity (LOQ).
(a) Spiked compound and sample (names of the tissues used)
(b) Spiked concentration
(c) Number of repeated analyses per test compound per spiked concentration
e. Report the dates of spiking, extraction, and analysis. If the analysis is not performed on the day of spiking or extraction, the stock conditions for that sample shall be described.
f. Calibration curves and representative chromatogram of residues in each tissue for the control group, recovery samples and dose groups, concentration calculation using raw data and example of recovery rate

(3) Results and discussion
(i) Recovery rate (%) of the analyte in each tissue, milk or eggs (report per analysis)
(ii) Stock stability of the analyte in each tissue, milk or eggs over time; stock duration and conditions (temperature, etc.)
(iii) Residue level in each tissue at each dose level (including control samples) (Report analytical values for each sample and clearly state whether the values were corrected by the recovery rate. In the case of multiple analytes, report analytical values for each analyte wherever possible. Residue levels in milk and eggs shall be reported at each dose level for each day of sampling.)
(iv) Presence or absence of residue transfer to milk, eggs, fat, meat, liver or kidney, time to reach the plateau

phase in milk and eggs, presence or absence of selective accumulation in specific tissues, and discussion on the degree of agreement with the results of Tests on metabolism in livestock (2-4-2).

(v) Discussion on the validity of study results for the method of application applied for registration

(4) Conclusion

A conclusion shall be reached as to the presence or absence of transfer of residues of the pesticide of interest to tissues through feed and discussion on the degree of transfer (for the method of calculating residue levels in particular livestock commodities, see Appendix 2.)

(5) Reference literature

(i) Typical chromatogram, spectrum, etc. (if applicable)

(ii) A list of study reports, etc. referred to in the conduct of the study

7. Literature

(1) OECD Guidance Document: Overview for Residue Chemistry Studies (2006)

(2) OECD Guidance Document on the Definition of Residue (2006)

(3) OECD Guidelines for the testing of chemicals: Metabolism in Livestock (2007)

(4) OECD Guidelines for the testing of chemicals: Residues in Livestock (2007)

(5) FAO: Submission and evaluation of pesticide residues data for the estimation of maximum residue levels in food and feed (2002)

(Appendix 1) Calculation of dietary burden estimates (estimated maximum dietary burden and the mean dietary burden)

(Example)

The maximum dietary burden is estimated by multiplying the residue level in each feed commodity determined in Tests on residues in crops (3-1-1) with the corresponding diet content shown in the Table of Feed Amounts (Attached Table 1) and adding the products in descending order of residue level (on a dry weight basis) until the total of the diet contents reach 100%. In consideration of the actual practice for nutritionally balanced feeding, however, the diet content that accounts for the highest percentage within a Codex commodity group (hereinafter referred to as "commodity group") shall not be exceeded.

(1) Calculation of the estimated maximum dietary burden

(i) The feed items from which dietary burdens are calculated are categorised in the commodity groups shown below, and based on the results of Tests on residues in crops (3-1-1), residue levels in crops shall be allocated to feed commodities of the corresponding commodity groups.

Codex code	Commodity group	Residue level used for calculation
AL	Legume forages and fodders	HR
AF/AS	Forages and fodders; cereals and grasses	HR
AM/AV	Miscellaneous fodder and forage crops	HR
CM/CF	Milled cereal products; cereal grain milling fractions	STMR-P
AB	By-products derived from fruit and vegetable processing	STMR-P
SM	Miscellaneous secondary food commodities of plant origin	STMR-P
VR	Root crops	HR
VD	Pulses	STMR
GC	Cereal grains	STMR

(ii) As provided in the above table, the residue levels used for calculation should be the highest residue (HR) and supervised trials median residue (STMR) values for agricultural commodities, or be the supervised trials median residue-processed (STMR-P) value, which allows for the processing factor, for processed commodities. However, in cases where study results are not available from more than three Tests on residues in crops (3-1-1), the estimated maximum residue limit (MRL) should be used in place of the HR value. If the MRL is not determined, the HR value should be used.

(iii) The residue level in each feed commodities shall be expressed on a dry weight basis.

(iv) Diet contents shall be allocated in descending order of dry-weight residue level. If more than one feed commodity are derived from the intended crops (including those under application for registration; the same applies hereinafter) within a commodity group, diet contents shall be allocated up to the maximum diet contents for feed commodities derived from the intended crops within a commodity group.

Example: For AF/AS, "other grasses (green forage)" is allocated with 5% (no change), and then "rice straw" with 50% (= 55% – 5%).

(v) Continue allocation until a total of 100% is achieved. If the total diet contents exceed 100%, delete entries, starting with the one with the lowest dry-weight residue, until 100% is achieved, in such a way as to retain the highest possible dietary burden.

Example: Reduce the barley content to 45% (= 100 - 55) to achieve a total diet contents of 100%.

(vi) If the total diet contents of the feed commodities derived from the intended crops do not add up to 100%, it is assumed that the animals are fed with feed commodities derived from unintended crops.

(vii) If there is a possibility that the same pesticide may present as a residue in imported feed, this should be taken into consideration in the calculation.

Example) calculation of the maximum burden of pesticide A in diet (beef cattle)

Feed commodities	Codex code	Residue in crops (mg/kg)		Dry matter (%)	Dry weight residue (mg/kgdw)	Maximum diet content (%)
Other grasses (green forage)	AF/AS	5	HR	25	20.00	5
Rice straw	AF/AS	0.7	HR	90	0.78	55
Barley	GC	0.05	STMR	88	0.06	70

For AF/AS, the contribution (5%) of green forage, for which the residue level is greater, is subtracted (= 55% – 5%).

Feed commodities	Codex code	Residue in crops (mg/kg)		Dry matter (%)	Dry weight residue (mg/kg dw)	Diet content (%)	Burden (mg/kg)
Other grasses (green forage)	AF/AS	5	HR	25	20.00	5	1
Rice straw	AF/AS	0.7	HR	90	0.78	50	0.389
Barley	GC	0.05	STMR	88	0.06	45	0.026
Total						100	1.414

To make the total diet content100% (= 100% – 55%)

(2) Calculation of the mean dietary burden

(i) Select the feed commodities from which the dietary burden is calculated, in the same manner as described for the estimated maximum dietary burden in (1), from the Table of Feed Amounts based on the results of Tests on residues in crops (3-1-1).

(ii) The selected commodities shall be categorised in commodity groups and crop residue levels allocated to the commodities of the corresponding commodity groups.

(iii) The residue levels used for the calculation shall be STMR or STMR-P, as provided in the following table. The residue level in each feed commodities shall be expressed on a dry weight basis.

Codex code	Commodity group	Residue level used for calculation
AL	Legume forages and fodders	STMR
AF/AS	Forages and fodders; cereals and grasses	STMR
AM/AV	Miscellaneous fodder and forage crops	STMR
CM/CF	Milled cereal products; cereal grain milling fractions	STMR-P
AB	By-products derived from fruit and vegetable processing	STMR-P
SM	Miscellaneous secondary food commodities of plant origin	STMR-P
VR	Root crops	STMR
VD	Pulses	STMR
GC	Cereal grains	STMR

(iv) Calculate the mean dietary residue level in the same manner as described for the estimated maximum dietary burden.

(v) Diet contents shall be allocated in descending order of dry-weight residue level. If there is more than one feed commodity from the intended crops within a commodity group, diet contents shall be allocated up to the maximum diet contents for feed commodities derived from the intended crops within the commodity group.

(vi) Continue allocation until a total of 100% is achieved. If the total diet contents exceed 100%, delete entries,

starting with the one with the lowest dry-weight residue, until 100% is achieved, in such a way as to retain the highest possible dietary burden.

Example: Reduce the rice straw content to 25% (= 100 - 75) to achieve a total diet contents of 100%.

(vii) If the total diet contents of the feed commodities derived from the intended crops do not add up to 100%, it is assumed that the animals are fed with feed commodities derived from unintended crops.

(viii) If there is a possibility that the same pesticide may present as a residue in imported feed, this should be taken into consideration in the calculation.

Example) Calculation of the mean residue level of pesticide A in diet (beef cattle)

Feed commodities	Codex code	Residue in crops (mg/kg)		Dry matter (%)	Dry weight residue (mg/kgdw)	Maximum diet content (%)
Other grasses (green forage)	AF/AS	3	STMR	25	12.00	5
Barley	GC	0.05	STMR	88	0.06	70
Rice straw	AF/AS	0.02	STMR	90	0.02	55

Feed commodities	Codex code	Residue in crops (mg/kg)		Dry matter (%)	Dry weight residue (mg/kgdw)	Diet content (%)	Burden (mg/kg)
Other grasses (green forage)	AF/AS	3	STMR	25	12.00	5	0.6
Barley	GC	0.05	STMR	88	0.06	70	0.0398
Rice straw	AF/AS	0.02	STMR	90	0.02	25	0.0056
Total						100	0.6453

To make the total diet content100% (= 100% – 75%)

(Appendix 2) Calculation of residue levels in livestock commodities from dietary burden estimates (example)

The maximum and mean residue levels in livestock commodities when the livestock was fed with feed commodities treated with pesticides shall be calculated based on the dietary burden estimates obtained by the method described in Appendix 1 and on the results of livestock residue studies.

In principle, the calculated maximum residue levels in livestock commodities are used in the estimation of MRLs in livestock commodities, and the calculated mean residue levels are used in the assessment of exposure in humans.

(1) Calculation of maximum residue levels in livestock commodities

(i) Based on the results of livestock residue studies, residue levels in livestock commodities (Y) (mg/kg) are plotted against dose levels (X) (test substance concentrations per dry weight of feed (mg/kg)).

a. In the case of estimating the maximum residue levels in meat, fat, liver, kidney and eggs, use the residue levels of the animal that showed the highest residue at each dose level in the livestock residue study.

b. In the case of estimating the maximum residue level in milk, use the median residue level for a plateau of the mean residue levels determined on each sampling day for all animals at each dose level in the livestock residue study.

Example) Pesticide B was administered to lactating dairy cattle (4 animals/group). Residue level in tissues of animals slaughtered within 24 hours after the last administration.

Tissue	Residue level in tissues, mg/kg		
	0.1 mg/kg (X_1) dose	0.3 mg/kg (X_2) dose	1 mg/kg (X_3) dose
Meat	<0.02 (4)	0.021, 0.024, 0.026, 0.031	0.040, 0.046, 0.051, 0.057
Liver	0.029, 0.030, 0.032, 0.033	0.081, 0.089, 0.096, 0.114	0.161, 0.217, 0.264, 0.298
Kidney	<0.02 (4)	<0.02 (4)	0.025, 0.031, 0.030, 0.028
Fat	0.297, 0.303, 0.327, 0.338	0.921, 1.045, 1.051, 1.245	2.649, 2.737, 3.396, 3.493

Use the underlined values (maximum values) for plotting.

(ii) Based on the results in (1), calculate the residue levels in livestock commodities equivalent to the estimated maximum dietary burden (A). In cases where the estimated maximum dietary burden differs between beef cattle and lactating dairy cattle, or between laying hens and broilers, the higher value serves in the calculation of the residue levels in meat, fat, liver, kidney, milk and eggs.

a. In cases where the estimated maximum dietary burden (A) is located between two dose levels, the residue levels in tissues can be estimated by linearly regressing the data points located between the two dose levels closest to each other.

(Example) Calculation of the residue level in meat when A=0.50 mg/kg ($X_2<A<X_3$)

Linearly regress (X_2,Y_2) = (0.3, 0.031) and (X_3,Y_3) = (1, 0.057), and based on the regression equation, calculate the residue level in meat when X=0.50 mg/kg.

Y= 0.037X + 0.020. When X=0.50, Y=0.038 mg/kg.

b. In cases where the estimated maximum dietary burden (A) is less than the lowest dose level (X_1), the residue levels in tissues can be estimated by linearly regressing data points between the origin and (X_1,Y_1).

(Example) Calculation of the residue level in the liver when A=0.05 mg/kg ($A< X_1$)

Linearly regress (X_0,Y_0) = (0, 0) and (X_1,Y_1) = (0.1, 0.033), and based on the regression equation, calculate the residue level in the liver when X=0.05 mg/kg.

Y= 0.33X. When X=0.05, Y=0.017 mg/kg.

c. In cases where the estimated maximum dietary burden (A) is greater than the maximum dose levels (X_3), residue levels in tissues can be estimated by linearly regressing the data points between the origin and (X_3,Y_3).

(Example) Calculation of the residue level in the liver when A=1.2 mg/kg ($A> X_3$)

Linearly regress (X_0,Y_0) = (0, 0) and (X_3,Y_3) = (1, 0.298), and based on the regression equation, calculate the residue level in the liver when X=1.0 mg/kg.

Y= 0.298X. When X=1.2, Y=0.358 mg/kg.

d. Data should not extrapolated when the maximum dose level (X_3) determined by livestock residue studies is exceeded by 30% or more.
e. In cases where the dose levels closest to each other are less than the LOQ, the residue levels shall be reported as less than LOQ.

(Example) Calculation of the residue level in the kidney when A=0.20 mg/kg (X_1<A<X_2)
Since Y1<0.02 and Y2<0.02, residue level in the kidney Y is <0.02 when X=0.20 mg/kg.

(2) Calculation of the mean residue level in a livestock commodities
(i) Based on the results of livestock residue studies, plot the residue levels in livestock commodities (Y) (mg/kg) against the dose levels (X) (concentration of the test substance per dry weight feed (mg/kg)).
a. To estimate the mean residue level in meat, fat, liver, kidney and eggs, use the mean residue level from all animals per each dose level of the livestock residue study.
b. To estimate the mean residue level in milk, use the median residue level at each dose level that occurred over the plateau phase, out of the mean residue levels calculated on each day of sampling for all animals used in the livestock residue study.

Example) mean residue level in meat, liver, and fat when the above pesticide B is administered to laying hens.

Tissue	Pesticide B, mg/kg		
	0.1 mg/kg(X_1) dose	0.3 mg/kg(X_2) dose	1 mg/kg(X_3) dose
Meat	<0.02	0.026	0.049
Liver	0.031	0.095	0.235
Kidney	<0.02	<0.02	0.029
Fat	0.316	1.066	3.069

→ Use the mean residue levels obtained from 4 animals for plotting

(ii) The procedures that follow are the same as described for the calculation of the maximum residue levels in livestock commodities.

Attached Table 1 Table of feed amounts

Beef cattle

Codex code	Feed commodity	Intended crop	Maximum diet content (%)	DM (%)
AB	Beet pulp (sugar beet)	Sugar beet	5	88
AF/AS	Rice straw	Rice	55	90
AF/AS	Fermented rice roughage (silage)	Rice WCS	5	40
AF/AS	Italian rye grasses (hay)	Grasses	30	86
AF/AS	Other grasses(green forage)	Grasses	5	25
AF/AS	Other grasses (hay)	Grasses	40	88
AF/AS	Other grasses (silage)	Grasses	5	40
AL	Alfalfa (hay, hey cube)	Legume forages and fodders	10	89
AL	Other legumes (hay)	Legume forages and fodders	5	85
CM/CF	Barley mixed bran	Barley	10	90
CM/CF	Rice bran	Rice	20	90
CM/CF	Bran (wheat)	Wheat	55	88
CM/CF	Corn gluten feed	Corn	25	40
CM/CF	Corn jam meal	Corn	5	85
CM/CF	Hominy feed (corn)	Corn	35	88
GC	Barley	Barley	70	88
GC	Wheat	Wheat	25	89
GC	Milo	Edible sorghum	35	86

GC	Corn	Corn for feed	75	88
GC	Feed rice (unhulled rice)	Rice	30	88
GC	Rye	Rye	35	88
SM	Beer lees (barley)	Barley	45	92
SM	Soybean oil cake	Soybean	65	92
SM	Soybean coat (soy hull pellet)	Soybean	5	90
SM	Soybean curd lees	Soybean	40	92
SM	Corn distiller's grains with solubles	Corn	10	92
SM	Rapeseed oil cake	Rapeseed	15	88
SM	Coconut meal (copra flakey)	Palm	5	90
VD	Soybean (whole fat soybean)	Soybean	15	89

Lactating dairy cattle

Codex code	Feed commodity	Intended crop	Maximum diet content (%)	DM (%)
AB	Beet pulp (sugar beet)	Sugar beet	40	88
AF/AS	Rice straw	Rice	25	90
AF/AS	Fermented rice roughage (silage)	Rice WCS	55	40
AF/AS	Italian rye grasses (green forage)	Grasses	10	17
AF/AS	Other grasses (green forage)	Grasses	10	25
AF/AS	Italian rye grasses (hay)	Grasses	30	86
AF/AS	Italian rye grasses (silage)	Grasses	35	29
AF/AS	Orchard grasses (hay)	Grasses	5	84
AF/AS	Orchard grasses (silage)	Grasses	20	27
AF/AS	Timothy (hay)	Grasses	70	85
AF/AS	Timothy (silage)	Grasses	35	40
AF/AS	Rye (hay)	Grasses	5	88
AF/AS	Rye (silage)	Grasses	5	28
AF/AS	Other grasses(hay)	Grasses	70	88
AF/AS	Other grasses(silage)	Grasses	80	40
AF/AS	Oats(green forage)	Oats for feed	5	30
AF/AS	Oats(hay)	Oats for feed	5	90
AF/AS	Oats(silage)	Oats for feed	5	35
AF/AS	Dent corn (green forage)	Corn for feed	20	40
AF/AS	Dent corn (silage)	Corn for feed	50	40
AF/AS	Sorgo (green forage)	Sorghum	40	35
AF/AS	Sorgo (hay)	Sorghum	5	88
AF/AS	Sorgo (silage)	Sorghum	10	21
AL	Alfalfa (hay, hey cube)	Legume forages and fodders	25	89
AL	Alfalfa (silage)	Legume forages and fodders	20	40
AL	Other legumes (hay)	Legume forages and fodders	25	85
AL	Other legumes (silage)	Legume forages and fodders	60	30
CM/CF	Rice bran	Rice	10	90
CM/CF	Bran (wheat)	wheat	45	88
CM/CF	Corn gluten feed	Corn	25	40
CM/CF	Corn gluten meal	Corn	15	40
GC	Oats	Oats	5	89
GC	Barley	Barley	40	88
GC	Wheat	Wheat	10	89
GC	Milo	Edible sorghum	30	86
GC	Corn	Corn for feed	80	88
GC	Feed rice (unhulled rice)	Rice	20	88
GC	Rye	Rye	15	88
SM	Beer lees (barley)	Barley	40	92
SM	Soybean oil cake	Soybean	60	92
SM	Soybean curd lees	Soybean	20	92

SM	Corn distiller's grains with solubles	Corn	15	92
SM	Rapeseed oil cake	Rapeseed	25	88
SM	Coconut meal (copra flake)	Palm	5	90
VD	Soybean (whole fat soybean)	Soybean	10	89

Pigs

Codex code	Feed commodity	Intended crop	Maximum diet content (%)	DM (%)
CM/CF	Rice bran	Rice	10	90
CM/CF	Bran (wheat)	Wheat	15	88
CM/CF	Corn gluten feed	Corn	10	40
CM/CF	Corn gluten meal	Corn	5	40
GC	Barley	Barley	30	88
GC	Wheat	Wheat	35	89
GC	Milo	Edible sorghum	55	86
GC	Corn	Corn for feed	85	88
GC	Feed rice (unhulled rice)	Rice	45	88
GC	Rye	Rye	35	88
SM	Soybean oil cake	Soybean	70	92
SM	Rapeseed oil cake	Rapeseed	20	88
SM	Alfalfa meal	Legume forages and fodders	5	89
SM	Coconut meal (copra flake)	Palm	15	90

Broilers

Codex code	Feed commodity	Intended crop	Maximum diet content (%)	DM (%)
CM/CF	Rice bran	Rice	5	90
CM/CF	Bran (wheat)	Wheat	5	88
GC	Barley	Barley	10	88
GC	Wheat	Wheat	10	89
GC	Milo	Edible sorghum	65	86
GC	Corn	Corn for feed	70	88
GC	Feed rice (unhulled rice)	Rice	40	88
SM	Soybean oil cake	Soybean	35	92
SM	Corn distiller's grains with solubles	Corn	5	92
SM	Rapeseed oil cake	Rapeseed	5	88
SM	Alfalfa meal	Legume forages and fodders	5	89

Laying hens

Codex code	Feed commodity	Intended crop	Maximum diet content (%)	DM (%)
CM/CF	Barley mixed bran	Barley	5	90
CM/CF	Rice bran	Rice	20	90
CM/CF	Bran	Wheat	30	88
CM/CF	Corn gluten feed	Corn	10	40
CM/CF	Corn gluten meal	Corn	10	40
GC	Milo	Edible sorghum	55	86
GC	Corn	Corn for feed	80	88
GC	Feed rice (unhulled rice)	Rice	65	88
SM	Sesame oil cake	Sesame	5	91
SM	Soybean oil cake	Soybean	30	92
SM	Corn distiller's grains with solubles	Corn	5	92
SM	Rapeseed oil cake	Rapeseed	15	88

Attached Table 2 Processing factors (default values) used where processing studies have not been conducted.

Feed commodity	Processing factor
Rapeseed oil cake	2
Sesame oil cake	2
Coconut meal (copra flake)	2
Soybean oil cake	2
Soybean coat (soy hull pellet)	10
Soybean curd lees	2
Beet pulp (sugar beet)	10
Beer lees (barley)	1
Barley mixed bran	2
Corn gluten feed	1
Corn gluten meal	1
Corn distiller's grains with solubles	1
Bran (wheat)	5
Rice bran	10
Soybean (whole fat soybean)	1

Test on residues in soil (3-3-1)

1. Objective

The objective of these tests is to obtain scientific information regarding the degree of residue of agricultural chemicals in soil under field conditions.

2. Test field

(1) When the test fields are domestic and include soil from at least 2 locations with different properties in terms of soil texture, parent material, and other aspects, select locations that are not contaminated with agricultural chemicals, etc. that might inhibit analyses.

(2) If fields with differing soil properties cannot be used, due to unavoidable circumstances, fields in which differing conditions other than soil properties (such as weather conditions) prevail may be used. Use paddies for studies of substances that are to be used in paddies, and use upland fields for studies of substances that are to be used in upland fields, so that studies are conducted under conditions in which the representative crops, on which the relevant agricultural chemical is to be used, would be cultivated.

3. Handling and preparation of the test substance

(1) The test substance should be employed soon after it is prepared.

(2) The test substance should be stored under appropriate conditions. If the substance is to be stored for a long period after being opened, its stability during the storage period should be confirmed.

(3) Apply the test substance properly, in a formulation and according to a method of usage (in terms of time, number of applications, quantity, etc.) that is relevant to the application for registration, and using the tools customarily employed.

(4) Do not apply the test substance during rainy weather, or when rainfall is expected shortly after application. However, when rain will not affect results, due to use of a greenhouse, etc., this limitation is unnecessary.

4. Collecting samples (soil)

(1) Method of collection

(i) Gather samples once from 4 different places in each field, and mix well.

(ii) Gather cylindrical samples of at least 200 g, down to a depth of approximately 10 cm below the surface. When the test fields are paddies, collect paddy water as well.

(2) Collection schedule and the number of times collected

Collection samples once each immediately prior to and following addition of the test substance, and at least 4 times thereafter.

5. Handling of samples

(1) Transporting samples

(i) Care should be taken in transporting samples so that they do not deteriorate or become contaminated. They should be transported rapidly, at low temperatures, but not frozen.

(ii) Handle samples properly, by affixing identifying labels, etc., in order to prevent confusion among samples during transport.

(2) Handling samples after transportation

Immediately upon receiving samples, verify their authenticity according to their identification labels, etc.

Handle them properly, so as to avoid confusion among samples, and use them promptly for analyses.

6. Study period

In general, set the study period as the time until the analytical values of the target substances (when there are multiple target substances, compute the total amount of active ingredient, based on the analytical values) in test soil decline to approximately 10% of the concentration immediately following addition (if the values do not decline to approximately 10%, the time until they decline to less than 1/2 of the concentration immediately following addition).

7. Analysis of samples

(1) Target substances for analysis

The substances to be analyzed are the active ingredients of agricultural chemicals related to the test substance, as well as substances generated in the course of the biological and chemical changes they undergo. However, this does not apply to substances that leave extremely minute residues, and which have been deemed non-harmful due to their extremely low toxicity, etc.

(2) Method of analysis

(i) Adopt a method by which the target substance can be accurately analyzed.

(ii) Express the amount of target substance residue as concentration in dry soil (mg/kg).

(iii) Analyze each sample at least twice, and use the mean value from these analyses as the measured value.

(iv) Confirm the precision of the analytical method by means of the coefficient of variation within the range of concentrations in which detection of the target substances is anticipated.

(v) The sensitivity of the analytical method should be consistent with study objectives, and should be expressed according to the limit of quantification, that is, the minimum concentration at which a recovery rate can be obtained that is sufficient for all operations involved in analysis of samples.

(vi) Confirm the recovery rate of the analytical method, within the limit of quantification and the range of concentrations in which detection of the target substances is anticipated, using samples that have been collected from untreated plots, and to which a known quantity of the target substance has been added.

(vii) In general, use samples for analysis promptly after they have been collected. If temporary storage of samples is unavoidable, store them under appropriate conditions, conduct storage stability examinations to determine the stability of target substances during the storage period.

(viii) Conduct storage stability examinations of stored samples, using samples that have been collected from untreated plots, and to which a known quantity of the target substance has been added, under the same conditions, for at least the same period, and according to the same methods by which test samples are analyzed.

8. Reporting

(1) Institution that created study report (site for field test or analysis)

(2) Test substance

(3) Study conditions

(4) Target substances for analysis

(5) Method of analysis (summary and details)

(6) Limit of quantification and recovery rate in each analysis

(7) Method of preparing samples, etc.

(8) Results of analysis (the analytical values at each sample collection time)

(9) Estimated half-life and derivation method

Test on residues in succeeding crops (3-3-2)

1. Objective

The objective of these tests is to obtain scientific information regarding the degree to which agricultural chemicals conveyed via soil leave residue in crops.

2. Test crops

(1) When the test substance is for application to paddies, select at least 2 plants, belonging to different families, from among wheat and other small-type grain, soybeans, and root vegetables.

(2) When the test substance is for application to upland fields, select at least 1 type of root vegetable, and otherwise, at least 1 type of crop thought suitable to be a succeeding crop.

3. Test plot (field) selection

The test plots should be the fields where the test substance was applied to preceding crops relevant to the application for registration in accordance with the application method relevant as well, prior to cultivation of the test crops. When it is difficult to ensure such fields, there is no objection to conducting the studies with pots using soil collected from the fields where the test substance was applied to preceding crops relevant to the application for registration in accordance with the application method relevant as well. Otherwise, conduct as in studies of residues in crops.

4. Cultivation of test crops

Conduct as in studies of residue in crops.

5. Handling and conduct of the test substance

Conduct as in studies of residue in crops.

6. Collecting samples

Conduct as in studies of residue in crops.

8. Handling samples

Conduct as in studies of residue in crops.

9. Analysis of samples

Conduct as in studies of residue in crops.

10. Reporting

Conduct as in studies of residue in crops.