The Chronic Toxicity Test, Test of Impact on Reproductive Potential and Future Generations, Teratogenicity Test, Mutagenicity Test, Carcinogenicity Test, Test of in vivo Fate, and Pharmacological Test, for Chemical Substances

I.

The standardized methods for the chronic toxicity test, the test of impact on reproductive toxicity and future generations, the teratogenicity test, the mutagenicity test, the carcinogenicity test, test of *in vivo* fate and pharmacological test are as follows.

II. General Rules

1. **Test animals**

   As a rule, mammals are selected to be test animals, and animals with a clearly known origin, species and breed are to be used. With the exception of certain special tests, it is necessary to use animals that are not much affected by age (affected by youth or old age). When the metabolic pattern of the test substance in people is known, it is desirable to use an animal with a metabolic pattern similar to that of people.

   As a rule, test animals of the same type, species and breed are used across all tests. Furthermore, it is desirable to use species for which the types and frequency of naturally occurring pathologies under appropriate breeding conditions are known.

2. **Management of breeding**

   When breeding animals over a long period, be careful to maintain appropriate management conditions in particular (the breeding environment: temperature, humidity, ventilation, lighting, and food for the animals, etc.) and to avoid the outbreak of infectious diseases.

3. **Test substances**

   When administering a test substance by adding it to the food for the animals, closely monitor the homogeneity, additive concentration and safety of the test substance after it has been added, and confirm these factors at fixed intervals. When dissolving the test substance in a solvent, to form a suspension or emulsion, clearly determine the concentration and safety of the test substance.

4. **Control group**

When administering a test substance by adding it to the food for the animals, establish a control group and give it food, which does not contain the test substance. When administering a test substance using a solvent, suspension agent, emulsion agent, it is desirable to establish a control group which is raised by being given feed, containing solvents, suspension agents or emulsion agents only. If the added test substance is highly concentrated, it is necessary to take into account nutritional balance.

The objective of this test is to determine the toxicity of the test substance by observing changes in the function, shape, of a living organism that become apparent when a test substance is administered to the animal continuously over a long period.

1. **Test Animals**

1-1. Animal type and sex

Use male and female animals of two or more types (mice, rats, etc.) of the same kind as are used in 1 to 3-month short-term preliminary tests. It is desirable for one of these types to be a non-rodent.

1-2. Age

For types of animals with a short life span, such as mice, rats, etc., use 5 to 6-week old animals with uniform weight. For types of animals with a relatively long life span, use animals of an age that in general corresponds to that of mice, rats, etc.

1-3. Number of animals

In the case of mice, rats, etc., use 20 or more males and 20 or more females in each group. In the case of non-rodents, use 4 or more males and 4 or more females in each group. If mice, rats, etc. are to be put down during the testing period in order to conduct tests on them, add the number of animals that will be required for that purpose in advance.

2. **Test Substance**

2-1. Method of administration

As a rule, the test substance is administered orally. It is desirable to administer

the test substance by adding it to food for the animal or the animal's drinking water. The concentration of the test substance added to the food for the animal must be 5W/W% or less. However, if due to the properties of the test substance it can not be administered orally, administer it parenterally. In the case of forced administration, administer the test substance at a set time each day.

2-2. Dosage

Administer the dosage in three stages or more in order to determine the relationship between dosage and effect.
Carry out 1 to 3-month short-term preliminary tests in advance and determine the maximum dosage for which some toxic effect caused by the test substance has been established without causing a large number of deaths.
Make the minimum dosage the amount which produces no effect on the animals during the period of the test. Establish a separate control group.
Calculate the actual amount of the test substance assimilated from the food intake of the animals or from the water intake and the concentration of the test substance.

2-3. Administration period

Make the administration period 12 months or more.[*1]

3. **Observation and Measurement Items**

As a rule, conduct observations on the following items.

3-1. General condition and death rate

3-2. Weight, food intake and water intake, food intake efficiency[*2]

3-3. Blood tests

3-3-1. Hematologic test[*3]
3-3-2. Blood biochemistry test[*4]

3-4. Urine test[*5]

3-5. Pathology tests

3-5-1. Observation with the naked eye and internal organ weight[*6]
3-5-2. Observation with a microscope (carry out a test using an electronic microscope or a histochemical test as necessary)[*7]

3-6. Other necessary items

Study the cause of death of animals that died during the testing. Also, promptly put down and do an autopsy on animals whose general condition is very bad and are about to die.

*[*1]: When using mice or rats, it is desirable to use at least five males and at least five females to study at least once during the administration period the same test items that are in the tests that will be carried out at the end of the test period.*

*[*2]: It is desirable to measure water intake only when administering the test substance by mixing it into the animals' drinking water and calculate food intake efficiency during the period of growth of the animals.*

*[*3]: The hematologic tests that are usually carried out are as follows. For the measurements of each item the test methods and measurement units widely used internationally are adopted. In addition, it is desirable to perform tests on any other items for which a relationship to toxicity is indicated.*
Red blood cell count, reticulocyte count, hemoglobin, hematocrit, white blood cell count, white blood cell percentage, platelet count, etc.

*[*4]: The blood biochemistry tests that are usually carried out are as follows. For the measurements of each item, the test methods and measurement units widely used internationally are adopted. In addition, it is desirable to perform tests on any other items for which a relationship to toxicity is indicated.*
Total protein, A/G ratio, blood sugar, triglyceride, phospholipid, total cholesterol, urea nitrogen, creatinine, uric acid, sodium, potassium, chlorine, calcium, phosphorus, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), lipotropic hormone (LPH), alkaline phosphatase, creatine phosphokinase, γ-guanosine triphosphate (GTP), ornithine decarboxylase, etc.

*[*5]: Carry out semiquantitative tests of urine volume, photogrammetry, occult blood, total protein, sugar, ketone body, urobilinogen and bilirubin and carry out tests of sediment with a microscope as necessary.*

*[*6]: Conduct autopsies on all the animals used in the testing (including those that died or were put down during the testing period) and carry out sufficient observations with the naked eye of all internal organs and tissues. Store all of the organs and tissues described in [*7] in an appropriate storage liquid for each group.*
*Measure the weight of organs and tissues marked with an asterisk in [*7].*

*[*7]: The organs and tissues for which a histopathological test is necessary are as follows. Implement this test for a maximum dosage group and a control group, and implement a test of other dosage groups for organs and tissues that showed changes in the maximum dosage group.*
Brain, spinal cord, peripheral nerve, pituitary gland*, eyeballs, nasal cavity (#), lungs* (including the bronchial tubes), tongue, esophagus, stomach, small intestine, large intestine, skin, salivary gland, lymph node, thyroid gland (including the parathyroid gland), thymus gland, heart*, liver*, pancreas, spleen*, kidney*, adrenal gland*, bladder, testicles*, seminal vesicle, prostate gland, mammary gland (females), ovaries*, uterus, sternum (including the bone marrow), vertebrae and femur (including the joint), and organs and tissues for which changes have been confirmed with the naked eye.*
(#) In the case of an inhalation test, the nasal cavity, pharynx, larynx, and trachea.

IV. Test of Impact on Reproductive Potential and Future Generations

Objective

The objective of this test is to determine the damage to reproductive potential and to the emergence of future generations caused by the test substance by administering the test substance to male and female animals over many generations.

4. **Test Animals**

1-1. Animal type

1-1-1. Use at least one type of animal (mice or rats, etc.) and choose the test animals from among the animals used in the V. Teratogenicity test. When selecting the type, species and breed of the test animals, take into account knowledge concerning reproduction, such as knowledge about

fertility, etc., the frequency of occurrence of naturally occurring abnormalities, sensitivity to substances known to have reproductive or birth toxicity. Also, it is desirable to select animals for which the frequency of occurrence of naturally occurring deformities is low.

1-1-2. When using the same type of animal as for the chronic toxicity tests, it is desirable to select animals of the same species.

1-1-3. When using animal types other than rats or mice, it is necessary to carry out appropriate modifications to these guidelines in order to achieve the objective of this test.

1-2. Number of animals

With rats or mice, prepare the number of females that are expected to be necessary to produce 20 pregnant animals, and the equivalent number of males, in the control group to which the test substance is not administered.

5. **Test substance**

2-1. Method of administration

As a rule, the test substance is administered orally. It is desirable to administer the test substance by adding it to food for the animal or the animal's drinking water. The concentration of the test substance added to the food for the animal must be 5W/W% or less. However, if due to the properties of the test substance it can not be administered orally, administer it parenterally.

2-2. Dosage

In order to determine the dosage-response relationship and estimate the maximum dosage with no effect, establish dosage test groups with at least three stages. Make the maximum dosage the amount that causes the parental generation of animals (F_0) to show slight indications of toxicity, such as declining food intake or suppression of growth in weight, but does not cause a death rate of more than 10%.

Make the minimum dosage the amount that does not have any toxic effects on reproductive potential or the emergence of future generations. Set up a control group separately.

6. **Crossbreeding and administration of the test substance**

3-1.

For F_0, commence administration of the test substance by the time the animals are 5-8 weeks old, as a rule, and after administering it every day for more than 10 weeks (for mice, 8 weeks), begin crossbreeding.

Make the cohabitation period of the same males and females 2 or 3 weeks and during that period verify every day whether copulation has occurred.

3-2.

Separate out the females that have mated, and create the first generation (F_1) through natural birth.

When adjusting the number of progeny in the litter, at a relatively early time after birth, randomly leave a fixed number of animals composed of approximately the same number of males and females for one mother. Leave the progeny to be raised by the mother animal.

Continue to administer the test substance to the father animals until crossbreeding to produce the F_1 generation is completed, and to the mother animal until weaning of the F_1 generation.

3-3.

Randomly select the animals to produce the next generation at the time of weaning of the F_1 generation and perform an autopsy examination on the remaining animals. After administering the test substance to the animals used to produce the next generation over a period of at least 10 weeks (for mice, 8 weeks) in the same way as for F_0 after weaning, as a rule, take at least 20 pairs of males and females from different litters, and crossbreed them in the same way as for F_0 to produce the second generation (F_2). As a rule, raise the F_2 generation by administering the test substance from after weaning until sexual maturation.

7. **Observation Items**

4-1. F_0

4-1-1. Observe general condition and whether there are deaths, measure weight and food intake (as necessary water intake), and calculate the

intake of the test substance.

4-1-2. Calculate the copulation rate and conception rate for the parent animal. Search for birth abnormalities and calculate the birthrate for the mother animals.[*1]

4-1-3. Do an autopsy on the mother animal at the time of weaning of the F_1 generation and observe the internal organs.

4-1-4. At an appropriate time, put down the males and those females that did not copulate, conceive or give birth, and observe their internal organs.[*2]

4-2. F_1

4-2-1. Examine the number born, survival rate, sex, weight, and changes in the external appearance of the new progeny.

4-2-2. After birth, observe general condition, whether there are deaths, growth, and the development of form and function. Measure weight at least once a week. Calculate survival rate at appropriate intervals and calculate the weaning rate at the time of weaning.[*3]

4-2-3. Conduct the same kind of examination of F_1 animals used in crossbreeding to produce the F_2 generation as was done for the F_0 generation. Do an autopsy on the remaining F_1 generation at the time of weaning.

4-3. F_2

Carry out the same observations as for the F_1 generation. As a rule, do an autopsy when the animals reach sexual maturity. As necessary, conduct a detailed examination using histological or biochemical methods.

4-4. How to draw conclusions from the observations

Study the observed abnormalities and the relationship between the symptoms of toxicity and amount of the test substance administered using appropriate statistical methods, and state an opinion concerning the maximum dosage with no effect. When doing so, it is desirable to make the progeny of one litter the sample unit until weaning.

8. **Test Extensions, etc.**

As necessary, perform tests to carry out repeated crossbreeding of F_0 and F_1 in

order to produce second generation progeny and later generations, conduct long-term observations of F_2 after the animals reach sexual maturity, and furthermore conduct tests of crossbreeding and reproductive potential in order to produce F_3. Also, when it is necessary to make clear whether reproductive failure caused by administration of the test substance is mainly due to an impact on males or females, carry out crossbreeding of males to which the test substance has been administered and females to which the test substance has not been administered, and crossbreeding of males to which the test substance has not been administered and females to which the test substance has been administered.

*[*1]: Normally the following calculation methods are used.*
Copulation rate = (Number of animals that copulated/Number of animals cohabitating)×100
Conception rate = (Number of pregnant animals/Number of animals that copulated)×100
Birthrate = (Number of females that gave birth to progeny/Number of pregnant females)×100

*[*2]: Males are normally put down at the end of the crossbreeding period. Females for which copulation could not be confirmed are put down at the end of the crossbreeding period, and females that did not conceive or give birth are put down when two or three days have passed from the expected date of birth as calculated from the date of copulation.*

*[*3]: Normally the following calculation methods are used.*
Survival rate = (Number of progeny at date of search/Number of progeny at time of birth, Number of progeny four days after birth or immediately after selection, or Number of progeny at the time of weaning)×100
The denominator varies depending on the time of the search.
Weaning rate = (Number of progeny at time of weaning/ Number of progeny four days after birth or immediately after selection)×100

V. Teratogenicity Test

Objective

The objective of this test is to administer the test substance to pregnant animals during the period that the internal organs of the fetus are forming, and determine what damage to the birth of the fetus is caused by the test substance, in particular the teratogenicity of the test substance.

9. **Test Animals**

1-1. Animal type

1-1-1. Use one or more types of rodents, such as rats or mice, and non-rodents such as rabbits.
When selecting the animal type, species and breed, take into consideration knowledge concerning reproduction, such as knowledge about fertility, the frequency of occurrence of naturally-occurring abnormalities, sensitivity of the animal to substances known to have reproductive or birth toxicity, etc. Also, it is desirable to select animals with a low frequency of occurrence of naturally-occurring abnormalities.

1-1-2. When using the same animal type as for the chronic toxicity test, it is desirable for the species of the animals to be the same.

1-1-3. When using animal types other than rats, mice or rabbits, it is necessary to carry out appropriate modifications to these guidelines in order to achieve the objective of this test.

1-2. Number of animals

With rats and mice, use 20 or more animals for each dosage group as the number of individuals that conceived. With rabbits, use 12 or more animals.

10. **Test substance**

2-1. Method of administration

As a rule, the test substance is administered forcibly and orally.

2-2. Dosage

In order to determine the dosage-response relationship and estimate the maximum dosage with no effect, establish dosage test groups with, as a rule, at

least three stages. Make the maximum dosage the amount that causes the mother animals to show slight indications of toxicity, such as declining food intake or suppression of increase in weight, but does not cause a death rate of over 10%.
In the case that the mother animal shows no indications of toxicity even from the maximum amount of the test substance that can be administered (make the limit 1000mg/kg), make that amount the maximum dosage. Make the minimum dosage the amount for which toxic impact on the birth of the fetus is not shown. Set up a control group separately to administer the solvent only.

2-3. Administration period

Administer the test substance every day during the period that the internal organs of the fetus are forming. Normally, when the date of verification of copulation is defined to be day 0 of pregnancy, the administration period is from the 6^{th} day to the 15^{th} day of pregnancy for mice, from the 7^{th} day to the 17^{th} day of pregnancy for rats, and from the 6^{th} day to the 18^{th} day of pregnancy for rabbits. However, for rats it is also acceptable to make the administration period from the 6^{th} day to the 15^{th} day of pregnancy.

11. **Observation Items**

3-1 Mother animal

3-1-1. Observe general condition throughout the test period and measure weight and food intake.
3-1-2.Do an autopsy on all of the animals approximately one day before the expected date of birth, study the establishment of pregnancy, count the number of corpus lutea and implantations, and observe the internal organs with the naked eye.

3-2. Fetus

Determine whether the fetus has survived and estimate the time of death of the dead progeny. Measure the weight of the living progeny and determine their sex. In addition, conduct an examination with the naked eye of the external appearance and internal organs, and an examination of the shape and ossification of bones through skeletal staining transparent sample.

3-3. How to draw conclusions from the observations

Study the observed abnormalities and the relationship between the symptoms of toxicity and amount of the test substance administered using appropriate statistical methods, and state an opinion concerning the maximum dosage with no effect. When doing so, it is desirable to make the progeny of one litter the sample unit.

VI. Mutagenicity Test

Objective

The objective of this test is to detect genetic toxicity of test substance with a relatively simple short-term test, and based on that to make a forecast of carcinogenicity and genetic impact on the next generation.

Selection of test method

There are various methods for the Mutagenicity Test[*1], but conduct the "1. Reverse Mutagenicity Test using Bacteria" as a test that gives an indication of genetic mutagenicity inducibility, and the "2. Chromosomal Aberration Test using Cultured Mammalian Cells" as a test that gives an indication of chromosome aberrations. If either of these tests gives a positive result, conduct "3. Micronucleus Test on Rodents."

12. **Reverse Mutagenicity Test on Bacteria**

    1-1. Objective

    Using bacteria, search for whether the test substance has genetic mutagenicity inducibility.

    1-2. Strain of bacteria to be used

    Conduct the test using the following five strains.

    (1) *Salmonella typhimurium* TA98

    (2) *Salmonella typhimurium* TA100

    (3) *Salmonella typhimurium* TA1535

    (4) *Salmonella typhimurium* TA1537, TA97 or TA97a

    (5) *Escherichia coli* WP2 *uvrA*, *Escherichia coli* WP2 *uvrA*/pKM101 or *Salmonella typhimurium* TA102

When chemical compounds that crosslink with DNA are detected, for *Salmonella typhimurium*, include TA102, and for *Escherichia coli*, add the WP2 or WP2/pKM101 strains which have a wild excision repair capacity. As necessary add other strains of bacteria.

1-3. Test method

Implement the test using either the pre-incubation method or the plate method. If there is a scientifically valid reason, it is acceptable to use another method. Whichever method is used, conduct tests on the case due to metabolic activation and the case not due to metabolic activation. In the case due to metabolic activation, use S9 mix with coenzymes, added to rodent (normally rat) liver homogenate 9,000×g supernatant fraction (S9) treated with an appropriate drug-metabolizing enzyme inducing agent (for example, combined use of Phenobarbital and 5,6-Benzoflavone). Make the final concentration of the S9 within the 5% to 30% range (normally10%).

1-4. Dosage stages

Use a dosage that can be analyzed in five stages or more at appropriate intervals. Conduct a dose-range finding test in advance and establish the maximum dosage, taking into account growth inhibition and solubility. As a rule, make the maximum dosage the dosage for which growth inhibition appears. If growth inhibition does not appear, make the maximum dosage 5 mg/plate. If it is a very insoluble substance and no growth inhibition can be seen at all, the dosage which forms a precipitation can be made the maximum dosage.

1-5. Control

Establish a group treated with a solvent as a negative control and a group treated with an appropriate known mutagen as a positive control.

1-6. Number of plates

As a rule, use two or more plates for each dosage of the test substance and the negative and positive control.

1-7. Observation of the reverse mutagenicity colony

After cultivating all of the plates, as a rule at 37°C for 48-72 hours, measure the number of reverse mutagenicity colonies on each plate and record the number. At the same time, observe growth inhibition, and if growth inhibition is

discovered, record the dosage. Also record the dosage if precipitation of the test substance is discovered.

1-8. Reproducibility

As a rule, the test results must be reproducible. However, if all strains of bacteria are being used and the dose-range finding study is being conducted, including negative control and positive control, using two or more plates for each dosage, it is possible to use it to verify reproducibility.

1-9. Judgment of the results

If the number of reverse mutagenicity colonies clearly increases relative to the negative control, or dosage dependence or reproducibility are discovered in its chemical action, determine that it is positive. If reproducibility is not discovered in the results of the dose-range finding test and this test, implement a test to verify the reproducibility. When it is impossible to give a clear positive or negative conclusion, a confirmation test must be carried out under appropriate experimental conditions.

1-10. Display of results

Show the number of reverse mutagenicity colonies for each plate and display their average value for each dosage.

13. **Chromosome Aberration Test Using Cultured Mammalian Cells**

2-1. Objective

The objective of this test is to use cultivated mammalian cells to search for whether the test substance has chromosomal structural aberration inducibility. When polyploids appear, record this fact.

2-2. Cells used

Use Chinese hamster fibroblast cell lines (for example, CHL/IU, CHO), human peripheral blood lymphocytes, or other primary, secondary, or established cells. Study the modal number, whether there is mycoplasma contamination, the cell cycle of the cells being used in the test.

2-3. Test method

Use cells in the growth phase and firstly, as a short-term treatment process, in

the case due to metabolic activation and the case not due to metabolic activation, treat with the test substance for 3-6 hours and make the chromosome sample approximately 1.5 cell cycles after the commencement of treatment. If the result of the short-term treatment process is negative, next implement a test using a 1.5-cell cycle continuous treatment process in the case not due to metabolic activation. Some test substances will cause a marked delay in the cell cycle. Sometimes continuous treatment longer than 1.5 cell cycles is necessary in the case not due to metabolic activation, and a sample making time longer than 1.5 cell cycles is necessary in the case due to metabolic activation. For this reason, conduct a confirmation test as necessary.
In the case due to metabolic activation, use S9 mix with coenzymes, added to rodent (normally rat) liver homogenate 9,000×g supernatant fraction (S9) treated with an appropriate drug-metabolizing enzyme inducing agent (for example, combined use of Phenobarbital and 5,6-Benzoflavone). Make the final concentration of the S9 within the 1% to 10% range (normally 5%).

2-4. Preparation of the test substance

Dissolve the test substance in an appropriate solvent or suspend it in an appropriate medium. If the test substance is a liquid, it is acceptable to directly add it to the test system. If the test substance is soluble in water, use a normal saline solution to dissolve it. If the test substance is not soluble in water, use dimethylsulfoxide (DMSO) to dissolve it.
As necessary, use carboxymethylcellulose (CMC) sodium to prepare a homogenous suspension.

2-5. Dosage stages

Use a dosage for which it is possible to do a chromosome analysis of three stages or more at appropriate intervals (as a rule, common ratio 2). It is desirable to establish the maximum dosage by conducting in advance a cell growth inhibition test using a maximum dosage of 5mg/mL or 10mM (the lowest of the two). As a rule, regardless of the solubility of the test substance in the culture solution, make the maximum dosage the dosage that clearly suppresses cell growth 50% or more. In tests using short-term treatment processes and the tests using continuous treatment processes, measure cell growth at the time the chromosome sample is made. If cell growth suppression of 50% or more is not confirmed, make the maximum dosage 5mg/mL or 10mM (the lowest of the two). If cell toxicity is not confirmed and precipitation of the test substance is

confirmed at the time of completion of treatment, the dosage which forms a precipitation can be made the maximum dosage.

2-6. Control

As a negative control, establish a solvent treatment group; as a positive control, establish a treatment group using an appropriate known chromosome aberrations inducing substance.

2-7. Number of plates

As a rule, use two plates for each dosage group of the test substance and the negative and positive control groups.

2-8. Observation of chromosome aberrations

Slide samples must be coded and observed under the condition that treatment conditions cannot be identified. With respect to chromosome structural aberration, observe at least 200 well-spread metaphase cells (for which the number of chromosomes is equal to mode ± 2) for each dosage, and record the number of cells with chromosome structural aberration and the number of cells for each kind of aberration. If two plates are used, as a rule, observe at least 100 metaphase cells per plate. Classify gaps separately from other aberrations and record them, but do not include them in structural aberrations. Gaps are defined as achromatic regions that are narrower than chromatids. With respect to the chromosome numerical aberration, observe 200 or more metaphase cells for each dosage and record the incidence of polyploids.

2-9. Judgment of the results

Positive determination is given when the incidence of cells with chromosome aberrations clearly increases as compared to the negative control, and dose dependency or reproducibility is observed in its action. When it is impossible to give a clear positive or negative conclusion, a confirmation test must be carried out under appropriate experimental conditions.

2-10. Display of results

Display the number of cells having chromosome structural aberrations, their incidence (%), and the number of cells for each type of structural aberration for all of the plates in the test by short-term treatment process and the test by continuous treatment process. Also, display the average value of the plate for

each group. Display the number and incidence (%) of polyploids. Display data on cell growth in each dosage group and the negative control group for the cell growth inhibition test, the test by short-term treatment process and the test by continuous treatment process. If precipitation of the test substance is seen, record that dosage.

14. **Micronucleus Test on Rodents**

3-1. Animals and observed cells

Use young adult rodents and observe the immature red blood cells in the bone marrow or the peripheral blood. Generally, mice or rats are used. If rats are used, be careful of the appearance of artificial micronuclei due to mast cell granules when using bone marrow, and of the elimination of red blood cells in the spleen that have micronuclei when using peripheral blood, and use a more appropriate observation method.

3-2. Sex and number of animals

Use five or more animals of each sex for each group. However, if no clear difference between the sexes with respect to toxicity can be seen, it is sufficient to use only one sex (five or more animals).

3-3. Preparation of the test substance

If the test substance is a solid, dissolve it in an appropriate solvent or suspend it in a medium. If the test substance is a liquid, it is acceptable to administer it directly or prepare it by diluting it in an appropriate medium[*2]. If the test substance is a gas, use purified air to dilute it. If the stability of the test substance after preparation is known, use the test substance within the stable period; if the stability is unknown, prepare the test substance at time of use.

3-4. Control group

Establish a solvent or medium as the negative control[*3] and an appropriate known micronuclei inducing substance[*4] as the positive control.

3-5. Route of administration

As a rule, use forced oral administration or intraperitoneal administration.

However, other routes may be used if there are scientific reasons to do so, such as the assumption of a specific exposure route (inhalation exposure, etc.).

3-6. Number of times to administer the test substance

Administer the test substance once or repeatedly.

3-7. Dosage stages

Make the maximum dosage the dosage for which cell toxicity, such as a decline in immature red blood cells in the bone marrow is confirmed, or the dosage at which some indications of toxicity are confirmed, or increasing the dose above the current level is expected to cause fatality, or the maximum dosage that can be administered technically[*5]. Make the maximum dosage when there are no indications of toxicity 2,000mg/kg/day for one-time administration or repeated administration within 14 days, and 1,000mg/kg/day for long-term repeated administration in excess of 14 days. If the test substance is a gas, make the maximum dosage the concentration that can be safely exposed[*6]. Establish a dosage of three stages or more at appropriate intervals (as a rule, common ratio 2 but common ratio $\sqrt{10}$ or less is acceptable).

3-8. Sample making time

3-8-1. When using bone marrow

For one-time administration, establish at least two sample making times at appropriate intervals during the 24-48 hours after administration, put down the animal and make a bone marrow smear sample[*7]. For repeated administration, make a sample once 18-24 hours after the final administration[*8].

3-8-2. When using peripheral blood

For one-time administration, establish at least two blood collection times at appropriate intervals during the 36-72 hours after administration, and make a sample[*7]. For repeated administration, make the sample once, 24-48 hours after the final administration[*8].

3-9. Observation

Before observation, encode all of the slide samples including the negative control and the positive control, and then conduct observations under the

condition that treatment conditions cannot be identified. Observe 2,000 or more immature red blood cells for each individual and find the incidence of cells possessing micronuclei. As an indicator of bone marrow cell growth suppression, find the incidence of immature red blood cells with respect to all red blood cells by observing 200 or more red blood cells per individual when bone marrow is used and 1,000 or more red blood cells when peripheral blood is used[*9].

3-10. Display of results

For each individual, display in tabular form the incidence of cells possessing micronuclei with respect to observed immature red blood cells and the incidence of immature red blood cells with respect to all red blood cells, and also display the average value for each group.

3-11. Judgment of the results

On the assumption that the test substance was appropriately administered at a sufficiently high dosage and the expected results were obtained in the negative and positive control group, judge the results by using appropriate statistical processing, including use of the background data of the negative control group[*10]. If no clear difference between the sexes in the results when using both sexes can be confirmed, it is acceptable to combine data from both sexes to conduct statistical processing. When it is impossible to give a clear positive or negative determination, because statistical significance is not the only judgment criteria, it is desirable to implement another test taking into account experimental conditions and make a final judgment.

3-12. Evaluation of results

Study the results of the determination for test substances which gave a positive result in any of the *in vitro* tests and a negative result in this test, using available knowledge about *in vivo* fate.

*[*1]: The results obtained here are the minimum information about the mutagenicity of chemical substances. Test methods for forecasting carcinogenicity and genetic impact on the next generation include the various short-term test methods given by way of example below.*

1. Tests that serve as indicators of DNA damage
in vitro and *in vivo* test systems

1) ^{32}P post-label method
2) Test to detect oxidative DNA damage
3) Single-cell gel electrophoresis method (comet assay)

2. Tests that serve as indicators of DNA repair

A. *in vitro* test systems

1) DNA repair test using hay bacteria
2) Umu test using *Salmonella typhimurium*
3) SOS test using *Escherichia coli*
4) Unscheduled DNA synthesis (UDS) test using cultured mammalian cells

B. *in vivo* test systems

1) Unscheduled DNA synthesis (UDS) test using rodents

3. Tests that serve as indicators of genetic mutagenicity inducibility

A. *in vitro* test systems

1) Reverse mutagenicity test using *Salmonella typhimurium* (Ames Test), *Escherichia coli,* etc.

2) Genetic mutagenicity test using cultured mammalian cells (mouse lymphoma L5178Y, human lymphocyte TK6, Chinese hamster cell lines V79, CHO, etc.)

B. *in vivo* test systems

1) Sex-linked recessive lethal test and wing spot test, both using drosophila
2) Test using transgenic animals
3) Specific locus test and spot test using mice
4) Genetic mutagenicity test using mammalian endogenous genes (*hprt*, etc.)

4. Tests that serve as indicators of chromosome aberration inducibility

A. *in vitro* test systems

1) Chromosome aberrations test including heteroploidy, using yeast
2) Chromosome aberrations test using cultivated human lymphocytes
3) Chromosome aberrations test using Chinese hamster, etc. cell lines
4) Micronuclei test using cultured mammalian cells

B. *in vivo* test systems

1) Micronuclei test using rodents
2) Chromosome aberrations test using rodent bone marrow cells
3) Dominant lethal test
4) Reciprocal translocation test

5. Other tests

A. *in vitro* test systems

1) Somatic recombination test using yeast
2) Gene conversion test using yeast
3) Sister chromatid exchange test using cultured mammalian cells
4) Transformation test using cultured mammalian cells

B. *in vivo* test system

1) Sister chromatid exchange test using rodents
2) Morphological abnormality test of sperm using rodents

*[*2]: Select a solvent or medium that does not react with the test substance and use a dosage that does not show toxicity. Generally, the use of water-based solvents such as a normal saline solution is recommended.*

*[*3]: In the case of a short-term test using peripheral blood (administer 1-3 times), it is possible to make the pre-administration sample the negative control.*

*[*4]: Examples of positive control substances:*

Ethyl methanesulfonate, Mitomycin C, Cyclophosphamide, Triethylene melamine

The dosage to be administered is not extremely high, but the dosage that shows clear micronuclei inducibility is recommended.

*[*5]: Make the maximum amount of liquid administered 20mL/kg when the main constituent of the medium is water, and 10mL/kg if it is not.*

*[*6]: For the maximum exposable concentration for mist and dust, use 5mg/L; for gas and steam, use the highest technically possible concentration that can safely be exposed and can maintain the appropriate oxygen concentration (19-21%).*

*[*7]: Even in the case of one-time administration, if as a result of studying the sample making time with a preliminary test the most sensitive time is confirmed and it is confirmed that positive results have been achieved, it is possible to make the sample once only at this time. Make the sample making time in this case the time confirmed as showing the most remarkable increase in micronuclei inducibility frequency.*
However, if an obvious increase in micronuclei inducibility frequency is not confirmed at any time, make the sample making time 24-30 hours after administration when using bone marrow, and 36-48 hours after administration when using peripheral blood.

*[*8]: Concerning the positive control, make the sample once at an appropriate time.*

*[*9]: For sample staining, normally the acridine orange fluorescent staining method or the Giemsa staining method are used for bone marrow samples, and the acridine orange supravital staining method is used for peripheral blood samples.*

*[*10]: If the test substance was appropriately administered at a sufficiently high dosage and the expected results were obtained in the negative and positive control groups, in case a statistically significant difference is not confirmed among all of the treatment groups with respect to the negative control group, determine it to be negative. Conversely, if there is a statistically significant difference in the number of cells possessing micronuclei and there is dosage dependency or reproducibility in results, determine it to be positive.*

VII. Carcinogenicity Test

Objective

The objective of this test is to determine whether the test substance is carcinogenic when it is administered to the animal continuously over its lifetime.

15. **Test Animals**

    1-1. Animal type and sex

    Use two or more types of male and female animals (mice, rats, etc.).
    In general, use types of animals whose natural rate of incidence for tumors under normal breeding conditions as well as sensitivity to known carcinogenic substances are well-known, animals with inbred genealogies or their first filial generation. In this case, selection of animals with a low rate of incidence for tumors is desirable.

    1-2. Age

    Use animals 5 to 6 weeks old with uniform weight.

    1-3. Number of animals

    Use 50 or more males and 50 or more females in each group.

16. **Test Substance**

    2-1. Method of administration

    As a rule, the test substance is administered orally. It is desirable to administer the test substance by adding it to food or drinking water. The concentration of the test substance added to the food for the animal must be 5W/W% or less.

However, if due to the properties of the test substance it can not be administered orally, administer it parenterally. In the case of forced administration, administer the test substance at a set time each day.

2-2. Dosage

Administer the dosage in three stages or more in order to know the relationship between dosage and response.
Carry out 1 to 3-month short-term tests in advance and take as the maximum dosage the greatest dosage amount that will maintain weight loss to around 10% compared to the control group, that does not result in poisoning-related death, and does not generate marked changes to the animal's general condition.
As a rule, median dosage and minimum dosage are established from maximum dosage using common ratio 2 or 3.
Actual intake is calculated using the animal's food intake or water intake and the concentration of the test substance.

2-3. Administration period

For most of the animal's lifetime (over 18 months for mice and hamsters, over 24 months for rats).
The death rate due to causes other than neoplastic lesions that stem from administering the test substance for 18 months for mice and hamsters and 24 months for rats must be, however, under 50%.

17. **Observation and Measurement Items**

3-1. General observation [*1]

3-2. Weight, food intake and water intake, food intake efficiency [*2]

3-3. Pathology tests

3-3-1. Observation with the naked eye [*3]

3-3-2. Observation with a microscope (carry out a test using an electronic microscope or a histochemical test as necessary) [*4]

3-4. Blood tests [*5]

3-5. Other necessary items

Study the cause of death of animals that died during the testing. Promptly put down and do an autopsy on animals whose general condition is very bad and are about to die.

*[*1]: Calculate the survival rate by observing the animal's general condition and incidence of death.*

*[*2]: It is desirable to measure water intake only when administering the test substance by mixing it into the animals' drinking water and calculate food intake efficiency during the period of growth of the animals.*

*[*3]: Do autopsies on all the animals used in the testing (including those that died or were put down during the testing period) and carry out sufficient observations with the naked eye of all internal organs and tissues. Store all of the organs and tissues described in [*4] in an appropriate storage liquid for each group.*

*[*4]: In addition to all neoplastic lesions that are visible to the eye, conduct microscopic studies for the following organs and tissues for all groups including the control group and maximum dosage group (*i).*
*For organs and tissues affected by maximum dosage, implement studies for other dosage groups as well (*ii):*
Brain, spinal cord, peripheral nerve, pituitary gland, eyeballs, nasal cavity (#), lungs (including bronchial tubes), oral cavity and tongue, esophagus, stomach, duodenum, jejunum, ileum, appendix, colon, rectum, external auditory canal, skin, salivary gland, lymph node, thyroid gland (including parathyroid gland), thymus gland, heart, liver, pancreas, spleen, kidney, adrenal gland, bladder, testicles, seminal vesicle, prostate gland, mammary gland (females), ovaries, uterus, vagina, sternum (including bone marrow), vertebrae, and femur (including the joints).
(#): In the case of inhalation test, nasal cavity, pharynx, larynx, and trachea.

*(*i): If the number of animals alive in the maximum dosage group is extremely small compared to the control group, conduct studies for the following dosage groups as well.*
*(*ii): If the maximum dosage group corresponds with the above (*i), assume the organs and tissues are those which are affected in the maximum dosage group and the following dosage groups.*
Effects that are recognized for the maximum dosage group and the following dosage groups include not just neoplastic lesions but also general toxic changes.

*[*5]: During the autopsy, create a blood smear sample for all groups and measure the blood cell count and conduct a blood biochemistry test as necessary.*

VIII. Test of *in vivo* fate

Objective

The objective of this test is to understand the behavior of the test substance inside a living organism by administering the test substance to animals and studying the substance's absorption, distribution, accumulation, metabolism, excretion, etc.

18. **Test Animals**

    1-1. Animal type and sex

    Use one or more types of male and female rats, rabbits, dogs or monkeys. For types of animals, it is desirable to use the same types as those used for other toxicity tests. Furthermore, use of two or more types of animals is desirable.

    1-2. Age

    Use young animals that have achieved maturation, and record their ages. Use animals that are young or meet some other condition as necessary.

    1-3. Number of animals

    In the case of rats, etc., use four or more males and four or more females in each group.
    In the case of dogs, monkeys, etc., two or more in each group.

19. **Test Substance**

    2-1. Method of administration

    As a rule, the test substance is forcibly administered orally.
    If oral administration poses difficulties for achieving the test objectives, administer parenterally.

    2-2. Dosage

    Administer the dosage in at least two stages if administration is a one-time event.

In this case, assume that maximum dosage is the amount by which signs of toxicity become evident as a result of repeated administration, and that minimum dosage is the amount by which effects are not manifested in animals. If possible, it is desirable to consider dosages close to the amount estimated from predicted intake given the natural environment and food.

2-3. Administration period

Administration takes place once. In addition, it is desirable to consider repeated administration over a fixed period of time.
Conduct the accumulation test via continuous administration for a sufficient period of time.

20. **Study**

For this test, administer labeled and unlabeled test substances to animals, and examine the absorption rate and absorbed amount of the test substance as well as the distribution pattern, accumulation, metabolism pattern and rate, excretion routes and rate, along with excreted amount of the test substances or major metabolites (hereinafter referred to as “test substance, etc.”) with respect to organs, tissues and body fluids. In addition, it is desirable to examine the effects of the test substance, etc. on body components and vital functions (enzyme activity) believed to be linked to the toxicity of the test substance, etc.
Furthermore, while the test will focus on *in vivo* examination, *in situ* as well as *in vitro* examinations will also be used as necessary.
Record the method of analysis and collection rate and detection limit for the test substance from biological samples.
If an isotope labeled compound is used as a test substance, assume that the greatest amount of information on metabolism may be obtained from the labeled areas, and record the method of preparation, purity, isotope concentration, specific radioactivity, etc. In addition, it is desirable to confirm whether the detected radioactivity is due to the test substance itself and to identify the chemical structure when metabolites are used.

3-1. Absorption

Estimate the absorption rate, absorbed amount, and absorption index of the test

substance in the gastrointestinal tract using either the method in 3-1-1. or 3-1-2. [*1].

3-1-1. Measure over time the residual volume of the test substance in the gastrointestinal tract, the excreted amount and residual volume of the test substance, etc. in the urine, bile, feces and exhaled air, and estimate the absorption rate, absorbed amount, and absorption index of the test substance in the gastrointestinal tract.

3-1-2. In conjunction to determining Cmax, Tmax, α1/2 and β1/2 for the concentration of the test substance, etc. in the blood (concentration in the blood, plasma or serum), compare this concentration to changes in the blood concentration of the group for which the substance was administered intravenously, and estimate the absorption rate, absorbed amount, and absorption index of the test substance in the gastrointestinal tract.

3-2. Distribution

Measure over time the concentration and amount of the test substance, etc. for as many organs and tissues as possible, and clarify the distribution pattern [*2]. In addition, calculate the biological half-life, and predict accumulation for the major organs and tissues.
Conduct studies using autoradiography as necessary.
Furthermore, a study on reversible association with plasma proteins is also desirable.

3-3. Accumulation

Study over time the accumulation of the test substance based on the results of the distribution with a focus on the organs and tissues in which possibility of accumulation exists. In addition, it is desirable to examine over time the reduction in accumulated amounts after suspension of the test substance's administration.

3-4. Metabolism

Conduct a distribution analysis for urine, feces, exhaled air, etc., isolate metabolites if the test substance is metabolized in the body, identify major metabolites, and determine their rate of production. In addition, predict the major

metabolic routes using *in vitro* tests.
It is desirable to study the bonding with biological polymers during interaction with body components whose link to toxicity has been indicated; reduction of endogenous non-protein thiol compounds in the liver, kidney, etc.; the effects on the enzyme system for metabolizing drugs.

3-5. Excretion

Measure over time the excretion of the test substance, etc. into feces, urine, exhaled air, etc. over the shorter of either a seven-day period or the period it takes for approximately 95% of the administered amount to be excreted, and determine the excretion rate and excretion index of the test substance, etc.
In addition, it is desirable to clarify the major excretion routes of the test substance, etc. [*3].

21. **Other Tests**

In cases where abnormalities believed to be caused by the test substance, etc. are confirmed via other toxicity tests and study of the behavior of the test substance in a living organism in more detail is believed to be useful based upon the explanations of the aforementioned abnormalities, it is desirable to conduct certain tests under specified conditions [*4].

*[*1]: With regard to the excretion route of the test substance, etc., it is possible to estimate the absorption rate, absorbed amount, and absorption index via comparisons with the amount excreted into urine, exhaled air, etc.*
Take note of the first-time passage effect and enterohepatic circulation.

*[*2]: Studies on the following organs and tissues shown below using radioactive isotope labeled compounds exist, although results also depend on sensitivity to the measurement method used:*
Cerebrum, cerebellum, medulla, spinal cord, sciatic nerve, eyeballs, lungs, heart, liver, spleen, stomach, small intestine, appendix, colon and rectum, pituitary gland, thyroid gland, thymus gland, adrenal gland, salivary gland, pancreas, mesenteric lymph nodes, testicles, epididymis, seminal vesicle, prostate gland, ovaries, uterus, mesentery, diaphragm, muscles, femur, fatty tissue, skin, hair, blood.

*[*3]: Take into consideration excretion of the test substance, etc. into breast milk and excretion from the skin as necessary.*

*[*4]: For example:*
1) If abnormalities are confirmed via the chronic toxicity test, along with measuring the distribution of the test substance, etc. within the body, also examine the production and distribution of active metabolites with regard to specific organs and tissues for which abnormalities have been confirmed.
2) If teratogenicity is confirmed via the teratogenicity test, administration of the test substance to pregnant animals, study of the distribution of the test substance, etc. to fetus and the placental transfer along with examination of fetus' metabolism if possible are desirable.

IX. Pharmacological Test

Objective

The objective of this test is to clarify the pharmacological properties of the test substance.

22. **Test Items**
    Conduct test on the effects on major vital functions. It is desirable to examine the effects on the functions of the organs and tissues believed to be linked to the toxic effects from the results of other toxicity tests.

23. **Test Animals**
    Select mammals and their sex that are appropriate for each test. Depending on the test, animals other than mammals may be used.

24. **Test Substance**

    3-1. Method of administration

3-1-1. In the case of *in vivo*, the test substance as a rule is administered orally. If, however, a precise observation of the effects of the test substance cannot be made if administered orally, administer it parenterally.

3-1-2. Whether it is *in vivo, in situ or in vitro*, administer the test substance as a solution using water, cooking oil or other appropriate solvents. If this is impossible, use appropriate suspending agents or emulsifying agents.

3-1-3. The test substance is usually administered once, but administer it repeatedly or continuously depending on the objective of the test.

3-2. Dosage

With respect to the relation between dosage and response, the dosage should be sufficient for gaining an understanding of the impacts derived from the test substance.

25. **Selection of Test Method**

While a wide array of test types exists, use a method that will clarify the specific actions of the test substance. At the same time, it is necessary to examine the action sites and action mechanism.