**General Tests**

**Test Methods**

In this Test Methods, "the test article etc." refers to the test article and the test sample.

Freeze-dried products not supplied with a diluent shall be tested by dissolving in physiological saline to make the volume indicated on the direct container unless otherwise specified.

**Properties Test**

The Properties Test is a method to examine the properties of the test article etc. by visual and olfactory senses.

In the tests given in 1.1 and 1.2, when the volume of small container used exceeds 20 mL or it is not made of clear and colorless glass, the test article shall be transferred to a clear and colorless glass container with a diameter of 18–20 mm before the tests.

The criterion for judgment shall be given in the monographs.

1 Test procedure

1.1 Color test

Inspect the color tone of the test article etc. against a white background under natural light or at 1,000 lux or higher under a white light source.

1.2 Transparency test

Inspect the transparency under natural light or at 1,000 lux or higher under a white light source.

In this test, transparent means no scattering of light rays by particles in the liquid, opaque means that the background is invisible, and translucent means none of the above.

1.3 Foreign matter test

Inspect foreign matters with the unaided eye against a white or black background under natural light or at 1,000 lux or higher under a white light source.

1.4 Odor test

Bring your nose to the mouth of the container and smell it.

1.5 Uniformity test for each container

For at least seven portions of final products, examine each container for its properties.

For freeze-dried products examine the properties before and after dissolution.

**Test for pH**

The Test for pH is a method to determine the hydrogen ion concentration of the test article etc. using a glass electrode system. The hydrogen ion concentration is usually represented by pH.

The criterion for judgment shall be given in the monographs.

1 Test procedure

The pH determination method specified in General Tests of the *Japanese Pharmacopoeia* shall apply.

**Vacuum Degree Test**

The Vacuum Degree Test is a method to determine the vacuum degree of dried final products sealed under reduced pressure.

1 Test procedure

All the dried final products and test samples shall be tested by non-polar discharge using a tesla coil at a distance of 5 to 10 mm in a dark place.

2 Judgment

The test results show discharge: the final products and test samples shall comply with the test.

**Test for Moisture Content**

The Test for Moisture Content is a method to measure the weight loss of the dried product of test article etc. by drying under heating and reduced pressure to determine the moisture content of the test article etc.

1 Test procedure

The test shall be performed as follows or the method specified in the guideline of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (GL26: Testing of residual moisture).

The test shall be performed at a relative humidity of not higher than 45%. Dry a weighing bottle and measure the weight of the bottle precisely. Crush the test article etc., precisely weigh approximately 100 mg of it in the weighing bottle, and use as the test material.

Place the weighing bottle in a vacuum dryer with its surface of mouth slightly opened and dry the bottle at a pressure of not more than 0.65 kPa and at 60°C for 3 hours, if necessary, over phosphorus oxide (V) or silica gel.

When drying is completed, place dried air in a vacuum dryer to return the pressure to normal level. Take out the weighing bottle and place in a desiccator containing phosphorus oxide (V) or silica gel, allow to cool to room temperature and weigh precisely.

Calculation of the moisture content

The moisture content is calculated by the following formula:

|  |  |  |
| --- | --- | --- |
| Moisture content (%) = | weight loss of the test material by drying | × 100 |
| weight of the test material before drying |

2 Judgment

The test results show that the moisture content is not more than 3% unless otherwise specified: the test article etc. shall comply with the test.

**Sterility Test**

The Sterility Test is a method to determine the absence of detectable microorganisms (bacteria or fungi) in the test article etc. by the following tests or the Sterility Test under the General Tests of the *Japanese Pharmacopoeia* unless otherwise specified.

1 Test for freedom from bacteria

1.1 Medium

Unless otherwise specified, the fluid thioglycolate medium consisting of the following components shall be used. The volume of medium used shall be not less than 15 mL per test tube .

1.1.1 Fluid thioglycolate medium

1.1.1.1 Composition

|  |  |
| --- | --- |
| L-Cystine | 0.5 g |
| Agar | 0.75 g |
| Sodium chloride | 2.5 g |
| Glucose | 5.0 g |
| Yeast extract | 5.0 g |
| Casein peptone | 15.0 g |
| Sodium thioglycolate | 0.5 g |
| 0.1 w/v% resazurin sodium solution | 1.0 mL |
| Water | 1,000 mL |

Adjust the pH so that after sterilization it is 6.9–7.3, sterilize by autoclaving at 121ºC for 15 minutes, and store in a dark place at 2ºC to 25ºC. Do not use the medium, if more than the upper one-third of the medium has acquired a pink color. A dried product of suitable quality may be used.

1.1.1.2 Suitability of the medium

The media used shall comply with the following tests:

1.1.1.2.1 Growth promotion test

When inoculated with not more than 100 CFU of *Clostridium sporogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively, or with not more than 100 CFU of *Streptococcus equi* subspecies *zooepidemicus* and *Clostridium sporogenes*, respectively, and cultured at 30ºC to 35ºC for 72 hours, clearly visible growth must be observed.

1.1.1.2.2 Sterility test

Culture portions of the media at 30–35ºC for 14 days. No growth of microorganisms shall be observed.

1.2 Culture materials

The test article and the test sample shall be used. Freeze-dried products not supplied with diluents shall be dissolved in suitable solubilizing solvent, such as phosphate-buffered saline, to make the volume specified in the Dosage and Administration.

1.3 Quantity of test article etc.

When testing the test article, unless otherwise specified, take a sufficient quantity of it from the container to perform the test. When testing the test sample, unless otherwise specified, at least 7 containers shall be tested.

1.4 Quantity of inoculum for each medium

When testing the test article, use 4 tubes of medium for each material. Inoculate 1 mL of material into two tubes, respectively, while inoculate 0.5 mL of material to other two tubes, respectively.

The test sample shall be tested according to the reference volume of each small container and quantity of inoculum for each medium, as shown in Table 1.

Table 1 Reference Volume of Small Container and Quantity of Inoculum and Number of Tubes of Medium for Each Container (Test for Freedom from Bacteria)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Reference volume | Quantity of inoculum per container | Number of tubes of medium per container | Quantity of inoculum per medium | |
| < 3 mL  3 mL≤ R < 5 mL  5 mL≤ R <10 mL  ≥ 10 mL | 1/4 quantity  1 mL  1.5 mL  3 mL | 1 tube  2 tubes  2 tubes  2 tubes | 1/4 quantity  0.5 mL  1 mL  0.5 mL  2 mL  1 mL | × 1 tube  × 2 tubes  × 1 tube  × 1 tube  × 1 tube  × 1 tube |

1.5 Culture and observation

The test article etc. shall be inoculated to a medium, mixed thoroughly, cultured at 30ºC to 35ºC for at least 14 days, and observed for bacterial growth between the 3rd day and 5thday of culture and between the 7th day and 9th day, and on the 14th day.

If the preparation causes turbidity in the medium or if otherwise necessary, subculture in a new medium on the 7th day, culture at the same temperature for at least 8 days, and observed.

1.6 Judgment

The test results show no bacterial growth: the test article shall comply with the test.

2 Test for freedom from fungi

2.1 Medium

Unless otherwise specified, fluid thioglycolate medium shall be used. For the test article etc. not including thimerosal, unless otherwise specified, soybean-casein digest broth shall be used.

The volume of medium used shall be not less than 15 mL per test tube.

2.1.1 Fluid thioglycolate medium

2.1.1.1 Composition

The composition shall be applied to 1.1.1.1.

2.1.1.2 Suitability of the medium

The medium shall comply with the following tests.

2.1.1.2.1 Growth promotion test

When inoculated with not more than 100 CFU of *Aspergillus brasiliensis*, *Bacillus subtilis* and *Candida albicans*, respectively, or with not more than 100 CFU of *Streptococcus equi* subspecies *zooepidemicus*, *Aspergillus brasiliensis* and *Candida albicans*, respectively, and cultured at 20ºC to 25ºC for five days, clearly visible growth must be observed.

2.1.1.2.2 Sterility test

The test shall be performed as specified in 1.1.1.2.2. The culture temperature shall be 20ºC to 25ºC.

2.1.2 Soybean-casein digest broth

2.1.2.1 Composition

|  |  |
| --- | --- |
| Casein peptone | 17.0 g |
| Soybean peptone | 3.0 g |
| Sodium chloride | 5.0 g |
| Dipotassium hydrogen phosphate | 2.5 g |
| Glucose | 2.3 g |
| Water | 1,000 mL |

Adjust the pH so that after sterilization it is 7.1–7.5, sterilize by autoclaving at 121ºC for 15 minutes, and store in a dark place at 2ºC to 25ºC. A dried product of suitable quality may be used.

2.1.2.2 Suitability of the medium

The suitability shall be applied to 2.1.1.2.

2.2 Culture materials

The culture materials shall be applied to 1.2.

2.3 Quantity of the test article etc.

The quantity of the test article etc. shall be applied to 1.3.

2.4 Quantity of inoculum for each medium

When testing the test article, use 4 tubes of medium for each material, and inoculate 1 mL of each material.

The test sample shall be tested according to the reference volume of each small container and quantity of inoculum for each medium, as shown in Table 2.

Table 2 Reference Volume of Small Container and Quantity of Inoculum and Number of Portions of Medium for Each Container (Test for Freedom from Fungi)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Reference volume | Quantity of inoculum per each container | Number of tubes  of medium per container | Quantity of inoculum per medium | |
| < 3 mL  3 mL≤ R < 5 mL  5 mL≤ | 1/2 volume  1 mL  2 mL | 2 tubes  2 tubes  2 tubes | 1/4 volume  0.5 mL  1 mL | × 2 tubes  × 2 tubes  × 2 tubes |

2.5 Culture and observation

The test article etc. shall be inoculated to a medium, mixed thoroughly, cultured at 20ºC to 25ºC for at least 14 days, and observed for bacterial growth between the 3rd day and 5th day of culture and between the 7th day and 9th day, and on the 14th day.

If the product causes turbidity in the medium or if otherwise necessary, subculture in a new medium on the 7th day, culture at the same temperature for at least 8 days, and observed.

2.6 Judgment

The criterion for judgment shall be applied to 1.6.

3 Retest

When doubt exists about the result for Tests 1 and 2, the test shall be repeated using at least twice the amount of the test article etc.

**Test for Freedom from Mycoplasma Contamination**

The Test for Freedom from Mycoplasma Contamination is conducted by the following method to verify the absence of Mycoplasma in the test article etc. or the method specified in the guideline of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (GL34: Testing for the detection of Mycoplasma contamination).

1 Medium

Unless otherwise specified, use broth medium for Mycoplasma with the following composition. The volume of broth medium shall be 100 mL per test tube.

1.1 Broth medium for Mycoplasma

1.1.1 Composition

1.1.1.1 Basal medium

Each 1,000 mL consists of:

|  |  |
| --- | --- |
| 50 w/v% beef heart infusion broth | 100 mL |
| Proteose peptone | 10 g |
| Sodium chloride | 5 g |
| Glucose | 1 g |
| L-monosodium glutamate | 0.1 g |
| L-arginine hydrochloride | 1 g |
| Water | Residual quantity |

Sterilize by filtration through a membrane filter with a pore size of 220 nm or sterilize by autoclaving at 121ºC for 15 minutes. Adjust the pH of the solution so that it is 7.2–7.4 after sterilization.

A dried product of suitable quality may be used.

1.1.1.2 Medium additives

To 77 mL of basal medium in 1.1.1.1, add the following additives:

|  |  |
| --- | --- |
| Horse serum | 10 mL |
| Heat-inactivated porcine serum | 5 mL |
| 25 w/v% fresh yeast extract | 5 mL |
| 1 w/v% β-nicotinamide adenine dinucleotide oxidized form | 1 mL |
| 1 w/v% L-cysteine hydrochloride test solution | 1 mL |
| 0.2 w/v% phenol red solution | 1 mL |

Previously filter sterilize the additives and aseptically add to the sterilized basal medium.

Note that the additives that can be sterilized by high pressure may be autoclaved.

Benzylpenicillin potassium, 500 units/mL of the medium, and/or Thallium acetate, 0.02 w/v%, may be added.

1.1.2 Performance

When inoculated with not more than 100 CFU of *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma hyopneumoniae* and *Mycoplasma orale*, respectively, and cultured at 35ºC to 37ºC for seven days, clearly visible growth must be observed.

1.2 Agar medium for Mycoplasma

1.2.1 Composition

1.2.1.1 Basal medium

Add 1 g of agar to 78 mL of basal medium given in 1.1.1.1.

1.2.1.2 Medium additives

Phenol red solution shall be removed from the media additives specified in 1.1.1.2.

After sterilization, 5 mL portions of media dissolved with the aid of heat shall be dispensed into sterilized Petri dishes with a diameter of 45–55 mm, cooled, solidified, and used as agar plates for Mycoplasma.

1.2.2 Performance

As specified in 1.1.2, when inoculated with not more than 100 CFU of *Mycoplasma* strains, respectively, and cultured in an air containing 5 vol% carbon dioxides at 35ºC to 37ºC for 10 days, these strains must form their own specific colonies.

2 Culture materials

The test article or the test sample shall be used. Freeze-dried productions not supplied with diluents shall be dissolved in suitable solubilizing solvent, such as phosphate-buffered saline, to make the volume specified in the Dosage and Administration. For preparations for oral administration (administration by drinking water) and preparations for administration by puncture, these preparations shall be diluted with suitable solubilizing solvent, such as phosphate-buffered saline, so that the quantity of inoculum is equal to that of a single dose.

3 Quantity of the test article etc.

For the test article, a test shall be performed per container. For the test sample, mix equal quantities of it taken from at least 2 small containers to perform the test.

4 Quantity of inoculum for each medium

A 1 mL portion of the test article shall be inoculated into 100 mL of broth medium. Then, a 0.1 mL portion of each broth medium shall be inoculated into agar plates.

5 Culture and observation

The test article etc. shall be inoculated into the broth medium, mixed thoroughly, and then cultured at 35ºC to 37ºC for 14 days. If the test article etc. includes live cells, the pH of cultured medium shall be adjusted timely.

Inoculate the agar plate for Mycoplasma with the culture medium on the 3rd, 7th, 10th, and 14th days of culture, cultured in an air containing 5 vol% carbon dioxides at 35ºC to 37ºC for 10 days to observe the plates for Mycoplasma colonies.

In this case, the plates inoculated as the control with the medium and *Mycoplasma* *synoviae* shall be observed in a similar manner.

6 Judgment

The test results show no Mycoplasma colonies in the plates inoculated with the test article etc. and medium and show colonies in the plate inoculated with *Mycoplasma* *synoviae*: the test article shall comply with the test.

If the test results show no colonies in plate inoculated with *Mycoplasma* *synoviae*, or show Mycoplasma colonies in the plate inoculated with the medium: the test shall be repeated.

7 Retest

When doubt exists about the result for tests, the test shall be repeated using at least twice the quantity of the test article etc.

**Test for Freedom from Salmonella Contamination**

Unless otherwise specified, the Test for Freedom from Salmonella Contamination is a method to examine the absence of Salmonella detectable in the test sample by the following test:

1 Medium

Unless otherwise specified, soybean-casein digest broth (SCD broth medium), selenite medium, BTB lactose agar medium (Drigalski improved medium), and DHL agar medium shall be used.

The volume of broth medium used in this test shall be 100 mL per test tube.

1.1 Soybean-casein digest broth

1.1.1 Composition

Dissolve a dried product of appropriate quality as specified and sterilize by autoclaving at 121ºC for 15 minutes. The pH after the sterilization shall be 7.1–7.3.

1.1.2 Performance

When inoculated with not more than 100 CFU of *Escherichia coli* and *Salmonella typhimurium*, respectively, and cultured at 35ºC to 37ºC for 18–24 hours, clearly visible growth must be observed.

1.2 Selenite medium

1.2.1 Composition

Dissolve a dried product of appropriate quality as specified. The pH shall be 7.1–7.3.

1.2.2 Performance

When inoculated with not more than 100 CFU of *Salmonella typhimurium* and cultured at 35ºC to 37ºC for 18–24 hours, clearly visible growth must be observed.

1.3 BTB lactose agar medium

1.3.1 Composition

Dissolve a dried product of appropriate quality as specified and sterilize by autoclaving at 121ºC for 15 minutes. The pH after the sterilization shall be 7.3–7.5.

1.3.2 Performance

When inoculated with not mor~~e~~ than 100 CFU of *Escherichia coli*, *Salmonella pullorum* and *Salmonella typhimurium*, respectively, and cultured at 35ºC to 37ºC for 18–24 hours, these microorganisms shall form their own specific colonies.

1.4 DHL agar medium

1.4.1 Composition

Dissolve a dried product of appropriate quality as specified. The pH shall be 6.9–7.1.

1.4.2 Performance

When inoculated with not more than 100 CFU of *Escherichia coli*, *Salmonella pullorum,* and *Salmonella typhimurium*, respectively, and cultured at 35ºC to 37ºC for 18–24 hours, these microorganisms shall form their own specific colonies.

2 Culture material

The test sample shall be used. Freeze-dried products not supplied with diluents shall be dissolved in suitable solubilizing solvent, such as phosphate-buffered saline, to make the volume specified in the Dose and Administration. For products for oral administration (administration by drinking water) and products for administration by puncture, these products shall be diluted with suitable solubilizing solvent, such as phosphate-buffered saline, so that the quantity of inoculum is equal to that of a single dose.

3 Quantity of the test article etc.

Mix equal quantities of the test article taken from at least two small containers to perform the test.

4 Culture and observation

The 5 mL portions of the test article etc. shall be inoculated into SCD broth medium and selenite medium, respectively, mixed thoroughly, and cultured for enrichment at 35ºC to 37ºC for 18–24 hours. A 0.1 mL portion of each culture medium shall be inoculated into BTB lactose agar medium and DHL agar medium, respectively, cultured at 35ºC to 37ºC for 18–24 hours, and observed for the presence of Salmonella colonies.

5 Judgment

The test results show that no colonies of Salmonella are observed: the test article shall comply with the test.

6 Retest

When doubt exists about the result for Tests, the test shall be repeated using at least twice the amount of the test article etc.

**Microbial Limit Test**

The Microbial Limit Test is a method to verify that the viable count of bacteria in the test article etc. is below a certain level.

1 Medium

Unless otherwise specified, heart infusion agar medium shall be used.

1.1 Heart infusion agar medium

1.1.1 Composition

Dissolve a dried product of appropriate quality as specified and sterilize in an autoclave at 121ºC for 15 minutes. The pH after the sterilization shall be 7.3–7.5.

1.1.2 Performance

When inoculated with not more than 100 CFU of *Escherichia coli* and cultured at 35ºC to 37ºC for 18–24 hours, the microorganism shall form their own specific colonies.

2 Culture materials

The test article or the test sample shall be used. For the test sample, the following preparations shall be used:

2.1 Freeze-dried product

Prepared by diluting with the supplied diluent or suitable solubilizing solvent, such as phosphate-buffered saline, so that the quantity of inoculum is equal to that of a single dose

2.2 Liquid product

Prepared by diluting with suitable solubilizing solvent, such as phosphate-buffered saline, in cases where the quantity of inoculum is not equal to that of a single dose.

3 Quantity of the test article etc.

For the test article a test shall be performed per container. For the test sample, mix equal quantities of it taken from at least two small containers to perform the test.

4 Quantity of inoculum for each medium

Dispense 1 mL portions of the test article or single-dose portions of the test sample to four Petri dishes with a diameter of 90 mm, add 15 mL portions of agar medium dissolved below 50ºC, mix thoroughly, and allow to solidify.

5 Culture and observation

Culture two of four plates at 35ºC to 37ºC for 48 hours and other two plates at 22ºC to 24ºC for four days and count the number of colonies grown.

6 Judgment

The test results indicate that the mean number of colonies at any culture temperatures is not more than 10 per 1 mL portion of the test article, while that is not more than one per single-dose portion of the test sample: the test article etc. shall comply with the test.

7 Retest

When doubt exists about the test results, the test shall be repeated using at least twice the number of Petri dishes for the test article etc. or using twice the number of containers for the test sample.

**Test for Freedom from Extraneous Viruses for Live Vaccines and Sera**

The Test for Freedom from Extraneous Viruses for Live Vaccines and Sera is a method to verify that no extraneous viruses in the test material are detected in live vaccines and sera.

1 Test material

1.1 Live vaccine

Unless specified in the monographs, the test article and liquid product shall be used as the test materials as they are. If the test sample is freeze-dried, dissolve in the supplied diluent or phosphate-buffered saline. To the dissolved test sample, add each immune serum specified in monographs, allow to neutralize at 37ºC for one hour or at 4ºC overnight, neutralize completely, and use as the test material.

Unless specified in the individual monographs, adjust the quantity of test material to be inoculated once so that it contains viral amount equivalent to the amount for one-dose before neutralization.

1.2 Sera, etc.

The test article etc. shall be dialyzed at 2ºC to 5ºC overnight using at least a 100-fold volume of phosphate-buffered saline to remove the preservative, and it shall be used as the test material.

2 Test procedure

2.1 Inoculation test using embryonated chicken egg

2.1.1 Inoculation into allantoic cavity

2.1.1.1 Observation of embryo

2.1.1.1.1 Test procedure

As specified in 1.1 in the Materials for Live Vaccine Production, embryonated chicken eggs aged 9–11 days shall be used. The 0.1 mL portions of the test material shall be injected into the allantoic cavity of 10 embryonated chicken eggs and cultured at 37ºC for seven days. On the final day of culture, the eggs shall be opened to examine abnormalities of the chicken embryos.

2.1.1.1.2 Judgment

The chicken embryos are properly developed, and no abnormalities are observed: the test material shall comply with the test.

2.1.1.2 Hemagglutination test

2.1.1.2.1 Test procedure

To allantoic fluid collected on the final day of culture given in 2.1.1.1, an equal quantity of 0.5 vol% chicken red blood cell suspension shall be added, allowed to stand at 4ºC for 60 minutes, and observed for the presence of hemagglutination.

2.1.1.2.2 Judgment

No hemagglutination is observed in the allantoic fluid: the test material shall comply with the test.

2.1.2 Inoculation onto chorioallantoic membrane

2.1.2.1 Test procedure

As specified in 1.1 in the Materials for Live Vaccine Production, embryonated chicken eggs aged 10–12 days shall be used. The 0.1 mL portions of the test material shall be inoculated onto the chorioallantoic membrane of 10 embryonated chicken eggs and cultured at 37ºC for five days. On the final day of culture, these eggs shall be opened to examine abnormalities of chicken embryos and chorioallantoic membranes.

2.1.2.2 Judgment

The chicken embryos are properly developed, and no abnormalities are observed in the chorioallantoic membranes: the test material shall comply with the test.

2.2 Inoculation test using cells derived from chicken

2.2.1 Inoculation test using chicken kidney cells

2.2.1.1 Observation of cultured cells

2.2.1.1.1 Test procedure

As specified in 2.2.1 in the Materials for Live Vaccine Production, primary cells of chicken kidneys shall be used. These cells shall be cultured in at least four Petri dishes with a size of not less than 20 cm2. The 0.2 mL portions of the test material shall be inoculated and cultured at 37ºC for seven days. The culture suspension and cells shall be harvested, and 0.2 mL portions shall be inoculated and passage into the cultured cells in at least four Petri dishes, cultured at 37ºC for seven days, and observed for the presence of CPE.

2.2.1.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.2.1.2 Hemadsorption test

2.2.1.2.1 Test procedure

On the final day of observation given in 2.2.1.1, the culture suspension shall be removed from the cultured cells in the Petri dishes, overlaid with 0.1 vol% chicken red blood cell suspension, allowed to stand at 4ºC for 60 minutes, and observed microscopically for the presence of hemadsorption.

2.2.1.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.2.2 Inoculation test using chicken embryonic cells

2.2.2.1 Observation of cultured cells

2.2.2.1.1 Test procedure

As specified in 2.1.1 in the Materials for Live Vaccine Production, primary cells of chicken embryo susceptible to avian leukosis virus shall be used. These cells shall be cultured into at least eight Petri dishes with a size of not less than 20 cm2, and the 0.2 mL portions of the test material shall be inoculated within 24 hours and cultured at 37ºC. These cells shall be passaged every three to five days to the third passage, and the cells of each passage shall be observed for the presence of CPE.

2.2.2.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.2.2.2 Hemadsorption test

2.2.2.2.1 Test procedure

For the third-passaged cells in at least four Petri dishes given in 2.2.2.1, culture suspension shall be removed and overlaid with 0.1 vol% chicken red blood cell suspension, allowed to stand at 4ºC for 60 minutes, and observed for the presence of hemadsorption.

2.2.2.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.2.2.3 COFAL test

2.2.2.3.1 Test procedure

After washing the third-cultured cells in four Petri dishes given in 2.2.2.1, add one-twentieth volume of culture medium of Veronal-buffered saline containing 1 w/v % gelatin. Freeze-thaw the harvested cell suspension three times and collect centrifugal supernatant to prepare the cell extract. Using this as an antigen, perform a COFAL test by the Kolmer’s method in pigeon, rabbit, or hamster serum specific to avian leukosis virus, three units of hemolytic elements, four units of guineapig complements and 2 vol% sheep erythrocytes.

2.2.2.3.2 Judgment

The COFAL test indicates that the cell extract is negative for the virus: the test material shall comply with the test.

2.2.2.4 Test for freedom from Reticuloendotheliosis virus

2.2.2.4.1 Test procedure

The test material given in 2.2.2.1.1 shall be inoculated. The cultured primary cell suspension shall be inoculated into a newly cultured primary cells of chicken embryos and cultured at 37ºC for four days. When the cell suspension is passaged to the third generation, the cell suspension shall be inoculated into the cultured cells in at least four Petri dishes with cover glasses and cultured at 37ºC for four days, tested by fluorescence antibody assay with anti-reticuloendotheliosis virus serum, and then observed.

2.2.2.4.2 Judgment

No specific fluorescent antigens are observed in the cultured cell: the test material shall comply with the test.

2.3 Inoculation test using cells derived from swine

2.3.1 Inoculation test using swine kidney cells

2.3.1.1 Observation of cultured cells

2.3.1.1.1 Test procedure

Primary or passaged swine kidney cells shall be used.

A 4 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 37ºC for five days, and observed for the presence of CPE. Then, the cells shall be passaged to the next generation, cultured at 37ºC for seven days, and observed for the presence of CPE.

2.3.1.1.2 Judgment

No CPE is observed in the cultured cells during the observation period: the test material shall comply with the test.

2.3.1.2 Hemadsorption test

2.3.1.2.1 Test procedure

On the final day of observation in 2.3.1.1, the cell surfaces shall be washed twice with phosphate-buffered saline. The cultured cells shall be divided into three groups, overlaid with 0.1 vol% red blood cell suspensions of guinea pig, goose, and chicken aged 7 days or younger, respectively, allowed to stand at 4ºC for 60 minutes and at 37ºC for 30 minutes, and observed microscopically for the presence of hemadsorption.

2.3.1.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.3.1.3 Test for freedom from Classical swine fever virus

The test shall be performed as specified in 2.3.1.3.1 or 2.3.1.3.2 as follows:

2.3.1.3.1 Fluorescent antibody assay

2.3.1.3.1.1 Test procedure

Culture supernatant shall be collected on the 5th day of inoculation of the test material given in 2.3.1.1.1, a 1 mL portion shall be inoculated into the cells in 2.3.1.1 cultured on a cover glass with a size of 3 cm2 or larger, cultured at 37ºC for 24–48 hours, tested with anti-classical swine fever virus serum by fluorescent antibody assay, and then observed.

2.3.1.3.1.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

2.3.1.3.2 END method and interference method

2.3.1.3.2.1 Test procedure

On the 5th day of inoculation of the test material given in 2.3.1.1.1, culture supernatant shall be collected, the 0.1 mL portions of it shall be dispensed into at least 20 small test tubes (wells), and the 0.5 mL portions of primary cells of swine testis suspended in the cell growth medium shall be added. After allowing to culture at 37ºC for four days, the cultured cells shall be divided into two groups and tested by the END method and interference method using the WEE virus.

2.3.1.3.2.2 Judgment

No Classical swine fever virus is observed in the cultured cells: the test material shall comply with the test.

2.3.1.4 Test for freedom from Porcine circovirus

2.3.1.4.1 Test procedure

PPK-3F cells shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2per 1 mL of the inoculum, treated with glucosamine, cultured at 37ºC for five days, and passaged to the next generation. The passaged cells shall be cultured at 37ºC for four days, passaged to the next generation, and the passaged cells shall be cultured at 37ºC for three days, and tested with anti-porcine circovirus serum by fluorescent antibody assay.

2.3.1.4.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

2.3.2 Inoculation test using swine testis cells

2.3.2.1 Observation of cultured cells

2.3.2.1.1 Test procedure

Primary or passaged cells of swine testis shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 37ºC, and observed for 10 days.

2.3.2.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.3.2.2 Hemadsorption test

2.3.2.2.1 Test procedure

On the final day of observation in 2.3.2.1, the cell surfaces shall be washed twice with phosphate-buffered saline. The cultured cells shall be divided into three groups, overlaid with 0.1 vol% red blood cell suspensions of guinea pig, goose, and chicken aged 7 days or younger, respectively, allowed to stand at 4ºC for 60 minutes and at 37ºC for 30 minutes, and observed microscopically for the presence of hemadsorption.

2.3.2.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.4 Inoculation using cells derived from bovine

2.4.1 Inoculation test using bovine kidney cells

2.4.1.1 Observation of cultured cells

2.4.1.1.1 Test procedure

Passaged cells of bovine kidney shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2per 1 mL of the inoculum, cultured at 34ºC to 36ºC for five days, observed for the presence of CPE, and passaged to the next generation. When the cells are grown as monolayers, the cell surfaces shall be washed with phosphate-buffered saline. To the cells, culture medium for maintenance shall be added, cultured at 37ºC for five days, and observed for the presence of CPE.

2.4.1.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.4.1.2 Hemadsorption test

2.4.1.2.1 Test procedure

On the final day of observation in 2.4.1.1, the cultured cells shall be divided into two groups, overlaid with 0.1 vol% guinea pig and goose red blood cell suspensions, respectively, allowed to stand for 60 minutes, and observed microscopically for the presence of hemadsorption.

2.4.1.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.4.2 Inoculation test using bovine testis cells

2.4.2.1 Test procedure

Passaged bovine testis cells shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 34ºC to 36ºC for five days, and observed for the presence of CPE. Then, these cells shall be passaged in 10 small test tubes, cultured for five days, and observed for the presence of CPE. After removing the culture suspension, a 0.5 mL portion of culture medium for maintenance containing approximately 105TCID50/mL of Bovine viral diarrhea virus Nose strain shall be added to each test tube, roller-cultured at 34ºC to 36ºC for seven days, and observed for the presence of CPE.

2.4.2.2 Judgment

During the observation period, CPE is not observed in the cultured cells before inoculation with Bovine viral diarrhea virus, but CPE is observed in the cultured cells after inoculation with the virus: the test material shall comply with the test.

2.5 Inoculation test using cells derived from canine

2.5.1 Inoculation test using canine kidney cells

2.5.1.1 Observation of cultured cells

2.5.1.1.1 Test procedure

Primary or passaged canine kidney cells shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 36ºC for five days, and observed for the presence of CPE. Then, these cells shall be passaged to the next generation, cultured at 36ºC for 10 days, and observed for the presence of CPE.

2.5.1.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.5.1.2 Hemadsorption test

2.5.1.2.1 Test procedure

On the final day of observation in 2.5.1.1, after removing the culture suspension, the cells shall be overlaid with 0.1 vol% guinea pig red blood cell suspensions, allowed to stand at 4ºC for 60 minutes, and observed microscopically for the presence of hemadsorption.

2.5.1.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.6 Inoculation test using cells derived from feline

2.6.1 Inoculation test using feline kidney cells

2.6.1.1 Observation of cultured cells

2.6.1.1.1 Test procedure

Primary or passaged cells of feline kidney shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 36ºC for five days, and observed for the presence of CPE. Then, these cells shall be passaged to the next generation, cultured at 36ºC for 10 days, and observed for the presence of CPE.

2.6.1.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.6.1.2 Test for freedom from Canine parvovirus and Feline panleukopenia virus

2.6.1.2.1 Test procedure

To the culture medium obtained on the final day of observation given in 2.6.1.1, add an equal quantity of borate buffered saline containing 0.2 w/v% bovine serum albumin.

Additionally, add 0.5 vol% swine red blood cells prepared with a quantity of VAD 6.0 solution equal to that of the mixture, allow to stand at 4ºC for 18 hours, and observe for the presence of hemagglutination.

2.6.1.2.2 Judgment

No hemagglutination is observed: the test material shall comply with the test.

2.7 Inoculation test using cells derived from other animals

2.7.1 Inoculation test using guinea pig kidney cells

2.7.1.1 Observation of cultured cells

2.7.1.1.1 Test procedure

Primary cells of guinea pig kidney shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 37ºC for 10 days, and observed for the presence of CPE.

2.7.1.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.7.1.2 Hemadsorption test

2.7.1.2.1 Test procedure

On the final day of observation in 2.7.1.1, the cell surfaces shall be washed twice with phosphate-buffered saline. The cultured cells shall be divided into three groups, overlaid with 0.1 vol% red blood cell suspensions of guinea pig, goose, and chicken aged 7 days or younger, respectively, allowed to stand at 4ºC for 60 minutes and at 37ºC for 30 minutes, and observed microscopically for the presence of hemadsorption.

2.7.1.2.2 Judgment

No hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.7.2 Inoculation test using MA-104 cells

2.7.2.1 Test for freedom from Rotavirus

The test shall be performed as specified in 2.7.2.1.1 or 2.7.2.1.2 as follows:

2.7.2.1.1 Observation of cultured cells

2.7.2.1.1.1 Test procedure

A 0.1 mL portion of the test material shall be inoculated in at least 10 test tubes of cultured cells and allowed to adsorb at 37ºC for 60 minutes. The cell surfaces shall be washed with phosphate-buffered saline. Culture medium containing an appropriate amount of trypsin shall be added, roller-cultured at 37ºC for seven days, and observed for the presence of CPE.

2.7.2.1.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

2.7.2.1.2 Fluorescent antibody assay

2.7.2.1.2.1 Test procedure

A 0.1 mL portion of the test material shall be inoculated into the cells cultured on a cover glass with a size of 3 cm2 or larger, cultured at 37ºC for 48 hours, tested with anti-rotavirus serum by fluorescent antibody assay, and then observed.

2.7.2.2.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

2.8 Animal inoculation test

2.8.1 Cattle or sheep inoculation test

2.8.1.1 Test for freedom from Bovine leukemia virus

2.8.1.1.1 Test procedure

Healthy cattle weighing 100–200 kg or healthy sheep weighing 30–50 kg shall be used.

A 10 mL portion of the test material shall be intramuscularly injected into one cattle or sheep, and serum shall be obtained through blood sampling in the 2nd and 3rd months. The obtained serum shall be tested by passive hemagglutination assay, enzyme immunoassay, or agar gel immunoprecipitation assay to detect the bovine leukemia virus antibody.

2.8.1.1.2 Judgment

No antibody against bovine leukemia virus is detected: the test material shall comply with the test.

2.8.2 Suckling mice inoculation test

2.8.2.1 Test procedure

Mice aged 3 days or younger shall be used.

The 0.02 mL portions of the test material shall be injected into the brain of 10 mice.

The centrifugal supernatant of pooled brain mixed emulsion collected on the 5th day shall subsequently be injected into the brain of 10 mice, and observed for 10 days.

2.8.2.2 Judgment

No mice show death or neurological symptoms: the test material shall comply with the test.

**Test for Thimerosal Content**

The Test for Thimerosal Content is a method to determine the thimerosal content of the test article etc. from the absorbance of the chelate compound with maximum specific absorption at a wavelength of 480 nm formed by the reaction of thimerosal with dithizone in a sulfuric acid solution.

1 Standard solution and test solutions

1.1 200 μg/mL thimerosal standard solution

Weigh precisely 20 mg of thimerosal and add water to make exactly 100 mL.

1.2 1 mol/L sulfuric acid test solution

Add 20 mL of sulfuric acid in 340 mL of water.

1.3 Dithizone test solution

Dissolve 2 mg of dithizone in carbon tetrachloride to make 100 mL.

If necessary, add 100 mL of 100-fold diluted ammonia solution (28), transfer dithizone to the water layer, wash several times with 20–30 mL of carbon tetrachloride, neutralize with hydrochloric acid, and transfer dithizone to carbon tetrachloride layer to adjust the concentration is 0.002 w/v%.

1.4 Ammonia test solution

Add water to 60 mL of ammonia solution (28) to make 100 mL.

2 Test procedure

Pipet 2.5, 5 and 7.5 mL of thimerosal standard solution, add water to make exactly 10 mL, and use as the standard dilutions of 50, 100 and 150 µg in 1 mL, respectively.

Pipet 0.5 mL of the test article etc., add water to make exactly 5 mL, and use as the test material.

Proceed with the standard solutions as directed above.

To these solutions, add 5 mL portions of 1 mol/L sulfuric acid test solution and 10 mL portions of dithizone test solution, and shake for 5 minutes vigorously. Allow to stand, and then collect the resulting carbon tetrachloride layer. Shake with 10 mL of water and allow to stand. Discard the resulting water layer, shake the rest with 10 mL of the ammonia test solution, and allow to stand and discard the water layer. Repeat this procedure of washing three times; then add 10 mL of water, shake, and mix.

Discard the resulting water layer and filter the remaining carbon tetrachloride layer through a filter paper. Measure the absorbance of the filtrate at a wavelength of 480 nm.

Since mercury dithizonate is unstable in direct sunlight and heat, extract at a liquid temperature below 20ºC, kept in a dark place, and measure the absorbance as quickly as possible.

Prepare a calibration curve from the absorbance of the standard dilutions and insert the absorbance of the test material in the calibration curve to calculate the thimerosal content of the test article etc. Correct by measuring the absorbance of water treated in the same manner.

3 Judgment

Unless otherwise specified, the test results indicate that the thimerosal content is not more than 0.01 w/v%: the test article etc. shall comply with the test.

**Test for Phenol Content**

The Test for Phenol Content is a method to determine the phenol content of the test article etc. from its absorbance at a wavelength of 550 nm by utilizing the color development by the reaction with 4-nitroaniline and nitrous acid.

The criterion for judgment shall be given in the monographs.

1 Standard solution and test solutions

1.1 5 mg/mL phenol standard solution

Weigh precisely 0.5 g of phenol and add water to make exactly 100 mL.

1.2 4-nitroaniline-sodium nitrite test solution

Before use, add 0.75 mL of sodium nitrite test solution to 25 mL of 4-nitroaniline test solution as below and mix thoroughly to prepare the solution.

1.2.1 4-nitroaniline test solution

Dissolve 1.5 g of hydrochloric acid in 40 mL of 4-nitroaniline and add water to make 500 mL. If necessary, heat in a water bath.

1.2.2 sodium nitrite test solution

Dissolve 10 g of sodium nitrite in water to make 100 mL.

1.3 50 w/v% sodium acetate test solution

Dissolve 50 g of sodium acetate trihydrate in water to make 100 mL.

1.4 sodium carbonate test solution

Dissolve 10.5 g of anhydrous sodium carbonate in water to make 100 mL.

2 Test procedure

Pipet 1, 2, 3, 4, and 5 mL of phenol standard solutions, add water to make exactly 5 mL, and use as standard dilutions at concentrations of 1, 2, 3, 4, and 5 mg/mL.

Pipet 1 mL each of the test article etc. and standard solutions, add water to make exactly 50 mL, and use as the test materials.

For the test article etc., if necessary, pipet a 1 mL portion and add water to make approximately 10 mL. To this solution, add approximately 10 mL of 5 w/v% trichloroacetic acid solution, and add water to make exactly 50 mL. Allow to stand at room temperature for 30 minutes, filter the solution, and use the filtrate obtained as the test materials.

Pipet 1 mL each of the test materials and add water to make 30 mL. To these solutions, add 1 mL of a 50 w/v% sodium acetate solution and then 1 mL of 4-nitroaniline-sodium nitrite test solution and shake thoroughly. Add 2 mL of the sodium carbonate test solution and water to make 50 mL and then shake thoroughly. Allow to stand at room temperature for 10 minutes. Take portions of the mixtures to measure absorbance at a wavelength of 550 nm immediately.

Prepare a calibration curve from the absorbance of the standard dilutions and insert the absorbance of the test material in the calibration curve to calculate the phenol content of the test article etc. Compensate by separately measuring the absorbance of water treated in the same manner for reference.

**Test for Formalin Content**

The Test for Formalin Content is a method to determine the formalin content of the test article etc. from its absorbance at a wavelength of 410 nm by means of the property that the formaldehyde contained in the formalin gives the yellowish orange color of 3.5-diacetyl-1.4-dihydrolutidine resulting from the reaction with acetylacetone in the presence of excess ammonia under slightly acidic conditions. The formalin content shall be determined by the following method or the method specified in the guideline of International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (GL25: Testing of residual formaldehyde).

The criterion for judgment shall be given in the monographs.

1 Standard solution and test solutions

1.1 Formalin standard solution

Precisely dilute formalin 500-fold with water.

1.2 Acetic acid-ammonium acetate buffer solution (pH 6.25)

Mix 40 mL of the following acetic acid solution and 1,000 mL of ammonium acetate solution and store in a dark place.

1.2.1 Acetic acid solution

Add water to 12.9 mL of acetic acid (100) to make 100 mL.

1.2.2 Ammonium acetate solution

Dissolve 173.4 g of ammonium acetate in water to make 1,000 mL.

1.3 Acetylacetone test solution

Mix 7 mL of acetylacetone and 14 mL of ethanol (99.5) and add water to make 1,000 mL.

2 Test procedure

Dilute the test article etc. accurately with water so that it contains 0.01–0.05 % formalin and use as the test materials. Pipet 0.5, 1, 1.5, 2, and 2.5 mL of formalin standard solutions accurately, and add water to make exactly 10 mL, respectively. Use these dilutions as 0.01, 0.02, 0.03, 0.04, and 0.05 vol% standard dilutions.

Pipet 0.1 mL each of the test material and standard dilutions and add 2 mL of acetic acid-ammonium acetate buffer solution to these dilutions, respectively. Add 2 mL of acetylacetone test solution and mix, heat at 60ºC for 15 minutes, and then cool for 5 minutes in cold water. Allow to stand for 20 minutes and measure the absorbance at a wavelength of 410 nm.

Prepare a calibration curve from the absorbance of the standard dilutions, and insert the absorbance of the test material in the calibration curve to calculate the formalin content of the test article etc. Compensate by separately measuring the absorbance of water treated in the same manner for reference.

**Test for Aluminum Content**

The Test for Aluminum Content is a method to determine the aluminum content of the test article etc. from the absorbance at a wavelength of 510 nm by utilizing the color development of chelate compound formed by the reaction of stilbazo with the insoluble salt of aluminum contained in the test article etc. which is dissolved in nitric acid.

The criterion for judgment shall be given in the monographs.

1 Standard solution and test solutions

1.1 Aluminum standard solution (4 μg/mL)

Weigh precisely 895 mg of aluminum (III) chloride hexahydrate, add water to make exactly 100 mL. Pipet 2 mL of the solution and add water to make exactly 500 mL.

1.2 1 mol/L acetate buffer (pH 5.55–5.75)

Add nine parts of 1 mol/L sodium acetate solution to one part of 1 mol/L acetic acid solution as below and mix.

1.2.1 1 mol/L acetic acid solution

Add water to 6.0 mL of acetic acid (100) to make 100 mL.

1.2.2 1 mol/L sodium acetate solution

Dissolve 13.6 g of sodium acetate trihydrate in water to make 100 mL.

1.3 Stilbazo test solution

Weigh approximately 60 mg of stilbazo and grind it in a mortar. Add water to make 100 mL and filter and use the filtrate. However, when pipet 1 mL of the filtrate, add 10 mL of 1 mol/L acetate buffer and 14 mL of water to the filtrate and allow to stand at approximately 25°C for 20 minutes,the absorbance of the mixture at 420 nm shall be 0.85 or more. Store the solution in a dark place, protected from sunlight, and use within 2 weeks after preparation.

2 Test procedure

Shake the test article etc. to make a homogeneous suspension and pipet 1 mL of the suspension. To this suspension, add 0.2 mL of nitric acid solution and boil to dissolve. Dilute the test article etc. with water so that the aluminum content is not more than 4 μg in 1 mL and use as the test material.

Dilute the aluminum standard solution with water to make 2 μg/mL and 4 μg/mL standard dilutions.

Pipet 1 mL each of the test dilutions and the standard dilutions. To the test solutions, add 2.5 mL of water, 1 mL of 1 mol/L acetic acid buffer, and 0.5 mL of the stilbazo test solution accurately. Allow the mixtures to stand at room temperature for 20 minutes and then determine the absorbance at a wavelength of 510 nm with a spectrophotometer immediately.

Prepare a calibration curve from the absorbance of the standard solution, insert the measurement of the test material in the calibration curve to calculate the aluminum content in the test material and calculate the aluminum content in each mL of the test article.

Compensate by separately measuring the absorbance of water treated in the same manner for reference.

**Test for Macrogol Content**

The Test for Macrogol Content is a method to determine the macrogol content of the test article etc. from its absorbance at 470 nm of the yellowish orange color of precipitated complex salt formed by the addition of phosphomolybdic acid to a polyoxyethylene nonionic surfactant and treatment with ammonium thiocyanate and tin (II) chloride.

The criterion for judgment shall be given in the monographs.

1 Standard solution and test solutions

1.1 Macrogol standard solution (10 μg/mL)

Weigh precisely 200 mg of Macrogol 4,000 or Macrogol 6,000 and dissolve in water to make exactly 1,000 mL. Pipet exactly 5 mL of this solution and add water to make exactly 100 mL.

1.2 Barium chloride test solution

Dissolve 10 g of barium chloride in water to make exactly 100 mL.

1.3 Phosphomolybdic acid test solution

Dissolve 10 g of phosphomolybdic acid in water to make 100 mL.

1.4 Ammonium thiocyanate test solution

Dissolve 10 g of ammonium thiocyanate in water to make 100 mL.

1.5 Tin (II) chloride test solution

Dissolve 2 g of tin (II) chloride dihydrate in 10 mL of hydrochloric acid and add water to make 100 mL. Filter if necessary.

1.6 Dilute hydrochloric acid test solution

Add water to 23.6 mL of hydrochloric acid to make 100 mL.

2 Test procedure

Take exactly an appropriate volume of the test article etc., add the equal volume of acetone, and allow to stand at room temperature for 10–15 minutes. For the period of time, agitate occasionally. Centrifuge it at 3,000 rpm for 10 minutes, dilute the supernatant with water so that it contains 10–50 μg of Macrogol in 1 mL, and use as the test material.

Pipet exactly 1, 2, 3, 4, and 5 mL of macrogol standard solution, add water to each portion of it to make exactly 5 mL, and use as the standard dilutions at concentrations of 2, 4, 6, 8 and 10 μg/mL. Pipet exactly 1 mL of the test material and add water to make exactly 5 mL. To this portion of it and each standard dilution, add three drops of dilute hydrochloric acid test solution, two drops of barium chloride test solution, and two drops of phosphomolybdic acid test solution, mix thoroughly, and allow to react at 40℃ for 10 minutes. Centrifuge for 10 minutes, discard the supernatant, and wash by centrifuge several times. If necessary, invert the centrifuge tube for 1–2 minutes to remove water. Add 1.2 mL of sulfuric acid to each solution, and heat to dissolve the precipitate. Add water to make 6 mL. Add 1 mL of an ammonium thiocyanate test solution and 0.5 mL of a tin (II) chloride test solution, add water to make exactly 10 mL, and mix thoroughly. After 20 minutes, measure the absorbance at 470 nm.

Prepare a calibration curve from the absorbance of the standard dilutions, and insert the absorbance of the test material in the calibration curve to calculate the macrogol content of the test article etc. Correct by measuring the absorbance of water treated in the same manner.

**Test for Protein Nitrogen Content**

The Test for Protein Nitrogen Content is a method to determine the protein nitrogen content of the test article etc. by measuring nitrogen in heated trichloroacetic acid-precipitable protein in the test article by the micro-Kjeldahl method.

The criterion for judgment shall be given in the monographs.

1 Test solutions and reagents

1.1 50 w/v% trichloroacetic acid test solution

Dissolve 500 g of trichloroacetic acid in water to make 1,000 mL.

1.2 5 w/v% trichloroacetic acid test solution

Dilute the test solution in 1.1 tenfold with water.

1.3 1 mol/L sodium hydroxide test solution

Dissolve 4.0 g of sodium hydroxide in water to make 100 mL.

1.4 Decomposition accelerator

Use a powdered mixture of 100 g of potassium sulfate and 10 g of copper (II) sulfate pentahydrate.

1.5 30 w/v% sodium hydroxide test solution

Dissolve 300 g of sodium hydroxide in water to make 1,000 mL.

1.6 Indicator

Dissolve 0.15 g of bromocresol green and 0.1 g of methyl red in 90 vol% ethanol to make 200 mL.

1.7 Boric acid test solution

Dissolve 40 g of boric acid in water to make 1,000 mL.

1.8 0.005 mol/L sulfuric acid (standard solution for volumetric analysis)

Contain 0.4904 g of sulfuric acid in 1,000 mL.

Preparation of 0.05 mol/L sulfuric acid

After adding 3 mL of sulfuric acid to an appropriate volume of water, make it 1,000 mL with water and standardize the solution as follows:

Standardization of 0.05 mol/L sulfuric acid

Heat sodium carbonate at 500℃ to 650℃ for 40–50 minutes, allow to cool in a desiccator (silica gel), weigh precisely approximately 0.08 g of it, dissolve in 30 mL of water, add three drops of methyl red standard solution, titrate with the prepared sulfuric acid, and calculate the normality factor. The endpoint shall set boil the content carefully, stopper the flask loosely, allow to cool, and continue the titration until the color of the solution changes to persistent orange to orange-red.

0.05 mol/L sulfuric acid 1mL = 5.299 mg of Na2CO3

Preparation of 0.005 mol/L sulfuric acid

Before use, dilute 0.05 mol/L sulfuric acid with water to make exactly 10 times the initial volume.

2 Test procedure

Pipet exactly a quantity of the test article etc. corresponding to 10–200 μg of protein nitrogen, add one-tenth of its volume of a 50 w/v% trichloroacetic acid test solution, heat in a boiling water bath for 15 minutes, and allow to cool to room temperature. With regard to antitoxins and sera listed in the monograph of drugs, this heat treatment is replaced by warming at an appropriate temperature for 15 minutes. Centrifuge the mixture, add an appropriate amount of a 5 w/v% trichloroacetic acid solution to the precipitate, and wash by centrifuge. Then, dissolve the precipitate in a small volume of 1 mol/L sodium hydroxide test solution to serve as the test material.

Place approximately 70 mg of decomposition accelerator and test material in a decomposition flask and pour 1 mL of sulfuric acid along the inner wall of the flask. Heat for approximately 5 hours to decompose until the solution changes to a clear blue, and the inside wall of the flask is free from a carbonaceous material. During the decomposition process, if necessary, add hydrogen peroxide (30) in the decomposition flask. After allowing to cool, add approximately 5 mL of water to cool the solution and rinse in the distillation flask washed beforehand by passing steam through it. Place 6 mL of a 30 w/v% sodium hydroxide test solution in the distillation flask.

To the absorption flask, add 5 mL of a boric acid test solution and some drops of the indicator to immerse the lower end part of the condenser tube.

Pass stream through the distillation flask and continue until the distillate measures 70 to 80 mL. Remove the absorption flask from the lower end of the condenser tube, rinse the lower end part with a small quantity of water, and titrate the distillate with 0.005 mol/L sulfuric acid until the color of the solution changes from green through pale grayish blue to pale grayish red-purple. Perform a blank determination in the same manner with water and make any necessary correction.

1 mL of 0.005 mol/L sulfuric acid = 140.07 μg of N

**Test for Freedom from Abnormal Toxicity**

The Test for Freedom from Abnormal Toxicity is a method by conducting the following tests 1 and 2 to confirm that the injection of the test article etc. into the experimental animals causes no abnormalities:

1 Test on guinea pigs

1.1 Test materials

1.1.1 Injection materials

The test article etc. shall be used.

1.1.2 Test animals

Guinea pigs weighing approximately 350 g that show no abnormal signs and a normal increase in body weight during an observation period of at least four days prior to use shall be used.

1.2 Test procedure

To two guinea pigs, the 5 mL portions of the injection material shall be given by intraperitoneal injection, and the animals shall be observed for seven days after the injection. Regarding those approved in particular, however, follow the approved observation period.

1.3 Judgment

None of the animals shall show any abnormal signs during the observation period: the test article etc. shall comply with the test.

2 Test on mice

2.1 Test materials

2.1.1 Injection materials

The test article etc. shall be used.

2.1.2 Test animals

Mice aged approximately 5 weeks that show no abnormal signs and a normal increase in body weight during an observation period of at least four days prior to use shall be used.

2.2 Test procedure

To 10 mice, the 0.5 mL portions of the injection material shall be given by intraperitoneal injection, and the animals shall be observed for seven days after the injection. Regarding those approved in particular, however, follow the approved observation period.

2.3 Judgment

None of the animals shall show any abnormal signs during the observation period: the test article etc. shall comply with the test.

**Toxicity Limit Test**

The Toxicity Limit Test is a method by conducting either of the following tests 1 or 2 to confirm that the injection of the test article etc. into the experimental animals causes no safety concerns.~~:~~

1 Test on mice

1.1 Test materials

1.1.1 Injection materials

The test article etc. shall be used.

1.1.2 Test animals

Mice aged approximately 5 weeks that show no abnormal signs and a normal increase in body weight during an observation period of at least four days prior to use shall be used.

1.2 Test procedure

To 10 mice, the 0.5 mL portions of the injection material shall be given by intraperitoneal injection, the body weight shall be measured before the injection and on Day 3 of injection, and the animals shall be observed for seven days after the injection.

1.3 Judgment

If the body weight on Day 3 of injection shall be no less than that measured before the time of injection, and none of the animals shall show any abnormal signs during the observation period: the test article etc. shall comply with the test.

2 Test on guinea pigs

2.1 Test materials

2.1.1 Injection materials

The test article etc. shall be used.

2.1.2 Test animals

Guinea pigs weighing approximately 350 g that show no abnormal signs and a normal increase in body weight during an observation period of at least four days prior to use shall be used.

2.2 Test procedure

To 5 guinea pigs, the 5 mL portions of the injection material shall be given by intraperitoneal injection, the body weight shall be measured before the injection and on Day 3 of injection, and the animals shall be observed for seven days after the injection.

2.3 Judgment

If the body weight on Day 3 of injection shall be no less than that measured before the time of injection, and none of the animals shall show any abnormal signs during the observation period: the test article etc. shall comply with the test.

**Test for Freedom from Extraneous Viruses**

The Test for Freedom from Extraneous Viruses is a method to verify that no detectable extraneous viruses are observed in the master seed virus, master cell seed and master seed coccidia.

1 The test material

1.1 Master seed virus

To a 1 mL portion of the test article, add immune serum, neutralize at 37ºC for 1 hour or at 4ºC overnight, neutralize completely, and use as the test material.

1.2 Master cell seed

Divide the cultured cells grown as monolayers into two groups for culture. Freeze-thaw the cells in one group three times. With the freeze-thawed mixture, mix the culture supernatant from the other group and use the obtained mixture as the test material.

1.3 Master seed coccidia

The test article processed in a method approved as suitable, such as centrifugation, shall be used as the test material.

2 Common test for freedom from extraneous viruses

Master seed virus, master cell seed and master seed coccidia shall be tested as specified in 2.1 and 2.2 or either thereof in consideration of the appropriate range of viruses that may infect the animal species of origin and viruses that are pathogenic to the animals targeted for vaccination.

2.1 Inoculation test using susceptible cell

2.1.1 Test materials

2.1.1.1 Cultured cells

Use cells susceptible to an appropriate range of viruses that may infect the animal species from which the cultured cells are derived and cells susceptible to an appropriate range of viruses that may infect the animal species targeted for vaccination.

2.1.2 Test procedure

2.1.2.1 Culture

The test material shall be inoculated into the cultured cell sheet of at least 70 cm2per 1 mL of the inoculum, cultured at 37ºC for seven days, and the cells shall be passaged to the next generation.

The passaged cells shall be cultured at 37ºC for seven days, and then shall be passaged to subsequent generations at 37ºC for seven days. If using cells derived from fish, these cells shall be cultured in a manner specified in the respective sections of the part of vaccine (seed lot product).

2.1.2.2 Observation of cultured cells

The cells shall be observed for the presence of CPE at least every two days during all the culture periods.

2.1.2.3 Cell staining

On the final day of culture, cultured cell sheets of at least 12 cm2 shall be subjected to Hematoxylin-Eosin stain or May-Grünwald-Giemsa stain, and observed microscopically for CPE, inclusion bodies and any cell abnormalities due to other extraneous factors.

2.1.2.4 hemadsorption

On the final day of culture, the cultured cell sheet shall be divided in three groups so that the size of each sheet is at least 18 cm2.The cell surfaces shall be washed twice with phosphate-buffered saline, and then overlaid with 0.1 vol% red blood cell suspensions of guinea pig, goose, and chicken aged 7 days or younger, respectively, allowed to stand at 4ºC for 30 minutes followed at 20ºC to 25ºC for 30 minutes. Then washed with phosphate-buffered saline, and observed microscopically for the presence of hemadsorption.

2.1.3 Judgment

During the period of observation, no CPE and inclusion bodies and other cell abnormalities are observed in the cultured cells, and no hemadsorption is observed in the cultured cells: the test material shall comply with the test.

2.2 Inoculation test using embryonated chicken egg

2.2.1 Inoculation into allantoic cavity

2.2.1.1 Observation of embryo

2.2.1.1.1 Test procedure

As specified in 1.1 in the Materials for Live Vaccine Production, embryonated chicken eggs aged 9–11 days shall be used. The 0.1 mL portions of the test material shall be injected into the allantoic cavity of 10 embryonated chicken eggs and cultured at 37ºC for seven days. On the final day of culture, these eggs shall be opened to examine abnormalities of chicken embryos.

2.2.1.1.2 Judgment

If chicken embryos are properly developed and no abnormalities are observed: the test material shall comply with the test.

2.2.1.2 Hemagglutination test

2.2.1.2.1 Test procedure

To the allantoic fluid collected on the final day of culture in 2.2.1.1.1, an equal volume of 0.5 vol% chicken red blood cell suspension shall be added, allowed to stand at 4ºC for 60 minutes, and observed for the presence of hemagglutination.

2.2.1.2.2 Judgment

No hemagglutination is observed in the allantoic fluid: the test material shall comply with the test.

2.2.2 Inoculation onto chorioallantoic membrane

2.2.2.1 Test procedure

As specified in 1.1 in the Materials for Live Vaccine Production, embryonated chicken eggs aged 10–12 days shall be used. The 0.1 mL portions of the test material shall be inoculated onto the chorioallantoic membrane of 10 embryonated chicken eggs and cultured at 37ºC for five days. On the final day of culture, these eggs shall be opened and observed to examine abnormalities of chicken embryos and chorioallantoic membranes.

2.2.2.2 Judgment

If chicken embryos are properly developed and no abnormalities are observed in the chorioallantoic membranes: the test material shall comply with the test.

3 Test for freedom from specific viruses

In principle, for the master seed virus, master cell seed, and master seed coccidia, the Test for Freedom from Specific Viruses shall be performed in addition to the Common Test for Freedom from Extraneous Viruses in consideration of the appropriate range of viruses that may infect the animal species of origin and viruses that are pathogenic to the target animals. Viruses specified in 3.2 Individual Test for Freedom from Specific Viruses shall be tested as directed in the Individual Test for Freedom from Specific Viruses.

3.1 General test for freedom from specific viruses

3.1.1 Fluorescent antibody assay

3.1.1.1 Test materials

3.1.1.1.1 Cultured cells

Use cells susceptible to an appropriate range of viruses that may infect the animal species from which the cultured cells are derived and cells susceptible to an appropriate range of viruses that may infect the animal species targeted for the vaccination. If using cells derived from fish, these cells shall be cultured in a manner specified in the respective sections of the part of vaccine (seed lot product).

3.1.1.2 Test procedure

3.1.1.2.1 Culture

The test material shall be inoculated into the cultured cell sheet of at least 70 cm2 per 1 mL of the inoculum, cultured at 37ºC for seven days, and the cells shall be passaged to the next generation. The passaged cells shall be cultured at 37ºC for seven days, and then the cells shall be passaged to the following generation and cultured at 37ºC for seven days. If using cells derived from fish, these cells shall be cultured in a manner specified in the respective sections of the part of the vaccine (seed lot product).

3.1.1.2.2 Fluorescent antibody assay

A fluorescent antibody assay shall be performed with antisera against the viruses specified in the respective sections of the part of vaccine (seed lot product) fluorescent antibody assay.

3.1.1.3 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

3.1.2 Inoculation to chicken

3.1.2.1 Test animals

Chickens aged 2 weeks or older derived from 1.1 in the Materials for Live Vaccine Production shall be used.

3.1.2.2 Test procedure

The test material shall be inoculated at a dose of 1 mL intramuscularly and at a dose of 0.03 mL ophthalmically or in other manners approved as suitable and shall be re-inoculated two weeks later. Obtain the sera at the time of initial inoculation and five weeks after initial inoculation to measure the antibodies against the viruses specified in the respective sections of the part of vaccine (seed lot product)

3.1.2.3 Judgment

No specific antibodies are detected in the sera obtained at the time of initial inoculation and at five weeks after initial inoculation: the test material shall comply with the test.

3.2 Individual test for freedom from specific viruses

3.2.1 Test for freedom from Avian leukosis virus

3.2.1.1 Test procedure

As specified in 2.1.1 in the Materials for Live Vaccine Production, primary cells of chicken embryo susceptible to avian leukosis virus shall be used.

A 1 mL portion of the test material shall be inoculated into the cell sheet of at least 70 cm2 cultured within 24 hours. These cells shall be cultured at 37ºC for three to five days and passaged to the next generation. The passaged cells shall be cultured at 37ºC three to five days, passaged to subsequent generations, and cultured at 37ºC for three to five days.

After washing the third cultured cell sheet of at least 70 cm2, add one-twentieth volume of culture medium of Veronal-buffered saline containing 1 w/v% gelatin. Freeze-thaw the harvested cell suspension three times and collect centrifugal supernatant to prepare cell extract. Using this as an antigen, perform a COFAL test by the Kolmer’s method in pigeon, rabbit, or hamster serum specific to avian leukosis virus, three units of hemolytic elements, four units of guinea-pig complements, and 2 vol% sheep red blood cells suspension.

3.2.1.2 Judgment

The COFAL test indicates that the cell extract is negative for the virus: the test material shall comply with the test.

3.2.2 Test for freedom from Reticuloendotheliosis virus

3.2.2.1 Test procedure

As specified in 2.1.1 in the Materials for Live Vaccine Production, primary cells of chicken embryo susceptible to avian leukosis virus shall be used.

A 1 mL portion of the test material shall be inoculated into the cell sheet of at least 70 cm2 cultured within 24 hours, the primary cell suspension cultured at 37ºC for three to five days shall be inoculated into a newly cultured primary cells of chicken embryo, and cultured at 37ºC for four days. The cell suspension shall be passaged in the same manner to the third generation, cultured at 37ºC for four days, tested with anti-reticuloendotheliosis virus by fluorescent antibody assay, and then observed.

3.2.2.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

3.2.3 Test for freedom from Classical swine fever virus

The test shall be performed as specified in 3.2.3.1 or 3.2.3.2 as follows:

3.2.3.1 Fluorescent antibody assay

3.2.3.1.1 Test procedure

Primary or passaged cultured cells of swine kidney shall be used.

A 4 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum and cultured at 37ºC for five days.

On the 5th day of inoculation of the test material, culture supernatant shall be collected, a 1 mL portion of it shall be inoculated into other cultured cell sheet of at least 3 cm2, cultured at 37ºC for 24–48 hours, tested with anti-classical swine fever virus serum by fluorescent antibody assay, and then observed.

3.2.3.1.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

3.2.3.2 END method and interference method

3.2.3.2.1 Test procedure

Primary or passaged swine kidney cells shall be used.

A 4 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum and cultured at 37ºC for five days.

On the 5th day of inoculation of the test material, culture supernatant shall be collected, 0.1 mL portions of it shall be dispensed into at least 20 small test tubes (wells), and 0.5 mL portions of primary cells of swine testis suspended in the cell growth medium shall be added. After allowing to stand to culture at 37ºC for four days, the cultured cells shall be divided into two groups and tested by the END method and interference method using WEE virus.

3.2.3.2.2 Judgment

No Classical swine fever virus is detected in the cultured cells: the test material shall comply with the test.

3.2.4 Test for freedom from Porcine circovirus

3.2.4.1 Test procedure

PPK-3F cells shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, treated with glucosamine, cultured at 37ºC for five days, and the cells shall be passaged to the next generation. The passaged cells shall be cultured at 37ºC for four days, and these cells shall be passaged to the next generation. The passaged cells shall be cultured at 37ºC for three days, tested with anti-porcine circovirus serum by fluorescent antibody assay, and then observed.

3.2.4.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

3.2.5 Test for freedom from Bovine viral diarrhea virus

3.2.5.1 Test procedure

Passaged bovine testis cells shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 34ºC to 36ºC for five days, and observed for the presence of CPE. Then, these cells shall be dispensed and passaged in 10 small test tubes, cultured for five days, and observed for the presence of CPE. After removing the culture suspension, a 0.5 mL portion of culture medium for maintenance containing approximately 105TCID50/mL of Bovine viral diarrhea virus Nose strain shall be added to each test tube, roller-cultured at 34ºC to 36ºC for seven days, and observed for the presence of CPE.

3.2.5.2 Judgment

During the observation period, CPE is not observed in the cultured cells before inoculation with the Bovine viral diarrhea virus, but CPE is observed in the cultured cells after inoculation with the virus: the test material shall comply with the test.

3.2.6 Test for freedom from Canine parvovirus and Feline panleukopenia virus

3.2.6.1 Test procedure

Primary or passaged cells of feline kidney shall be used.

A 2 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 20 cm2 per 1 mL of the inoculum, cultured at 36ºC for five days, passaged to the next generation, and cultured at 36ºC for 10 days.

To the culture medium obtained on the final day of culture, add an equal quantity of borate buffered saline containing 0.2 w/v% bovine serum albumin. Additionally, add 0.5 vol% swine red blood cells prepared with a quantity of VAD 6.0 solution equal to that of the mixture, allow to stand at 4ºC for 18 hours, and observe the presence of hemagglutination.

3.2.6.2 Judgment

No hemagglutination is observed: the test material shall comply with the test.

3.2.7 Test for freedom from Rotavirus

The test shall be performed as specified in 3.2.7.1 or 3.2.7.2 as follows:

3.2.7.1 Observation of cultured cells

3.2.7.1.1 Test procedure

MA104 cells shall be used.

A 0.1 mL portion of the test material shall be inoculated in at least 10 portions of cultured cells and allowed to adsorb at 37ºC for 60 minutes. The cell surfaces shall be washed with phosphate-buffered saline. Culture suspension containing an appropriate amount of trypsin shall be added, roller-cultured at 37ºC for seven days, and observed for the presence of CPE.

3.2.7.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

3.2.7.2 Fluorescent antibody assay

3.2.7.2.1 Test procedure

A 0.1 mL portion of the test material shall be inoculated into the cultured cell sheet of at least 3 cm2, cultured at 37ºC for 48 hours, tested with anti-rotavirus serum by fluorescent antibody assay, and then observed.

3.2.7.2.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

3.2.8 Test for freedom from bovine leukemia virus

3.2.8.1 Test procedure

Healthy cattle weighing 100–200 kg or healthy sheep weighing 30–50 kg shall be used.

A 10 mL portion of the test material shall be intramuscularly injected into one cattle or sheep, and serum shall be obtained through blood sampling in the 2nd and 3rd months. The obtained serum shall be tested by passive hemagglutination assay, enzyme immunoassay, or agar gel immunoprecipitation assay to detect the bovine leukemia virus antibody.

3.2.8.2 Judgment

No antibody against bovine leukemia virus is observed: the test material shall comply with the test.

3.2.9 Test for freedom from Japanese encephalitis virus or Rabies virus

3.2.9.1 Test procedure

Mice aged 3 days or younger shall be used.

The 0.02 mL portions of the test material shall be injected into the brain of 10 mice. The centrifugal supernatant of pooled brain mixed emulsion collected on the 5th day shall subsequently be injected into the brain of 10 mice, and observed for 10 days.

3.2.9.2 Judgment

No mice show death or neurological symptoms: the test material shall comply with the test.

3.2.10 Test for freedom from Avian encephalomyelitis virus

3.2.10.1 Test procedure

As specified in 1.1 in the Materials for Live Vaccine Production, embryonated chicken eggs aged 6 days shall be used.

The 0.1 mL portions of the test material shall be inoculated into the yolk sac of 10 embryonated chicken eggs and cultured at 37ºC for 12 days. On the final day of culture, 5 eggs shall be opened, and observed to examine abnormalities of chicken embryos. The remaining eggs shall be hatched and observed for 10 days.

3.2.10.2 Judgment

No deaths or neurological symptoms are observed in the chicken embryos and chickens: the test material shall comply with the test.

3.2.11 Test for freedom from Koi herpesvirus

The test shall be performed as specified in 3.2.11.1 or 3.2.11.2 as follows:

3.2.11.1 Observational test of cultured cells

3.2.11.1.1 Test procedure

KF-1 or CCB cells shall be used.

A 0.1 mL portion of the test material shall be inoculated in at least 10 portions of cultured cells and allowed to adsorb at 25ºC for 60 minutes. The cell surfaces shall be washed with phosphate-buffered saline, cultured at 25ºC for 21 days, and observed for the presence of CPE.

3.2.11.1.2 Judgment

No CPE is confirmed in the cultured cells during the observation period: the test material shall comply with the test.

3.2.11.2 Fluorescent antibody assay

3.2.11.2.1 Test procedure

A 0.1 mL portion of the test material shall be inoculated into KF-1 cell or CCB cell sheet of at least 3 cm2, cultured at 25ºC for seven days, tested with anti-Koi herpesvirus serum by fluorescent antibody assay, and then observed.

3.2.11.2.2 Judgment

No specific fluorescent antigens are observed in the cultured cells: the test material shall comply with the test.

**Target Animal Immunogenicity Test**

The Target Animal Immunogenicity Test is a method to verify that master seeds of live vaccines retain the immunogenicity to the animals targeted for vaccination even through passages.

1 Test materials

1.1 Test article

For live virus vaccines, use master seed virus obtained by 5 passages as the test article. For live bacterial vaccines, use master seed bacteria obtained by 10 passages as the test article. For live coccidia vaccines, use master seed coccidia obtained by 10 passages as the test article. However, this number of passages does not apply if approved by the Minister of Agriculture, Forestry and Fisheries.

1.2 Test animals

Animals used for the test shall be known for their breeds and strains (SPF, etc.), healthy and eligible for vaccination, and suitable for immunogenicity evaluation.

2 Test procedure

2.1 Setting of test groups (including the control group)

The animals shall be assigned to two test groups (at least three animals per group for mammals and at least 10 birds per group for chickens) and one control group.

2.2 Administration method

Administration shall be given in the same manner as applied for clinical use. In principle, administration shall be given up to once, but if unable to evaluate the immunogenicity by a single administration, administration can be given at appropriate intervals as frequent as it should be for evaluation.

2.3 Dose

Administration shall be given at a dose estimated to be a single dose applied for clinical use. The level of antigen administered shall be the same between individual animals in the test groups.

2.4 Evaluation method

Using approved methods (methods that can verify protection against infection by antibody response, cellular immunity, or challenge test etc.), evaluate the immunogenicity of passaged master seed in an objective manner.

3 Judgment

The immunogenicity of passaged master seed shall be tested in an appropriate manner.

**Target Animal Safety Test**

The Target Animal Safety Test is a method to examine the absence of pathogenic factors against the animals targeted for vaccination in the master seeds of live vaccines.

1 Test materials

1.1 Test article

Use master seed as the test article. If a sufficient quantity of master seeds for the test is not available, use the working seeds whose number of passages is the lowest as the test article.

1.2 Test animals

1.2.1 Master seeds of live vaccines for mammals

Use at least four healthy target animals aged day~~s~~ at which the clinical symptoms related to the safety evaluation are most likely to occur within the scheduled vaccination days old.

1.2.2 Master seeds of live vaccines for birds

Use at least 20 healthy chickens aged day~~s~~ at which the clinical symptoms related to the safety evaluation are most likely to occur within the scheduled vaccination days old.

2 Test procedure

2.1 Administration

2.1.1 Test on master seeds of live vaccine targeted mammals

Test animals shall be divided into two groups, each with at least two animals. One group shall be given the test article prepared from the master seeds via the planned route of administration. The other group shall not be given the test article as the control group.

2.1.2 Test on master seeds of live vaccine targeted birds

Test animals shall be divided into two groups, each with at least ten animals. One group shall be given the test article prepared from the master seeds via the planned route of administration. The other group shall not be given the test article as the control group.

2.2 Matters to be observed

Animals shall be observed for clinical symptoms for 21 days after administration.

3 Judgment

No clinical abnormalities shall be observed during the observation period.

**Test for Absence of Reversion to Virulence**

The Test for Absence of Reversion to Virulence is a method to ensure that the virulence of the strain used for live vaccine production is not reversed or enhanced in the animals targeted for inoculation.

1 Test materials

1.1 Test article

The master seeds for live vaccine production shall be used as the test article. The test article shall be adjusted to include the maximum amount of the strain in the shipment. However, if a sufficient quantity of master seeds is not available for the test, use the working seeds whose number of passages is the lowest as the test article. In this case, the test article shall also be adjusted to include the maximum amount of the strain in the shipment.

1.2 Passage material

From the test animals receiving the test article, sample the parts inbody and etc. at which microorganisms derived from the strain used for production are frequently isolated, and prepare the passage materials. For each passage, microorganisms derived from the strain used for production shall be quantified using a suitable method. Passage materials shall not be grown or cultured in the laboratory.

1.3 Test animals

Target healthy animals suitable for the test shall be used as the test animals.

2 Test procedure

2.1 Administration route

Administer the test article to at least two test animals(birds) via the route planned in the application or natural infection route as appropriate to investigate reversion or enhancement of virulence. The route of administration planned in the application shall be appropriate for investigating the reversion to or enhancement of virulence.

2.2 Material sampling

Based on the properties of the test article, sample the passage materials from the test animals at an appropriate time.

2.3 Passage

From the initial inoculation with the test materials up to the 4th-passaged materials, at least two test animals(birds) each shall be used. For the final 5th-passaged material, at least eight test animals(birds) shall be used.

If the microorganisms derived from the strain used for production are not recovered during the course of passage, use the last passage material recovered to repeat passage with 10 animals (birds). During the repeated passage, if microorganisms derived from the virus strain used for production are recovered from at least one animal(bird), the passage shall be continued using the materials recovered during the repeated passage as the materials for the next passage. Even if the passage is repeated, the number of passages in the test shall be regarded as once.

2.4 Test period

Test animals used from initial inoculation up to the 4th-passaged materials shall be observed within 21 days, and those inoculated with up to the 5th-passaged materials shall be observed for 21 days, respectively.

2.5 Observation

During the test period, the test animals shall be clinically observed for the occurrence of clinical symptoms of the target diseases associated with the reversion to or enhancement of virulence.

2.6 Comparative test

During the observation period, if clinical symptoms associated with the reversion to or enhancement of virulence are identified in the test animals used for inoculated with the 5th-passaged materials during their observation period, the test article and passage materials used for the final passage shall be adjusted to the maximum amount of the strain in the shipment and inoculated to at least eight test animals (birds) per group to compare the clinical symptoms associated with the reversion to or enhancement of virulence.

3 Judgment

In all the test animals inoculated with up to the 4th-passaged material, no clinical symptoms associated with the reversion to or enhancement of virulence shall be observed during the observation period. Also, in the comparative test, no differences in clinical symptoms associated with the reversion to or enhancement of virulence shall be identified between the animals inoculated with the test article and those inoculated with the final passage material.

**Test for Stability Confirmation of Recombinant Gene**

The Test for Stability Confirmation of Recombinant Gene is a method to confirm that the master seeds produced by genetic recombination technology have the stability of the modified nucleic acids and properties during the passages.

1 Test materials

1.1 Test article

For genetically modified viruses, master seed viruses and their 5th-passaged viruses shall be used as the test article. For genetically modified bacteria, master seed bacteria and their 10th -passaged bacteria shall be used as the test article. However, if a sufficient quantity of master seeds is not available for the test, use the working seeds whose number of passages is the lowest as the test article unless otherwise approved by the Minister of Agriculture, Forestry and Fisheries.

1.2 Cultured cells or medium

Cultured cells or medium used as materials for production of vaccine seeds shall be used.

2 Test procedure

2.1 Test for gene stability confirmation

The nucleic acids at the modified sites in the master seeds and their passaged ones shall be compared for their states and sequences using an approved test method.

2.2 Test for property stability confirmation

The properties obtained by modification of nucleic acids in the master seeds and their passaged ones shall be compared by an approved test method and evaluated objectively.

3 Judgment

3.1 Test for gene stability confirmation

Compare the master seeds and their passaged ones and there are no differences in the states of nucleic acids at the modified sites and sequences: the test article shall comply with the test.

3.2 Test for property stability confirmation

Compare the master seeds and their passaged ones and there are no changes in their properties: the test article shall comply with the test.