Livestock Promotional Subsidy from
the Japan Racing Association

MINIMUM REQUIREMENTS FOR VETERINARY BIOLOGICAL PRODUCTS

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CONTENTS

General Rules

1 These standards (”Minimum Requirement for Veterinary Biological Products”） specify the manufacturing methods, properties, quality, storage, and other matters for veterinary biological products listed in the monograph of drugs (hereafter referred to as “monographs”). These standards are abbreviated to “veterinary biological standards” in this document. Monographs include bulk material or final bulk that serve as intermediate products.

2 The *Japanese Pharmacopoeia* is specified by the Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (Law No. 145, 1960, hereafter referred to as "Law"), and the Japanese Industrial Standards are specified by the Industrial Standardization Law (Law No. 185, 1949).

3 “Standard names” imply names listed in the monograph of drugs.
For bulk material or final bulk, however; standard names shall be specified by appending “bulk material” or “final bulk” to the names listed in the respective monograph of drugs. Standard names are considered nonproprietary names when applied to Article 50 of the Law.

4 Veterinary biological products are to be judged according to the provisions given in the General Rules, pertinent monograph, and the provisions of the General Tests. For bulk material or final bulk that served as intermediate products in the pertinent monograph, these products shall be judged according to the rules specified in the sections related to bulk material or final bulk.

5 The details of the manufacturing methods and test items in the test methods may be changed under the condition in which products manufactured after changes are made are equal to or better than those manufactured in a specified manner only when such changes are approved as specified in Article 14, Article 19 (2), Article 23 (2)-5, or Article 23 (2)-17 of the Law.

6 Standard names enclosed in single quotation marks (‘ ’) indicate that the properties and quality conform to those specified in these standards; however, the standard names shown in the title of pertinent monograph shall not be enclosed in these marks, and those enclosed in double quotation marks (“ ”) indicate substances that exhibit specific biological activities.

7 The following abbreviations are used as main units of measurement:

Centimeter	cm	Kilogram	kg

Millimeter	mm	Gram	g
Nanometer	nm	Milligram	mg
Square centimeter	cm^2	Microgram	μg
Liter	L	Mole per liter	mol/L
Deciliter	dL	Pascal	Pa
Milliliter	mL	Gravity acceleration	*G*
Microliter	μL		

8 Mass percent, mass per volume percent, and volume percent shall be expressed as %, w/v% and vol%, respectively.

9 The Celsius scale is used for temperature measurement and "°C" is added after Arabic numerals.

10 Solutions without a solvent name indicate water solutions.

11 "Diluent" indicates an attached diluent or diluting solution approved as suitable for dissolving of the relevant product.

12 For veterinary biological products freeze-dried or intended to be dissolved for use whose label instructions state "Dissolve with diluent" only, these products shall be dissolved using diluent according to the method stated on the container.

13 Unless otherwise specified, pharmaceuticals or chemical agents that are listed in the *Japanese Pharmacopoeia* (JP) and that are used to manufacture drugs in the monographs shall comply with the JP requirements, whereas those not listed in the JP but specified in the Japanese Industrial Standards shall comply with their specifications depending on the purpose of use.

14 Inactivator and stabilizer specified by the term, for example, "use suitable agents" in the monograph of drugs shall be safe for use at the usual amount used and shall not inhibit the beneficial effects of the drug or interfere with pharmaceutical tests.

15 The actual amount of drugs in the monographs and attached diluents shall be sufficient for collecting the labeled amount and sufficient for the administration of the labeled dose.

16 “Original strain” indicates a virus strain, strain, or coccidia strain specified as the strain used for production in the monograph of drugs.

“Original virus,” “original bacteria,” or “original coccidia” indicates a passaged original strain used for the production of the seed virus, seed bacteria, or seed coccidia for vaccines. It is passaged according to the specified method in the part the vaccines in monograph of drugs or in the specifications and has the same property as that of the original strain.

“Seed virus,” “seed bacteria,” or “seed coccidia” indicates a passaged original strain directly used for production of vaccine. It is passaged according to the specified method in the monograph of drugs and has the same property as that of the original strain.

17 “Primary cultured cells” indicate cultured cells obtained from appropriate tissues through trypsin digestion and etc. and basically having the same property as that of the original cells.

“Passaged cells” indicate cultured cells having the continuous propagation ability.

18 “Seed lots” indicate homogeneous suspensions of specific viruses, bacteria, and cells etc. obtained from single cultures and stored under proper conditions to maintain their genetic properties.

19 “Seed lot product” indicates a vaccine produced using seed lots.

20 “Master seed” indicates a virus strain used for the production of seed lot products specified in the monograph of drugs and passaged not more than approved passages and it is permanently stored as a virus strain (hereafter referred to as “master seed virus”), as a bacterial strain (hereafter referred to as “master seed bacteria”), and as a coccidia strain (hereafter referred to as “master seed coccidia”).

“Working seed” indicates a virus strain derived from the master seed and not directly used for manufacturing the products (hereafter referred to as “working seed virus”), a bacteria strain derived from master seed and not directly used for manufacturing the products (hereafter referred to as “working seed bacteria”), or a coccidia strain derived from master seed and not directly used for manufacturing the products (hereafter referred to as “working seed coccidia”).

“Production seed” is a virus strain derived from the working seed and directly used for manufacturing the products (hereafter referred to as “production seed virus”), a bacteria strain derived from working seed and directly used for manufacturing the products (hereafter referred to as “production seed bacteria”), or a coccidia strain derived from working seed and directly used for manufacturing the products (hereafter referred to as “production seed coccidia”).

21 “Cell line” indicates cultured cells having the continuous propagation ability and used for manufacturing seed lot products.

“Master cell seed” indicates cultured cells that have been passaged not more than approved passages.

“Working cell seed” indicates a cell line derived from the master cell seed and not directly used for manufacturing the products.

“Production cell seed” is a cell line derived from the working cell seed and directly used for manufacturing the products.

22 “Master primary cell seed” indicates primary cultured cells generated through not more than five passages of cells that have been collected from animals.

“Working primary cell seed” indicates primary cultured cells derived from the master primary cell seed and not directly used for manufacturing the products.

“Production primary cell seed” is primary cultured cells derived from the working primary cell seed and directly used for manufacturing the products.

23 “Bulk material” indicates a veterinary biological product containing a single active ingredient that is not dispensed as is into final containers.

24 “Final bulk” indicates a bulk drug prepared in one vessel and ready for immediate filling into final containers. The content shall be completely homogeneous in terms of pharmaceutical properties and qualities. Shaking is allowed to maintain homogeneity.

25 “Final product” indicates the products filled into small containers from a final bulk, freeze-dried if necessary, and hermetically sealed.

26 “Lot” usually indicates a group of final products derived from the final bulk. Also, the “lot” of bulk material or final bulk in the monograph of drugs that serves as veterinary biological products indicates a group of products having a uniform character and quality that has been filled in a series of manufacturing processes within a specified period.

27 In general, the same manufacturing number or manufacturing code is assigned to the products of one lot. However, additional numbers or marks shall be assigned to the individual final lots or freeze-dried lots, if dispensed and hermetically sealed under different operating conditions (the amount to be dispensed may vary) or if freeze-dried under different drying conditions, respectively.

28 Generally, tests on specifications for bulk material or final bulk and final lot products required in the monographs are collectively performed with samples from the same lot. For final products having individual final lots or individual freeze-dried lots, however, the following tests shall be conducted on individual lots: properties test, sterility test, viable count test, spore count test, test for freedom from contaminant microorganisms, test for virus content, vacuum degree test, test for moisture content, and additionally specified tests. Other tests shall be conducted by taking and mixing equal volumes of the test sample of individual lots.

29 "Test for freedom from abnormal toxicity," "Toxicity limit test," "Safety test," and "Potency test" for final lot products specified in the monographs may be omitted when the requirements otherwise specified by the Director of the National Veterinary Assay Laboratory are met.

30 "Test sample" indicates a final product used for tests or the final product dissolved with diluent.

"Test article" indicates a product subject to tests on the final bulk or earlier stages that has not been manipulated in any way.

"Test material" indicates a test sample or article that has been diluted or otherwise manipulated.

31 Generally, tests on veterinary biological products freeze-dried or intended to be dissolved drug for use in the monographs, unless these tests are the "Vacuum degree test," "Test for moisture content," "uniformity test per container of freeze-dried product before dissolution" in the "Properties test," and otherwise specified, shall be conducted on solutions diluted or suspended according to the method stated on the container using the diluents.

32 The "Inactivation test" evaluates the loss or reduction in biological activity of viable microorganisms used in the production of a veterinary biological product below the level specified in the requirements.

The "Detoxication test" evaluates the loss of toxicity of toxic substances present during the production process of a veterinary biological product below the level specified in the requirements.

The "Test for freedom from (name of microorganism or substance)" determines the absence of substances or microorganisms indicated in ( ) below the level specified in the requirements.

33 Unless otherwise specified, tests shall be conducted at room temperature.

Room temperature is defined as 15℃ to 25℃.

34 Unless otherwise specified, tests shall be conducted in accordance with the “Reagents, Test Solutions, Etc.” of the General Tests.

The term “water” for use in the tests indicates purified water specified by the JP.

35 To “accurately weigh” means weighing to the specified digit.

To “exactly measure” means measuring the specified whole volume using pipettes, measuring flasks, or burettes.

To “precisely weigh” means weighing to the lowest digit, for example, 0.1 mg, 0.01 mg, or 0.001 mg.

36 Numerical values shall indicate the specified value with a ± 5% variation.

“Approximately” in collecting the desired amount or quantity indicates a variation in numerical value within ±10%.

37 When a test result is judged by comparing a specified value (hereafter referred to as “specified value”) with actually measured values obtained from a test (hereafter referred to as “experimental values”) in the Physiochemical Test of the General Tests, experimental values shall be obtained to one digit lower than that for the specified value and rounded off to the same digit with the specified value.

38 Animals used in tests must be healthy. If animals exhibit incidental abnormalities during the test, the veterinary biological product shall not meet the test requirements unless the abnormalities can be demonstrated to be unrelated to the product tested.

39 The test methods or the details of the test methods specified in the Minimum Requirement for Veterinary Biological Products may be changed so far as it is equally or more precise and accurate than the specified method. However, if the test results are different from the ones by using the specified method of the Minimum Requirements for Veterinary Biological Products, the final judgement of the test shall be made in accordance with said specified method.

40 Unless otherwise specified, storage temperature shall be kept ≤ 10℃ for freeze-dried products and 2℃ to 10℃ for liquid products in a light-shielded environment. However, this shall not apply to bulk material or final bulk.

41 “Delivery from the warehouse” means the shipping of the veterinary biological products from the storage of the manufacturing facility for sales or transport. Veterinary biological products shall be stored at a constant temperature before shipping

42 Unless otherwise specified, the shelf life and expiry date shall be set based on the month following the month of the day when production is completed. However, for veterinary biological products to be subjected to national certification tests according to Article 43 (1) of the Law, the date when the national certification test is completed may be substituted for the date when production is completed.

43 In cases where veterinary biological products should be subject to the national certification test but could not be submitted for application within the period specified in Article 152, Section 3, of the Regulatory Rules for Veterinary Medicinal Products (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 107, 2004) under unavoidable circumstance when Director of National Veterinary Assay Laboratory determines that particularly there is any reason for shortening the expiry date as the result of the national certification test, the shelf life of such products shall be defined as the date specified by the director notwithstanding above provisions.

44 Unless specified otherwise, entries on the immediate container for monographs required by Article 50 (9) of the Law shall be those listed below.

(1) Storage conditions

(2) Expiry date

(3) Live or inactivated for vaccines

However, these entries may be omitted when they are provided on the outer container or package if the following conditions apply:

-The product is provided in an ampoule or direct container or package with a volume of 2 mL or less.

-The product is provided in an ampoule or container with a volume of more than 2 mL having an area on which these entries are directly printed but less than 10 mL having an area insufficient to clearly label the entries specified in the provisions of Article 50 of the Law.

-The product is designed for in vitro diagnostic purposes and provided in the outer container or package stating “For in vitro diagnostic purposes.”

45 When the monographs or pharmaceuticals used for the production of the monographs are derived from animals, these animals must be healthy, unless otherwise specified.

Monograph of Drugs

Vaccines

Akabane Disease Vaccine, Live, Seed

1 Definition

Akabane Disease Vaccine, Live, Seed is a freeze-dried vaccine of virus suspension obtained by propagating attenuated Akabane virus that meets the seed lot specifications in cell lines that meet these specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Attenuated Akabane virus TS-C2 strain or strain approved as equivalent thereof

2.1.2 Properties

Inoculation of this virus strain into pregnant cows causes neither abortion, stillbirth, or congenital defects in newborns.

2.1.3 Master seed virus

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu-1 cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

Dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70℃ or lower or stored freeze-dried at 5℃ or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4 Working seed virus

2.1.4.1 Propagation, passage and storage

The working seed virus shall be propagated and passaged in HmLu-1 cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70℃ or lower or be stored freeze-dried at 5℃ or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5 Production seed virus

2.1.5.1 Propagation and storage

The production seed virus shall be propagated in HmLu-1 cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70℃ or lower or stored freeze-dried at 5℃ or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Cell line

HmLu-1 cells or cell lines approved as suitable for production shall be used.

2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.3 Master cell seeds

2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70℃ or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.4 Working cell seeds

2.2.4.1 Propagation, passage and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.

The working cell seeds shall be stored frozen at -70℃ or lower. The working cell seeds shall be tested as specified in 3.2.2.

2.2.5 Production cell seeds

2.2.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3 if it needs to be stored.

2.3 Bulk material

2.3.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as an individual cultured cells. No abnormalities shall be detected prior to the inoculation of virus.

2.3.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1. The filtrate or supernatant after centrifugation of culture medium collected from individual cultured cells at the peak of virus propagation shall be mixed, and the mixture shall serve as the bulk material.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

A stabilizer approved as appropriate shall be added to the bulk material to serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.1.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.1.1.3Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For Lymphocytic choriomeningitis virus, Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 1.1 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.7 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cell line

3.2.1 Tests on master cell seeds

3.2.1.1 Test for confirmation of cell properties

The test given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Test for identification of the animal species of the cell

The test given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.5 Test for freedom from extraneous viruses

3.2.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2 Test for freedom from specific viruses

3.2.1.5.2.1 General test for freedom from specific viruses

For Lymphocytic choriomeningitis virus, Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 1.2 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2.2 Individual test for freedom from specific viruses

For Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus

and Rabies virus, the tests given in 1.2, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.6 Test for karyological (chromosomal) characterization

The test given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.7 Test for freedom from tumorigenicity

The test given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 Test for confirmation of cell properties

The test given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3 Tests on production cell seeds

3.2.3.1 Test for confirmation of cell properties

The test given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on bulk material

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Test for virus content

3.3.2.1 Materials

3.3.2.1.1 Test materials

The test article shall be diluted ten-fold with virus growth medium (Note) and each level dilution shall serve as a test material.

3.3.2.1.2 Cultured cells

HmLu-1 cells shall be cultured in small test tubes for one to three days, and monolayer cultures shall be used.

3.3.2.2 Test procedures

A 0.1 mL portion of the test material shall be inoculated into at least four tubes of cultured cells and allowed to stand for adsorption at 37℃ for 60 minutes. A 0.5 mL portion of virus growth

medium shall be added, roller-cultured at 34°C to 36°C for seven days and observed.

3.3.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$. The virus content in 1 mL shall be $10^{6.5}TCID_{50}$ or higher.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid with a specific color. No foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.4.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.4.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.4.4 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.5 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply. However, this test may be omitted if it is conducted during the intermediate process including the bulk material.

3.4.6 Test for virus content

When the test is performed as specified in 3.3.2, the virus content of the test sample shall be $10^{5.0}$ $TCID_{50}$ or higher per animal.

3.4.7 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.4.8 Safety test

3.4.8.1 Materials

3.4.8.1.1 Injection materials

The test sample shall be used as an injection material.

3.4.8.1.2 Test animals

Cattle weighing 100–200 kg shall be used.

3.4.8.2 Test procedures

A dose of injection material for one animal shall be administered subcutaneously to one animal and observed for 14 days.

3.4.8.3 Judgment

During the observation period, a slight fever (40.5°C or lower), if observed, shall not persist for

more than three days, and no other abnormalities shall be identified.

3.4.9 Potency test

3.4.9.1Materials

3.4.9.1.1 Test animals

The animals used in the test specified in 3.4.8 shall be used.

3.4.9.1.2 Virus for neutralization test

Akabane virus JaGAr39 strains propagated in HmLu-1 cells shall be used.

3.4.9.1.3 Cultured cells

HmLu-1 cells shall be cultured in small test tubes for one to three days, and monolayer cultures shall be used.

3.4.9.2 Test procedures

The serum obtained 14 days after the completion of test specified in 3.4.8 shall be subject to the neutralization test.

The serum shall be heat-inactivated, and then shall be diluted two-fold with virus growth medium. With 0.5 mL of each diluted serum, 0.5 mL of the virus for the neutralization test with approximately 200 $TCID_{50}$ per 0.1 mL shall be mixed and reacted at 37℃ for 60 minutes. The 0.1 mL of the mixtures of each shall be inoculated into four tubes of cultured cells and allowed for adsorption at 37℃ for 60 minutes. To each tube, 0.5 mL of virus growth medium shall be added and roller-cultured at 34℃ to 36℃ for seven days for observation.

3.4.9.3 Judgment

The maximum dilution factor of serum at which CPE is observed in at least two tubes of cultured cells shall be defined as the neutralizing antibody titer.

The neutralizing antibody titer in test animals shall be at least two-fold or higher.

4 Storage and expiry date

The expiry date shall be two years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note Virus growth medium

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Glucose	1.0 g
Yeast extract	0.5 g
Bovine serum	5-20 mL
Eagle’s MEM	residual quantity

pH shall be adjusted to 7.2-7.6 with sodium hydrogen carbonate.

Bovine serum to be used shall be negative for neutralizing antibodies against the Akabane virus.
Minimal amounts of antibiotics may be added.

Bovine Respiratory Syncytial Virus Infection Vaccine, Live, Seed

1 Definition

Bovine respiratory syncytial virus infection vaccine, live, seed is a freeze-dried vaccine of the virus suspension obtained by propagating attenuated bovine respiratory syncytial virus that meets the seed lot specifications in cell lines that meet these specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Attenuated bovine respiratory syncytial virus rs-52 strain or strain approved as equivalent thereof

2.1.2 Properties

This virus shows no pathogenic effects when inoculated in cows. The propagative ability of this virus at 30ºC in HAL cells established from cells derived from hamster lung is 100-fold higher than that of the virulent virus.

2.1.3 Master seed virus

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HAL cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4 Working seed virus

2.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HAL cells or cells approved as suitable. The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5 Production seed virus

2.1.5.1 Propagation and storage

The production seed virus shall be propagated in HAL cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Cell line

HAL cells or cell lines approved as suitable for production shall be used.

2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.3 Master cell seeds

2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.4 Working cell seeds

2.2.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.

The working cell seeds shall be stored frozen at -70ºC or lower. The working cell seeds shall be tested as specified in 3.2.2.

2.2.5 Production cell seeds

2.2.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3 if they need to be stored.

2.3 Bulk material

2.3.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected in production cell seeds prior to the inoculation of the virus.

2.3.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1. The filtrate or supernatant

after centrifugation of culture medium collected from individual cultured cells at the peak of the virus propagation shall be mixed, and the mixture shall serve as the bulk material.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

A stabilizer approved as appropriate shall be added to the bulk material to serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.1.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For the lymphocytic choriomeningitis virus and Bluetongue virus, the tests given in 1.1 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.7 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cell line

3.2.1 Tests on master cell seeds

3.2.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Test for identification of the animal species of the cell

The tests given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.5 Test for freedom from extraneous viruses

3.2.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2 Test for freedom from specific viruses

3.2.1.5.2.1 General test for freedom from specific viruses

For the Lymphocytic choriomeningitis virus, Bluetongue virus, and Bovine respiratory syncytial virus, the tests given in 1.2 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.2, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.6 Test for karyological (chromosomal) characterization

The tests given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.7 Test for freedom from tumorigenicity

The tests given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3 Tests on production cell seeds

3.2.3.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on bulk material

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Test for virus content

3.3.2.1 Materials

3.3.2.1.1 Test materials

The test article shall be diluted ten-fold with the virus growth medium (Note 1) and each level dilution shall serve as the test material.

3.3.2.1.2 Cultured cells

Vero cells shall be cultured in small test tubes for three to four days, and monolayer cultures shall be used.

3.3.2.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions of cultured cells and allowed to stand for adsorption at 37ºC for 60 minutes. The 0.5 mL portions of the virus growth medium shall be added, roller-cultured at 34ºC for 14 days, and observed.

3.3.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{6.3}TCID_{50}$ or higher.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid with a specific color. No foreign matter and foreign odor shall be observed. The property of the product per small container shall be uniform.

3.4.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.4.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.4.4 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.5 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

However, this test may be omitted if it is conducted during the intermediate process, including the bulk materials.

3.4.6 Test for virus content

When the test is performed as specified in 3.3.2, the virus content of the test sample shall be $10^{5.0}$ $TCID_{50}$ or higher per animal.

3.4.7 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.4.8 Safety test

3.4.8.1 Materials

3.4.8.1.1 Injection materials

The test samples shall be used as injection materials.

3.4.8.1.2 Test animals

Cattle weighing 100–200 kg shall be used.

3.4.8.2 Test procedures

A dose of injection material for one animal shall be administered intramuscularly to one animal

and observed for 14 days.

3.4.8.3 Judgment

During the observation period, a slight fever (40.5ºC or lower), if observed, shall not persist for more than three days, and no other abnormalities shall be identified.

3.4.9 Potency test

3.4.9.1 Materials

3.4.9.1.1 Injection materials

The test samples shall be used as injection materials.

3.4.9.1.2 Test animals

Hamsters weighing approximately 100 g shall be used.

3.4.9.1.3 Virus for neutralization test

The Bovine respiratory syncytial virus NMK7 strain propagated in passage cells of bovine kidneys.

3.4.9.1.4 Cultured cells

Vero cells shall be cultured in small test tubes for three to four days, and monolayer cultures shall be used.

3.4.9.2 Test procedures

The 2 mL portions of the injection materials shall be injected intramuscularly twice at intervals of 14 days in five test animals, and serum shall be obtained from individual animals 14 days after the second injection to conduct a neutralization test.

The test serum shall be heat-inactivated and diluted with the dilution for neutralization test (Note 2) in two-fold serial dilutions. A 0.5 mL portion of diluted serum and a 0.5 mL portion of the virus suspension for neutralization test containing approximately 200$TCID_{50}$ in 0.1 mL shall be mixed and reacted at 22ºC for 24 hours. The 0.1 mL portion of each mixture shall be inoculated into four tubes of cultured cells and allowed to stand for adsorption at 37ºC for 60 minutes. Then, the 0.5 mL portions of the virus growth medium shall be added, roller-cultured at 34ºC for 10 days, and observed.

3.4.9.3 Judgment

The maximum dilution factor of serum at which inhibition of CPE is observed in at least two tubes of cultured cells shall be defined as the neutralizing antibody titer. A two-fold or greater increase in the neutralizing antibody titer shall be defined as positive.

The neutralizing antibody positive rate in test animals shall be 80% or higher.

4 Storage and expiry date

The expiry date shall be one year and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Virus growth medium

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Monosodium glutamate	5 g
Glucose	1 g
Fetal bovine serum	10–20 mL
Yeast extract	0.5 g
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.2–7.6 with sodium hydrogen carbonate.

Fetal bovine serum to be used shall be negative for neutralizing antibodies against the bovine respiratory syncytial virus.

Minimal amounts of antibiotics may be added.

Note 2 Dilution for neutralization

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Fetal bovine serum	100 mL
Kanamycin sulfate	100 mg (potency)
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.2–7.4 with sodium hydrogen carbonate.

Fetal bovine serum to be used shall be negative for neutralizing antibodies against the bovine respiratory syncytial virus.

Minimal amounts of antibiotics may be added.

Akabane Disease, Chuzan Disease, Aino Virus Infection, Peaton Virus Infection Vaccine (adjuvant), Inactivated, Seed

1 Definition

This Vaccine, Inactivated, Seed is made by mixing the inactivated virus suspensions obtained by propagating the Akabane virus, Kasba virus, Aino virus and Peaton virus that meet the seed lot specifications in cell lines that meet these specifications, respectively, and by adding an adjuvant.

2 Production methods

2.1 Virus strain used for production

2.1.1 Akabane virus

2.1.1.1 Name

Akabane virus E-24 strain or strain approved as equivalent thereof

2.1.1.2 Properties

This virus shall be propagated in the following cells in which CPE is confirmed: primary cultured cells of bovine kidney, primary cultured cells of swine kidney, HmLu-1 cells, HmLu-SC cells, and Vero cells.

2.1.1.3 Master seed virus

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.1.4 Working seed virus

2.1.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HmLu cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.1.5 Production seed virus

2.1.1.5.1 Propagation and storage

The production seed virus shall be propagated in HmLu cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 Kasba virus

2.1.2.1 Name

Kasba virus K-47 strain or strain approved as equivalent thereof

2.1.2.2 Properties

Inoculation of this virus strain into the brain of calves causes fever, anorexia, and leukopenia, followed by neurological symptoms.

This virus shall be propagated in the following cells in which CPE is confirmed: BHK-21 cells, BHK-SC cells, HmLu-1 cells, HmLu-SC cells, and Vero-T cells.

2.1.2.3 Master seed virus

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.2.4 Working seed virus

2.1.2.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HmLu cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.2.5 Production seed virus

2.1.2.5.1 Propagation and storage

The production seed virus shall be propagated in HmLu cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.3 Aino virus

2.1.3.1 Name

Aino virus JaNAr28 strain or strain approved as equivalent thereof

2.1.3.2 Properties

When this virus strain is inoculated intravenously in cows, viremia is present, but no clinical symptoms such as fever are observed.

This virus shall be propagated in the following cells in which CPE is confirmed: BHK-21 cells, HmLu-1 cells, HmLu-SC cells, and Vero cells.

2.1.3.3 Master seed virus

2.1.3.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.3.4 Working seed virus

2.1.3.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HmLu cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.3.5 Production seed virus

2.1.3.5.1 Propagation and storage

The production seed virus shall be propagated in HmLu cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.4 Peaton virus

2.1.4.1 Name

Peaton virus NS/3 strain or strain approved as equivalent thereof

2.1.4.2 Properties

When this virus strain is inoculated intravenously in cows, viremia is present, but no clinical symptoms such as fever are observed.

This virus shall be propagated in the following cells in which CPE is confirmed: BHK-21 cells,

HmLu-1 cells, HmLu-SC cells, and Vero cells.

2.1.4.3 Master seed virus

2.1.4.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4.4 Working seed virus

2.1.4.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HmLu cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.4.5 Production seed virus

2.1.4.5.1 Propagation and storage

The production seed virus shall be propagated in HmLu cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Cell line

HmLu cells or cells approved as suitable shall be used.

2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.3 Master cell seeds

2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.4 Working cell seeds

2.2.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.

The working cell seeds shall be stored frozen at -70ºC or lower.

The working cell seeds shall be tested as specified in 3.2.2.

2.2.5 Production cell seeds

2.2.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.

The production cell seeds shall not be stored.

2.3 Bulk material

2.3.1 Akabane virus bulk material

2.3.1.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.1.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.1.

2.3.1.3 Inactivation

Virus suspension shall be inactivated by the addition of an inactivator approved as appropriate and shall serve as the inactivated virus suspension.

The inactivated virus suspension shall be tested as specified in 3.4.1.1.

2.3.1.4 Bulk material

The inactivated virus suspension shall be concentrated by a method approved as suitable and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.

2.3.2 Kasba virus bulk material

2.3.2.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.2.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.2.1. The filtrate, supernatant

after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.2.

2.3.2.3 Inactivation

Virus suspension shall be inactivated by the addition of an inactivator approved as appropriate and shall serve as the inactivated virus suspension.

The inactivated virus suspension shall be tested as specified in 3.4.1.2.

2.3.2.4 Bulk material

The inactivated virus suspension shall be concentrated by a method approved as suitable and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.

2.3.3 Aino virus bulk material

2.3.3.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.3.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.3.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.3.

2.3.3.3 Inactivation

Virus suspension shall be inactivated by the addition of an inactivator approved as appropriate and shall serve as the inactivated virus suspension.

The inactivated virus suspension shall be tested as specified in 3.4.1.3.

2.3.3.4 Bulk material

The inactivated virus suspension shall be concentrated by a method approved as suitable and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.

2.3.4 Peaton virus bulk material

2.3.4.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.4.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.4.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.4.

2.3.4.3 Inactivation

Virus suspension shall be inactivated by the addition of an inactivator approved as appropriate and shall serve as the inactivated virus suspension.

The inactivated virus suspension shall be tested as specified in 3.4.1.4.

2.3.4.4 Bulk material

The inactivated virus suspension shall be concentrated by a method approved as suitable and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.

2.4 Final bulk

The Akabane virus bulk material, Kasba virus bulk material, Aino virus bulk material and Peaton virus bulk material shall be adjusted for their antigen levels and mixed, and then mixed with an aluminum gel adjuvant or adjuvant approved as suitable and shall serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.6.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.3.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

The tests given in 1.1 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cell line

3.2.1 Tests on master cell seeds

3.2.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Test for identification of the animal species of the cell

The tests given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.5 Test for freedom from extraneous viruses

3.2.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2 Test for freedom from specific viruses

3.2.1.5.2.1 General test for freedom from specific viruses

The tests given in 1.1 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis

virus, and Rabies virus, the tests given in 1.2, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.6 Test for karyological (chromosomal) characterization

The tests given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.7 Test for freedom from tumorigenicity

The tests given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on virus suspension

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Test for virus content

3.3.2.1 Akabane virus

3.3.2.1.1 Materials

3.3.2.1.1.1 Test materials

The test article shall be diluted ten-fold with the virus growth medium approved as suitable, and each level dilution shall serve as the test material.

3.3.2.1.1.2 Cultured cells

HmLu-1 cells shall be used.

3.3.2.1.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions (wells) of cultured cells and allowed to stand for adsorption at 37ºC for 60 minutes. The 1.0 mL portions of the virus growth medium approved as suitable shall be added, cultured at 37ºC for seven days, and observed.

3.3.2.1.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{6.5}TCID_{50}$ or higher unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.3.2.2 Kasba virus

3.3.2.2.1 Materials

3.3.2.2.1.1 Test materials

The test article shall be diluted ten-fold with the virus growth medium approved as suitable, and each level dilution shall serve as the test material.

3.3.2.2.1.2 Cultured cells

Vero-T cells shall be used.

3.3.2.2.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions (wells) of cultured cells and allowed to stand for adsorption at 36ºC for 60 minutes. The 1.0 mL portions of the virus growth medium approved as suitable shall be added, cultured at 37ºC for seven days, and observed.

3.3.2.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{7.5}TCID_{50}$ or higher unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.3.2.3 Aino virus

3.3.2.3.1 Materials

3.3.2.3.1.1 Test materials

The test article shall be diluted ten-fold with the virus growth medium approved as suitable, and each level dilution shall serve as the test material.

3.3.2.3.1.2 Cultured cells

HmLu-1 cells shall be used.

3.3.2.3.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions (wells) of cultured cells and allowed for adsorption at 36ºC for 60 minutes. The 1.0 mL portions of the virus growth medium approved as suitable shall be added, cultured at 37ºC for seven days, and observed.

3.3.2.3.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{6.5}TCID_{50}$ or higher unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.3.2.4 Peaton virus

3.3.2.4.1 Materials

3.3.2.4.1.1 Test materials

The test article shall be diluted ten-fold with the virus growth medium approved as suitable, and each level dilution shall serve as the test material.

3.3.2.4.1.2 Cultured cells

HmLu-1 cells shall be used.

3.3.2.4.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions (wells) of cultured cells and allowed to stand for adsorption at 37ºC for 60 minutes. The 1.0 mL portions of the virus growth medium approved as suitable shall be added, cultured at 37ºC for seven days, and observed.

3.3.2.4.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{7.5}TCID_{50}$ or higher unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.4 Tests on inactivated virus suspension

3.4.1 Inactivation test

3.4.1.1 Akabane virus

3.4.1.1.1 Materials

3.4.1.1.1.1 Test materials

A 5 mL portion of the test article shall be dialyzed overnight at 4ºC using at least a 100-fold volume of phosphate-buffered saline to remove the inactivator, and it shall be used as the test material.

3.4.1.1.1.2 Cultured cells

HmLu-1 cells shall be cultured, and monolayer cultures shall be used.

3.4.1.1.2 Test procedures

Whole test material shall be inoculated to the cultured cell sheet of at least 3 cm^2 per 1 mL portion, allowed to stand for adsorption at 34ºC for 60 minutes, cultured with culture medium approved as suitable at 37ºC for seven days, and observed.

3.4.1.1.3 Judgment

The cultured cells in which no CPE is observed shall be judged to be negative for active virus.

No active virus shall be observed in the test material.

3.4.1.2 Kasba virus

3.4.1.2.1 Materials

3.4.1.2.1.1 Test materials

A 5 mL portion of the test article shall be dialyzed overnight at 4ºC using at least a 100-fold volume of phosphate-buffered saline to remove the inactivator, and it shall be used as the test material.

3.4.1.2.1.2 Cultured cells

Vero-T cells shall be cultured, and monolayer cultures shall be used.

3.4.1.2.2 Test procedures

Whole test material shall be inoculated to the cultured cell sheet of at least 3 cm^2 per 1 mL portion, allowed to stand for adsorption at 34ºC for 60 minutes, cultured with culture medium approved as suitable at 34 – 36ºC for five days, and then the cells shall be passaged to the next generation. After the cell layers are formed, culture media shall be sampled. The culture medium approved as suitable shall be added, cultured at 34ºC to 36ºC for five days, passaged to subsequent generations, and then cultured in the same manner as the second generation and observed.

3.4.1.2.3 Judgment

The cultured cells in which no CPE is observed shall be judged to be negative for active virus. No active virus shall be observed in the test material.

3.4.1.3 Aino virus

3.4.1.3.1 Materials

3.4.1.3.1.1 Test materials

A 5 mL portion of the test article shall be dialyzed overnight at 4ºC using at least a 100-fold volume of phosphate-buffered saline to remove the inactivator, and it shall be used as the test material.

3.4.1.3.1.2 Cultured cells

HmLu-1 cells shall be cultured, and monolayer cultures shall be used.

3.4.1.3.2 Test procedures

Whole test material shall be inoculated to the cultured cell sheet of at least 3 cm^2 per 1 mL portion, allowed to stand for adsorption at 34ºC for 60 minutes, cultured with culture medium approved as suitable at 34ºC to 36ºC for seven days, and observed.

3.4.1.3.3 Judgment

The cultured cells in which no CPE is observed shall be judged to be negative for active virus. No active virus shall be observed in the test material.

3.4.1.4 Peaton virus

3.4.1.4.1 Materials

3.4.1.4.1.1 Test materials

A 5 mL portion of the test article shall be dialyzed overnight at 4ºC using at least a 100-fold volume of phosphate-buffered saline to remove the inactivator, and it shall be used as the test material.

3.4.1.4.1.2 Cultured cells

HmLu-1 cells shall be cultured, and monolayer cultures shall be used.

3.4.1.4.2 Test procedures

Whole test sample shall be inoculated to the cultured cell sheet of at least 3 cm^2 per 1 mL portion, allowed to stand for adsorption at 34ºC for 60 minutes, cultured with culture medium

approved as suitable at 34ºC to 36ºC for seven days, and observed.

3.4.1.4.3 Judgment

The cultured cells in which no CPE is observed shall be judged to be negative for active virus. No active virus shall be observed in the test article.

3.5 Tests on bulk material

3.5.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6 Tests on final product

3.6.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be a homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of the product per small container shall be uniform.

3.6.2 Test for pH

When the test is performed as specified in the Test for pH of the General Tests, the pH shall have an intrinsic value.

3.6.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6.4 Test for formalin content

For products inactivated with formalin when the test given in the Test for Formalin Content of the General Tests is applied, the formalin content shall be no higher than 0.05 vol%.

3.6.5 Test for aluminum content

When the test is performed as specified in the Test for Aluminum Content of the General Tests, the content of aluminum shall have a specific value. When the Minister of Agriculture, Forestry and Fisheries specifies the test procedure, follow it.

3.6.6 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.6.7 Potency test

3.6.7.1 Materials

3.6.7.1.1 Injection materials

The test samples shall be used as injection materials.

3.6.7.1.2 Test animals

Guinea pigs weighing approximately 350 g shall be used.

3.6.7.1.3 Virus for neutralization test

3.6.7.1.3.1 Akabane virus

Akabane virus JaGAr 39 strain propagated in HmLu cells or strain approved as equivalent thereof shall be used.

3.6.7.1.3.2 Kasba virus

Kasba virus K-47 strain propagated in BHK-21 cells or strain approved as equivalent thereof shall be used.

3.6.7.1.3.3 Aino virus

Aino virus JaNAr 28 strain propagated in HmLu cells or strain approved as equivalent thereof shall be used.

3.6.7.1.3.4 Peaton virus

Peaton virus NS/3 strain propagated in HmLu cells or strain approved as equivalent thereof shall be used.

3.6.7.1.4 Cultured cells

HmLu-1 cells and Vero-T cells shall be used.

3.6.7.2 Test procedures

A 0.5 mL each of the injection materials shall be injected intramuscularly twice at intervals of three weeks in five test animals, and serum shall be obtained from individual animals 10 days after the second injection to conduct a neutralization test.

The test serum shall be heat-inactivated, and then diluted with culture medium approved as suitable in two-fold serial dilutions. Mix equal quantities of each diluted serum and virus suspension for neutralization test containing approximately 200 $TCID_{50}$ in 0.1 mL and neutralize at 37ºC for 60 minutes. For the Kasba virus, the mixture shall be neutralized for 90 minutes. The mixtures obtained shall be inoculated at a dose of 0.1 mL into four portions (wells) of cultured HmLu-1 cells for the Akabane virus, Aino virus, and Peaton virus, respectively, and into four portions (wells) of cultured Vero-T cells for the Kasba virus, respectively, and allowed to stand for adsorption at 37ºC for 60 minutes. Then, culture media approved as suitable shall be added at a dose of 1.0 mL and cultured at 37ºC for seven days for the Akabane virus, Kasba virus, and Peaton virus and at 34ºC to 36ºC for seven days for the Aino virus.

3.6.7.3 Judgment

The maximum dilution factor of serum at which CPE is observed in at least two portions of cultured cells shall be defined as the neutralizing antibody titer.

A 16-fold or greater increase in the neutralizing antibody titer against the Akabane virus and Peaton virus, a 32-fold or greater increase in the neutralizing antibody titer against the Kasba virus and an 8-fold or greater increase in the neutralizing antibody titer against the Aino virus shall be regarded as positive for neutralizing antibodies.

The neutralizing antibody positive rates in test animals shall be 80% or higher against each virus.

4 Storage and expiry date

The expiry date shall be two years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Infection Bovine Rhinotracheitis, Bovine Viral Diarrhea (bivalent), Bovine Parainfluenza, Bovine Respiratory Syncytial Virus Infection Vaccine (adjuvant), Inactivated, Seed

1 Definition

This Vaccine, Inactivated, Seed is made by mixing the inactivated virus suspensions obtained by propagating Infections bovine rhinotracheitis virus, Bovine viral diarrhea virus type 1, Bovine viral diarrhea virus type 2, Bovine parainfluenza type 3 virus, and Bovine respiratory syncytial virus that meet the seed lot specifications in cell lines that meet these specifications, respectively, and by adding an adjuvant.

2 Production methods

2.1 Virus strain used for production

2.1.1 Infectious bovine rhinotracheitis virus

2.1.1.1 Name

Infectious bovine rhinotracheitis virus No. 758-KB strain or strain approved as equivalent thereof

2.1.1.2 Properties

This virus shows the pathogenic effects on cows, such as fever, respiratory symptoms, and leukopenia.

This virus shall be propagated in cultured cells derived from bovine and cultured cells derived from swine in which CPE is confirmed.

2.1.1.3 Master seed virus

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.1.4 Working seed virus

2.1.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in MDBK-NSC cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.1.5 Production seed virus

2.1.1.5.1 Propagation and storage

The production seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 Bovine viral diarrhea virus type 1

2.1.2.1 Name

Bovine viral diarrhea virus type 1 Nose-KB strain or strain approved as equivalent thereof

2.1.2.2 Properties

Inoculation of this virus into the nasal cavity of cows causes respiratory symptoms, fever viremia, and leukopenia.

This virus shall be propagated in cultured cells derived from bovine in which CPE is confirmed, and if co-infected with cells infected with the non-cytopathogenic strain, CPE is inhibited.

2.1.2.3 Master seed virus

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.2.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.2.4 Working seed virus

2.1.2.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in MDBK-NSC cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC

or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.2.5 Production seed virus

2.1.2.5.1 Propagation and storage

The production seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.3 Bovine viral diarrhea virus type 2

2.1.3.1 Name

Bovine viral diarrhea virus type 2 KZ-91-KB strain or strain approved as equivalent thereof

2.1.3.2 Properties

Inoculation of this virus into the nasal cavity of calves causes respiratory symptoms, fever, viremia, and leukopenia.

This virus shall be propagated in cultured cells derived from bovine in which CPE is confirmed, and if co-infected with cells infected with the non-cytopathogenic strain, CPE is inhibited.

2.1.3.3 Master seed virus

2.1.3.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.3.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.3.4 Working seed virus

2.1.3.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in MDBK-NSC cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.3.5 Production seed virus

2.1.3.5.1 Propagation and storage

The production seed virus shall be propagated in MDBK-NSC cells or cells approved as

suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.4 Bovine parainfluenza type 3 virus

2.1.4.1 Name

Bovine parainfluenza type 3 virus BN_1-1-KB strain or strain approved as equivalent thereof

2.1.4.2 Properties

This virus shows the pathogenic effects on cows, such as fever and respiratory symptoms.

This virus shall be propagated in cultured cells derived from bovine and cell lines derived from African green monkey kidneys (hereinafter referred to as “Vero cells” in this paragraph) in which CPE is confirmed.

2.1.4.3 Master seed virus

2.1.4.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.4.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4.4 Working seed virus

2.1.4.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in MDBK-NSC cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.4.5 Production seed virus

2.1.4.5.1 Propagation and storage

The production seed virus shall be propagated in MDBK-NSC cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.5 Bovine respiratory syncytial virus

2.1.5.1 Name

Bovine respiratory syncytial virus rs-52-KB strain or strain approved as equivalent thereof

2.1.5.2 Properties

Inoculation of this virus on cows cause no pathogenic effects.

This virus shall be propagated in the following cells in which CPE is confirmed: cells derived from bovine, cells derived from hamster lung and Vero cells.

2.1.5.3 Master seed virus

2.1.5.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu-SC cells or cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.5.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.5.4 Working seed virus

2.1.5.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HmLu-SC cells or cells approved as suitable.

The working seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5.5 Production seed virus

2.1.5.5.1 Propagation and storage

Production seed virus shall be propagated in HmLu-SC cells or cells approved as suitable.

The production seed virus shall be stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Infectious bovine rhinotracheitis virus, Bovine viral diarrhea virus type 1, Bovine viral diarrhea virus type 2, and Bovine parainfluenza type 3 virus

2.2.1.1 Cultured cells

MDBK-NSC cells or cell lines approved as suitable shall be used.

2.2.1.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.1.3 Master cell seeds

2.2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.1.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower.

The master cell seeds shall be tested as specified in 3.2.1.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines.

The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.1.4 Working cell seeds

2.2.1.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.1.2.

The working cell seeds shall be stored frozen at -70ºC or lower.

The working cell seeds shall be tested as specified in 3.2.2.1.

2.2.1.5 Production cell seeds

2.2.1.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.1.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3.1 if they need to be stored.

2.2.2 Bovine respiratory syncytial virus

2.2.2.1 Cultured cells

HmLu-SC cells or cell lines approved as suitable shall be used.

2.2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.2.3 Master cell seeds

2.2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower.

The master cell seeds shall be tested as specified in 3.2.1.2.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production

cell seeds.

2.2.2.4 Working cell seeds

2.2.2.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.2.

The working cell seeds shall be stored frozen at -70ºC or lower.

The working cell seeds shall be tested as specified in 3.2.2.2.

2.2.2.5 Production cell seeds

2.2.2.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3.2 if they need to be stored.

2.3 Bulk material

2.3.1 Infectious bovine rhinotracheitis virus

2.3.1.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.1.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.1.

2.3.1.3 Inactivation

The virus suspension inactivated by treating it with a method approved as suitable shall be used as the bulk material.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.1.

2.3.2 Bovine viral diarrhea virus type 1

2.3.2.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected in production cell seeds prior to the inoculation of the virus.

2.3.2.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.2.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.2.

2.3.2.3 Inactivation

The virus suspension inactivated by treating it with a method approved as suitable shall be used

as the bulk material.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.1.

2.3.3 Bovine viral diarrhea virus type 2

2.3.3.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.3.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.3.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.3.

2.3.3.3 Inactivation

The virus suspension inactivated by treating it with a method approved as suitable shall be used as the bulk material.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.1.

2.3.4 Bovine parainfluenza type 3 virus

2.3.4.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.4.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.4.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.4.

2.3.4.3 Inactivation

The virus suspension inactivated by treating it with a method approved as suitable shall be used as the bulk material.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.1.

2.3.5 Bovine respiratory syncytial virus

2.3.5.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected in production cell seeds prior to the inoculation of the virus.

2.3.5.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.5.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at a time deemed appropriate shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.1 and 3.3.2.5.

2.3.5.3 Inactivation

The virus suspension inactivated by treating it with a method approved as suitable shall serve as the bulk material.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.2.

2.4 Final bulk

Mix Infectious bovine rhinotracheitis virus bulk material, Bovine viral diarrhea virus type 1 bulk material, Bovine viral diarrhea virus type 2 bulk material, Bovine parainfluenza type 3 virus bulk material, and Bovine respiratory syncytial virus bulk, add adjuvants and preservatives approved as suitable, and then adjust the concentration of the mixture to serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.5.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Infectious bovine rhinotracheitis virus

3.1.1.1.1 Identification test

The tests given in 1.4.2.3.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.1.4 Test for freedom from extraneous viruses

3.1.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.1.4.2 Test for freedom from specific viruses

3.1.1.1.4.2.1 General test for freedom from specific viruses

For the Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 1.1 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.1.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom

from Extraneous Viruses of the General Tests shall apply.

3.1.1.2 Bovine viral diarrhea virus type 1

3.1.1.2.1 Identification test

The tests given in 1.4.2.3.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.2.4 Test for freedom from extraneous viruses

3.1.1.2.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.2.4.2 Test for freedom from specific viruses

3.1.1.2.4.2.1 General test for freedom from specific viruses

For the Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.2.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.3 Bovine viral diarrhea virus type 2

3.1.1.3.1 Identification test

The tests given in 1.4.2.3.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.3.4 Test for freedom from extraneous viruses

3.1.1.3.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.3.4.2 Test for freedom from specific viruses

3.1.1.3.4.2.1 General test for freedom from specific viruses

For the Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 3.1 in the

Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.3.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4 Bovine parainfluenza type 3 virus

3.1.1.4.1 Identification test

The tests given in 1.4.2.3.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.4.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.4.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4.4 Test for freedom from extraneous viruses

3.1.1.4.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.4.2 Test for freedom from specific viruses

3.1.1.4.4.2.1 General test for freedom from specific viruses

For the Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 1.1 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.5 Bovine respiratory syncytial virus

3.1.1.5.1 Identification test

The tests given in 1.4.2.3.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.5.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.5.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.5.4 Test for freedom from extraneous viruses

3.1.1.5.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the

General Tests shall apply.

3.1.1.5.4.2 Test for freedom from specific viruses

3.1.1.5.4.2.1 General test for freedom from specific viruses

For the Lymphocytic choriomeningitis virus and Bluetongue virus, the tests given in 1.1 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.5.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.5, 3.2.7, 3.2.8 and 2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cultured cells

3.2.1 Tests on master cell seeds

3.2.1.1 MDBK-NSC cells

3.2.1.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.1.2 Test for identification of the animal species of the cell

The tests given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.1.5 Test for freedom from extraneous viruses

3.2.1.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the

General Tests shall apply.

3.2.1.1.5.2 Test for freedom from specific viruses

3.2.1.1.5.2.1 General test for freedom from specific viruses

For the Bovine respiratory syncytial virus and Bluetongue virus, the tests given in 1.2 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.1.5.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.2, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.1.6 Test for karyological (chromosomal) characterization

The tests given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.1.7 Test for freedom from tumorigenicity

The tests given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.1.2 HmLu-SC cells

3.2.1.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2.2 Test for identification of the animal species of the cell

The tests given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.2.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.2.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.2.5 Test for freedom from extraneous viruses

3.2.1.2.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.2.5.2 Test for freedom from specific viruses

3.2.1.2.5.2.1 General test for freedom from specific viruses

For the Bovine respiratory syncytial virus, Bluetongue virus, and Lymphocytic choriomeningitis virus, the tests given in 1.2 and 3.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.2.5.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Rotavirus, Bovine leukemia virus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.2, 3.2.5, 3.2.7, 3.2.8 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.2.6 Test for karyological (chromosomal) characterization

The tests given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.2.7 Test for freedom from tumorigenicity

The tests given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 MDBK-NSC cells

3.2.2.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.2.2 HmLu-SC cells

3.2.2.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3 Tests on production cell seeds

3.2.3.1 MDBK-NSC cells

3.2.3.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3.2 HmLu-SC cells

3.2.3.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on virus suspension

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Test for virus content

3.3.2.1 Infectious bovine rhinotracheitis virus

3.3.2.1.1 Materials

3.3.2.1.1.1 Test materials

The test article shall be diluted ten-fold with cell growth medium (Note 1) and each level dilution shall serve as the test material.

3.3.2.1.1.2 Cultured cells

Cell line suspension derived from bovine kidneys (hereinafter referred to as “MDBK cells” in this paragraph) shall be used.

3.3.2.1.2 Test procedures

The 100 μL portions of the test material shall be inoculated into 100 μL portions of cultured cells dispensed into at least 4 wells in a 96-well microplate, cultured in an air containing 5 vol% carbon dioxide at 37ºC for seven days, and observed.

3.3.2.1.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{8.0}TCID_{50}$ or higher.

3.3.2.2 Bovine viral diarrhea virus type 1

3.3.2.2.1 Materials

3.3.2.2.1.1 Test materials

The test article shall be diluted ten-fold with cell growth medium and each level dilution shall serve as the test material.

3.3.2.2.1.2 Cultured cells

Bovine testis cell suspension shall be used.

3.3.2.2.2 Test procedures

The 100 μL portions of the test sample shall be inoculated into 100 μL portions of cultured cells dispensed into at least 4 wells in a 96-well microplate, cultured in an air containing 5 vol% carbon dioxide at 37ºC for seven days, and observed.

3.3.2.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{7.6}TCID_{50}$ or higher.

3.3.2.3 Bovine viral diarrhea virus type 2

3.3.2.3.1 Materials

3.3.2.3.1.1 Test materials

The test article shall be diluted ten-fold with cell growth medium and each level dilution shall serve as the test material.

3.3.2.3.1.2 Cultured cells

Bovine testis cell suspension shall be used.

3.3.2.3.2 Test procedures

The 100 μL portions of the test sample shall be inoculated into 100 μL portions of cultured cells dispensed into at least 4 wells in a 96-well microplate, cultured in an air containing 5 vol% carbon dioxide at 37ºC for seven days, and observed.

3.3.2.3.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{6.4}TCID_{50}$ or higher.

3.3.2.4 Bovine parainfluenza type 3 virus

3.3.2.4.1 Materials

3.3.2.4.1.1 Test materials

The test article shall be diluted ten-fold with cell growth medium and each level dilution shall serve as the test material.

3.3.2.4.1.2 Cultured cells

MDBK cell suspension shall be used.

3.3.2.4.2 Test procedures

The 100 μL portions of the test sample shall be inoculated into 100 μL portions of cultured cells dispensed into at least 4 wells in a 96-well microplate, cultured in an air containing 5 vol% carbon dioxide at 37ºC for seven days, and observed.

3.3.2.4.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{7.9}TCID_{50}$ or higher.

3.3.2.5 Bovine respiratory syncytial virus

3.3.2.5.1 Materials

3.3.2.5.1.1 Test materials

The test article shall be diluted ten-fold with cell growth medium and each level dilution shall serve as the test material.

3.3.2.5.1.2 Cultured cells

Vero cell suspension shall be used.

3.3.2.5.2 Test procedures

The 100 μL portions of the test sample shall be inoculated into 100 μL portions of cultured cells

dispensed into at least 4 wells in a 96-well microplate, cultured in an air containing 5 vol% carbon dioxide at 34ºC for seven days, and observed.

3.3.2.5.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$. The virus content in 1 mL shall be $10^{6.6}TCID_{50}$ or higher.

3.4 Tests on bulk material

3.4.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.2 Inactivation test

3.4.2.1 Infectious bovine rhinotracheitis virus, Bovine viral diarrhea virus type 1, Bovine viral diarrhea virus type 2 and Bovine parainfluenza type 3 virus

3.4.2.1.1 Materials

3.4.2.1.1.1 Test materials

The test article shall be dialyzed overnight at 4ºC using at least a 100-fold volume of phosphate-buffered saline to remove the inactivator, and it shall be used as the test material.

3.4.2.1.1.2 Cultured cells

MDBK cells shall be cultured for one to three days, and monolayer cultures shall be used.

3.4.2.1.2 Test procedures

A 1 mL portion of the test material shall be inoculated to the cultured cell sheet of at least 3 cm^2, allowed to adsorb for 90 minutes at 37ºC, cultured with the virus growth medium (Note 2) at 37ºC for seven days, and observed.

3.4.2.1.3 Judgment

The cultured cells in which no CPE is observed shall be judged to be negative for active virus. No active virus shall be observed in the test materials.

3.4.2.2 Bovine respiratory syncytial virus

3.4.2.2.1 Materials

3.4.2.2.1.1 Test materials

The test article shall be dialyzed overnight at 4ºC using at least a 100-fold volume of phosphate-buffered saline to remove the inactivator, and it shall be used as the test material.

3.4.2.2.1.2 Cultured cells

Vero cells shall be cultured for one to three days, and monolayer cultures shall be used.

3.4.2.2.2 Test procedures

A 1 mL portion of the test sample shall be inoculated to the cultured cell sheet of at least 3 cm^2 or more, allowed to adsorb for 90 minutes at 37ºC, cultured with the virus growth medium at 34ºC for seven days, and observed.

3.4.2.2.3 Judgment

The cultured cells in which no CPE is observed shall be judged to be negative for active virus. No active virus shall be observed in the test materials.

3.5 Tests on final product

3.5.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be a homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of the product per small container shall be uniform.

3.5.2 Test for pH

When the test is performed as specified in the Test for pH of the General Tests, the pH shall have an intrinsic value.

3.5.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.5.4 Test for formalin content

When the test given in the Test for Formalin Content of the General Tests is applied, the formalin content shall be no higher than 0.08 vol%.

3.5.5 Test for aluminum content

When the test is performed as specified in the Test for Aluminum Content of the General Tests, the content of aluminum shall be 1.35–1.65 mg in 1 mL.

3.5.6 Toxicity limit test

The test given in the Toxicity Limit Test 2 of the General Tests shall apply. However, verify by a t-test of population mean differences assuming equal variances (unpaired, two-tailed, 5% level of significance) that the body weight on the third day after injected at a dose of 1 mL is equal to or greater than that before injected.

3.5.7 Potency test

3.5.7.1 Potency on infectious bovine rhinotracheitis

3.5.7.1.1 Materials

3.5.7.1.1.1 Injection materials

The test samples shall be used as injection materials.

3.5.7.1.1.2 Test animals

Guinea pigs weighing approximately 300 g shall be used.

3.5.7.1.1.3 Virus for neutralization test

Infectious bovine rhinotracheitis virus No. 758 strain or strain approved as suitable shall be used.

3.5.7.1.1.4 Cultured cells

MDBK cell suspension shall be used.

3.5.7.1.2 Test procedures

Three mL each of the injection materials shall be injected twice at intervals of two weeks in five test animals. Each test material shall be injected intramuscularly at a volume of 1 mL into two sites and subcutaneously at a volume of 1 mL into one site. The serum shall be obtained from individual animals 10 days after the second injection to conduct a neutralization test.

The serum shall be heat-inactivated, and then shall be diluted two-fold with the virus growth medium. Mix equal quantities of each diluted serum and virus for neutralization test containing approximately 200 $TCID_{50}$ in 25 μL and neutralize at 37ºC for 18 hours. The 25 μL portions of the mixed solution shall be inoculated into 4 wells in a 96-well microplate, the 0.1 mL portions of the cultured cells shall be added, and cultured in an air containing 5 vol% carbon dioxide at 37ºC for seven days, and observed.

3.5.7.1.3 Judgment

The maximum dilution factor of serum at which inhibition of CPE is observed in at least two wells of cultured cells shall be defined as the neutralizing antibody titer.

When a 128-fold or greater increase in the neutralizing antibody titer is regarded as positive, the neutralizing antibody positive rate in test animals shall be 80% or higher.

3.5.7.2 Potency on bovine viral diarrhea

3.5.7.2.1 Materials

3.5.7.2.1.1 Injection materials

The test samples shall be used as injection materials.

3.5.7.2.1.2 Test animals

Rats weighing approximately 100 g shall be used.

3.5.7.2.1.3 Virus for neutralization test

3.5.7.2.1.3.1 Bovine viral diarrhea virus type 1

Bovine viral diarrhea virus type 1 Nose strain or strain approved as suitable shall be used.

3.5.7.2.1.3.2 Bovine viral diarrhea virus type 2

Bovine viral diarrhea virus type 2 KZ-91-cp strain or strain approved as suitable shall be used.

3.5.7.2.1.4 Cultured cells

MDBK cell suspension shall be used.

3.5.7.2.2 Test procedures

Two mL each of the injection materials shall be injected intramuscularly at a volume of 1 mL each in thighs of both hindlimbs in five test animals. Serum shall be obtained from individual animals 21 days after the injection to conduct a neutralization test.

The serum shall be heat-inactivated, and then shall be diluted two-fold with the virus growth medium. Mix equal quantities of each diluted serum and virus for neutralization test containing approximately 200 $TCID_{50}$ in 25 μL and neutralize at 37ºC for 60 minutes. The 25 μL portions of the mixed solution shall be inoculated into 4 wells in a 96-well microplate, the 0.1 mL portions of

the cultured cells shall be added, and cultured in an air containing 5 vol% carbon dioxide at 37ºC for seven days, and observed.

3.5.7.2.3 Judgment

The maximum dilution factor of serum at which inhibition of CPE is observed in at least two wells of cultured cells shall be defined as the neutralizing antibody titer.

When a two-fold or greater increase in the neutralizing antibody titer is regarded as positive, the neutralizing antibody positive rates in test animals shall be 80% or higher against bovine viral diarrhea virus type 1 and bovine viral diarrhea virus type 2, respectively.

3.5.7.3 Potency test on bovine parainfluenza

3.5.7.3.1 Materials

3.5.7.3.1.1 Injection materials

The injection materials specified in 3.5.7.1.1.1 shall be used.

3.5.7.3.1.2 Test animals

The test animals specified in 3.5.7.1.1.2 shall be used.

3.5.7.3.1.3 Virus for neutralization test

Bovine parainfluenza type 3 virus BN_1-1 strain or strain approved as suitable shall be used.

3.5.7.3.1.4 Cultured cells

Vero cell suspension shall be used.

3.5.7.3.2 Test procedures

The test method given in 3.5.7.1.2 shall apply.

However, the serum to be tested shall be neutralized at 37ºC for 60 minutes.

3.5.7.3.3 Judgment

The maximum dilution factor of serum at which inhibition of CPE is observed in at least two wells of cultured cells shall be defined as the neutralizing antibody titer.

When an at least four-fold increase in the neutralizing antibody titer is regarded as positive, the neutralizing antibody positive rate in test animals shall be 80% or higher.

3.5.7.4 Potency on bovine respiratory syncytial virus infection

3.5.7.4.1 Materials

3.5.7.4.1.1 Injection materials

The injection materials specified in 3.5.7.1.1.1 shall be used.

3.5.7.4.1.2 Test animals

The test animals specified in 3.5.7.1.1.2 shall be used.

3.5.7.4.1.3 Virus for neutralization test

Bovine respiratory syncytial virus NMK7 strain or strain approved as suitable shall be used.

3.5.7.4.1.4 Cultured cells

Vero cells which are grown as monolayers in a 96-well microplate shall be used.

3.5.7.4.2 Test procedures

A neutralization test shall be performed on the serum obtained in 3.5.7.1.2.

The serum shall be heat-inactivated, and then shall be diluted two-fold with the virus growth medium. Equal quantities of each diluted serum and virus for neutralization test containing approximately 200 $TCID_{50}$ in 25 μL shall be mixed and neutralized at 37ºC for 60 minutes. Removing the culture medium of Vero cells, the 25 μL portions of the mixture shall be added into 4 wells, respectively, and adsorbed in an air containing 5 vol% carbon dioxides at 37ºC for 60 minutes. After the adsorption, the 0.1 mL portions of the virus growth medium shall be added, cultured in an air containing 5 vol% carbon dioxides at 34ºC for seven days, and observed.

3.5.7.4.3 Judgment

The maximum dilution factor of serum at which inhibition of CPE is observed in at least two wells of cultured cells shall be defined as the neutralizing antibody titer.

When a two-fold or greater increase in the neutralizing antibody titer is regarded as positive, the neutralizing antibody positive rate in test animals shall be 80% or higher.

4 Storage and expiry date

The expiry date shall be one year and six months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Cell growth medium

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Fetal bovine serum	50–100 mL
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.2–7.6 with sodium hydrogen carbonate.

Fetal bovine serum used shall be suitable for cell maintenance and virus growth.

Minimal amounts of antibiotics may be added.

Note 2 Virus growth medium

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Fetal bovine serum	20–100 mL
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.2–7.6 with sodium hydrogen carbonate.

Fetal bovine serum used shall be suitable for cell maintenance and virus growth.

Minimal amounts of antibiotics may be added.

Equine Influenza Vaccine, Inactivated

1 Definition

Equine Influenza Vaccine, Inactivated is an inactivated vaccine produced by the purification and concentration of the virus suspension obtained by propagating Equine influenza viruses in embryonated chicken eggs.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Strains of Equine influenza A virus specified separately

2.1.2 Properties

The virus strains shall be propagated in the allantoic cavities of embryonated chicken eggs, and it shall agglutinate chicken red blood cells.

2.1.3 Passage and storage

The original strain and seed virus shall be propagated in the embryonated chicken eggs.

The original strain and seed virus shall not be passaged more than five times and three times, respectively.

This does not apply if especially specified.

The original strain and seed virus shall be stored frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

2.2 Materials for production

2.2.1 Embryonated chicken eggs

Embryonated chicken eggs aged 10–12 days shall be used.

2.3 Bulk material

2.3.1 Virus cultivation

Each strain of virus shall be separately inoculated into the allantoic cavities of embryonated chicken eggs, and the inoculated eggs shall be incubated. The allantoic fluid containing the propagated virus shall be harvested to serve as the virus suspension of each strain.

The virus suspension shall be tested as specified in 3.1.

2.3.2 Virus purification and concentration

Each virus suspension shall be purified and concentrated by the ultracentrifugal method, alcohol precipitation method, or other methods approved as suitable. In this case, a stabilizer approved as suitable may be added.

2.3.3 Inactivation

Each purified and concentrated virus suspension shall be inactivated by the addition of formalin to serve as the virus suspension of each strain.

The inactivated virus suspensions shall be tested as specified in 3.2.

2.3.4 Bulk material

Virus suspension shall be mixed and diluted with a dilution approved as suitable to adjust the concentration to serve as the bulk material.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

The bulk material shall be mixed to serve as the final bulk. In this case, a stabilizer approved as suitable may be added.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final products.

The final products shall be tested as specified in 3.4.

3 Test methods

3.1 Test on virus suspension

3.1.1 Hemagglutination titer test

3.1.1.1 Materials

3.1.1.1.1 Test materials

A series of two-fold dilutions of the test article shall be prepared using physiological saline. Each dilution shall be used as the test material.

3.1.1.2 Test procedures

To each 0.4 mL portion of the test material, add 0.4 mL portion of chicken red blood cells adjusted to 0.5 vol% and allow to react for 60 minutes.

3.1.1.3 Judgment

The highest dilution of test article that causes the hemagglutination is considered the hemagglutination titer.

The hemagglutination titer of the test article shall be within its specific range.

3.2 Test on inactivated virus suspension

3.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2 Inactivation test

3.2.2.1 Materials

3.2.2.1.1 Injection materials

The test article shall be used as the injection material.

3.2.2.1.2 Embryonated chicken eggs

Eggs aged 9–10 days shall be used as specified in 1.1 in the Materials for Live Vaccine Production.

3.2.2.2 Test procedures

The injection material shall be inoculated at a dose of 0.2 mL into the allantoic cavities of at least six embryonated chicken eggs. The inoculated eggs shall be incubated at 32℃ to 36℃. The allantoic fluid shall be passaged every three days to the second generation. To the second-passaged allantoic fluid, red blood cells given in 3.1.1.2 shall be added and observed.

If the allantoic fluid of each egg shows hemagglutination, equal volumes of these allantoic fluids shall be mixed and passaged to the third generation. The third-passaged allantoic fluid shall be retested in a similar manner and then judged.

3.2.2.3 Judgment

If no hemagglutination is observed, the test article shall be judged to be negative for active virus.

No active virus shall be observed in the test article.

3.3 Tests on bulk material

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 CCA test

3.3.2.1 Materials

The test article and standard influenza vaccine (for CCA) (Note 1) shall be used as the materials.

3.3.2.2 Test procedures

The CCA value of the test article shall be determined by the modified Miller-Stanley's method.

3.3.2.3 Judgment

The CCA value in 1 mL of the test article shall be within its specific range.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be a liquid or a homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.4.2 Test for pH

When the test is performed as specified in the Test for pH of the General Tests, the pH shall have an intrinsic value.

3.4.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.4 Test for formalin content

For products added with formalin, when the test given in the Test for Formalin Content of the General Tests is applied, the formalin content shall be no higher than 0.1 vol%.

3.4.5 Test for thimerosal content

For products added with thimerosal, the test given in the Test for Thimerosal Content of the General Tests shall apply.

3.4.6 Test for protein nitrogen content

When the test is performed as specified in the Test for Protein Nitrogen Content of the General Tests, the protein nitrogen content shall be 50 μg or lower in 1 mL.

3.4.7 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.4.8 Potency test

3.4.8.1 Materials

3.4.8.1.1 Injection materials

The test sample shall be diluted ten-fold with phosphate-buffered saline to serve as the injection material.

3.4.8.1.2 Test animals

Mice aged 4 weeks shall be used.

3.4.8.1.3 Hemagglutination antigens

Hemagglutination antigens of each virus strain contained in the test sample shall be used.

3.4.8.2 Test procedures

Twenty test animals inoculated with 0.5 mL portions of injection materials into the peritoneal cavities, and then divided into four groups. Sera obtained after 14 days shall be pooled for each group to perform a hemagglutination inhibition test. The sera to be tested shall be prepared by RDE, trypsin, potassium periodate, or a method approved as suitable and make two-fold dilutions. To 0.2 mL of each dilution, a 0.2 mL portion of hemagglutination antigens containing 8 units per 0.4 mL shall be added and treated at 37°C for 60 minutes. To the treated mixtures, 0.4 mL portions of red blood cells given in 3.1.1.2 shall be added, allowed to stand for 60 minutes, and observed for the presence of hemagglutination.

3.4.8.3 Judgment

The highest dilution of the pooled sera that causes the inhibition of hemagglutination is considered the hemagglutination inhibition titer. In at least two of four groups, the hemagglutination inhibition titer shall be no less than 8.

4 Storage and expiry date

The expiry date shall be one year and a half after the manufacturing unless otherwise specified

by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Standard influenza vaccine (for CCA)

A standard provided by the National Veterinary Assay Laboratory that contains 1,000 CCA of virus in 1 mL

Japanese Encephalitis Vaccine, Inactivated, Seed

1 Definition

Japanese encephalitis, vaccine, inactivated, seed is a vaccine of inactivated virus suspension obtained by propagating the Japanese encephalitis virus that meets the seed lot specifications in cell lines that meets the specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Japanese encephalitis virus Nakayama-Yakken strain or strain approved as equivalent thereof

2.1.2 Properties

This virus strain shall be propagated in primary cells of swine kidneys, and goose, day-old chick and pigeon red blood cells shall be hemagglutinated.

2.1.3 Master seed virus

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -60ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4 Working seed virus

2.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in cells approved as suitable.

The working seed virus shall be stored frozen at -60ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5 Production seed virus

2.1.5.1 Propagation and storage

The production seed virus shall be propagated in cells approved as suitable.

The production seed virus shall be stored frozen at -60ºC or lower or stored freeze-dried at 5ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Cell line

Vero cells or cell lines approved as suitable for production shall be used.

2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.3 Master cell seeds

2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines.

The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.4 Working cell seeds

2.2.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.

The working cell seeds shall be stored frozen at -70ºC or lower.

The working cell seeds shall be tested as specified in 3.2.2.

2.2.5 Production cell seeds

2.2.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3 if they need to be stored.

2.3 Bulk material

2.3.1 Culture of production cell seeds

The cultured cells obtained in one session shall be regarded as individual cultured cells. No abnormalities shall be detected in the cultured cells prior to the inoculation of the virus.

2.3.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1. The filtrate, supernatant after centrifugation, or concentrate of the culture medium collected from individual cultured cells at the peak of virus propagation shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.

2.3.3 Inactivation

The virus suspension shall be inactivated by the addition of formalin or inactivator approved as suitable and shall serve as the inactivated virus suspension.

The inactivated virus suspension shall be tested as specified in 3.4.

2.3.4 Bulk material

The inactivated virus suspension shall be mixed to make the final bulk. The bulk material shall be tested as specified in 3.5.

2.4 Final bulk

The bulk material shall be mixed, diluted with a dilution approved as suitable to adjust its concentration and shall serve as the final bulk.

In this case, preservatives approved as suitable may be added.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.6.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.3.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For the Equine infectious anemia virus, Porcine reproductive and respiratory syndrome virus, and Endogenous retrovirus (Types C and D particles) in a test using cells derived from monkeys, the test given in 1.1 and 3.1.1 of the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For the Classical swine fever virus, Porcine circovirus, Bovine viral diarrhea virus, Rotavirus, and Rabies virus, the test given in 1.1, 3.2.3, 3.2.4, 3.2.5, 3.2.7 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cell line

3.2.1 Tests on master cell seeds

3.2.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Test for identification of the animal species of the cell

The tests given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.5 Test for freedom from extraneous viruses

3.2.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2 Test for freedom from specific viruses

3.2.1.5.2.1 General test for freedom from specific viruses

For the Equine infectious anemia virus, Porcine reproductive and respiratory syndrome virus, and Endogenous retrovirus (Types C and D particles), the test given in 1.2 and 3.1.1 of the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2.2 Individual test for freedom from specific viruses

For the Classical swine fever virus, Porcine circovirus, Bovine viral diarrhea virus, Rotavirus, Japanese encephalitis virus, and Rabies virus, the test given in 1.2, 3.2.3, 3.2.4, 3.2.5, 3.2.7 and 3.2.9 of the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.6 Test for karyological (chromosomal) characterization

The tests given in 2.1.4.2.1.6.1 in the Seed Lot Specifications shall apply.

3.2.1.7 Test for freedom from tumorigenicity

The tests given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3 Tests on production cell seeds

3.2.3.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on virus suspension

3.3.1 Test for virus content

The test shall be performed through the inoculation test in mice or inoculation test in cultured cells.

3.3.1.1 Inoculation test in mice

3.3.1.1.1 Materials

3.3.1.1.1.1 Test materials

The test article shall be diluted ten-fold with phosphate-buffered saline or diluent solution approved as suitable, and each level dilution shall serve as the test material.

3.3.1.1.1.2 Test animals

Mice aged approximately 3 weeks shall be used.

3.3.1.1.2 Test procedures

A 0.03 mL each of the test materials shall be injected into the brain of at least four test animals

and observed for 14 days.

3.3.1.1.3 Judgment

Mice that showed encephalitis symptoms and died shall be judged to be infected to calculate LD_{50}.

The virus content in 1 mL shall be $10^{7.5}LD_{50}$ or higher.

3.3.1.2 Inoculation test in cultured cells

3.3.1.2.1 Materials

3.3.1.2.1.1 Test materials

The test article shall be diluted ten-fold with the virus growth medium (Note 1), and each level dilution shall serve as the test material.

3.3.1.2.1.2 Cultured cells

Primary cultured cells of chicken embryo in accordance with 2.1.1 of the Materials for Live Vaccine Production or cultured cells approved as suitable shall be cultured in small test portions, and cells that are grown as monolayers shall be used.

3.3.1.2.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions of cultured cells and allowed to stand for adsorption at 37ºC for 60 minutes. The 0.5 mL portions of the virus growth medium shall be added, cultured at 37ºC for seven days, and observed.

3.3.1.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in 1 mL shall be $10^{7.5}TCID_{50}$ or higher.

3.4 Tests on inactivated virus suspension

3.4.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.2 Inactivation test

3.4.2.1 Materials

3.4.2.1.1 Injection materials

The test article shall be used as the injection materials.

3.4.2.1.2 Test animals

Mice aged 3 weeks shall be used.

3.4.2.2 Test procedures

A 0.03 mL each of the injection materials shall be injected into the brain of 10 test animals, and observed for 14 days.

3.4.2.3 Judgment

If there are no test animals that showed encephalitis symptoms and died, the test article shall be judged to be negative for active virus.

No active virus shall be observed in the test article.

3.5 Tests on bulk material

3.5.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6 Tests on final product

3.6.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be a liquid with a specific color or a homogeneous suspension, and no foreign matter and foreign odor shall be observed. The property of the product per small container shall be uniform.

3.6.2 Test for pH

When the test is performed as specified in the Test for pH of the General Tests, the pH shall have an intrinsic value.

3.6.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6.4 Test for thimerosal content

For products added with thimerosal, the test given in the Test for Thimerosal Content of the General Tests shall apply.

3.6.5 Test for formalin content

For products added with formalin when the test given in the Test for Formalin Content of the General Tests is applied, the formalin content shall be no higher than 0.2 vol%.

3.6.6 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.6.7 Potency test

3.6.7.1 Materials

3.6.7.1.1 Injection materials

The test sample diluted ten-fold with phosphate-buffered saline shall be used as the injection materials.

3.6.7.1.2 Test animals

Mice aged 2–3 weeks shall be used.

3.6.7.2 Challenge virus

The Japanese encephalitis virus Nakayama-Yakken strain or strain approved as suitable (Note 2) shall be used.

3.6.7.3 Test procedures

Thirty and sixty animals shall be assigned to the test group and control group, respectively. On Day 1 and Day 4 of the test, 0.1 mL each of the injection materials shall be injected

intraperitoneally into the animals in the test group.

On Day 8 of the test, a 0.2 mL portion of challenge virus shall be injected intraperitoneally into 30 animals each of the test group and control group. Then, other 30 animals in the control group shall be divided into three groups (10 animals per group), and these groups shall be injected intraperitoneally with a 0.2 mL portion of the challenge virus diluted 10-fold, 100-fold, and 1,000-fold, respectively. The test group and control group shall be observed for 14 days.

3.6.7.4 Judgment

Animals that died showing encephalitis symptoms and animals that survived but still showing encephalitis symptoms shall be considered dead, and the mortality rate of each group and LD_{50} of challenge virus shall be calculated.

The tolerability of the test group must be more than 40%. In this case, the mortality rate of the control group injected with challenge virus must be more than 90%, and the challenge virus content must be less than 10^3LD_{50} in 0.2 mL.

4 Storage and expiry date

The expiry date shall be one year after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Virus growth medium

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Bovine serum or goat serum	0–50 mL
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.0–7.6 with sodium hydrogen carbonate.

Minimal amounts of antibiotics may be added.

Note 2 Challenge virus

The Japanese encephalitis virus Nakayama-Yakken strain or strain approved as suitable shall be inoculated into the brain of mice aged 3-4 weeks, sampled at the peak of disease onset, and diluted with phosphate-buffered saline or dilutions approved as suitable to serve as the 10-fold emulsion.

The supernatant obtained by centrifuging this solution shall be used as the challenge virus.

Mycoplasma gallisepticum Vaccine, Live, Seed

1 Definition

Mycoplasma gallisepticum vaccine, live, seed is a freeze-dried vaccine of culture bacteria medium of attenuated *Mycoplasma gallisepticum* that meets the seed lot specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Mycoplasma gallisepticum G210 strain or strain approved as equivalent thereof

2.1.2 Properties

This virus strain shall agglutinate chicken red blood cells and have the immunogenicity to prevent *Mycoplasma gallisepticum.*

2.1.3 Master seed bacteria

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

Master seed bacteria shall be propagated in a medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70ºC or lower or stored freeze-dried at 5ºC or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than the production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product. However, the number of passages shall apply if approved by the Minister of Agriculture, Forestry and Fisheries.

2.1.4 Working seed bacteria

2.1.4.1 Propagation, passage, and storage

The working seed bacteria shall be propagated and passaged in medium approved as suitable for production.

The working seed bacteria shall be frozen at -70ºC or lower or be stored freeze-dried at 5ºC or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.5 Production seed bacteria

2.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable for production.

The production seed bacteria shall be prepared before use for each vaccine production.

2.2 Materials for production

2.2.1 Medium

A medium approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 Cultivation

The production seed bacteria inoculated into a medium and cultured shall serve as the cultured bacterial medium. This may be concentrated by methods approved as suitable.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.2 Bulk material

Bulk material shall be obtained by adjusting, if necessary, the concentration of bacteria in the cultured bacterial medium. A stabilizer approved as suitable may be added.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

A stabilizer approved as appropriate shall be added to the bulk material to serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.2.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.4 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.5 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply.

3.2 Test on cultured bacterial medium

3.2.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply.

3.3 Tests on bulk material

3.3.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Viable count test

3.3.2.1 Materials

3.3.2.1.1 Test materials

The test article shall be diluted ten-fold with the dilution medium (Note 1) or medium approved as suitable, and each level dilution shall serve as the test material.

3.3.2.1.2 Medium

Dilution medium and agar medium (Note 2) or medium approved as suitable shall be used.

3.3.2.2 Test procedures

A 25 μL portion of the test material shall be inoculated into two agar plates or cultured by the pour plate culture method in an air containing 5 vol% carbon dioxide at 37ºC for 14 days.

3.3.2.3 Judgment

Calculate the viable count from the number of colonies formed. The viable count of the test material per 1 mL shall be $10^{9.2}$ or greater. This number shall not apply if otherwise specified.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid with a specific color. No foreign matter and foreign odor shall be observed. The property of the product per small container shall be uniform.

3.4.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.4.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.4.4 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply.

3.4.5 Viable count test

When the test is performed as specified in 3.3.2, the viable count of the test sample per bird shall be $10^{6.9}$ or greater. This number shall not apply if otherwise specified.

3.4.6 Identification test

This test may be omitted on products for which it is not specified.

3.4.6.1 Materials

3.4.6.1.1 Test materials

Dissolve the quantity of test samples for 1,000 birds in 30 mL of phosphate-buffered saline or diluent solution.

3.4.6.1.2 *Mycoplasma gallisepticum* positive serum

Mycoplasma gallisepticum positive serum (Note 3) shall be used.

3.4.6.2 Test procedures

Mix 30 μL of the test material and 30 μL of *Mycoplasma gallisepticum* positive serum on a reaction glass plate and observe the reaction while tilting the plate back and forth, left and right.

3.4.6.3 Judgment

Aggregated granules must be observed in one minute.

3.4.7 Marker test

This test may be omitted on products for which it is not specified.

3.4.7.1 Materials

3.4.7.1.1 Inoculation materials

The test materials given in 3.4.5 shall be used.

3.4.7.1.2 Embryonated chicken eggs

The embryonated chicken eggs aged six days in 1.1 in the Materials for Live Vaccine Production shall be used.

3.4.7.2 Test procedures

A 0.1 mL each of the test materials shall be injected into the yolk sac of 10 embryonated chicken eggs, cultured at 37ºC for 14 days, and observed.

3.4.7.3 Judgment

Chicken embryos in which the specific lesions are observed shall be regarded as infected, and EID_{50} shall be calculated. Of them, the embryos that died within 24 hours of inoculation shall be excluded from the calculation. The viable count per EID_{50} must be 10^5 or greater when calculated from the viable count obtained by the test given in 3.4.5.

3.4.8 Safety test

3.4.8.1 Materials

3.4.8.1.1 Inoculation materials

Dilute the test sample with phosphate-buffered saline or diluent so that a 0.1 mL portion of the dilution contains the quantity of test sample for 10 birds to serve as the inoculation materials.

3.4.8.1.2 Test animals

The chickens aged 2–4 weeks derived from 1.1 in the Materials for Live Vaccine Production.

3.4.8.2 Test procedures

Ten and five test animals shall be assigned to the test group and control group, respectively.

Inoculate 0.1 mL of inoculation materials ophthalmically into the test group and observe both groups for three weeks. On the final day of the test, the animals shall be autopsied and observed for the presence of lesions in the nasal cavity, suborbital sinus, trachea, and air sacs.

3.4.8.3 Judgment

During the period of observation, no clinical abnormalities shall be observed in the test and control groups. No abnormalities shall be observed in the autopsy.

3.4.9 Potency test

3.4.9.1 Materials

3.4.9.1.1 Inoculation material

Dilute the test sample with phosphate-buffered saline or diluent so that a 0.03 mL of the dilution contains the quantity of test sample for one bird to serve as the inoculation material.

3.4.9.1.2 Test animals

The chickens aged 2–4 weeks derived from 1.1 in the Materials for Live Vaccine Production shall be used.

3.4.9.1.3 Agglutination antigen

Inactivated antigen against *Mycoplasma gallisepticum* (Note 4) shall be used.

3.4.9.2 Test procedures

Ten and three animals shall be assigned to the test group and control group, respectively.

Inoculate 0.03 mL portions of inoculation material ophthalmically into the test group. After 4 weeks, the sera of individual animals obtained from the test and control groups are subjected to an agglutination reaction test using the agglutination antigen.

3.4.9.3 Judgment

At least 70% of the test group must be positive for agglutination antibodies in three minutes. In this case, all of the control group must be negative for agglutination antibodies.

4 Storage and expiry date

The expiry date shall be three years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Dilution medium

1,000 mL consists of the following:

Proteose peptone	7.4 g
Yeast extract	2.5 g
Dextrose	4.0 g

Sodium chloride	5.0 g
Sodium dihydrogen phosphate dihydrate	1.8 g
Phenol red	15 mg
Water	Residual quantity

After adjusting the pH to 8.0, sterilize by filtration.

Note 2 Agar medium

The agar medium used is obtained by adding 1.0 g of bactoagar to 90.0 mL of dilution medium from which phenol red is removed, sterilizing by autoclaving at 121ºC for 15 minutes, cooling to approximately 50ºC, and adding 10 mL of swine serum.

Note 3 *Mycoplasma gallisepticum* positive serum

A serum obtained by immunizing the chickens in 1.1 in the Materials for Live Vaccine Production with the R strain of *Mycoplasma gallisepticum* or strain approved as suitable and showing positive serum plate agglutination reactions. It shall have the potency to show agglutination in a minute.

Note 4 Inactivated antigen of *Mycoplasma gallisepticum*

Inactivated antigen of *Mycoplasma gallisepticum* used is obtained by harvesting bacteria from the liquid culture medium of *Mycoplasma gallisepticum* S6 strain and diluting with phosphate-buffered saline so that the viable count is 10^{10} per mL. Then, they shall be inactivated with thimerosal and stained with crystal violet.

Agglutination is observed in three minutes in a serum plate agglutination reaction test using sera immunized with *Mycoplasma gallisepticum* S6 strain; however, no agglutination is observed in three minutes in the test using serum obtained from non-immunized control chickens or chickens immunized with *Mycoplasma synoviae*.

Rabies (tissue culture) Vaccine, Inactivated, Seed

1 Definition

Rabies (tissue culture) vaccine, inactivated, seed is a vaccine of purified and inactivated virus suspension obtained by propagating rabies virus adapted to tissue culture that meets the seed lot specifications in cell lines that meet the specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Cell culture adapted RC·HL strain of Rabies virus

2.1.2 Properties

Injection of this virus into the brain of suckling mice aged 3 days or less causes the onset of the disease and death of the infected animals; however, the injection of this virus into the brain of mice aged 3 weeks or older, guinea pigs weighing approximately 300 g, rabbits weighing approximately 1.5 kg and dogs aged 1.5 months have almost no pathogenic effects.

This virus propagates in HmLu cells with CPE.

2.1.3 Master seed virus

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in HmLu cells, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored freeze-dried at 5ºC or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines.

The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4 Working seed virus

2.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in HmLu cells.

The working seed virus shall be stored freeze-dried at 5ºC or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5 Production seed virus

2.1.5.1 Propagation, passage, and storage

The production seed virus shall be propagated in HmLu cells.

The production seed virus shall be stored frozen at -70ºC or lower when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Cultured cells

HmLu cells shall be used.

2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.3 Master cell seeds

2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -100ºC or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds. When using the suspension culture method, approximately 3-fold increase in cell count than increase in the population doubling time shall be regarded as passage of one generation.

2.2.4 Working cell seeds

2.2.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.

The working cell seeds shall be stored frozen at -100ºC or lower.

The working cell seeds shall be tested as specified in 3.2.2.

2.2.5 Production cell seeds

2.2.5.1 Propagation, passage, and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.

The production cell seeds shall be stored frozen at -100°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3 if they need to be stored.

2.3 Bulk material

2.3.1 Culture of production cell seeds

In the monolayer culture method, cells obtained and cultured in one session shall be regarded as individually cultured cells, and in the suspension culture method, cells cultured in the final fermenter shall be regarded as individually cultured cells, respectively. No abnormalities shall be detected in the cultured cells prior to inoculation of the virus.

2.3.2 Virus cultivation

The production seed virus shall be inoculated into the cultured cells, cultured at 32ºC to 34ºC,

and harvested at the peak of CPE or G protein production. Centrifugal supernatant or filtrate of the culture medium obtained shall serve as the virus suspension.

The virus suspension shall be tested as specified in 3.3.

2.3.3 Purification

The virus suspension shall be purified and concentrated with macrogol or in a manner approved as suitable to serve as the purified virus suspension. In the suspension culture method, purification may be followed by the inactivation process given in 2.3.4.

2.3.4 Inactivation

The purified virus suspension shall be inactivated with β-propiolactone or in a manner approved as suitable to serve as the inactivated virus suspension.

The inactivated virus suspension shall be tested as specified in 3.4.

2.3.5 Adjustment of bulk material

Inactivated virus suspension shall be diluted with phosphate-buffered saline to adjust its concentration and serve as the bulk material. To inactivated virus suspension or inactivated and purified virus suspension, in the suspension culture method, add preservatives approved as suitable to serve as the bulk material.

The bulk material shall be tested as specified in 3.5.

2.4 Final bulk

The bulk material shall be mixed, diluted with phosphate-buffered saline to adjust its concentration in the suspension culture method, and serve as the final bulk. In this case, preservatives approved as suitable may be added.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.6.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.1.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For the Feline leukemia virus/Feline sarcoma virus and Lymphocytic choriomeningitis virus, the test given in 1.1 and 3.1.1 of the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Canine parvovirus, Feline panleukopenia virus, and Japanese encephalitis virus, the test given in 1.1, 3.2.5, 3.2.6 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.2.3 Efficiency and immunogenicity test

3.1.2.3.1 Tests using guinea pigs

3.1.2.3.1.1 Materials

3.1.2.3.1.1.1 Test materials

Dissolve the new and old working seed viruses in phosphate-buffered saline and adjust the concentrations so that the virus content in 1 mL is approximately $10^{7.0}TCID_{50}$. To the virus solutions, add β-Propiolactone so that the concentration is 0.0125 vol%, sensitize at 4ºC for 48 hours and inactivate the virus to serve as the test material.

3.1.2.3.1.1.2 Test animals

Guinea pigs weighing approximately 400 g shall be used.

3.1.2.3.1.1.3 Challenge virus

Rabies virus CVS strain (Note 1) shall be used. Adjust the concentration with physiological saline containing 2 vol% horse serum to be 10 LD_{50} of the virus in 0.2 mL when injecting into the masseter muscle of guinea pigs and serve as the challenge virus.

3.1.2.3.1.2 Test procedures

Inject the old and new test materials at a dose of 0.5 mL subcutaneously into the inner thigh of 10 test animals, respectively. These treated animals shall be regarded as the test groups. Another 10 animals shall be regarded as the untreated control group. On the 21st day after injection, inject

0.2 mL of challenge virus into masseter muscle of the three groups each, and observe the animals clinically for 14 days.

3.1.2.3.1.3 Judgment

The animals that did not develop the disease shall be regarded as tolerant, and the tolerability shall be calculated.

The tolerability of both groups shall be 70% or higher. In this case, the tolerability of the control group shall be 20% or lower.

3.1.2.3.2 Tests using dogs

3.1.2.3.2.1 Materials

3.1.2.3.2.1.1 Test materials

The test materials specified in 3.1.2.3.1.1.1 shall be used.

3.1.2.3.2.1.2 Test animals

Dogs aged approximately 4 months and negative for antibody against rabies virus shall be used.

3.1.2.3.2.2 Test procedures

Inject 1 mL portions of the old and new test material subcutaneously into three test animals each, and one month later, collect the blood from each animal, and measure the neutralizing antibody titer using the cell culture adapted RC-HL strain of rabies virus.

3.1.2.3.2.3 Judgment

The neutralizing antibody titers in both study groups must be greater than ten-fold geometric mean, respectively.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cell line

3.2.1 Tests on master cell seeds

3.2.1.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Test for identification of the animal species of the cell

The tests given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests

shall apply.

3.2.1.5 Test for freedom from extraneous viruses

3.2.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2 Test for freedom from specific viruses

3.2.1.5.2.1 General test for freedom from specific viruses

For the Feline leukemia virus/Feline sarcoma virus and Lymphocytic choriomeningitis virus, the test given in 1.2 and 3.1.1 of the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2.2 Individual test for freedom from specific viruses

For the Bovine viral diarrhea virus, Canine parvovirus, Feline panleukopenia virus, Japanese encephalitis virus, and Rabies virus, the test given in 1.2, 3.2.5, 3.2.6 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.6 Test for karyological (chromosomal) characterization

The tests given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.7 Test for freedom from tumorigenicity

The tests given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3 Tests on production cell seeds

3.2.3.1 Test for confirmation of cell properties

The tests given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on virus suspension

3.3.1 Test for virus content

3.3.1.1 Materials

3.3.1.1.1 Test materials

The test article shall be diluted ten-fold with cell growth medium (Note 2) and each level dilution shall serve as the test material.

3.3.1.1.2 Cultured cells

Primary cultured cells of chicken embryo shall be used.

3.3.1.2 Test procedures

The 0.1 mL portions of the test material shall be inoculated into at least four portions (wells) of cultured cells and allowed to stand to culture at 37ºC for two days. On the second day, the culture medium shall be replaced by virus growth medium 1 (Note 3), allowed to stand to culture at 32ºC for eight days, and observed.

Note that if a 96-well microplate is used, each 25 μL portion of the test material shall be inoculated into 10 wells of cultured cells, then 0.2 mL portions of the virus growth medium 2 (Note 4) shall be added, cultured at 37ºC for 10 days and observed.

3.3.1.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate $TCID_{50}$.

The virus content in the test article shall be $10^{7.5}$ $TCID_{50}$ or greater in 1 mL in the monolayer culture method, and $10^{7.0}$ $TCID_{50}$ or greater in 1 mL in the suspension culture method.

3.4 Tests on inactivated virus suspension

3.4.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.2 Inactivation test

3.4.2.1 Materials

3.4.2.1.1 Injection materials and inoculation materials

The test article shall be used as the injection material and inoculation material.

3.4.2.1.2 Test animals and cultured cells

Suckling mice aged three days or less and primary cultured cells of chicken embryo shall be used.

Note that if the primary cultured cells of chicken embryo are used, the 10 mL portions of the cells shall be dispensed into a culture bottle, and cells that are grown as monolayers of approximately 36 cm^2 shall be used.

3.4.2.2 Test procedures

The 0.02 mL portions of the injection materials shall be injected into the brain of 10 test animals, and observed for 14 days.

The 2 mL portions of the inoculation material shall be inoculated into at least four portions of cultured cells and adsorbed at 37ºC for 60 minutes. The inoculation material shall be removed, the

10 mL portions of the virus growth medium 1 shall be added, allowed to stand to culture at 37ºC for 10 days, and observed.

3.4.2.3 Judgment

If the test animals show no symptoms of rabies virus, and the cultured cells in which no CPE is observed, the material shall be judged to be negative for active virus.

No active virus shall be observed in the test article.

3.5 Tests on bulk material

3.5.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.5.2 Potency test

3.5.2.1 Materials

The test article, reference vaccine (Note 5), antibody adsorption plate (Note 6), and enzyme labeled antibody (Note 7) shall be used.

3.5.2.2 Test procedures

To 6 mL of the test article, add 327.6 mg of disodium hydrogen phosphate dodecahydrate and 38.6 mg of potassium dihydrogen phosphate, and mix thoroughly by shaking. Dissolve the reference vaccine in the method specified by the National Veterinary Assay Laboratory. These test articles and reference vaccine shall be sonicated for 30–60 seconds each, and then centrifuged at approximately 200 G for 5 minutes. Previously filter each centrifuged supernatant through a membrane filter with a pore size of 450 nm pre-treated with phosphate-buffered saline containing 1 w/v% bovine serum albumin, and 4 mL of the filtrate obtained shall be gel-filtered (Note 8). Collect 8 mL of the filtrate containing the peak of the first fraction and prepare original (high dose), two-fold dilution (medium dose) and four-fold dilution (low dose) using the antigen diluting solution (Note 9). To two wells in an antibody adsorption plate, add 100 μL portions of antigen solutions to serve as the negative control wells. Seal the plate and allow to react at 37ºC for 60 minutes. After washing five times with washing solution (Note 10), add 100 μL portions of enzyme-labeled antibody to the wells each. The plate shall be sealed, and shielded from light, and allowed to react at 37ºC for 90 minutes. After washing five times with washing solution, add 100 μL of substrate chromogenic solution (Note 11) to the wells each. The plate shall be allowed to react at room temperature for 30 minutes avoiding light. Immediately after the reaction is completed, add the 50 μL portions of the stop solution (Note 12) to the wells each, and measure the absorbance at the main wavelength of 492 nm and at the reference-wavelength of 630 nm, respectively. The value obtained by subtracting the absorbance value of each negative control well from that of each well to which the test article and reference vaccine are added shall be defined as the absorbance value of each antigen. Measure the absorbance twice for each antigen solution after gel filtration and then calculate the relative titer of the test article against the reference

vaccine from the absorbance values of the test article and the reference vaccine according to the relative titer calculation method (Note 13).

3.5.2.3 Judgment

The relative titer of the test article against the reference vaccine shall be 0.683 or higher.

3.6 Tests on final product

3.6.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be a liquid with a specific color. No foreign matter and foreign odor shall be observed. The property of the product per small container shall be uniform.

3.6.2 Test for pH

When the test is performed as specified in the Test for pH of the General Tests, the pH shall have an intrinsic value.

3.6.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6.4 Test for thimerosal content

The test given in the Test for Thimerosal Content of the General Tests shall apply.

3.6.5 Test for macrogol content

When the test is performed as specified in the Test for Macrogol Content of the General Tests, the macrogol content shall be 0.5 mg or lower in 1 mL.

3.6.6 Test for protein nitrogen content

When the test is performed as specified in the Test for Protein Nitrogen Content of the General Tests, the protein nitrogen content shall be 100 μg or lower in 1 mL.

3.6.7 Safety test

3.6.7.1 Materials

3.6.7.1.1 Injection materials

The test samples shall be used as injection materials.

3.6.7.1.2 Test animals

Dogs weighing approximately 5–10 kg and cats weighing approximately 1 kg shall be used.

3.6.7.2 Test procedures

A 5 mL each of the injection materials shall be injected subcutaneously into two dogs and 2 mL each into two cats and observed clinically for 10 days.

3.6.7.3 Judgment

During the period of observation, no abnormalities shall be observed in the test group.

3.6.8 Potency test

The test given in 3.5.2 shall apply.

4 Storage and expiry date

The expiry date shall be two years after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Rabies virus CVS strain

A sample of brain inoculated with rabies virus CVS strain in 3-week-old mice exhibiting symptoms. This sample is emulsified with physiological saline containing 2 vol% horse serum to make homogenates.

When this homogenate is administered into the masseter muscle of guinea pigs weighing approximately 400 g, the virus content shall be $10^{2.5}LD_{50}$ or higher in 0.2 mL.

Note 2 Cell growth medium

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Fetal bovine serum or calf bovine serum	50 mL
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.1–7.3.

Minimal amounts of antibiotics may be added.

Note 3 Virus growth medium 1

1,000 mL consists of the following:

Tryptose phosphate broth	2.95 g
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.4–7.6.

Minimal amounts of antibiotics may be added.

Note 4 Virus growth medium 2

1,000 mL consists of the following:

Glucose	5 g
L-glutamine	0.4 g
Fetal bovine serum or Calf serum	5–25 mL
Eagle's MEM	Residual quantity

The pH shall be adjusted to 7.1–7.3.

Minimal amounts of antibiotics may be added.

Note 5 Reference vaccine

The reference vaccine used is obtained by adjusting the concentration of rabies (tissue culture) vaccine, inactivated, for reference provided by the National Veterinary Assay Laboratory as specified by the National Veterinary Assay Laboratory.

Note 6 Antibody adsorption plate

The antibody adsorption plate used is obtained by adding 100 μL portions of the dilution of purified monoclonal antibody against Rabies virus G protein of the concentration specified by the National Veterinary Assay Laboratory to each wells of a 96-well plate, sealing the plate, and allowing to adsorb at 37ºC for 18 hours.

The antibody adsorption plate shall be used after washing four times with phosphate-buffered saline.

Note 7 Enzyme labeled antibody

The enzyme labeled antibody used is obtained by adjusting the concentration of peroxidase-labeled monoclonal antibody against Rabies virus G protein with the following dilution solution to the concentration specified by the National Veterinary Assay Laboratory.

In 100 mL:

Bovine serum albumin	0.3 g
Polysorbate 20	0.05 mL
Phosphate-buffered saline	Residual quantity

Sterilize by filtration through a membrane filter with a pore size of 450 nm.

Note 8 Gel filtration

Using a column specified by the National Veterinary Assay Laboratory, separate the fractions by liquid chromatography at a flow rate of 1 mL per minute with antigen diluting solution as the mobile phase.

The detection wavelength shall be 280 nm.

Note 9 Antigen diluting solution

In 1,000 mL:

Disodium hydrogen phosphate dodecahydrate	54.66 g
Potassium dihydrogen phosphate	6.44 g
Water	Residual quantity

After adjusting the pH to 7.2, filter through a membrane filter with a pore size of 450 nm.

Note 10 Washing solution

The washing solution used is obtained by adding polysorbate 20 to phosphate-buffered saline so that the concentration is 0.05 vol%.

Note 11 Substrate chromogenic solution

In 1,000 mL:

Citric acid	21.0 g
Anhydrous monobasic sodium phosphate	28.4 g
Water	Residual quantity

The dissolved solution shall be filtered through a membrane filter with a pore size of 450 nm. To 20 mL of the solution, add 10 mg of o-phenylenediamine dihydrochloride to dissolve. Immediately before use, add 5 μL of hydrogen peroxide (30).

Note 12 Stop solution

The stop solution used is obtained by adding 150 mL of sulfuric acid to 1,000 mL of water.

Note 13 Relative tier calculation method

1 Validity test

Multiply each absorbance value obtained by 1,000 and convert it to the ordinary logarithm. Evaluate the total values of the doses for the reference vaccine and the test article and calculate the following formula. The low, medium, and high doses of the reference vaccine shall be represented by S_1, S_2, and S_3, respectively, and those of the test article shall be represented by T_1, T_2, and T_3, respectively.

Difference between the test article and reference vaccine SPa $=(S_1+S_2+S_3)-(T_1+T_2+T_3)$

Linearity SPb $=(S_3-S_1)+(T_3-T_1)$

Curvilinearity SPc $=[(S_1+S_3)-2S_2]+[(T_1+T_3)-2T_2]$

Line non-parallelism SPb′$=(S_3-S_1)-(T_3-T_1)$

Curve non-parallelism SPc′$=[(S_1+S_3)-2S_2]-[(T_1+T_3)-2T_2]$

If SPb obtained by the above formula is greater than or equal to 0.50, the absolute value of SPc is less than or equal to 0.86, that of SPb′ is less than or equal to 0.49, and that of SPc′ is less than or equal to 0.86: the validity test is met.

2 Calculation of relative tier

If the validity test given in 1 is judged as compliant, calculate the relative titer by the following formula:

$M = - (4 \times SPa \times \log 2) / (3 \times SPb)$

Furthermore, calculate the relative titer of the test article to the reference vaccine by

finding the true number of M (inverse logarithm of M, 10^M).

$P= 10^M$

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:

$$\text{Moisture content (\%)} = \frac{\text{weight loss of the test material by drying}}{\text{weight of the test material before drying}} \times 100$$

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	1/4 quantity	1 tube	1/4 quantity	× 1 tube
3 mL≤ R < 5 mL	1 mL	2 tubes	0.5 mL	× 2 tubes
5 mL≤ R <10 mL	1.5 mL	2 tubes	1 mL	× 1 tube
			0.5 mL	× 1 tube
≥ 10 mL	3 mL	2 tubes	2 mL	× 1 tube
			1 mL	× 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 5th day 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	1/2 volume	2 tubes	1/4 volume	× 2 tubes
3 mL≤ R < 5 mL	1 mL	2 tubes	0.5 mL	× 2 tubes
5 mL≤	2 mL	2 tubes	1 mL	× 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 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.

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 cm^2. 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 cm^2, 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 cm^2 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 cm^2 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 cm^2 per 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 cm^2 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 cm^2 per 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 cm^2 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 $10^5 TCID_{50}$/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 cm^2 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 cm^2 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 cm^2 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 cm^2 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 Na_2CO_3

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 cm^2 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 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 cm^2 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 cm^2.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 cm^2 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 cm^2 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 cm^2, 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 cm^2 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 cm^2 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 cm^2, 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 cm^2 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 cm^2 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 cm^2 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 $10^5 TCID_{50}$/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 cm^2 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 cm^2, 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 cm^2, 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 days 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 days 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.

Reagents, Test Solutions, Etc.

Reagents, test solutions, etc. are used for the tests in this standard. In addition to those specified herein, reagents and test solutions shall proceed as directed in Reagents, Test Solutions, etc. specified in the General Tests, Processes and Apparatus in the *Japanese Pharmacopoeia*. In this standard, the reagents noted with [JP], [JP monograph], [Special grade], or [1st grade] represent that they conform to the Reagents, Test solutions specified in the General Tests, Processes and Apparatus in the *Japanese Pharmacopoeia*, to the pharmaceuticals specified in the Official Monographs in the same, and to the reagents noted with the Special grade or 1st grade in the Japanese Industrial Standards. The reagent is marked with an asterisk (*) in the name of the reagent shall meet the suitable quality for their intended uses.

A

Acetic acid (100) [Glacial acetic acid, Special grade]

Acetone [Special grade]

Acetylacetone [Special grade]

Acriflavine*

0.2 w/v% acriflavine

Dissolve 2.0 g of acriflavine in water to make 1,000 mL and sterilize at 121 ℃ for 15 minutes.

Agar [Agar, JP monograph]

Agar (for sterility test)

An agar that conforms to the following specifications shall be used:

(1) Nitrogen content: not more than 0.5%.

(2) When conducting a test by the method specified in the Japanese Industrial Standards, the jelly strength at a concentration of 1.5% is 300 to 500 g/cm^2.

Agar medium containing 0.02 w/v% acriflavine

Add acriflavine to nutrient agar medium so that it is contained at a rate of 0.02 w/v%.

Agar medium containing 10 vol% horse serum

Add horse serum in nutrient agar medium so that it is contained at a concentration of 10 vol%.

Aluminum (III) chloride hexahydrate [Aluminum chloride, Special grade]

4-Aminobenzoic acid [p-Aminobenzoic acid, Special grade]

2-Amino-2-hydroxymethyl-1,3-propanediol [tris (hydroxymethyl) aminomethane, Special grade]

Ammonia water (28) [Ammonia water, Special grade]

Ammonium acetate [Special grade]

Ammonium thiocyanate [Special grade]

Anhydrous calcium chloride See calcium chloride, anhydrous.

Anthrone [Special grade]

L-arginine monohydrochloride [Special grade]

B

Bacto peptone*

Barbital [JP monograph]

Barbital sodium salt [JP]

Barium chloride [Special grade]

Benzylpenicillin potassium [JP monograph]

N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid*

Boric acid [JP monograph]

Bovine serum albumin

Bovine serum albumin is a pale-yellow or pale-brown powder purified from bovine serum by a method that does not affect albumin and other plasma proteins, and it conforms to the following specifications:

(1) The 10 w/v% solution is clear, and its pH ranges from 5.0 to 5.5.

(2) When conducting a test using electrophoresis, albumin must be not less than 97% of total protein.

5.5 w/v% bovine serum albumin solution

Dissolve 5.5 g of bovine serum albumin in water to make 100 mL.

Bromocresol green [Special grade]

C

Calcium chloride, anhydrous [Special grade]

Calcium chloride dihydrate [Calcium chloride, Special grade]

3 w/v% calcium chloride solution

Dissolve 3.0 g of calcium chloride dihydrate in water to make 100 mL and sterilize at 121℃ for 15 minutes.

Carbon tetrachloride [Special grade]

Casein peptone

Description: Casein peptone is a grayish yellow powder. It has a characteristic odor but has no putrefactive odor. It is soluble in water but insoluble in ethanol (95) or diethyl ether.

Digestibility: Dissolve 1 g of casein peptone in 10 mL of water and use as the sample solution for conducting the following tests:

(1) On 1 mL of sample solution, superimpose 0.5 mL of solution prepared by adding 1 mL of acetic acid (100) to 10 mL of dilute ethanol: no ring or precipitate is produced at the zone of contact. When mixing this solution, no turbidity is produced.

(2) To 1 mL of sample solution add 4 mL of saturated zinc sulfate solution: a small amount of precipitate is produced.

(3) Filter the mixture prepared in (2). To 1 mL of the filtrate add 3 mL of water and 0.2 mL of bromine test solution: a red-violet color develops.

Casein peptone (for sterility test)

In addition to its specifications, casein peptone used conform to the following specifications:

(1) The 2 w/v% solution is pale yellow clear, and its pH ranges from 6.5 to 7.0.

(2) When measured by the Van Slyke method and Kjeldahl method, amino nitrogen is 25% to 50% of total nitrogen.

(3) When measured by the microbioassay or other assays, the content of tryptophan is not less

than 1.5%.

(4) Prepare the following five types of media A–E, adjust the pH to 7.2 to 7.4, and conduct the following tests a–e: the media conform to the specifications:

Medium	Sample	Sodium chloride	Others	Water
A	2.0 g	0.5 g	–	100 mL
B	1.0 g	0.5 g	–	100 mL
C	0.1 g	0.5 g	–	100 mL
D	1.0 g	0.5 g	Glucose 0.5 g	100 mL
E	2.0 g	0.5 g	Agar 1.5 g	100 mL

a Fermentable carbohydrates test

To 5 mL of Medium A, add an appropriate amount of phenol red test solution, put a Durham tube, inoculate a loopful of *Escherichia coli* incubated at approximately 36 ºC for 24 hours, and incubate it at 35ºC to 37°C for 48 hours: the bacteria grow well, and no acid or gas is produced.

b Hydrogen sulfide production test

To 5 mL of Medium B, inoculate *Salmonella typhi*, place a piece of lead acetate paper between the cotton stopper and the mouth of the test tube at a height of about 5 cm above the medium surface, and incubate at 35ºC to 37°C for 24 hours: the bacteria grow well, and when incubated for 48 hours, the piece of paper shows a marked dark brown color.

c Indole production test

Inoculate *Escherichia coli* in 5 mL of Medium C at 35ºC to 37°C for 24 hours: *Escherichia coli* grows well; layer 0.5 mL of Indole test solution 0.5 mL: a red-violet color develops clearly at the zone of contact.

d Acetyl methyl carbinol production test

Inoculate *Klebsiella pneumoniae* in 5 mL of Medium D at 35°C to 37 ºC for 24 hours: *Klebsiella pneumoniae* grows well; add 5 mL of 10 w/v% sodium hydroxide solution, shake, and allow to stand at approximately 25 ºC for 5 hours: a pale-red color develops.

e Bacterial growth-promoting ability test

(a) Puncture *Brucella melitensis* in a stab medium of Medium E at 35°C to 37 ºC for 48 hours: bacteria grow along the puncture line.

(b) Inoculate *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella Typhi, Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Staphylococcus epidermidis* in a slant medium of Medium E at 35°C to 37 ºC for 24 hours: all bacteria grow well.

(c) To Medium E, add defibrinated blood from a rabbit so that it is contained at a concentration of approximately 5 vol%, inoculate *Streptococcus pneumoniae* or β-hemolytic *Streptococcus pneumoniae*, mix well, fix on a plate, and incubate at 35°C to

37 ºC for 48 hours: both bacteria grow well, showing a characteristic α or β- hemolytic zone.

(d) To Medium E, add defibrinated bovine blood so that it contained at a concentration of approximately 10 vol%, fix on a plate, heat at 80ºC to 90 ºC until the medium turns to chocolate brown in color, grow *Neisseria gonorrhoeae*, and incubate gas at 35°C to 37 ºC for 48 hours: the bacteria grow well when incubated in an incubator maintaining 10% carbon dioxide.

Chicken water*

Chloroform [Special grade]

Copper (II) sulfate pentahydrate [Copper sulfate, Special grade]

L-cystine [Special grade]

L-cysteine hydrochloride monohydrate [Special grade]

1 w/v% L-cysteine monohydrochloride test solution

Dissolve 1.0 g of L-cysteine hydrochloride monohydrate in water to make 100 mL.

D

Dibasic sodium phosphate anhydrous See sodium dihydrogen phosphate, anhydrous.

Dipotassium hydrogen phosphate [Dipotassium hydrogen phosphate, Special grade]

Disodium hydrogen phosphate dodecahydrate [Disodium hydrogen phosphate 12-water, Special grade]

Dithizone [Special grade]

E

Eagle's MEM

Dissolve a dried product of suitable quality according to the direction and sterilize.

Earle's solution

Calcium chloride dihydrate	0.2 g
Potassium chloride	0.4 g
Magnesium sulfate heptahydrate	0.2 g
Sodium chloride	6.8 g
Sodium hydrogen carbonate	2.2 g
Sodium dihydrogen phosphate dihydrate	0.163 g
Glucose	1.0 g
Phenol red	0.05 g

Dissolve all the above-listed ingredients in water to make 1,000 mL, and filter sterilize.

A dried product of suitable quality may be used if it is prepared by dissolving according to the

direction and sterilized.

Ethanol (95) [ethyl alcohol, Special grade]

Ethanol (99.5) [Anhydrous ethanol, Special grade]

90 vol% ethanol

Add water to 180 mL of ethanol (99.5) to make 200 mL.

F

Formalin [JP monograph]

F10 medium

Dissolve the dried F10 medium of suitable quality according to the direction and sterilize.

G

Gelatin [JP monograph]

Glacial acetic acid See acetic acid (100).

Glucosamine*

Glucose [JP monograph]

Glycerin [Special grade]

H

Hank's solution

Sodium chloride	8.0 g
Potassium chloride	0.4 g
Disodium hydrogen phosphate dodecahydrate	0.06 g
Glucose	1.0 g
Magnesium chloride hexahydrate	0.2 g
Calcium chloride dihydrate	0.14 g
Sodium hydrogen carbonate	0.35 g
Phenol red	0.02 g

Dissolve all the above-listed ingredients in water to make 1,000 mL, filter sterilize.

A dried product of suitable quality may be used if it is prepared by dissolving according to the direction and sterilized.

Heart infusion broth

Hydrochloric acid [Special grade]

Hydrogen peroxide (30) [Hydrogen peroxide solution, Special grade]

I

Indole test solution*

K

Kaolin [JP monograph]

25 w/v% kaolin solution

Suspend 25 g of kaolin in water to make 100 mL.

L

Lactalbumin hydrolysate*

Lactose*

Lactose agar*

Liquid paraffin [Light liquid paraffin, JP monograph]

M

Macrogol 4,000 [JP monograph]

Macrogol 6,000 [JP monograph]

Magnesium chloride hexahydrate [Magnesium chloride, Special grade]

Magnesium sulfate heptahydrate [Magnesium sulfate, Special grade]

Malachite green oxalate [Malachite green (oxalate), Special grade]

2 w/v% malachite green solution

Dissolve 2.0 g of malachite green oxalate in water to make 100 mL.

Meat extract broth*

Meat peptone*

Meat water*

Methyl red [Special grade]

Methyl red test solution

Dissolve 0.1 g of methyl red in ethanol (95), and filter if necessary.

Monosodium glutamate*

Mucin*

N

Neutral red [Special grade]

1 w/v% β-Nicotinamide-adenine dinucleotide [oxidized form] solution*

Nitric acid [Special grade]

4-Nitroaniline [p-nitroaniline, 1st grade]

Nutrient agar medium

Dissolve 15 g of agar in 1,000 mL of nutrient broth with the aid of heat, add water to make up for the loss, adjust the pH to between 6.4 and 7.0, and filter. Dispense the filtrate and sterilize by autoclaving at 121℃ for 15 minutes.

Nutrient broth

Dissolve 5 g of meat extract and 10 g of peptone in 1,000 mL of water by gentle heating. Adjust the pH of the mixture between 6.4 and 7.0 after sterilization, cool, add water to make up for the loss, and filter. Sterilize the filtrate by autoclaving for 30 minutes at 121℃.

Nutrient broth containing 10 vol% horse serum

Add horse serum in nutrient broth so that it is contained at a concentration of 10 vol%.

P

Peptone*

Peptone for toxins*

Peroxidase-conjugated anti-mouse immunoglobulin*

Phenol [Special grade]

Phenol red [Special grade]

0.2 w/v% phenol red solution

Dissolve 2.0 g of phenol red in water to make 1,000 mL.

Phosphate-buffered saline (pH 7.4–7.45)

Dissolve 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of dibasic sodium phosphate anhydrous, and 0.2 g of potassium dihydrogen phosphate in water to make 1,000 mL and sterilize.

1/60 mol/L phosphate-buffered saline (pH 7.0)

Dissolve 14.45 g of dibasic sodium phosphate anhydrous, 8.83 g of potassium dihydrogen phosphate, and 85.0 g of sodium chloride in water to make 10,000 mL and sterilize.

Phosphate-buffered saline containing 2 vol% horse serum

Add horse serum in phosphate-buffered saline so that it is contained at a concentration of 2 vol%.

1/60 mol/L phosphate-buffered saline containing 0.2 w/v% gelatin (pH 7.0)

Add gelatin in 1/60 mol/L phosphate-buffered saline so that it is contained at a concentration of 0.2 w/v%, heat until dissolved and sterilize.

Phosphate-buffered saline containing 0.5 w/v% phenol (pH 7.0)

1.45 g of potassium dihydrogen phosphate, 15.28 g of disodium hydrogen phosphate dodecahydrate, 4.8 g of sodium chloride, and 5.0 g of phenol in water to make 1,000 mL, and filter sterilize.

Phosphomolybdic acid n-hydrate [Phosphomolybdic acid, Special grade]

Phosphorus (V) oxide [Phosphorus (V) oxide, Special grade]

Physiological saline [JP monograph]

Physiological saline containing 0.3 vol% formalin

Add 3.0 mL of formalin to physiological saline to make 1,000 mL.

Physiological saline containing 50 vol% glycerin

Add 50 mL of physiological saline in 50 mL of glycerin.

Physiological saline containing 2 vol% horse serum

Add horse serum in physiological saline so that it is contained at a concentration of 2 vol%.

Physiological saline containing 0.1 w/v% magnesium sulfate

Dissolve 1.0 g of magnesium sulfate heptahydrate in physiological saline to make 1,000 mL and sterilize.

Physiological saline containing 0.5 w/v% phenol

Dissolve 5.0 g of phenol in physiological saline to make 1,000 mL.

Polysorbate 80 [JP monograph]

Potassium chloride [Special grade]

Potassium dihydrogen phosphate [Potassium dihydrogen phosphate, Special grade]

Potassium periodate [Potassium periodate, Special grade]

Potassium sulfate [JP monograph]

Potato extract*

R

RDE*

Resazurin [JP]

0.1 w/v% resazurin solution

Dissolve 1.0 g of resazurin in water to make 1,000 mL.

S

Silica gel [JP]

Skimmed milk powder*

Sodium acetate trihydrate [Sodium acetate (trihydrate), Special grade]

Sodium azide

Description: Sodium azide is white or practically white crystals. It is easily soluble in water, and practically insoluble in diethyl ether. The solution in water is alkaline.

Qualitative test: When treated with iron (III) nitrate test solution, sodium azide solution turns

to reddish brown in color. When sodium azide is heated in a colorless flame, the flame turns yellow in color.

Content: Not less than 97.0%

10 w/v% sodium azide solution

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

Sodium carbonate, anhydrous [Anhydrous sodium carbonate, Special grade]

Sodium carbonate decahydrate [Sodium carbonate, Special grade]

Sodium carboxymethyl cellulose [JP monograph]

Sodium chloride [Special grade]

Sodium dihydrogen phosphate, anhydrous [Disodium hydrogen phosphate anhydrous, Special grade]

Sodium dihydrogen phosphate dihydrate [Sodium dihydrogen phosphate, Special grade]

Sodium hydrogen carbonate [Special grade]

Sodium hydroxide [Special grade]

Sodium nitrite [Special grade]

Sodium thioglycolate [Special grade]

Soybean-casein digest agar

Dissolve a dried soybean-casein digest agar of suitable quality according to the direction and sterilize.

Soy peptone*

Stilbazo

(1) A black-brown powder.

(2) Shows yellow at pH 3–7, orange at pH 9 and red at pH 11 when dissolved in water. No insoluble matter is produced when 50 mg of stilbazo is dissolved in 100 mL of water.

(3) When igniting 0.5 g of sodium hydroxide with 1 mL of stilbazo and weighing, the weight of the residue shall not exceed 10 mg.

(4) The absorbance of 0.002 w/v% solution of stilbazo at 410 nm must be not less than 0.7.

(5) Add water to 5 mL of 0.05 w/v% solution of stilbazo, 10 mL of 0.0001 mol/L aluminum chloride and 10 mL of 1 mol/L of acetate buffer solution to make 100 mL. The absorbance of the diluted solution at wavelength 505 nm and optical path length 10 mm is higher by 0.42 or more than that of stilbazo alone.

Sulfuric acid [Special grade]

T

Thallium acetate*

Thiamine chloride [JP monograph]

Thimerosal $C_9H_9HgNaO_2S$

Description: Thimerosal is a white or pale-yellow crystalline powder, having a slight characteristic odor. The pH of the solution at a concentration of 1.0 g/100 mL ranges from 6.0 to 7.0.

Purity

(1) Clarity and color of solution: Colorless and clear (solution at a concentration of 1.0 g/10 mL)

(2) Diethyl ether-soluble substances: Weigh precisely about 0.5 g of powdered thimerosal, put in a 50-mL glass-stoppered conical flask, add 20 mL of anhydrous diethyl ether, stopper, shake for 10 minutes, filter in a weight-known beaker through filter paper previously washed with diethyl ether, wash the residues with 5 mL of anhydrous diethyl ether, combine the filtrate and washings, evaporate the combined solution on a water bath, and dry in a desiccator (in vacuum, silica gel) for 24 hours: the mass of the residue is not more than 0.60%.

(3) Other soluble mercury salt: Dissolve 0.10 g of thimerosal in 10 mL of water, add 3 drops of acetic acid (31) in 5 mL of this solution: a white precipitate is produced. To this solution, add 1 drop of sodium sulfide, and allow to stand for 10 minutes: no dark color develops in the solution.

(4) Readily carbonizable substances: Take 0.20 g of thiomersal and conduct the test as specified the General Tests, Processes and Apparatus in the *Japanese Pharmacopoeia*. The solution has no more color than that of J. the matching fluid for color. The test must be conducted at a standard temperature.

Loss on drying: Not more than 0.5% (1 g, in vacuum, silica gel, 5 hours)

Content: Not less than 98.0%

Assay: Weigh precisely about 0.3 g of previously dried thimerosal for the assay, put in a 300 mL Kjeldahl flask, add 10 mL of sulfuric acid and 4 mL of fuming nitric acid, heat gently on a sand bath, and gradually increase the heat until the contents in the flask become almost colorless and white smoke is produced. After cooling, transfer the contents with 100 mL of water to a beaker and heat on a water bath for 15 minutes while occasionally shaking. Then add 0.5 g of urea, shake, and add potassium permanganate test solution dropwise until a slight pale red color develops. After cooling, add hydrogen peroxide test solution dropwise until the red color of the solution disappears, and titrate with 0.1 mol/L ammonium thiocyanate solution (indicator: 2 mL of ammonium iron (III) sulfate test solution).

1 mL of 0.1 mol/L ammonium thiocyanate solution = 20.240 mg $C_9H_9HgNaO_2S$

Storage: Preserve in a light-resistant tight container.

Thiourea [Special grade]

Tin (II) chloride dihydrate [Stannous chloride, Special grade]

Trichloroacetic acid [trichloroacetic acid, Special grade]

Tris-HCl buffer solution

Dissolve 20.5 g of 2-Amino-2-hydroxymethyl-1,3-propanediol, 13.6 g of sodium acetate trihydrate, 2.2 g of calcium chloride dihydrate and 0.68 g of zinc chloride in 700 mL of water, add hydrochloric acid, and adjust the pH to 7.4 to make 1,000 mL in total.

Tris(hydroxymethyl)aminomethane See 2-Amino-2-hydroxymethyl-1, 3-propanediol.

Trypsin*

Tryptose phosphate broth*

V

Veronal-buffered saline (pH 7.2)

Add 85.0 g of sodium chloride, 5.75 g of barbital and 3.75 g of barbital sodium salt to water to make 1,000 mL, and filter sterilize.

Veronal-buffered saline containing 1 w/v% gelatin (pH 7.5)

Add gelatin in veronal-buffered saline so that it is contained at a concentration of 1 w/v%, heat until dissolved, and sterilize.

Y

Yeast extract

A peptone-like substance which represents all the soluble product of yeast cells (*Saccharomyces*) prepared under optimum conditions, clarified, and dried by evaporating to a powder. Yeast extract (1 g) represents not less than 7.5 g of yeast. A reddish yellow to brown powder with a characteristic odor. Soluble in water and forms a yellow to brown solution.

(1) The nitrogen content of yeast extract is 7.2% to 9.0%.

(2) The loss on drying at 105℃ at constant mass of yeast extract is not more than 5%.

(3) The residue of yeast extract when ignited is not more than 15%.

(4) Heat 5 w/v% yeast extract solution until boiling: no precipitate is produced.

(5) Chloride (as sodium chloride): not more than 5%.

Z

Zinc chloride [Special grade]

Specifications

Materials for Live Vaccine Production

Embryonated eggs, cultured cells, and bovine serum used as materials for live vaccine production shall comply with the following specifications. However, embryonated eggs and cultured cells used for seed lot products shall comply with the Seed Lot Specifications.

1 Embryonated eggs

1.1 Embryonated chicken eggs

Embryonated chicken eggs used for live vaccine production must be derived from SPF chicken flocks that have been confirmed to be free from the pathogens listed in Table 1 by the inspection and treatment listed in the same table, or by the inspections and treatment approved as equivalent thereof.

1.2 Embryonated quail eggs

Embryonated quail eggs used for live vaccine production must be derived from SPF quail flocks that have been confirmed to be free from the pathogens listed in Table 2 by the inspection and treatment listed in the same table, or by the inspections and treatment approved as equivalent thereof.

1.3 Embryonated duck eggs

Embryonated duck eggs used for live vaccine production must be derived from SPF duck flocks that have been confirmed to be free from the pathogens listed in Table 3 by the inspection and treatment listed in the same table, or by the inspections and treatment approved as equivalent thereof.

2 Cultured cells

2.1 Cells derived from chicken embryos

2.1.1 Primary cultured cells of chicken embryos

Primary cultured cells of chicken embryos (chicken embryo fibroblasts) used for live vaccine production must be prepared from a chicken embryo derived from embryonated chicken eggs compliant with the specification given in 1.1.

2.1.2 Primary cultured cells of chicken embryo kidneys

Primary cultured cells of chicken embryo kidneys used for live vaccine production must be prepared from a chicken embryo kidney derived from embryonated chicken eggs compliant with the specification given in 1.1.

2.1.3 Primary cultured cells of chicken embryo livers

Primary cultured cells of chicken embryo livers used for live vaccine production must be prepared from a chicken embryo liver derived from embryonated chicken eggs compliant with the

specification given in 1.1.

2.2 Cells derived from chickens

2.2.1 Primary cultured cells of chicken kidneys

Primary cultured cells of chicken kidneys used for live vaccine production must be prepared from a chicken kidney derived from embryonated chicken eggs compliant with the specification given in 1.1.

2.3 Cells derived from quail embryos

2.3.1 Primary cultured cells of quail embryos

Primary cultured cells of quail embryos (quail embryo fibroblasts) used for live vaccine production must be prepared from a quail embryo derived from embryonated quail eggs compliant with the specification given in 1.2.

2.4 Cells derived from duck embryos

2.4.1 Primary cultured cells of duck embryos

Primary cultured cells of duck embryos (duck embryo fibroblasts) used for live vaccine production must be prepared from a duck embryo derived from embryonated duck eggs compliant with the specification given in 1.3.

2.5 Cells derived from ducks

2.5.1 Primary cultured cells of duck kidneys

Primary cultured cells of duck kidneys used for live vaccine production must be prepared from a duck kidney derived from embryonated duck eggs compliant with the specification given in 1.3.

2.6 Cells derived from swine

2.6.1 Primary cultured cells of swine kidneys

Primary cultured cells of swine kidneys used for live vaccine production must be prepared from a lesion-free kidney removed from swine kept under health care for more than seven days prior to slaughter and have no abnormal findings including fever.

2.6.2 Primary cultured cells of swine testis

Primary cultured cells of swine testis used for live vaccine production must be prepared from a lesion-free testis removed from swine kept under health care for more than seven days prior to removal and have no abnormal findings including fever.

2.7 Cells derived from bovine

2.7.1 Primary cultured cells of bovine kidneys

Primary cultured cells of bovine kidneys used for live vaccine production must be prepared from a lesion-free kidney removed from bovine kept under health care for more than seven days prior to slaughter and have no abnormal findings including fever.

2.7.2 Primary cultured cells of bovine testis

Primary cultured cells of bovine testis used for live vaccine production must be prepared from a

lesion-free testis removed from bovine kept under health care for more than seven days prior to slaughter and have no abnormal findings including fever.

3 Bovine serum

Bovine serum used for live vaccine production shall be separated from fresh blood of healthy bovine or bovine fetus, sterilized by filtration, dispensed, and heat-inactivated. When this is used as the test material and tested as specified in the Sterility Test and Test for Freedom from Mycoplasma Contamination of the General Tests and in 2.4.1 and 2.4.2 in the Test for Freedom from Extraneous Viruses for Live Vaccines and Sera of the General Tests, this test material shall comply with these tests. Bovine serum used for materials for production of live vaccines for cattle also shall comply with 2.8.1 in the Test for Freedom from Extraneous Viruses for Live Vaccines and Sera of the General Tests when tested as specified.

Table 1　Inspection and Treatment of SPF Chicken Flocks

Pathogen	Antigen under inspection[1]	Inspection timing and number of chickens inspected				Inspection method[2]	Treatment
		1st		2nd and subsequent			
		Timing	Number of chickens (%)	Timing	Number of chickens (%)		
Newcastle disease virus	Ishii strain	8–12 weeks old	20	Every 3 months	10	HI	All the chickens positive to the inspection and cohabitant chickens[3] sacrificed
Avian infectious bronchitis virus	M-41 strain	〃	〃	〃	〃	ELISA	〃
Avian leukosis virus	Sub-A, B	〃	〃	〃	〃	SN	〃
Avian encephalomyelitis virus	Van Roekel strain	〃	〃	〃	〃	ELISA	〃
Avian nephritis virus	G-4260 strain	〃	〃	〃	〃	FA	〃
Infectious laryngotracheitis virus	NS-175 strain	〃	〃	〃	〃	ELISA	〃
Reticuloendotheliosis virus	T strain	〃	〃	〃	〃	FA	〃
Marek's disease virus	JM strain	〃	〃	〃	〃	FA	〃
Infectious bursal disease virus	J1 strain	〃	〃	〃	〃	ELISA	〃
Avian reovirus	Uchida strain	〃	〃	〃	〃	DID	〃
Avian adenovirus	Ote strain	〃	〃	〃	〃	DID	〃
EDS-76 virus	JPA-1 strain	〃	〃	〃	〃	HI	〃
Avian influenza virus	5331 strain	〃	〃	〃	〃	DID	〃
Chicken anemia virus	Gifu-1 strain	〃	〃	〃	〃	FA	〃
Turkey rhinotracheitis virus	MM-1 strain	〃	〃	〃	〃	FA	〃
Avian paramyxovirus	Yucaipa strain	〃	〃	〃	〃	HI	〃
Haemophilus paragallinarum type A	221 strain	〃	〃	〃	〃	HI	〃
Haemophilus paragallinarum type C	S1 strain	〃	〃	〃	〃	HI	〃
Salmonella pullorum	9-25 strain	〃	〃	〃	〃	AGG	〃
Mycoplasma gallisepticum	S6 strain	〃	〃	〃	〃	AGG	〃
Mycoplasma synoviae	WVU-1853 strain	〃	〃	〃	〃	AGG	〃
Salmonella (except for *Salmonella pullorum*)		〃	〃	〃	〃	Bacterial isolation	〃
Chickenpox virus		Everyday	100	Everyday	100	Clinical observation	Chickens positive to the inspection sacrificed

Note　Health conditions and abnormalities of the chickens shall be completely recorded. For dead chickens, histopathological examinations shall be performed.

1) For antigens under inspection, other appropriate strains may be used.

2) Other similar inspection methods may be used, if any. The inspections shall be performed by a method whose validity has been verified and guaranteed. HI: Hemagglutination test　ELISA: Enzyme-linked immunosorbent assay　SN: Serum neutralization test　FA: Fluorescence antibody test　DID: Double immunodiffusion test　AGG: Agglutination test

3) Cohabitant chickens refer to the chickens not completely isolated from those positive to the inspection.

Seed Lot Specifications

1 Vaccine seeds

1.1 Range of passage numbers

To obtain the final product, the master seed virus shall not be passaged more than 5 times, the master seed bacteria shall not be passaged more than 10 times, the master seed coccidia shall not be passaged more than 10 times, unless otherwise approved by the Minister of Agriculture, Forestry and Fisheries.

1.2 Preparation method

Master seeds shall be prepared in accordance with the approved method in consecutive process to ensure their homogeneity and stability and prevent contamination.

Master seed virus shall be dispensed at a virus concentration at which it can be adequately neutralized by antiserum in the Test for Freedom from Extraneous Viruses.

1.3 Storage

Master seeds shall be stored under the approved conditions.

1.4 Origin of seeds and specifications and test methods

1.4.1 Records on origin

1.4.1.1 Origin

The following information on the origin of seeds shall be recorded: Method of isolation, place of isolation, timing of isolation, animal species of origin, and characteristics of isolates of viruses, bacteria, etc. isolated from animal species of origin.

If the master seed was distributed (or purchased), from whom it was distributed (or from whom it was purchased) and the time of distribution (or purchase) shall also be recorded.

If the seed was produced by the genetic recombination technology, the characteristics of the genetically modified microorganism (description of the host or the taxonomic species to which the host belongs, description of the donor nucleic acid, description of the vector, method of conditioning genetically modified microorganisms, method of identifying the genetically modified microorganism, differences from the host or the taxonomic species to which the host belongs, etc.) shall be recorded.

1.4.1.2 Passage history

With regard to passages after the isolation, the animals used, cultured cells, and media, cloning and attenuation methods shall be recorded. If the master seed virus has been distributed, its passage history before and after the distribution shall also be recorded.

1.4.2 Specifications and test methods

1.4.2.1 Live virus vaccines

1.4.2.1.1 Master seed virus

1.4.2.1.1.1 Identification test

The test shall be performed as specified in 1.4.2.1.1.1.1 or 1.4.2.1.1.1.2.

1.4.2.1.1.1.1 Fluorescent antibody assay

When the cultured cells inoculated with and without the test article are stained with a fluorescence-labeled antibody, respectively, fluorescence characteristic of the virus shall be detected in the cells inoculated with the test article but shall not be detected in those inoculated without it.

1.4.2.1.1.1.2 Serum neutralization test

During the growth of the test article using appropriate cultured cells, virus-specific cytopathogenic changes shall be detected, and the growth shall be neutralized by the specific antiserum.

1.4.2.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.1.1.4 Test for freedom from extraneous viruses

The tests given in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

1.4.2.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

1.4.2.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

1.4.2.1.1.7 Test for absence of reversion to virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

1.4.2.1.1.8 Test for stability confirmation of recombinant gene

If gene recombination technology is used for preparation of the master seed virus, the test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

1.4.2.1.2 Working seed virus

1.4.2.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.1.3 Production seed virus

For storage, the following tests shall be performed:

1.4.2.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.2 Live bacteria vaccine

1.4.2.2.1 Master seed bacteria

1.4.2.2.1.1 Identification test

The test for identification of bacteria species shall be performed in compliance with an appropriate morphological characterization test method, biochemical characterization test method or other approved test methods.

1.4.2.2.1.2 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.2.1.3 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

1.4.2.2.1.4 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

1.4.2.2.1.5 Test for absence of reversion to virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

1.4.2.2.1.6 Test for stability confirmation of recombinant gene

If gene recombination technology is used for preparation of the master seed bacteria, the test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

1.4.2.2.2 Working seed bacteria

1.4.2.2.2.1 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.2.3 Production seed bacteria

For storage, the following tests shall be performed:

1.4.2.2.3.1 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply.

If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.3 Inactivated virus vaccine

1.4.2.3.1 Master seed virus

1.4.2.3.1.1 Identification test

The test shall be performed as specified in 1.4.2.3.1.1.1 or 1.4.2.3.1.1.2.

1.4.2.3.1.1.1 Fluorescent antibody assay

Stain the cultured cells inoculated with and without the test article with a fluorescence-labeled antibody, respectively: the cells inoculated with the test article show fluorescence characteristic of the virus and those not inoculated do not show it.

1.4.2.3.1.1.2 Serum neutralization test

Propagate the test article with appropriate cultured cells: virus-specific cytopathic changes shall be identified, and the propagation shall be neutralized by a specific antiserum.

1.4.2.3.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.3.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.3.1.4 Test for freedom from extraneous viruses

The tests given in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

1.4.2.3.1.5 Test for stability confirmation of recombinant gene

If gene recombination technology is used for preparation of the master seed virus, the test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

1.4.2.3.2 Working seed virus

1.4.2.3.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.3.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.3.3 Production seed virus

For storage, the following tests shall be performed:

1.4.2.3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.3.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests

shall apply.

1.4.2.4 Inactivated bacteria vaccine

1.4.2.4.1 Master seed bacteria

1.4.2.4.1.1 Identification test

The test for identification of bacteria species shall be performed in compliance with an appropriate morphological characterization test method, biochemical characterization test method, or other approved test methods.

1.4.2.4.1.2 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.4.1.3 Test for stability confirmation of recombinant gene

If gene recombination technology is used for preparation of the master seed bacteria, the test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

1.4.2.4.2 Working seed bacteria

1.4.2.4.2.1 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.4.3 Production seed bacteria

For storage, the following tests shall be performed:

1.4.2.4.3.1 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.5 Recombinant protein vaccine

1.4.2.5.1 Expression system in recombinant virus

1.4.2.5.1.1 Master seed virus

1.4.2.5.1.1.1 Identification test

The test given in 1.4.2.1.1.1 shall apply.

1.4.2.5.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.5.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.5.1.1.4 Test for freedom from extraneous viruses

The tests given in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

1.4.2.5.1.1.5 Test for stability confirmation of recombinant gene

The test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

1.4.2.5.1.2 Working seed virus

1.4.2.5.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.5.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.5.1.3 Production seed virus

For storage, the following tests shall be performed:

1.4.2.5.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.5.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.5.2 Expression system in recombinant bacteria

1.4.2.5.2.1 Master seed bacteria

1.4.2.5.2.1.1 Identification test

The test for identification of bacteria species shall be performed in compliance with an appropriate morphological characterization test method, biochemical characterization test method, or other approved test methods.

1.4.2.5.2.1.2 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.5.2.1.3 Test for stability confirmation of recombinant gene

The test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

1.4.2.5.2.2 Working seed bacteria

1.4.2.5.2.2.1 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.5.2.3 Production seed bacteria

For storage, the following tests shall be performed:

1.4.2.5.2.3.1 Test for freedom from contaminant microorganisms

The test given in the respective sections of the parts of vaccine (seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

1.4.2.6 Live coccidia vaccine

1.4.2.6.1 Master seed coccidia

1.4.2.6.1.1 Identification test

The test for identification of coccidia shall be performed in compliance with an appropriate morphological characterization test method, PCR, enzyme electrophoresis, or other approved test methods.

1.4.2.6.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.6.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.6.1.4 Test for freedom from extraneous viruses

The tests given in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

1.4.2.6.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

1.4.2.6.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

1.4.2.6.1.7 Test for absence of reversion to virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

1.4.2.6.2 Working seed coccidia

1.4.2.6.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.6.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

1.4.2.6.3 Production seed coccidia

For storage, the following tests shall be performed:

1.4.2.6.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

1.4.2.6.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

2 Cell seeds

2.1 Cell line

2.1.1 Range of passage number

The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

When using the suspension culture method, approximately a three-fold increase in cell counts than an increase in the population doubling time shall be regarded as the passage of one generation. Note that this does not apply if otherwise approved by the Minister of Agriculture, Forestry and Fisheries based on the test results that assure their suitability as cells for production.

2.1.2 Preparation method

Cell suspension shall be dispensed in one continuous working session with a series of processes in accordance with the approved method to ensure their homogeneity and stability and prevent contamination.

2.1.3 Storage

Store under the approved conditions.

2.1.4 Origin, specifications and test methods of cell seeds

2.1.4.1 Records on origin

2.1.4.1.1 Origin

Record the name of animal and organ from which the cells are derived and backgrounds of establishment (passages, cloning, establisher, timing) to the extent possible.

2.1.4.1.2 Passage history

Record the passage history and cloning, etc. after established and after distributed (or purchased).

2.1.4.1.3 Culture medium

Record the culture media used for passage, propagation, and preservation.

2.1.4.2 Specifications and test methods

2.1.4.2.1 Master cell seeds

2.1.4.2.1.1 Test for confirmation of cell properties

When observed for microscopic findings, cell growth rate, acid production, morphological characteristics, and other characteristics from which the cells are considered normal as the cell lines, the cells shall comply with the test.

2.1.4.2.1.2 Test for identification of the animal species of the cell

When the test is performed by fluorescent antibody assay, the animal species shall be identical to “animal and organ from which the cells are derived” in 2.1.4.1.1 Origin.

2.1.4.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

2.1.4.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

2.1.4.2.1.5 Test for freedom from extraneous viruses

The tests given in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

2.1.4.2.1.6 Test for karyological (chromosomal) characterization

The test given in either of the following sections shall apply:

2.1.4.2.1.6.1 Test on master cell seeds and their passaged cells

The following tests shall be performed on each of the master cell seeds and the maximum passaged cells.

In a test on chromosomes in at least 50 dividing cells, the modal number (modal chromosome number) in the maximum passaged cells shall be within ±15% of that in the master cell seeds. All indicator chromosomes present in the master cell seeds shall also be identified in the maximum passaged cells.

2.1.4.2.1.6.2 Test on passaged cells

When the cultured cells are tested as specified in 2.1.4.2.1.6.2.1, 2.1.4.2.1.6.2.2, 2.1.4.2.1.6.2.3, 2.1.4.2.1.6.2.4, and 2.1.4.2.1.6.2.5, there are no differences between all the four cultures of the cells that passaged at not less than the number of passages used for production and master cell seeds: the cultured cells shall comply with the test.

2.1.4.2.1.6.2.1 Polyploidy test

A total of more than 300 cells from four cultures of the cells shall be examined for polyploidy.

2.1.4.2.1.6.2.2 Aneuploidy test

A total of more than 100 cells from four cultures of the cells shall be examined for aneuploidy.

2.1.4.2.1.6.2.3 Morphology abnormalities test

A total of more than 100 cells from four cultures of the cells shall be examined for morphology abnormalities of chromosomes.

2.1.4.2.1.6.2.4 Chromosome cleavage test

A total of more than 100 cells from four cultures of the cells shall be examined for chromosome cleavage.

2.1.4.2.1.6.2.5 Karyotype analysis test

A karyotype analysis test shall be performed on one cell from one of four cultures of the cells.

2.1.4.2.1.7 Test for freedom from tumorigenicity

If findings show that malignant tumors are suspected to be induced in the animals targeted for inoculation of seed lot products using the cell lines, culture by passage the master cell seeds and passage at least four cultures of the cells used for production of seed lot products up to or more than the maximum number: these cultured cells shall comply with the following test.

Athymic mice (nu/nu), or immunosuppressed mice or hamsters shall be used for the test. At least five animals shall be given by subcutaneous injection with more than 2×10^6 cells, respectively, and observed for 28 days.

During the period, no animal shall show evidence of tumor formation. As control, at least five animals shall be similarly injected with more than 2×10^6 HeLa cells, which are known to form tumors. At the end of observation for 28 days, at least 80% of the animals shall show tumor formation.

2.1.4.2.2 Working cell seeds

2.1.4.2.2.1 Test for confirmation of cell properties

When observed for microscopic findings, cell growth rate, acid production, morphological characteristics, and other characteristics from which the cells are considered normal as the cell lines, the cells shall comply with the test.

2.1.4.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

2.1.4.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

2.1.4.2.3 Production cell seeds

For storage, the following tests shall be performed:

2.1.4.2.3.1 Test for confirmation of cell properties

When observed for microscopic findings, cell growth rate, acid production, morphological characteristics, and other characteristics from which the cells are considered normal as the cell lines, the cells shall comply with the test.

2.1.4.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

2.1.4.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

2.2 Primary cells

2.2.1 Use conditions

For production of seed lot products, cell lines shall be used. Note that this does not apply if

primary cells compliant with 2.2.4 Specifications and test methods are available.

2.2.2 Range of passage number

To obtain the production primary cell seed, the cells prepared from the animals shall not be passaged more than 10 times. However, this does not apply if otherwise approved by the Minister of Agriculture, Forestry and Fisheries based on the test results that assure their suitability as cells for production.

2.2.3 Storage

Store under the approved conditions.

2.2.4 Specifications and test methods

2.2.4.1 Animals from which primary cells are collected

The animals shall comply with the SPF Animal Specifications.

2.2.4.2 Specifications and test methods for primary cells

2.2.4.2.1 Master primary cell seed

2.2.4.2.1.1 Test for confirmation of cell properties

When observed for microscopic findings, cell growth rate, acid production, morphological characteristics, and other characteristics from which the cells are considered normal as the primary cells, the cells shall comply with the test.

2.2.4.2.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

2.2.4.2.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

2.2.4.2.2 Working primary cell seed

2.2.4.2.2.1 Test for confirmation of cell properties

When observed for microscopic findings, cell growth rate, acid production, morphological characteristics, and other characteristics from which the cells are considered normal as the primary cells, the cells shall comply with the test.

2.2.4.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

2.2.4.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

2.2.4.2.3 Production primary cell seed

For storage, the following tests shall be performed:

2.2.4.2.3.1 Test for confirmation of cell properties

The test given in the culture observation of cells for production specified in the parts of vaccine

(seed lot product) shall apply. If no test method is given in the respective sections of the parts of vaccine (seed lot product), the test shall be performed in the approved manner.

2.2.4.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

2.2.4.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3 Embryonated eggs

3.1 Animals from which embryonated eggs are collected

The animals shall comply with the SPF Animal Specifications.

3.2 Specifications and test methods for embryonated eggs

3.2.1 Observation of embryonic eggs

The control embryonated eggs shall be incubated and observed without being inoculated with vaccine seeds under the same conditions as the culture of vaccine seeds. No abnormalities shall be detected in the embryos.

4 Chickens

4.1 Chickens

Chickens used for the production of seed lot products shall be derived from SPF chicken flocks as specified in 1.1 in the Materials for Live Vaccine Production. However, this does not apply to Chicken anemia virus among the pathogens listed in Table 1 in the Materials for Live Vaccine Production, if the absence of the virus is confirmed in the intermediate manufacturing process of productions, including the bulk material.

4.2 Specifications and test methods for chickens

4.2.1 Growth test

The control chickens shall be bred and observed without being inoculated with vaccine seeds under the same conditions as the culture of vaccine seeds. No abnormalities shall be detected.

5 Other materials

The materials for use necessary to manufacture or maintain the seeds such as the media, digestive fluid and other materials shall be free from microorganisms and foreign matter.

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