Corporation obtaining approval, the name of its representative, and the address of its main office

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Marek's disease virus serotype 1 strain 207 with transferring of the F protein gene originating from the Newcastle disease virus [NDV-F, Herpesviridae Alphaherpesvirinae Mardivirus Gallid herpesvirus 2 (Marek's disease virus serotype 1)] (Cellmune N)
Contents of the Type 1 Use of Living Modified Organism	(1) Transportation and storage (including transportation and storage of inoculated animals with the viable, modified live vaccine) (2) In the case of a study to collect data on clinical research, which must be submitted as specified by Article 14 Paragraph 3 of the Pharmaceutical Affairs Law (Law No. 145 of 1955) (hereinafter referred to as "clinical trial"), use of Living Modified Organism in accordance with the notification of the clinical trial plan submitted according to Article 80-2 Paragraph 2 of the said Law and the protocol prepared according to Article 7 of the Ordinance for good clinical practice of new animal drugs (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 75 of 1997) (3) Use in accordance with the application for approval as specified by Article 14 Paragraph 1 of the Pharmaceutical Affairs Law [except operations relevant to (4)] (4) Vaccination (to layer and broiler chickens) (5) Disposal of devices and residues after inoculation in accordance with the standard for disposal of infectious industrial waste provided in Article 12-2 of the Waste Disposal and Public Cleansing Law (Law No.137 of 1970) (6) Disposal excluding those in (5) (including cases that accompany the disposal of inoculated animals carrying the viable modified live vaccine) (7) Acts incidental to (1) to (6)
Method of the Type 1 Use of Living Modified Organism	-

Outline of the Biological Diversity Risk Assessment Report

I. Information Collected Prior to Assessing Adverse Effect on Biological Diversity

1. Information concerning a recipient organism or the species to which the recipient organism belongs

(1) Taxonomy and distribution in nature

Marek's disease virus (MDV) is a member of the Alphaherpesvirinae subfamily of the genus Herpesviridae. There are three serotypes of MDV, MDV serotype 1 (MDV1), serotype 2 (MDV2) and serotype 3 (MDV3), and only the MDV1 is pathogenic. MDV1 is distributed throughout the world, and many poultry farms are estimated contaminated with the virulent MDV1. According to the information supply investigations on veterinary food inspection in the FY2001 Ministry of Health, Labour and Welfare (MHLW) Statistical Database System, 10% of rejected chickens are reportedly affected with the Marek's disease.

The attenuated MDV1s have been applied as a live vaccine to Marek's disease (T lymphoma) as well as the serotype 2 (MDV2) and serotype 3 [MDV3: represents the herpesvirus of turkeys (HVT)], and a vaccine from any of these serotypes is administered individually or in combination to chickens.

The MDV1 (Herpesviridae Alphaherpesvirinae Mardivirus Gallid herpesvirus 2) CVI988 C17 strain used as the recipient organism of the recombinant virus is an avirulent strain derived from the attenuated MDV1 CVI988 strain isolated from healthy chickens (Reference 1) and obtained by passages in the chicken embryo primary cells and the duck embryo primary cells.

(2) History and current situation of use, etc.

The viruses derived from CVI988 strain have long been used as the live vaccine, and at present it is also administered into chickens in many poultry farms.

(3) Physiological and ecological (biological) properties

a) Basic properties

The MDV1 contains the double-stranded DNA of approx. 180kbp as the genome. The virus genome is encased in the nucleocapsid (85 to 100 nm in diameter), and the outside is additionally covered with envelope (150 to 160 nm in diameter). In the feather-follicle epithelium from which infectious virus is released, the size of viral particles having an envelope is reported to range from 273 to 400 nm (Reference 2).

The MDV1 genome is very similar to the herpes simplex virus serotype 1 (HSV1) in the genetic constitution (Figure 1). The genome is composed of L component and S component with their unique

sequences flanked by inverted repeat sequences. TRL (Terminal Repeat Long) and IRL (Internal Repeat Long) located at both ends of UL (Unique Long) are configured with the same nucleotide sequence, but the IRL sequence is reversed from the TRL sequence. The S component has a similar configuration.

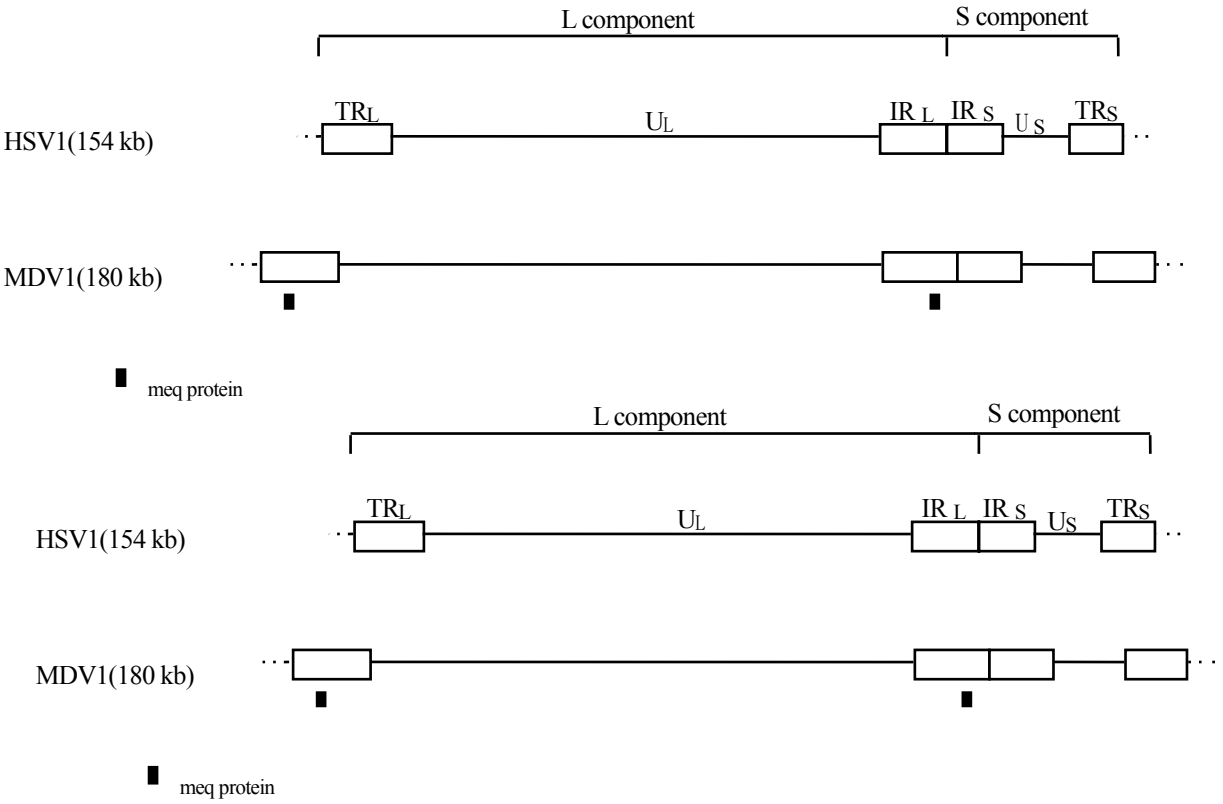

Figure 1 MDV1 genome constitution

Recently, the whole genome of very virulent MDV1-Md5 strain was analyzed and as a result, among the 103 estimated open reading frames (ORFs), 63 ORFs (61%) were found to be HSV1 homologues. Particularly, the genetic constitution in the unique region is found to be very similar to that of HSV1, and 55 of 63 ORFs encoded in UL and 7 of 9 ORFs encoded in US are found to be HSV1 homologues (Reference 3).

In addition to chickens, natural hosts of MDV1 are quails, turkeys and white-fronted geese. Experimentally infected birds include common pheasant and duck. In addition, it is reported that sparrows are not infected, and there are no reports that other birds including crows, doves and other wild fowl have been infected（References 2, 4). For mammals, it has been reported that monkeys (rhesus monkey, crab-eating macaque, bonnet macaque, marmoset), rats, hamsters and other mammals are not infected (Reference 2).

For the CVI988 strain, it did not infect rhesus monkeys, and it has been reported to cause no seroconversion even in the case of humans stuck with a needle by accident during vaccine administration

(Reference 1).

b) The habitat or conditions for existence

The recipient organism virus CVI988 C17 strain propagated in the chick embryo cell at 35℃ and 40℃, whereas it did not propagated below 30℃ and above 45℃ (Annex 1). In addition, it did not infect the cell lines from various mammals including humans (Annex 2).

c) Predacity or parasitism

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d) Mode of propagation or reproduction

MDV maturates in the feather-follicle epithelium of infected chickens, and it is shed as cell-free virus associated with dander to form a new source of infection. In practice, however, it does not cause any vertical transmission (Reference 2). The virus inhaled by chickens propagates first in the lungs then moves to lymphocytes where it persists for life (References 5, 6). This property is similarly observed in the CVI988 strain which is the original strain of the recipient organism virus, and the virus is reported to persist for two-years in the inoculated chickens (Reference 1).

MDV1 is cell-associated and it rarely produces cell-free viral particles except from the feather-follicle epithelium, and the process from invasion into cells to maturity of viral particles has not been clearly identified. The MDV3 (HVT), which produces cell-free viruses, enters cells by fusion of the viral envelope and the cell membrane in a manner similar to other herpesviruses. The mode of propagation of the virus after infection to cells has not been clarified, though at the cultured cell level, the virus moves between cells through cell-to-cell infection. The MDV1 encodes at least eight (8) membrane proteins, gB, gC, gE, gH, gI, gK, gL and gM; and four of them, gB, gE, gI, and gM, are essential for cell-to-cell infection (Reference 7).

The relationship between virus and cell may be broadly classified into three modes; the productive infection in which infectious viral particles are produced, the latent infection or a latent state in which expression of genes almost ceases, and the transforming infection in which cells are transformed.

The latent infection is a mode of infection characteristic to the herpesvirus in which some genes are transcribed but translation to viral antigen is not detected. In the productive infection, approximately 1,200 copies of virus DNA per cell are detected, whereas in the latent infection, only 5 copies are detected (Reference 2). Although where the MDV lies dormant in the inoculated chicken has not been identified, the lymphocyte is considered to be one of these locations.

Regarding crossability, the nucleotide sequence homology between MDV1 and MDV2 or MDV 3 (HVT) is low and the likelihood of recombination is considered to be extremely low. There have been no reports that any recombinant viruses between each serotypes have been isolated from chickens (Reference 8). In addition, there have been no reports on recombination within MDV1 strains.

e) Pathogenicity

The virulent MDV1 can cause T-lymphona as well as paralysis in the sciatic nerves and other peripheral nerves in infected chickens. In addition, it has been reported that the virus infects and causes tumors in quails, turkeys and white-fronted geese (References 2, 4). Infection with virulent MDV1 causes transformation of the T-lymphocyte and leads to tumors and, the meq gene has been identified as a gene involved in this transformation. The meq gene enhances the transcription required for latent infection, and it is thought to transform the T-lymphocyte by enhancing the transcription of IL-2 (Reference 9, Figure 2). For protection against possible infection with the virulent MDV1, live vaccine of either serotype of MDV1, MDV2 or MDV3 has been administered individually or in combination into chickens in many poultry farms.

The MDV1 CVI988 strain which is the original strain of the recipient organism virus, is an attenuated MDV1 strain isolated from healthy chickens and it does not exhibit any apparent pathogenicity to chickens (Reference 1).

The recipient organism virus can cause inflammatory reaction or immune reaction simply by the proliferation of MDV1 when inoculated into chickens which is the natural host, and it does not exhibit any pathogenicity including oncogenicity (Annex 3).

There have been no reports regarding possible effects on microorganisms (Reference 2).

f) Productivity of harmful substances

There have been no reports that MDV1 has ever produced any harmful substances except that it expresses a protein which cause tumorigenic transformation (Reference 2). It has not been reported that the CVI988 strain which is the original strain of the recipient organism virus, is oncogenic (Reference 1), and the CVI988 C17 strain which is the recipient organism virus, is also not oncogenic (Annex 3).

g) Other information

The MDV1 is cell-associated and always requires living cells for propagation and it cannot propagate and survive in any environments outside cells. However, the mature virus shed in dander from the infected chickens is reportedly able to maintain infectivity for up to 8 months at room temperature and 10 years at 4℃ in the dry condition (Reference 10). Nevertheless, the stability decreases as the humidity increases. In the phosphate buffer solution, the mature MDV1 virus becomes inactivated in 4 days at 25℃ and 18 hours at 37℃ (Reference 11). In addition, it becomes inactivated in 10 minutes by any ordinary disinfectant (Reference 10).

2. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

a) Composition and origins of component elements

i) gB promoter

It is cloned from the gB (glycoprotein B) promoter region in the MDV1 CVI988 C17 strain, the recipient organism virus (Annex 4). It is a 0.5kb fragment amplified through PCR (polymerase chain reaction), with the EcoRI site added at each 5' end. The gB promoter sequence is configured mostly with the 3'-terminal of UL28 gene, containing 20% of its ORFs.

ii) NDV-F protein gene

NDV-F protein is a cDNA (F protein gene) of F protein gene derived from the avirulent NDV-D26 strain. The nucleotide sequence and other related data are contained in Annex 5.

iii) Transcription termination factor

Transcription termination factor is a 0.25kb fragment derived from the commercially available expression plasmid pSVL (Annex 6). It contains the polyA addition signal and also the 3'-terminal sequence (77 bases) of large T antigen ORF of SV40 and the 3'-terminal sequence (61 bases) of VP1, the major virus capsid.

b) Function of component elements

i) gB promoter

The gB promoter has the function of effectively expressing the downstream ORF in the cells infected with MDV1. It is known that the homologous UL28 of HSV1 functions by incorporating the virus DNA into viral particles (Reference 12). However, it is not known how the protein encoded by UL28 functions in MDV1.

ii) NDV-F protein gene

In the NDV, the F protein cleaved by the protease fuses the virus envelope and cell membrane in the presence of the NDV-HN protein, thereby taking part in setting up an infection (Reference 13). The F protein alone cannot express the membrane fusion activity. Antibodies against the F protein are effective for protection from infection, and the F protein is important as a protective antigen.

iii) Transcription termination factor

Transcription termination factor acts to terminate the transcription to mRNA. In the sequence used as a termination factor, the 3'-terminal sequence of large T antigen is contained, though the region does not possess any function that affects the transformation of cells (Reference 14).

(2) Information concerning vector

a) Name and origin

pUC119 (plasmid for *E. coli*) (Annex 7)
Commercially available plasmid used widely in the field of gene technology (3,162bp).

b) Properties

The vector contains an ampicillin-resistant gene and a part of a β-galactosidase gene as selective markers. It has been reported that this vector does not transfer (Reference 15), and it does not possess any promoter that would function in eukaryotic cells.

(3) Method of preparing living modified organisms

a) Structure of the entire nucleic acid transferred in the recipient organism

The insertion vector plasmid pKA4BPF for the development of the recombinant virus (Annex 8) contains the expression cassette composed of gB promoter, F protein gene and transcription termination factor transferred in the US10 gene in the MDV1-A4 fragment cloned to pUC119.

b) Method of transferring nucleic acid transferred to the recipient organism

Homologous recombination was conducted by transferring the insertion vector plasmid pKA4BPF into the chicken embryo primary cell infected with the MDV1 CVI988 C17 strain, the recipient organism virus, based on the electroporation (a method to electrically make a hole in the cell) (Annex 9).

c) Processes of rearing of living modified organisms

By applying the enzyme immuno assay, the F protein expressing plaque is stained with the monoclonal antibody against the F protein to clone the recombinant virus. Cloning and passage were repeated to develop the recombinant live vaccine original strain (Annex 10). From the original strain, master virus bank (MVB) and working virus bank (WVB) were prepared (Annex 11).

(4) State of existence of nucleic acid transferred in cells (of the recipient organism) and stability of

expression of traits caused by the nucleic acid

The F protein gene expression cassette is confirmed to be transferred into the US10 gene of MDV1 DNA and stably maintained even through passages beyond manufacturing conditions, and the expression is found to be stable (Annex 12).

In addition, the transferred sequence is found identical to that of insertion vector plasmid, and it remains unchanged even through passages beyond the manufacture conditions (Annex 12).

When and where the F protein is expressed in the body of chicken has not been clarified. Although the F protein gene controlled by the gB promoter does not express while the virus is in the state of latent infection, it is thought to express in a manner similar to other viral antigens when the virus is activated.

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

Monitoring of the recombinant virus in the natural environment is attained by detecting the F protein gene in the DNA extracted from MDV1 isolated from specimens (Annex 13). The detection sensitivity of F protein gene based on the nested PCR is presented in Annex 13.

(6) Difference between the modified organism and **the recipient organism or the species to which the recipient organism belongs**

a) Differences in properties between the modified organism and the recipient organism used for the preparation or the species to which the recipient organism belongs

i) Mode of growth

In the chicken embryo primary cell, the recombinant virus successfully grew similarly to the recipient organism virus in the temperature range between 35 and 40℃. When cultured at 37℃, the recombinant virus continued to grow up to the fourth day, after which the growth leveled off. Below 30℃ and above 45℃, the recombinant virus did not grow (Annex 1).

In the inoculated chickens, the recombinant virus infected the peripheral blood mononuclear cell (PBMC) similarly to the recipient organism virus, and it was isolated from PBMC for at least 2 years (Annex 14). In addition, the recombinant virus was isolated from the whole body organs similarly to the recipient organism virus (Annex 15).

ii) Genetic properties

Unlike the recipient organism virus and normal MDV1, the recombinant virus contains the transferred F protein gene expression cassette (Annex 12). It has been confirmed that the genome of

the recombinant virus does not contain the sequence of the vector plasmid pUC119 used for the recombination (Annex 17).

iii) Pathogenicity

The recombinant virus does not exhibit any oncogenicity similarly to the recipient organism virus, and it did not exhibit any pathogenicity to chickens (Annex 16).

iv) Productivity of harmful substances

The recombinant virus expresses the NDV-F protein, though there have been no reports of toxicity of the NDV-F protein, and the recombinant virus did not exhibit any pathogenicity to chickens (Annex 16).

v) Infectivity (tissue affinity and persistent infectivity)

In the inoculated chickens, the recombinant virus was recovered from the whole body organs even at 10 weeks similarly to the recipient organism virus (Annex 15).

Broiler and layer chickens are typically raised for about 2 months and about 2 years respectively, and the recombinant virus has persisted in the inoculated chickens for 2 years similarly to the CVI988 strain (Annex 14).

The recombinant virus did not grow in the cell lines derived from mammals, similarly to the recipient organism virus (Annex 2).

In addition, the recombinant virus did not infect any mice and cats similarly to the recipient organism virus (Annex 18).

vi) Possibility of activation of endogenous virus and influencing pathogenicity

There have been no reports that the MDV1 activates the endogenous virus or confer the pathogenicity (Reference 2), and there is also no similar report regarding NDV. Consequently, it was considered that there is no possibility that the recombinant virus would activate the endogenous virus and confer the pathogenicity similarly to the recipient organism virus.

vii) Excretion from vaccinated animals and infectivity by cohabitation

The recombinant virus differed from the recipient organism virus (Reference 16) in that it was not shed in dander of inoculated chickens and it did not exhibit any infectivity by cohabitation (Annex 19, Annex 20, Annex 21).

Even in cases where the chickens inoculated with the recombinant virus were raised under stress-induced conditions in which feeding and water supply are restricted or the temperature of the environments is elevated, the virus was not shed into dander and feces, there was no infection by cohabitation (Annex 22).

The recombinant virus was not excreted into eggs (Annex 23) similarly to the normal MDV1 (Reference 2).

viii) Viability in the natural environment

The recombinant virus could not survive in muddy water, rainwater and blood clots (Annex 25).

ix) Crossability

Regarding crossability, the nucleotide sequence homology of MDV1 with MDV2 or MDV 3 (HVT) is low and it is considered that the chance of recombination taking place is extremely low. There have been no reports of isolation of a recombinant virus between individual serotypes from chickens (Reference 8). In addition, there are no reports of recombination within MDV1 strains. Even in the case where a field strain was inoculated into the chickens inoculated with the recombinant virus, there was no transmission of the transferred expression cassette to the field strain (Annex 24).

x) Inactivation

The cells infected with the recombinant virus were easily inactivated by alcohol, chlorine, and the invert soap widely used in poultry farms (Annex 26, Annex 27). In addition, the recombinant virus was inactivated by artificial gastric juice (Annex 28).

b) Discrimination of the modified organism from the recipient organism or biological species to which they belong

Discrimination of the modified organism is possible by detecting a part of the F protein gene based on the PCR method (Annex 13).

In addition, in contrast to MDV1, the plaque of the recombinant virus is specifically stained based on the enzyme immuno assay using the monoclonal antibody that identifies the F protein (Annex 12).

II. Review by Persons with Specialized Knowledge and Experience Concerning Adverse Effects on Biological Diversity

Persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) reviewed possible Adverse Effects on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organisms based on the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms." The results of the review are listed below.

○ Name: Marek's disease virus serotype 1 strain 207 with transferring of the F protein gene originating from the Newcastle disease virus (NDV-F, Herpesviridae Alphaherpesvirinae Mardivirus Gallid herpesvirus 2 (Marek's disease virus serotype 1) (Cellmune N)

○ Content of the Type 1 Use

(1) Transportation and storage (including transportation and storage of inoculated animals carrying the viable modified live vaccine)

(2) In the case of a study to collect data on clinical research, which must be submitted as specified by Article 14 Paragraph 3 of the Pharmaceutical Affairs Law (hereinafter referred to as "clinical trial"), use in accordance with the notification of the clinical trial plan submitted according to Article 80-2 Paragraph 2 of the said Law and the protocol prepared according to Article 7 of the Ordinance for good clinical practice of new animal drugs (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 75 of 1997)

(3) Use in accordance with the application for approval as specified by Article 14 Paragraph 1 of the Pharmaceutical Affairs Law [except operations relevant to (4)]

(4) Vaccination (to layer and broiler chickens)

(5) Disposal of devices and residues after inoculation in accordance with the standard for disposal of infectious industrial waste provided in Article 12-2 of the Waste Disposal and Public Cleansing Law (Law No.137 of 1970)

(6) Disposal excluding those in (5) (including cases that accompany the disposal of inoculated animals carrying the viable modified live vaccine)

(7) Acts incidental to (1) to (6)

○ Applicant: The Chemo-Sero-Therapeutic Research Institute

1. Item-by-item assessment of Adverse Effect on Biological Diversity

(1) Property of reducing other microorganisms

There are no reports that the MDV1, which is the taxonomical species to which the recipient organism belongs, possesses any property of reducing other microorganisms. The genetically modified live vaccine was developed only with the transferring of the NDV-F protein gene into the CVI988 C17strain which is the recipient organism virus, and so since there is no property reducing other microorganisms reported in the MDV1, and, also because only the NDV-F protein gene is transferred, this property of reducing microorganisms is considered to remain unchanged from the recipient organism virus. Based on the abovementioned understanding, no wild animals and plants which may be affected by the property of reducing other microorganisms were identified.

Consequently,, it was judged that the Type I Use in accordance with the Type 1 Use Regulation would not cause any risk of Adverse Effect on Biological Diversity attributable to the property of reducing other microorganisms.

(2) Pathogenicity

The genetically modified live vaccine is not shed from the inoculated chickens and it is also considered unlikely to affect birds susceptible to MDV1. Based on the theoretical consideration that the expression of the NDV-F protein would not affect the tropism of MDV1, there should be no effects on the Muridae family animals and domesticated cats which are not infected with MDV1 by nature, weasels and other small meat-eating mammals, and other animals which can prey on chickens.

Based on the abovementioned understanding,,, there were no wild animals and plants identified which would be affected by the pathogenicity. Consequently, it was judged that the Type 1 Use in accordance with the Type 1 Use Regulations would not cause any risk of Adverse Effect on Biological Diversity attributable to the pathogenicity.

(3) Productivity of harmful substances

There are no reports that the MDV1, the taxonomical species to which the recipient organism belongs, produces any harmful substances except that it expresses a protein which causes tumorigenic transformation. The CVI988 strain which is the original strain of the recipient organism virus has been reported not to be oncogenic, and the recipient organism virus CVI988 C17 strain also does not have any oncogenicity. The genetically modified live vaccine has been developed only with the transferring of the NDV-F protein gene into the recipient organism virus CVI988 C17 strain and the property of the lack of productivity of harmful substances is considered to remain unchanged from the recipient organism virus.

Based on the abovementioned understanding,, wild animals and plants which may be affected by the productivity of harmful substances were not identified. Consequently, it was judged that the Type I Use in accordance with the Type 1 Use Regulation would not cause any risk of Adverse Effect on Biological

Diversity attributable to the productivity of harmful substances.

(4) Property of transmitting nucleic acid horizontally

The genetically modified live vaccine is not shed from the inoculated chickens with infectivity and its ability to transmit nucleic acid horizontally is considered to be substantially degraded compared to MDV1 which is the taxonomical species to which the recipient organism belongs. In addition, based on the theoretical consideration that the tropism of MDV1 would not be affected by the expression of the NDV-F protein (Annex 2), even when the part of the chicken containing the recombinant virus is eaten by other animals, the genetically modified live vaccine would not establish any infection and thus it is considered not to offer any property of transmitting nucleic acid horizontally.

Based on the abovementioned understanding,, there were no wild animals and plants identified which would be affected by the property of transmitting nucleic acid horizontally. Consequently, it was judged that the Type 1 Use in accordance with the Type 1 Use Regulation would not cause any risk of Adverse Effect on Biological Diversity attributable to the property of transmitting nucleic acid horizontally.

2. Conclusion based on the Biological Diversity Risk Assessment Report

Regarding the property of reducing other microorganisms, there is no difference between the genetically modified live vaccine and MDV1, which is the taxonomical species to which the recipient organism belongs and it was judged that the Type 1 Use in accordance with the Type I Use Regulation would not cause any risk of Adverse Effect on Biological Diversity attributable to the property of reducing other microorganisms.

Regarding pathogenicity, the recipient organism virus and the genetically modified live vaccine are equivalent to each other and thus, it was judged that the Type 1 Use in accordance with the Type 1 Use Regulation would not cause any risk of Adverse Effect on Biological Diversity attributable to the pathogenicity.

Regarding the productivity of harmful substances, the genetically modified live vaccine is similar to the recipient organism virus in possessing no property of producing any harmful substances and thus, it was judged that the Type 1 Use in accordance with the Type 1 Use Regulation would not cause any risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances.

Regarding the property of transmitting nucleic acid horizontally, the genetically modified live vaccine is considered to have a degraded capability of transmitting nucleic acid horizontally compared to the recipient organism virus and thus, it was judged that the Type 1 Use in accordance with the Type 1 Use Regulation would not cause any risk of Adverse Effect on Biological Diversity attributable to the property of transmitting nucleic acid horizontally.

Based on the abovementioned understanding, it was judged that the Biological Diversity Risk Assessment Report

which concluded there is no risk that the use of this genetically modified vaccine in accordance with the Type 1 Use Regulation would cause Adverse Effect on Biological Diversity, is reasonable.