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

Applicant Name: Merial Japan Ltd.
Michel Lachaussee, President (Seal)
Address: Sanno Grand Bldg. 8F, 2-14-2 Nagata-cho, Chiyoda-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Canarypox virus ALVAC to which a protective antigen protein expression gene derived from feline leukemia virus (vCP97 strain) was transferred (FeLV-*env*, *gag*, *pol*, Canarypox virus)
Content 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 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 (excluding those involving human food) (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

Recipient organism :

a) Name of strain (product name) : ALVAC [also known as: CPpp (Canary Pox plaque purified)〕

b) If obtained from a public microorganism collection center, the name of the center, strain number, and date of receipt: -

c) Source of the recipient organism and lineage (Application Annex 1)

Poxviridae

Chordopoxvirinae

Avipoxvirus

Canarypox virus (Application Annex 2)

Strain name before attenuation Rentschler strain Lineage (see “d) Mode of propagation or reproduction”)

d) Distribution in nature: Wild type Canarypox virus is considered to exist worldwide (Application Annex 3)

Animal cultured cell :

a) Name: Chicken embryonic fibroblast (CEF) cells

b) If obtained from a public microorganism collection center, the name of the center, strain number, and date of receipt: -

c) Donor animal: Cultured cells obtained by enzymatic digestion of SPF chicken embryos (*Gallus gallus domesticus*) (Application Annex 4)

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

Recipient organism:

The recipient orgaism ALVAC has already been commercially available in Europe and the United States as a recombinant vaccine into which the antigen genes of rabies virus or West Nile virus are transferred. The feline leukemia (FeLV) recombinant vaccine has also been approved and is commercially available in 29 countries (Application Annex 5). Also in humans, recombinant vaccines, to which rabies, measles virus fusion and hemagglutinin, or HIV envelope glycoprotein, etc. has been transferred, are under development (Application Annex 6).

Cultured cell :

Cultured cells are used worldwide at universities, research facilities, and by the manufacturers of vaccines, etc., for virus studies and vaccine production, etc.

(3) Physiological and ecological (biological) properties

a) Basic properties

"Susceptibility": Cells or bodies to be entered are infected, and viruses of the next generation are copied.
"Pathogenicity": To infect animals and hinder their growth.

Wild type Canarypox virus infects the canary where it propagates. In cells under general culture conditions (37±1ºC, 5%CO_2), good growth can be obtained in chicken embryonic fibroblast cells and chicken embryonic kidney cells, as well as in chicken embryos and other avian embryos including duck, turkey, etc. There have been no reports of recombination of the Canarypox virus with others of the genus Avipoxvirus, which are genetically closely related.

The lineage of the recipient organism ALVAC (name during development: CPpp) is shown in section “d) Mode of propagation or reproduction”. The genome is a linear double-stranded DNA with nucleotides of approximately 325kb (Application Annex 7).

Each virus species of Poxviruses has an animal species in which it causes productive infection to produce infectious viruses (recipient organism specificity) (Response Annex 2, Application Annex 8). In mammalian cells, replication is stopped at an early stage after infection, and infectious virus particles are not produced in the form of abortive infection (Application Annex 8). The properties of ALVAC are shown in Application Annex 6.

The pathogenicity of ALVAC in its susceptible animal canaries appears as a mild inflammatory reaction at the site of inoculation; after inoculation in the skin, the virus can be isolated from the site and from organs by 16^{th} day from inoculation, and is passed on to noninoculated cage mate canaries. It is reported to be nonpathogenic to humans and other mammals (mice, guinea pigs, rabbits, monkeys, and cats) (Application Annex 10).

b) The habitat or conditions for existence

Canarypox virus grows well in cultured cells from certain avian species, chicken embryos, and embryos of ducks, turkeys, etc. With infection of chicken chorioallantoic membranes, large, white and raised rash appears. In cultured cells, marked CPE, cytoplasmic inclusions and elementary bodies

are detected. The genus Avipoxvirus is classified into ten kinds of major virus species including fowlpox, pigeon pox, turkey pox, and Canarypox. The viruses cannot exist independently in nature; based on the fact that susceptible cultured cells are limited to avian cells and the study results on natural and experimental infection with avipoxvirus, it has been confirmed that the viruses have recipient organism specificities (Application Annex 8).

The recipient organism ALVAC maintains the general properties of Canarypox virus; chicken embryonic fibroblast cells are used for its culture.

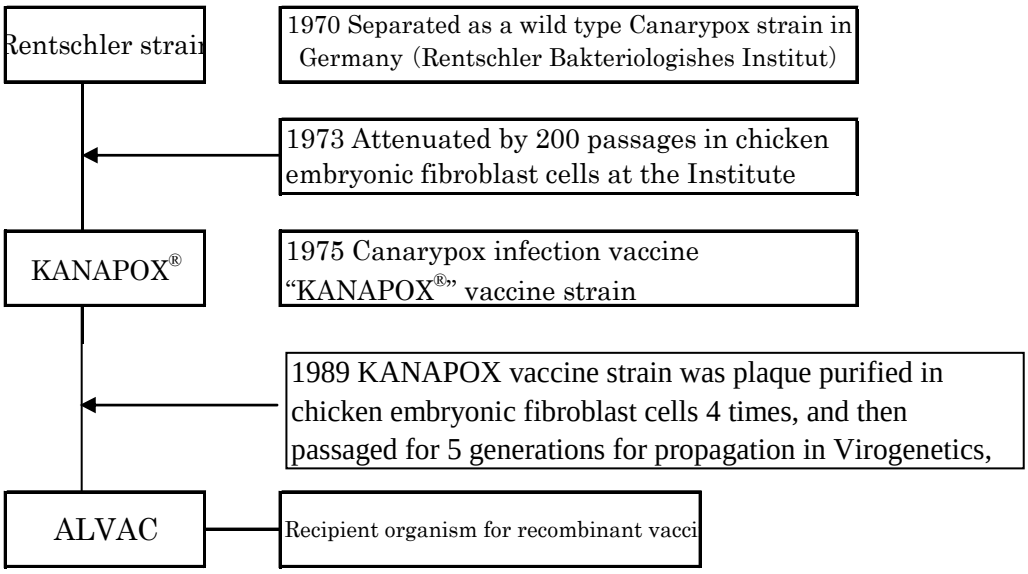
c) Predacity or Parasitism

-

d) Mode of propagation or reproduction (Application Annex 1)

KANAPOX® and ALVAC: The Rentschler strain, a wild type Canarypox virus isolated in Germany in 1970, was sufficiently attenuated by 200 passages in chicken embryonic fibroblast cells to prepare the CP1 strain for the production of Canarypox vaccine (trade name KANAPOX®, Merial France). This strain was plaque purified four times, and then passaged and propagated for five generations to become ALVAC (name during development: CPpp). The characteristic of ALVAC is that the pathogenicity of the wild type Canarypox virus is sufficiently weakened, and it is thus presumed that the genes related to the pathogenicity of the Rentschler strain before passage have been modified.

Lineage of ALVAC



Auxotrophy: ALVAC has the viral properties to live by replicating itself using the cells of other organisms, thus it cannot live alone in nature. Therefore, as the recipient organism auxotrophy, it requires avian cultured cells *in vitro*, and live cells of birds and the embryos of canaries, etc. *in vivo*.

Drug sensitivity : ALVAC is sensitive to formalin, phenol and chloroform. It is also sensitive to general disinfectants; in a comparative study of disinfectants using ALVAC and vCP97, it was confirmed that ALVAC is sensitive to cationic disinfectants, glutaraldehydes, and sodium hypochlorite in this order (Application Annex 32).

Mode of reproduction: ALVAC shows infectivity due to cohabitation in susceptible bird canaries; however, a generalized rash like field infection or edema, blisters, or pustules at the site of inoculation are not formed, showing no pathogenicity. In these avian cells, productive infection occurs with the replication of infectious viruses, while abortive infection occurs in mammalian cells if infection happens but the mature infectious viruses are not replicated and thus virus excretion and infection by cohabitation do not occur (Response Annex 22).

Crossability: Recombination by coinfection with the wild type Canarypox virus in canaries in the natural environment is possible, but in an infection study using virulent Canarypox virus and ALVAC, coinfection was not detected (Response Annex 6). In addition, FeLV is an endogenous retrovirus in cats, but it is considered that crossability by coinfection is not possible, because the growth part of the retrovirus is intranuclear (Response Annex 11) and ALVAC does not have the LTR sequence necessary to complement the endogenous virus.

e) Pathogenicity

The natural recipient organism of wild type Canarypox virus is canaries. The virus causes a local or whole-body rash, leading to death. Additionally, the virus induces pneumonia, with a fatality reaching 90% and over. It causes a local rash, but shows no systemic infection or fatality in one-day-old chickens, adult chickens, turkeys or ducks (Application Annex 8). Recipient organisms other than canaries susceptible to this virus include sparrows, which are classified as Passeriformes [house sparrows (Passer domesticus), white-crowned sparrows (Zonotrichia leucophrys) etc.], pigeons, chickens, and turkeys, based on cases of natural epidemics that have occurred so far and experimental infections, etc. Avipoxviruses including the Canarypox virus are highly recipient organism-specific and can infect only a specific bird from which they had been separated, thus they are classified into 10 species by the kind of birds they are derived from. As shown in Application Annex 14, susceptibility to avipoxvirus from Colaptes auratus was not found in 16 species of wild birds, chickens, pigeons, and turkeys. Also, as shown in Application Annex 15, the susceptibility to two strains of Canarypox virus was investigated in chickens, pigeons and wild birds: one of the strains showed pathogenicity in two species of sparrows, while susceptibility to the other strain was not observed in chickens, pigeons or any of the 11 species of wild birds. Spreading in birds is mainly due to contact with skin lesions or inhalation of scabs.

Since the recipient organism ALVAC is derived from the Canarypox infection live vaccine strain, it is not lethal when inoculated to the natural recipient organism canaries; only localized lesions, such as follicular necrosis, occur as a local reaction at the site of inoculation, and whole-body rash etc. characteristic to pathogenic Canarypox has not been observed (Application Annex 10). Moreover, vCP97 has been passaged for five generations in canaries by transdermal inoculation, and it has been confirmed that no back mutation occurs (Application Annex 9). Based on these results and the fact that recipient organism ALVAC hardly shows pathogenicity comparable to the attenuated Canarypox virus CP1 strain in the most suceptible canaries, it is considered very unlikely to spread to the wild birds with less susceptibility than canaries.

In mice (Application Annex 11), guinea pigs (Application Annexes 11 and 12), rabbits (Application Annex 12), cats (Application Annex 13) and humans (Application Annex 12), only a mild inflammatory reaction at the site of inoculation was seen; there was no replication of viruses, and pathogenicity was not reported.

Protection and treatment methods:
As mentioned above, ALVAC does not have pathogenicity. Therefore, there are no protective and treatment methods. Vaccines such as KANAPOX® are effective for protection against Canarypox caused by wild strains. There are no effective treatment methods.

f) Productivity of harmful substances

ALVAC: Expressed proteins that were predicted from the whole gene sequence of the Canarypox virus (Response Annex 7) were searched for allergenicity using the Allergy Database for Food Safety and for genotoxicity based on the Genotoxicity Database on Chemicals, but no substances were found to show allergenicity or genotoxicity. Although other harmful substances could not be searched, approximately 7 million doses of recombinant vCP97 vaccine have been used since its approval overseas in 2000 until 2006, and there have been no reports of the production of harmful substances due to this vaccine; thus, it is presumed that it has no influence due to the production of harmful substances.

g) Other information

There has been no information on excretion or infection due to cohabitation in cats, which are the target animal of inoculation with ALVAC or in other mammals.

2. Information concerning preparation of living modified organisms

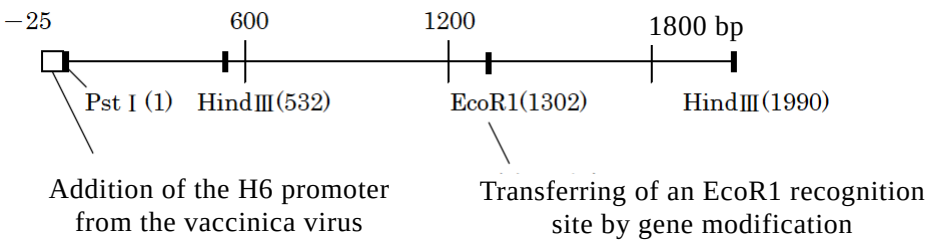
(1) Information concerning donor nucleic acid

a) Composition and origins of component elements

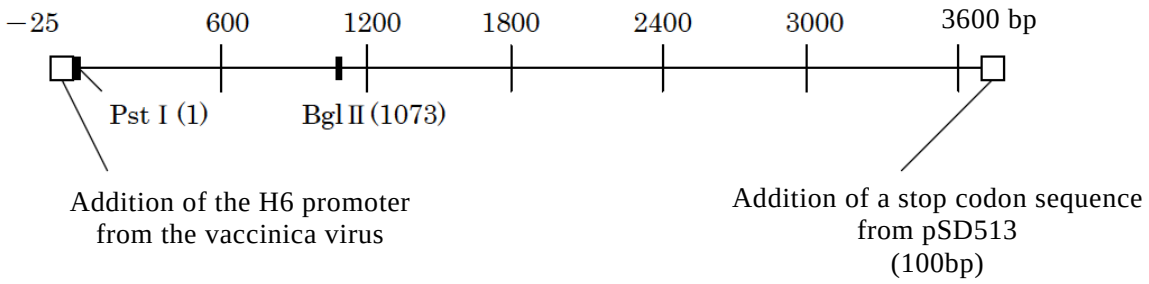
1) As the target genes, the *env* gene (1,930 bp), *gag* gene (1,683 bp), and *pol* gene partly (1,272 bp) derived from the Glasgow-1 strain, which belongs to the type A feline leukemia virus (FeLV, type C Oncovirus, Retroviridae), are used as virus protective antigens. Note that these genes were prepared from the plasmids that subcloned the viral DNA in the provirus state in cells infected with the FeLV-A/ Glasgow-1 strain (Application Annex 17). The H6 promoter (94bp) of vaccinia (Orthopoxvirus, Poxviridae) is used as the regulatory system and is transferred upstream of the FeLV gene. It was prepared from genomic DNA fragments digested with the restriction enzyme *Hind*Ⅲ-*Eco*R Ⅰ (Application Annex 18).

2) The restriction enzyme cleavage map is shown below (Application Annex 19).

i) *env* gene



ii) *gag* gene and *pol* gene (partial)



b) Function of component elements

Objective of development:

Currently, vaccines used for protection against viral diseases include killed vaccines and attenuated live vaccines. Killed vaccines are killed pathogens, which do not show pathogenicity but have the disadvantages of a local reaction at the inoculation site due to adjuvant and a comparatively short duration of immunity. Attenuated live vaccines use attenuated pathogens, induce cellular immunity in addition to humoral immunity, and provide a comparatively long duration of immunity, while carrying such risks as back mutation to recover pathogenicity, and spreading of the virus. Current vaccines against FeLV are only killed vaccines, which have the abovementioned disadvantages. A recombinant vaccine was developed to improve them. The poxvirus used for the development of this vaccine has enough safety information from long experience in the smallpox vaccine, and also has advantages including that it can induce not only humoral immunity but also cellular immunity, that there is no possibility of back mutation to recover pathogenicity because it is not a pathogenic virus to the target animal, that it does not contain adjuvants and is highly safe, and that it is not much affected by maternally transferred antibodies (Response Annex 16, Response Annex 32).

Dynamics of ALVAC in mammalian cells and the immunological properties:

• Dynamics in mammalian cells

Because the recipient organism of ALVAC is the canary, when it infects other mammals than the recipient organism, the compatibility between the virus and cells is low and thus abortive infection occurs when infectious viruses are not replicated via the synthesis and maturation of the nucleic acids or the proteins of the virus (Response Annex 30, Response Annex 31).

Concerning the observation of ALVAC dynamics in cells, there have been reports including that there was no viral replication in electromicroscopy of cells derived from dogs and cats after infection with the recombinant virus vCP97 (Response Annex 10), that passage cannot be performed in mammalian cells (Application Annex 25), and that replication of ALVAC does not occur in human cells infected with rabies G protein recombinant ALVAC (Response Annex 26). As summarized in Response Annex 30 and Response Annex 31, based on these reports, the non-productive phase of viral growth is supposeed to be before the expression of the late genes

that encode viral proteins, because the viral component proteins are not detected in the non-productive phase. The mechanism of abortive infection based on recipient organism specificity has not yet been elucidated, except that the involvement of the gene specifying the recipient organism range is suggested in some poxviruses (Response Annex 2)

.• Immunological properties of ALVAC

The immunological mechanism of ALVAC has been poorly understood and there have been few reports. However, some of the recent studies suggest that dendritic cells that play an important role in the antigen presentation to lymphocytes are involved in the mechanism of ALVAC to provide immunity (Response Annex 24).

As for the recombinant vaccine, neutralizing antibodies were not produced but defence against the challenge were observed in inoculated cats, suggesting the involvement of cellular immunity. In addition, enhancement of the neutralizing antibody response was observed after the challenge, also suggesting priming of the humoral immunity (Application Annex 27, Response Annex 32). Immunological properties of ALVAC in other mammals than cats have been confirmed *in vivo* as protein expression by several kinds of recombinant vaccines in addition to the feline leukemia-modified vaccine of this application (Response Annexes 25-29). Testing of the ALVAC recombinant vaccine has been performed in animals including cats, dogs, pigs and horses, and also in primates including monkeys, chimpanzees, and human volunteers. Both humoral and cellular immunity have been confirmed as an immune response in mammals.

Components of the modified organism:

1) The transferred gene expression cassettes are as follows:

i) *env* gene expression cassette

Vaccinia virus H6 promoter – regulates gene transcription at the early and late stages of viral infection.

FeLV *env* gene – encodes the precursor p85 of the viral envelope glycoprotein. p85 is degraded into gp70 and p15E by the action of protease. gp70 protrudes outside the outer envelope, and specifically binds to the viral receptor of a cell when the cell is infected with the virus.. p15E is within the outer envelope and is bound to gp70. The antibody specific to *env* protein plays a role to protect against infection by neutralizing the FeLV.

ii) *gag/pol* gene expression cassette

Vaccinia virus H6 promoter - regulates gene transcription at the early and late stages of viral infection.

FeLV *gag* gene – encodes the precursor of core protein to form the basic structure of the virus.

This is degraded into p27 (major core protein), p10 (nucleoprotein), p15C (core protein), and p12 (inner coat) by the action of protease.

FeLV *pol* gene (partial) – a part of the polymerase gene is used, which expresses 14 kDa protease to degrade the *gag* precursor.

2) By the expression of the target gene, envelope glycoprotein gp70, p15E, as well as core protein p27 (major core protein), p10 (nucleoprotein), p15C (core protein), and p12 (inner coat) of FeLV. As for the functions of these expressed proteins, gp70 shows type specific antigenicity of FeLV, has the function of a protective antigen against viral infection, and is involved in immunity against reinfection. p15E is supposed to interfere with the immune response of the recipient organism and promote persistent viral infection. p27, p10, p15c and p12 are all involved in the formation of the inner shell structure of the viral particle. p27 is involved in the construction of the viral particle and is detected in blood, tears and saliva in addition to the infected cells. These expressed proteins do not show allergenicity on searching of the allergen database. In addition, approximately 7 million doses of the recombinant vaccine have been used since 2000, and during that period there have been no reports that harmful substances were produced.

3) The target genes were transferred in the C3 and C5 sites of the open reading frame of ALVAC. It has been confirned that transferring of exogenous genes in the C3 and C5 sites does not affect growth in the susceptible chicken embryonic fibroblast cells (Application Annex 20), thus it is supposed not to change the metabolic pathway of the recipient organism.

(2) Information concerning vector

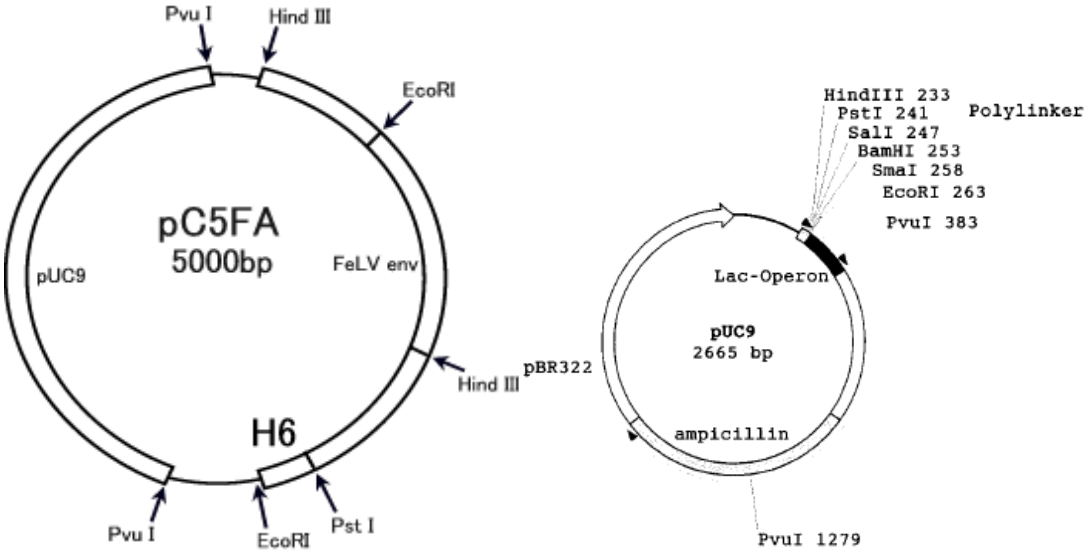
a) Name and origin

The name of the vector used in the preparation of vCP97 is pC5FA and pC3DOFGAGVQ. pC5FA was prepared by transferring the FeLV *env* expression cassette into pRW831, and pC3DOFGAGVQ was prepared by transferring the FeLV *gag/pol* expression cassette into pC3I. pRW831 contains a flanking region in pUC9 for the transfer into the C5 site of ALVAC, and pC3I contains a flanking region in pBS-SK (pBluescriptSK+) for the transferring into the C3 site of ALVAC. The primary sequences of the respective flanking regions are shown in Figures 1 and 2 of Application Annex 22, excluding the sequences from the initiation site of H6 promoter to the end of the transferred gene. pUC9 and pBS-SK are plasmid vectors from *E. coli*, and the nucleotide sequences of these vectors

are shown in Application Annex 19. pUC9 is disclosed in Genbank accession No. L09128, and pBS-SK is disclosed in the Web site of Stratagene Inc.

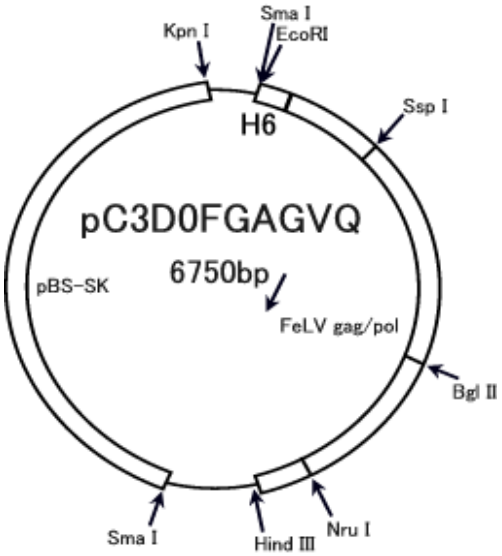
b) Properties

1）pC5FA



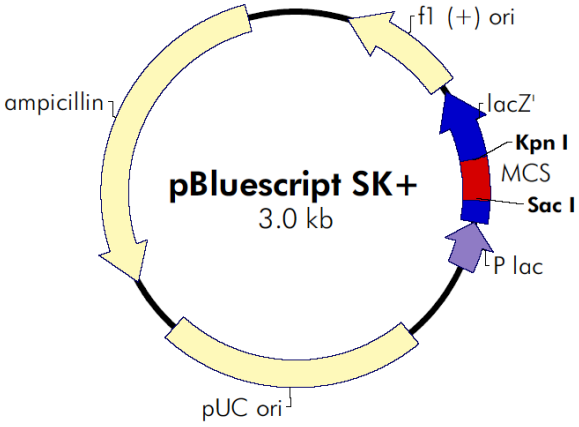
Information on pUC9 in the vector is as shown in the right figure (Application Annex 19).

2）pC3DOFGAGVQ



Information on pBS-SK in the vector is as shown below (Application Annex 19).

f1 (+) origin 138–444
β-galactosidase α-fragment 463–816
multiple cloning site 653–760
***lac* promoter 817–938**
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



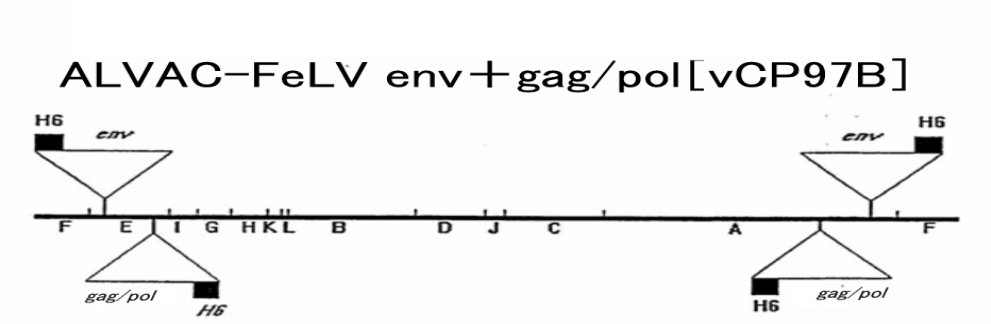
3) The presence or absence of infectivity of vectors and information concerning the recipient organism range if infectivity or pathogenicity is present:

Major parts of both pUC9 in pC5FA and pBS-SK in pC3DOFGAGVQ are comprised of plasmid vectors from *E. coli*; therefore, their respective infectivity, pathogenicity and transmissibility are supposed to be homologous to the respective plasmid vectors. pUC9 and pBS-SK are widely used as plasmid vectors for experiments in *E. coli* K12, and their whole gene sequences have been determined; pathogenicity and infectivity are not known, and they are generally recognized as types with a low possibility of conjugational transfer.

(3) Method of preparing living modified organisms

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

The state of transferring the *env* gene and the *gag/pol* genes respectively into the C3 and the C5 sites at the 3' and the 5' ends of ALVAC is shown (Application Annex 21).



The restriction enzyme cleavage maps of the *env* gene and the *gag/pol* gene are shown in Application Annex 19. The nucleotide sequences of the respective transferred genes are attached as Application Annex 22.

Additionally, for recipient organism ALVAC, the nucleotide sequence and the amino acid sequence of the C3 and the C5 sites where the target genes are transferred, are shown (Response Annex 3). The whole nucleotide sequence and the protein functions of Canarypox virus are also shown in Response Annex 7.

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

A. Preparation of FeLV-*env* transfer vector

The FeLV *env* gene was divided into three parts and amplified, and the three nucleic acids were then transferred into one plasmid to prepare pH6FA-3. Moreover, pH6FA-3 and pRW831 were digested with Hind III and EcoRI to prepare the vector pC5FA to transfer the FeLV *env* gene into ALVAC (Application Annex 23).

B. Preparation of the FeLV-*gag/pol* transfer vector

The FeLV *gag/pol* gene was divided into two parts and amplified, and then the two nucleic acids were transferred into one plasmid to prepare pC3FGAG, from which the *gag/pol* gene was spliced out again, and a stop codon was added to prepare pC3FGAGVQ. pC3FGAGVQ and pC3I were digested with Hind III and EcoRI to prepare the vector pC3DOFGAGVQ for the transferring of the FeLV *gag/pol* gene into ALVAC (Application Annex 23). Chicken embryonic fibroblast cells were transfected with the expression cassette plasmid into which the target gene and the promoter had been transferred by the calcium phosphate method, and at the same time were infected with the rescue virus (ALVAC), causing homologous recombination in the cytoplasm and thus transferring the target gene into ALVAC (Application Annex 23).

c) Processes of rearing of living modified organisms

vCP97 prepared by homologous recombination was plaque purified three times and cloned. The recombinant virus was gradually scaled up from 60 mm dishes to be grown in roller bottles, and was stored as Stock 183. Stock 349 of the same strain was further plaque purified, gradually scaled up, and the recombinant virus grown in roller bottles was stored as Stock 477. This virus was further grown in roller bottles to prepare the master seed virus (MSV) (Application Annex 23).

Manufacture is controlled by the seed lot system; vCP97 is the strain for manufacture, and it is specified that a strain that has passaged five generations (X+5) from MSV (X) is to be used in manufacture, for the inspection of items to be performed on MSV and the working seed virus (WSV), as well as the process to prepare WSV from MSV and to produce the drug substance (Application Annex 24).

(4) State of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid

The target genes are incorporated into the genomic DNA of the recipient organism Canarypox virus. The sites of transferring are the open reading frame C3 and C5 regions of the genomic DNA, and the *env* gene, *gag* gene and a part of the *pol* gene from FeLV-A, as well as H6 promoter from the vaccinia virus as the regulatory element are transferred. Application Annex 22 shows the whole nucleotide

sequence and the amino acid sequence of the region containing the C3 site transferred with the *env* of the target gene and the C5 site where the *gag*/*pol* gene is transferred in the ALVAC virus gene.

In this genomic sequence, it is supposed that the C3 site corresponds to CNPV 004 Ankyrin repeat protein in Response Annex 7 (p355 Table 1 CNPV ORFs), and similarly that the C5 site corresponds to CNPV 009 Ankyrin repeat protein. These Ankyrin repeat proteins are considered to be disrupted by substitution with the donated FeLV transferring gene. Other effects on the other characteristics that have not been tested are not known. In this way, transferring of the target genes into the C3 and C5 sites of the recipient organism does not affect growth in the susceptible chicken embryonic fibroblast cells, but target gene products are expressed (Application Annex 25); thus the target genes are supposed not to change the metabolic pathway of the recipient organism after transferring.

In addition, in studies conducted at our company, no differences in resistance were shown between viruses before (ALVAC) and after (vCP97) gene transferring concerning the viability in water (Application Annex 31), and concerning heat resistance in nonadherent conditions of 37 ºC and 60 ºC (Response Annex 23).
These results confirmed that the virus after transferring of the target genes is comparable to the recipient organism ALVAC before transferring and that there are no changes in properties due to the transferring of FeLV genes.

Moreover, because the target gene products gp70, p15E (Application Annex 27) and precursors of p85 and p63 (Response Annex 14) are expressed in mammalian cells, the target genes are supposed not to change the metabolic pathway of the recipient organism after transferring.

The expression stability in passages of five generations is confirmed in chicken embryonic fibroblast cells (Application Annex 26). In the passage using dilution, the *env* gene expression slightly decreased compared to the *gag* gene expression; however, this is supposed to be due to severe test conditions and the stability is considered to be adequate for the usual passage conditions (Response Annex 1). The expression of target genes was demonstrated in Vero cells and CRFK cells, and inoculated cats are confirmed to show defence against challenge by FeLV (Application Annex 27).

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

Identification of the recombinant virus is performed by the immunofluorescent antibody method using anti Canarypox virus monoclonal antibody labelled with Fluorescein-iothiocyanate (FITC) and anti gp70 glycoprotein monoclonal antibody labelled with Indocarbocyanine (Cy3) (Application Annex 28, Response Annex 19). The detection limit is 6 $TCID_{50}$.

(6) Difference from 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

Mode of growth: Wild type Canarypox virus grows in avian cells under general cell culture conditions (37±1 ºC, 5%CO_2). Although the recombinant virus vCP97 lacks C3 and C5, these parts are not involved in growth and its productivity is thus considered to be comparable to the wild strain, and is confirmed as such (Application Annex 25).

As for the mode of growth of vCP97 in the body of inoculated cats, survival for 2 days (less than 4 days) was suggested at the site of inoculation (Response Annex 9), but viremia in the body, excretion into the nasal discharge or feces and infectivity to susceptible cats by cohabitation were not observed (Application Annex 16). Canarypox virus is characterized by the fact that, while canaries and some birds are susceptible to it and the virus infects them and grows in them, it does not grow in mammals without susceptibility even if infection occurs (recipient organism specificity). In this way, the mode of productive infection is seen in birds and their cultured cells in which infectious viruses are replicated, while the mode in mammalian cells is abortive infection in which infectious viruses are not replicated and there is neither viral excretion nor infection due to cohabitation. The mode of abortive infection of recombinant virus vCP97 and Canarypox virus in mammalian cells is shown in Response Annexes 21 and 22 (modified from the original figures in Response Annex 2). The inactive phase of viral growth of this modified organism based on the summarized results of the respective data (Application Annexes 16, 27 and Response Annexes 10, 14 and 26) was supposed to be approximately 40 hours after viral inoculation, and as for gene expression, it was supposed to be before the expression of the late genes that encode viral component proteins (Response annexes 30, 31 and 32).

Genetic properties: As the genetic properties of the recombinant virus vCP97, it has two copies of the *env* gene, the *gag* gene and a part of the *pol* gene, respectively, of FeLV and four copies of vaccinia virus H6 promoter in the genome of Canarypox virus. In addition, the abovementioned genes are transferred into the deletion of the open reading frames C3 and C5 of Canarypox virus, thus the genome of the recombinant virus lacks them (Application Annex 19 and Annex 21).

Possibility of incorporation into the gene of an inoculated animal: In the life cycle of poxvirus (Response Annex 2), although the nucleic acids of DNA viruses usually replicate in the nucleus of a cell, exceptionally, gene expression, DNA replication and virus particle formation of poxvirus occur

in the cytoplasm. Therefore, ALVAC and the recombinant virus vCP97 are not dependent on the gene replication mechanism in the nucleus of recipient organism cells for the transcription from viral genomes, so it is supposed that there is no possibility for them to be incorporated by homologous recombination into the gene of the recipient organism cell.

Pathogenicity: The recombinant virus vCP97 and its recipient organism ALVAC cannot be replicated in the body of a mammal, so it has no pathogenicity to cats. In canaries, systemic events due to infection with the recombinant virus were not observed, and the only symptom was confirmed to be transient and mild inflammation at the site of inoculation (Application Annex 8).

Productivity of harmful substances and other major physiological properties: Expressed proteins that were predicted from the whole gene sequence of Canarypox virus in Response Annex 7 were searched for allergenicity using the Allergy Database for Food Safety, and for genotoxicity based on the Genotoxicity Database on Chemicals, and none of the expressed proteins predicted from the abovementioned gene sequence were found to show allergenicity or genotoxicity. Although other harmful substances could not be searched, there have been no reports of the production of harmful substances in many cases of the use of the vaccine since its approval overseas in 2000; thus, it is presumed that there is no influence due to the production of harmful substances by ALVAC and the recombinant virus vCP97.

Infectivity: The recombinant virus vCP97 and its recipient organism ALVAC can infect mammalian cats but cannot be replicated in them (Application Annex 13), thus cats have no susceptibility. Survival of the recombinant virus vCP97 at the site of inoculation is supposed to last for two days (less than four days) after inoculation (Response Annex 9).

Possibility of activation of endogenous virus and endowment of pathogenicity: Recombination may occur between endogenous retrovirus and highly homologous FeLV. The mode of growth of retroviruses is as shown in Response Annex 11; replication of the provirus occurs within the nucleus, and protein synthesis starts from the mRNA in the cytoplasm. Due to this difference in the parts of the cell where these viruses grow, the possibility of recombination at the gene level by coinfection is supposed to be extremely low. In addition, endogenous FeLV lacks the *env* gene and a part of LTR (Long terminal repeat), thus it cannot produce infectious particles. Note that there is a phenomenon where infectious viruses are produced by complementing the deleted part via further recombination with exogenous FeLV genome. However, the recombinant vCP97 virus does not have the LTR sequence and cannot complement the deleted part, thus it is supposed to be impossible to produce infectious FeLV.

In birds, as shown in Response Annexes 12 and 13, there have been reports that the gene sequence of

some retroviruses was detected in the gene of fowlpox virus, while there have been no such cases of poxvirus infection in cats. These indicate that there is very little possibility of recombination of ALVAC and the recombinant virus vCP97 with endogenous retroviruses in cats.

Possibility of recombination due to coinfection of the recombinant virus vCP97 with highly virulent Canarypox virus: Experimental coinfection of wild type Canarypox virus and the recombinant recipient organism (ALVAC-Luc) was carried out in canaries, and no possibility of the emergence of recombinant virus due to coinfection was observed (Response Annex 6).

As for the possibility of coinfection with the highly virulent Canarypox virus in cats, the Canarypox virus in the natural environment does not grow as an infectious virus in cats, therefore the risk of recombination of ALVAC and recombinant virus vCP97 with the highly virulent Canarypox virus by coinfection is supposed to be extremely low, excluding the need for monitoring.

Excretion from the body and infectivity by cohabitation: when cats of eight weeks old were inoculated with high doses of vCP97 and ALVAC of approximately 60-fold the vaccine specification values, neither of the inoculated viruses were isolated (Application Annex 13). These were not isolated from the control cats in cohabitation either. This study demonstrated that the recombinant virus vCP97 is not excreted by inoculated cats and is not spread to other cats.

Viability in the natural environment: It is considered that the recombinant virus vCP97 has comparable viability in the natural environment to that of ALVAC. More specifically, because the virus cannot exist alone in the natural world, susceptible cultured cells are limited to avian cells, and there is recipient organism specificity even within avipoxvirus, and it is highly unlikely for the virus to have contact with susceptible cells and susceptible animals and to survive for a long period under natural conditions (Application Annex 8). Comparison of the viability of recipient organism ALVAC and recombinant virus vCP97 in distilled water at 22ºC (Application Annex 31), heat resistance at 37ºC and 60ºC (Response Annex 23) and inactivation by disinfectants (Application Annex 32), confirmed that this recombinant virus vCP97 is comparable to recipient organism virus ALVAC.

b) Characteristics such as colony formation and chromogenicity for discrimination of the modified organism from the recipient organism

None

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.

1. Name: Canarypox virus ALVAC to which a protective antigen protein expression gene derived from feline leukemia virus (vCP97 strain) was transferred (FeLV-*env, gag, pol,* Canarypox virus)

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 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 (excluding those involving human food)

(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: Merial Japan Ltd.

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

1) Property of reducing other microorganisms

The virus strain used in the modified live vaccine has the *env* gene, the *gag* gene and a part of

the *pol* gene from feline leukemia virus as the donor nucleic acids and provides a protective effect against feline leukemia virus via the expression of these genes. It is unlikely that the Canarypox virus that expresses these genes will produce more substances than the parent strain that reduces other microorganisms.

In addition, there have been no reports of reduction of other microorganisms in the natural environment during overseas use experience.

Based on the abovementioned understanding, it was judged that the conclusion made by the applicant that no wild animals or wild plants likely to be affected by the Type 1 Use can be specified and that there is no risk of Adverse Effects on Biological Diversity attributable to the property of reducing other microorganisms, is valid..

2) Pathogenicity

It has been reported that infection with avipoxviruses including Canarypox virus is hard to occur in other wild birds than the bird species from which the virus is derived. The recipient organism strain Canarypox virus is from an attenuated vaccine strain, and severe pathogenicity is not found in the study of inoculation in canaries. Moreover, the modified live vaccine maintains similar traits as the recipient organism strain, and it is supposed not to show more pathogenicity than the recipient organism strain when it is inoculated to canaries. In the study of inoculation of the modified live vaccine in cats, neither pathogenicity nor viral excretion was observed in inoculated cats.

The reason This is supposed to be that replication of the infectious virus did not occur since the mammals, including cats are not the recipient organisms of Canarypox virus, thus it is supposed that the vaccine will not show pathogenicity to other wild mammals.

Based on the abovementioned understanding, it was judged that the conclusion made by the applicant that no wild animals or wild plants likely to be affected by the Type 1 Use can be specified and that there is no risk of Adverse Effects on Biological Diversity attributable to pathogenicity, is valid.

3) Productivity of harmful substances

There is little possibility of productivity of harmful substances by this recombinant gene, and the results of database search for allergenicity and genotoxicity do not indicate the possibility

that these harmful substances could be produced.

Moreover, productivity of harmful substances of the modified vaccine has not been reported overseas where there is experience of its use.

Based on the abovementioned understanding, it was judged that the conclusion made by the applicant that no wild animals and wild plants likely to be affected by the Type 1 Use can be specified and that there is no risk of Adverse Effects on Biological Diversity attributable to productivity of harmful substances, is valid.

4) Property of transmitting nucleic acid horizontally

There have been no cases of problems due to homologous recombination of poxvirus during the longstanding history of its use in smallpox vaccine. Therefore, there is little possibility of homologous recombination. even with coinfection of the modified vaccine strain and the highly virulent Canarypox virus. In addition, in the coinfection study in canaries with the highly virulent Canarypox virus strain and the modified vaccine strain, the results showed that there is little possibility of homologous recombination.

With infection in cats, the modified vaccine strain neither grew sufficiently nor replicated the infectious virus in the cat body, so there is little possibility of homologous recombination. with other poxviruses or retroviruses . Moreover, because there was no viral excretion from inoculated cats and there seems to be no possibility of transmission to birds, etc., which eat the cats inoculated with the vaccine, which do not produce the infectious virus, the influence on other wild birds or mammals is considered to be very small.

In addition, the modified live vaccine has comparative traits to those of the recipient organism strain for viability in distilled water and heat resistance, and it also is highly specific to infectious animals; therefore, the possibility of its long-term survival under natural conditions is supposed to be extremely low.

Based on the abovementioned understanding, it was judged that the conclusion made by the applicant that no wild animals or wild plants likely to be affected by the Type 1 Use can be specified, and that there is no risk of Adverse Effects on Biological Diversity attributable to the property of transmitting nucleic acid horizontally, is valid.

(2) Conclusion based on the Biological Diversity Risk Assessment Report

Based on the abovementioned understanding, it was judged that the conclusion of the Biological Diversity Risk Assessment Report that there is no risk that the use of this modified live vaccine in accordance with Type I Use Regulation can cause Adverse Effects on Biological Diversity, is reasonable.

References

1. Gene composition of FeLV (Stewart *et al*, J.Virol, 58:825-834, 1968) (Application Annex 17)
2. Protection of Cats against Feline Leukemia Virus by Vaccination with a Canarypox Virus Recombinant, ALVAC-FL (Tartaglia *et al*, J.Virol, 67:2370-2375, 1993) (Application Annex 27)
3. Masakazu Hatanaka (1997) Virology, poxvirus, Asakura Publishing Co., Ltd, Tokyo (Response Annex 2)
4. The Genome of Canarypox Virus (J Virol, Jan, 78(1) p353-366, 2004) (Response Annex 7)
5. Isolation and Molecular Biological Investigations of Avian Poxviruses from Chickens, a Turkey, and a Pigeon in Croatia (Avian disease 440-444, 2006) (Response Annex 13)
6. Toyoro Osato (1992) Medical virology, LD_{50} and $TCID_{50}$, Nankodo, Tokyo (Response Annex 20)
7. Canarypox Virus-Induced Maturation of Dendritic Cells Is Mediated by Apoptotic Cell Death and Tumor Necrosis Factor Alpha Secretion. (J Virol, Dec, 74(3) p11329-11338, 2000) (Response Annex 24)
8. Biological and immunogenic properties of a canarypox-rabies recombinant, ALVAC-RG (vCP65) in non-avian species (Vaccine, 13(6) p539-549, 1995) (Response Annex 26)
9. Recombinant canarypoxvirus vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge (ArchVirol Suppl, 18, p221-230, 2004) (Response Annex 27)
10. Recombinant Nipah Virus Vaccines Protect Pigs against Challenge. (J. Virol, Aug, 80(16) p7929-7938, 2006) (Response Annex 28)
11. Immune Responses to Human Immunodeficiency Virus (HIV) Type 1 Induced by Canarypox Expressing HIV-1_{MN}gp120, HIV_{SF2} Recombinant gp120, or Both Vaccines in Seronegative Adults (J.Inf Disease, 177 p1230-1246, 1998) (Response Annex 29)

Annex: References

Not made available or disclosed to unauthorized person