Environmental Assessment
for Field Testing
Canine Melanoma Vaccine, DNA

I. Proposed Action

APHIS is considering granting authorization to ship an unlicensed Canine Melanoma Vaccine, DNA, for field testing. Merial, Inc., Athens, Georgia, has requested authorization to conduct clinical studies that will provide efficacy and safety data in dogs administered this vaccine. The efficacy trial will measure the sparing effect of the vaccine on dogs diagnosed with melanoma. The safety of the vaccine will be evaluated in animals participating in the studies.

Under the provisions of the Virus-Serum-Toxin Act of 1913, as amended in 1985, the USDA must ensure that veterinary biologics are pure, safe, potent, and efficacious and not worthless, contaminated, dangerous, or harmful. Accordingly, APHIS has conducted a risk analysis and has concluded that the safety risks to animals, public health, and the environment are low. A copy of the risk analysis with confidential business information removed is available upon request.

II. Background

Canine malignant melanoma (CMM) is a common and aggressive form of cancer in dogs, with usually a poor prognosis (1). Treatments for this condition have included surgery, chemotherapy, and radiation therapy (2). However, these therapeutic approaches have not been fully successful. Usually, CMM is detected at a late stage when excision is not likely to be complete and metastasis to regional lymph nodes has already occurred (1). The potential benefits of vaccination as a therapeutic option against CMM have been reported in the literature (3-6).

CMM is the most common malignant neoplasm of the oral cavity, and accounts for 7% of all malignant tumors in dogs. Other species affected by malignant melanoma include cats, horses, and humans (1). It is not as common in cats as in dogs, but the prognosis is equally grave. Gray horses commonly develop melanoma. Oral melanoma comprises only 1-2% of all human melanomas and is uncommon compared to the cutaneous form, which occurs with far greater frequency.

The experimental vaccine to be used in the proposed field tests is a replication-incompetent plasmid which has been genetically modified to contain a therapeutic gene. The phenotypic result of this genotypic modification is a requirement for growth factors not readily found in the external environment. Lacking these, its growth is attenuated, even with a suitable bacterial host.
The proposed field safety test will be conducted in at least three different geographical locations and the product will be used according to instructions on the product circular. The potential for escape and dispersal of the experimental vaccine from the proposed release sites is low. The personnel to conduct the study are experienced in canine health management.

III. Need for the Proposed Action

There are no CMM vaccines approved for use in dogs. Although CMM is a progressive fatal disease, there are reports in the literature suggesting that vaccination can potentially improve survival time following treatment of the primary tumor growth, presumably through inhibiting progression of metastases. A replication-incompetent DNA vaccine can potentially be both safe and efficacious, inducing both antibodies and cytotoxic T-cells in a broad immune response. A vaccine found to be successful against CMM may have wider application in treating melanoma in other animal species and in humans. It is expected that the data from these monitored field trials will confirm the safety of this melanoma DNA vaccine for use in dogs and within the environment of the United States.

IV. Areas of Concerns

The three areas of concern to APHIS are: 1) animal safety, 2) public health, and 3) environmental safety. APHIS has conducted a risk analysis to assess whether risks are associated with the proposal to field test this experimental vaccine. The safety characteristics of this vaccine have been thoroughly evaluated. The conclusions derived from the risk analysis for each of the areas of concern are summarized below.

A. Animal Safety

The risk to target animals is low. The experimental vaccine is highly purified plasmid DNA not capable of replication in mammalian cells. In a previous study, the vaccine was found to be safe when administered at the recommended dose and in excess of that dose, to dogs afflicted with melanoma. Plasmid DNA can persist at the site of injection but there have been no signs of pathology or toxicity associated with that persistence. The minimum age for dogs with melanoma, the target population, is 2 years old, so younger dogs will not be exposed. Regarding non-target species, because the vaccine is not infectious, cannot replicate in eukaryotic cells, and is not pathogenic, the risk to non-target species is considered to be low.

B. Public Health

The risk to public health is likewise low. In these proposed clinical studies, the possibility of human exposure is slight and limited to veterinary personnel. Should accidental self-injection occur, it is not expected to cause a public health concern. The plasmid vector has been used previously with other gene inserts to create vaccines against melanoma. These have been reported to be used in human subjects without adverse effect.
There is one veterinary DNA vaccine licensed for use in the United States. It is for use against West Nile Virus in horses and has been demonstrated to be safe and effective. There have been no reports of adverse effects or hazards to public health for this registered product.

C. Environmental Safety

The risks to the environment are low. The potential for escape and dispersal of this replication-incompetent DNA vaccine is restricted. The plasmid cannot replicate within the vaccinated animal. Thus, the shed/spread capabilities of the vaccine are very limited even under direct contact exposure. In the case of an accidental spill, the plasmid is unstable in the environment and rapidly degraded in the stomach of any animal that may ingest it. Should there be plasmid uptake by soil bacteria, the plasmid will not be maintained in the absence of its growth requirements. There are no expected adverse ecological effects associated with the proposal to conduct field testing with this experimental vaccine.

V. Alternatives

Two alternatives were considered. The only alternative considered, other than the preferred action alternative, is not to approve the proposed field tests, the “no action” alternative. We have considered the applicants’ goals in light of the agency’s public interest and responsibilities and any potential environmental impact. Based upon the results of our risk analysis and the potential applications for this vaccine in disease control, APHIS adopts the alternative that the proposed field tests be approved.

VI. Conclusion

Based upon the risk analysis documented in this EA, APHIS has determined that implementation of the proposal would not significantly affect the quality of the human environment and that the preparation of an Environmental Impact Statement is not required (Finding of No Significant Impact).

References


3. Alexander AN, Huelsmeyer MK, Mitzey A, Dubielzig RR, Kurzman ID, Macewen EG, Vail DM; Development of an allogeneic whole-cell tumor vaccine expressing


Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for Melanoma DNA Vaccine

(Product Code 9240.D0, Unlicensed)
# Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for Melanoma DNA Vaccine

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Executive Summary

Melanoma is a progressive fatal disease of dogs. Survival time is dependent upon many factors some of which are not well defined. Existing treatments include aggressive surgical intervention, chemotherapy and radiation; however, their effectiveness is questionable.

Merial has prepared this document to provide information on the Melanoma DNA Vaccine that is being developed for therapeutic immunization of dogs diagnosed with melanoma. The document provides a risk analysis consisting of the summary information format that describes the vaccine composition and use followed by a risk assessment of the safety of the product in animals, humans and the environment.

The experimental Melanoma DNA vaccine is highly purified plasmid DNA consisting of the vector plasmid with the sequence of the gene inserted and is not capable of replication in mammalian cells, because its replication is restricted to E. coli bacteria. Vaccines containing the plasmid, and others utilizing the plasmid with similar gene inserts, have been tested in human subjects and in dogs. All of the studies to date, have indicated the vaccine is safe. No adverse effects on the test subjects or the environment have been observed.

Extensive testing of DNA vaccines over the last 10 years in animals and humans has demonstrated that the safety issues postulated during the early days of DNA vaccine technology (integration, autoimmunity, tolerance) have not materialized into real concerns. Biodistribution studies have demonstrated that after vaccination, although small amounts of plasmid are transiently and passively distributed throughout the body, accumulation in specific tissues does not occur. Plasmid levels in the tissues of vaccinated animals fall below the range detectable by PCR within weeks to months after vaccination. These facts in conjunction with the inability of the plasmid to replicate within the animal, indicate that the risk of plasmid shedding into the environment is exceedingly low.

In the event of an accidental spill into the environment, the risk to target and non-target animals is negligible as the plasmid is not infectious, it is not stable under environmental conditions and it would be unlikely to enter an animal via mucosal (oral) or dermal (skin) contact. The minute amount of plasmid, which might be ingested by an animal, would be degraded in the stomach and as such, be inconsequential. Similarly, the risk of uptake and maintenance by soil bacteria is inconsequential, as the plasmid would not be maintained in the absence of .

This product will be produced on medium devoid of and will be extensively purified and formulated without . The risk of contamination with adventitious agents of significance to mammals will be virtually eliminated.
Merial proposes to conduct clinical studies that will provide efficacy and safety data in dogs administered this vaccine. The efficacy trial will evaluate the impact of the vaccine on dogs diagnosed with melanoma. Safety will be assessed in the melanoma patients that are enrolled. The Risk Analysis for the environmental release of Melanoma DNA vaccine associated with these clinical studies, results in a calculated risk rating of "Low" as regards risks to animal, human and environmental safety.

Collectively, the information presented in this document indicates that the Melanoma DNA vaccine will have a high safety profile in animals and pose an exceedingly low risk to the safety of humans, animals and the environment. Based on this Merial recommends that the CVB issue a "finding of no significant impact" for the Melanoma DNA vaccine and authorize initiation of the proposed clinical studies.
I. INTRODUCTION

A. Objective

Merial plans to develop and license a vaccine for melanoma that contains plasmid DNA encoding a gene [redacted]. The vaccine is intended for the therapeutic immunization of dogs diagnosed with melanoma. This Risk Analysis provides the technological information that is known and available to Merial at this time with respect to the proposed vaccine.

Merial submits this Risk Analysis in support of the request that APHIS grant a “Finding of No Significant Impact” (FONSI) for this DNA vaccine under the National Environmental Policy Act.

B. Proposal

Merial has prepared a master seed bacteria (MSB) containing [redacted] plasmid DNA. This MSB will be used to prepare purified plasmid DNA from which Merial intends to formulate vaccines for the purpose of conducting experimental trials to demonstrate safety and efficacy of the melanoma vaccine in support of USDA licensure.

II. CHARACTERIZATION OF THE BACTERIAL (REPLICATION) HOST CELL

The E. coli replication host cell is strain [redacted] obtained from [redacted]. Details of the host cell are provided throughout this document.

A. Identification and Source

These cells were acquired commercially from Escherichia coli, resistant.
B. Purity

B.1. 9 CFR 113.27 Purity Test Results
The purity of the host bacteria in the MSB was established according to 9CFR 113.27(d).

In addition, the genus and species identification (i.e. demonstrate that the MSB is an *E. coli*) was established according to 9CFR 113.100 using metabolic markers specific of *E. coli*. The Gram staining results obtained for purity testing also contributed to the identification of the MSB as *E. coli*.

The MSB was found to be pure when tested in accordance to the procedures described above.

B.2. Lack of Endogenous Extraneous Plasmid(s) and Bacteriophage(s)
Most plasmids carry antibiotic resistance genes and are stable in the bacterial host only in the presence of the appropriate antibiotic pressure. The plasmid codes for the resistance gene. The plasmid containing the gene is referred to in this document as.

The absence of contamination by an extraneous plasmid also carrying the resistance was demonstrated by a plasmid identity test. This test, as per 9CFR 113.100, was based on an enzymatic restriction map of purified plasmid. The restriction profile matched the theoretical profile established from the known plasmid nucleotide sequence. The absence of extraneous DNA fragments after enzymatic digestion proves the absence of a contaminating plasmid.
Specific receptors are required for bacteriophages to penetrate into bacteria. No receptor for bacteriophages has been described on the strain. To the best of Merial’s knowledge, there is no known bacteriophage susceptibility associated with the strain.

C. Molecular Characterization

C.1. Conventional (non-engineered) Bacterial Replication Host
This section is not applicable to the strain.

C.2. Genetically Engineered Bacterial Replication Host

C.2.a. Parental Strain
The cells were acquired commercially. The complete lineage of this strain leading to is published in the scientific literature:

C.2.b. Summary of Construction Process

C.2.b.i. Cloning Strategies, Procedures, and Vectors
The strain of E. coli was constructed from

The strain was constructed as follows:
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was constructed by introduction of A

C.2.b.ii. Deleted Genes

C.2.b.ii.1. Site and Function of Deleted Gene(s) (if known)

genotype is A A

The following genes were deleted:
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C.2.b.ii.2. Consequences of Gene Deletion(s)
The consequences of the identified gene deletions are as follows:
- Deletion of [redacted] improves yield and quality of the plasmid preparations.
- Deletion of [redacted] decreases homologous recombination to 0.1% of normal, thereby ensuring plasmid insert stability.
- Deletion of [redacted] mutation prevents cleavage by an endogenous endonuclease system.
- Deletion of [redacted] renders the host strain more transformation efficient.
- Consequences of [redacted] are less well understood.

C.2.b.ii.3. Inserted Gene(s)
a) [redacted]: allows the host to grow without supplemental [redacted].

D. Known Bacteriophage Susceptibility of Strain, (if applicable)
Specific receptors are required in order for bacteriophages to penetrate into bacteria. No receptor for bacteriophages has been described on the [redacted] strain. To the best of Merial's knowledge, there is no known bacteriophage susceptibility associated with the [redacted] strain.

E. Antibiogram: Endogenous Chromosomal Antibiotic Resistance Factors Allowed
No known endogenous chromosomal antibiotic resistance is associated to the strain.

F. Previous Use of Bacterial Replication Host
The [redacted] strain is a supercompetent bacteria commercialized for in vitro use in molecular biology laboratories.

III. CONSTRUCTION AND CHARACTERIZATION OF THE PLASMID CONSTRUCT

A. Parental Plasmid Source

A.1. Source(s) of Parental Sequences
The plasmid backbone [redacted] was constructed [redacted] using the following components: a eukaryotic promoter and enhancer, [redacted], a [redacted] to facilitate cloning of a variety of DNA fragments, donor and acceptor splice sites and a sequence [redacted], the origin of replication and a gene conferring resistance. With the exception of the [redacted] resistance gene, all other gene segments were amplified by polymerase chain reaction (PCR) and sourced from [redacted].
A.2. Phenotype Conferred on Bacterial Replication Host

The plasmid confers a specific phenotype on the bacterial replication host.

A.3. Known Properties of Parental Plasmid

The plasmid is a eukaryotic expression vector, which has been optimized for the expression of a transgene following direct injection into skeletal muscles.

In order to increase the transgene expression, optimal promoter-enhancer, intron and transcriptional termination signals have been engineered into this plasmid.

A.4. Known Hosts for Parental Plasmid

E. coli bacteria.

A.5. Replication and Expression Control Elements

A.5.a. Prokaryotic

A.5.a.i. Origin of Replication

The plasmid uses the plasmid origin of replication. This origin of replication is shared with numerous classical plasmids used in molecular biology laboratories.

A.5.a.ii. Bacterial Promoter(s), Enhancers, and Other Control Elements

The only prokaryotic transcription activity associated to the gene is related to the phenotype in E. coli. Since it is inactive in eukaryotic cells (i.e., in the cells of the vaccinated animal), there is no expression of the gene in the vaccinated animal.

A.5.b. Eukaryotic (mammalian/avian/target host species) Control Elements

A.5.b.i. Promoters

The expression of the transgene in the vaccinated animal is driven by the strong sequence.

In order to increase the transfer of nuclear transcripts into the cytosol, the cloned sequences and the region.
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A.5.b.ii. Enhancers
As described above, the transgene expression is under the control of the enhancer/promoter sequences.

A.6. Identified Immunomodulatory Sequences and CpG Motifs
(immunostimulatory and immunosuppressive)
Based on mouse data, specific CpG sequences within the plasmid could modulate the immune system and modify subsequent immune responses. Based on data obtained in other species, it is likely that the actual immunomodulatory sequences are species-specific (i.e. sequences identified in the mouse are not active in larger animals). Since sequences active in vivo in cats and dogs have not yet been identified, the effect of Immunomodulatory sequences in those species could have an impact in the target species of the contemplated DNA vaccine is not available.

A.7. Diagrammatic Representation of Parental Plasmid with Restriction Map

B. Inserted Sequences

B.1. Summary of Construction Process

B.1.a. Step-by-Step Construction Events
The plasmid was cloned from a melanoma cell line in the molecular biology laboratory. cDNA clones were isolated from a melanoma cell line derived from the melanoma cell line. Recombinant cDNA clones were screened and reactive clones were plaque purified. The cDNA inserts were subcloned into the plasmid vector and designated cDNA.

detected mRNA transcripts in Northern blot analysis of a panel of melanoma cell lines. An fragment containing the cDNA was inserted into the site in of . Orientation was confirmed by bi-directional sequencing.

B.1.b. Parental and Intermediate (cloning) Vectors
The parental plasmid is as described in section III.A.1. The plasmid was used as an intermediate vector to express the candidate cDNA insert containing the gene as described in Section III.B.1.a.. The was originated by , and supplied by.

The plasmid was the source of the gene as described in Section III.A.1.
B.2. Antigenic Gene(s)

B.2.a. Source and Sequence(s)

The cDNA sequence was obtained from the cDNA clone isolated from a cell line derived from the melanoma cell line.

B.2.b. Control Sequence(s) (promoter and/or enhancer)

There are no known promoters or enhancers in the antigenic gene sequence.

B.2.c. Known Biologic Properties in Parental Strain/Species

The production of melanin is generally restricted to melanocytic cells containing melanosomes. These cells occur normally and are most commonly found in dermal and mucosal tissues. Melanosomes are abundant in typical melanoma cancer cells.

B.3. Immunomodulatory Gene(s) and Sequences:

Data is not available to confirm the presence of immunomodulatory sequences in the gene.
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B.4. Selection Sequences (Kanamycin and Neomycin acceptable, other antibiotic resistance factors may be considered)
The selection sequence is present.

B.5. Diagrammatic Representation of Target Protein

IV. PLASMID CONSTRUCT (Product)

A. Complete Characterization

A.1. Sequence

A.1.a. Antigenic Sequences

A.1.a.i. If Plasmid Does Not Encode Replicating Virus, Percentage Complete Viral Genome Encoded
The plasmid does not encode a virus. It encodes the entire protein as described in section III.B.5.

A.1.b. Ability of Plasmid to Recombine With Wild-Type Agent
The plasmid does not encode an infectious agent. The probability of a DNA molecule to integrate into the chromosome of muscle cells after intramuscular injection is at least 3000x lower than the natural mutation rate in an organism (Martin et al. Human gene Therapy, 10; 759-68, 1999; Ledwith et al., Intervirology, 43; 258-72, 2000).

A.1.b.i. Consequences of Recombinant Event
Consequences of a recombinant event involving the chromosomal DNA are subject to speculation. Problems associated with such events have not been reported, likely due to the extremely low probability of occurrence. Safety evaluations of injection sites have been performed. Plasmid DNA was found to persist in the muscle at the site of injection; however, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology was observed in the injected muscles (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000).

A.1.c. Immunomodulator Sequences/Genes
Immunomodulator genes are not known to be present in . Immunomodulator sequences (i.e., CpG) active in vivo in cats and dogs have not yet been identified. The effect of immunomodulatory sequences in that could have an impact in the target species of the contemplated DNA vaccine is not known.

A.1.d. Other Immunologically Reactive Sequences
Other immunologically reactive sequences in have not been recognized.
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A.1.e. Transcription Binding and Termination Sequences
The protein open reading frame is initiated by an codon and terminated by the codon. The gene is under the control of an upstream promoter and precedes a downstream region coding for a termination sequence.

A.1.f. Polyadenylation Sites
The stabilization of the transgene mRNA is ensured by the presence of the bovine growth hormone termination and polyadenylation signals downstream of the transgene coding sequence.

A.1.g. Anchor or Secretory Sequences
is protein bound in the melanosome membrane near the C terminus with the majority of the protein (N terminus) contained within the melanosome.

A.1.h. Sequences With Known Oncogenic Potential/Target Host Cell Genome Homology
The does not contain known sequences of oncogenic potential and does not contain sequences of origin.

A.2. Diagrammatic Representation of Plasmid DNA (Product size and restriction map)

A.3. Host Range for Replication
can only replicate in E. coli. It cannot replicate within the vaccinated animal. Potential replication in other bacteria is unknown.

A.4. Host Range for Expression
Expression of the can only occur in the bacteria host because it is under the control of a bacterial promoter.

Expression of the protein cannot occur in the bacterial host (because it is under the control of an eukaryotic promoter). It will however, occur within the cells of the vaccinated animal into which the plasmid has been administered.

B. Purity of Plasmid DNA (Product)

B.1. 9 CFR 113.26
Purified plasmid will be tested for detection of viable bacteria and fungi in accordance with 9 CFR 113.26.
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B.2. Contaminants

B.2.a. Endotoxin Range
It is proposed that the amount of endotoxin present in the final product should not exceed [redacted].

B.2.b. Cellular Components

B.2.b.i. Extraneous DNA
The presence of chromosomal DNA in the vaccine should not be a substantive issue because this vaccine is produced in an *E. coli* strain derived from the non-pathogenic [redacted] strain [redacted]. Furthermore, there is no data suggesting that residual chromosomal DNA is a safety issue in any of the existing, conventional USDA-licensed vaccines.

In order to support the consistency of its production process and demonstrate that the plasmid present in the melanoma DNA vaccine is well defined, Merial will demonstrate that only very small amounts of chromosomal DNA are present in the vaccine.

B.2.b.ii. RNA
Same comments as for *E.coli* chromosomal DNA impurities, bacterial RNA is not expected to impact the DNA vaccine safety profile. In order to support the consistency of its production process and demonstrate that the plasmid present in the melanoma DNA vaccine is well defined, Merial will demonstrate that only very small amounts of bacterial RNA are present in the vaccine.

B.2.b.iii. Debris (organelles, proteins, lipids, etc.)
The presence of bacterial proteins in the vaccine is not considered to impact the safety of the DNA vaccine for the following reasons:
- the vaccine will be produced on [redacted] medium [redacted]
- the only potential proteins in the vaccine will be derived from the *E. coli* host cell (i.e., derived from [redacted] bacteria), which does not contain known allergens
- the production process will not contain [redacted],
- no [redacted] will be used in the vaccine.

Based on the above, the likelihood of adverse reactions in the field should be minimal and protein contamination should have no impact on the vaccine safety.
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In order to support the consistency of its production process and demonstrate that the plasmid present in the Melanoma DNA vaccine is well defined, Merial will demonstrate that

B.3. Structural Integrity (circular plasmid)
As of today the importance of the ratio of supercoiled (sc) versus open circular (oc) plasmid isoforms on the efficacy of DNA vaccination is complex and only partially understood (Bergan et al. 2000; Middaugh et al., 1998; Evans et al., 2000).

In order to support the consistency of its production process and demonstrate that the plasmid present in the melanoma DNA vaccine is well defined, Merial will demonstrate that the plasmid ccc/oc in the final product meets or exceeds a minimal threshold of

C. Stability

C.1. Genetic
The nucleotide sequence of the plasmid will be confirmed (on both strands) on the and on thereby demonstrating genetic stability.

C.2. Phenotypic
The phenotypic stability of the Melanoma DNA vaccine will be demonstrated via . As the plasmid is the active ingredient in the melanoma DNA vaccine, and as it will be a highly purified, well-defined plasmid, Merial proposes that the DNA vaccine can be assessed based

The intended parameters are:
D. Undesirable Immune Reaction(s) Based on Immune Reaction Targeted

D.1. Th1: Evidence of Autoimmunity Induction Potential

D.1.a. Autoimmunity in General
The development of melanoma occurs due to failure of the immune system to recognize proteins expressed on the malignant cells as foreign to the host animal. Induction of an immune response against one or more of these proteins is desirable in order to induce . This may involve induction of an immune response against a protein otherwise considered to be “self” by the host animal (an auto-immune response). It this case, it is desirable to target a protein that does not result in a major adverse systemic or physiologic impact on the host.

has approximately 85% homology with . The difference between the two proteins is sufficient to induce an immune response in the dog against protein, but similar enough that on melanocytic cells will be targeted. In this respect, an auto-immune condition is induced to the benefit of the host. The side effect hypothesized would involve depigmentation of normal melanin laden tissues (refer to section III.B.2.c).

Identified as an issue, melanoma, this phenomena was not administered the plasmid to approximately 85 dogs. Two of these dogs exhibited mild depigmentation (ear and chin); otherwise, significant toxicities were not observed. If depigmentation does develop, the risk associated with this side effect is minimal in consideration of the short survival times and high mortality from melanoma.

D.1.b. Anti-DNA Antibodies
The possibility to induce anti-DNA antibodies through DNA vaccination has been presented as a theoretical critical issue in the early days of the technology development. Although no evidence of anti-DNA antibodies has been obtained in most laboratory animals, a single publication reports a slight increase of anti-DNA antibody titers when multiple injections of plasmid DNA were performed on lupus-prone mice (Gilkeson et al, J. Clin. Invest., 95; 1398-1402, 1995). However, these limited antibody titers had no impact on the onset and/or on the severity of the disease in this mouse model. We consider that this observation is anecdotal and likely to be specifically related to the mouse model used in this published study.

In human medicine, anti-DNA antibodies have been monitored over the course of numerous clinical trials but no clear seroconversion has been observed.
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As such, Merial (in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002) considers that the induction of anti-DNA antibodies is a theoretical issue, without practical implications for the development of a melanoma vaccine.

D.1.c. Tolerance
Another safety issue related to auto-immunity, which has been raised for DNA vaccines, is tolerance. It has been hypothetized that due to the low but long term expression of the transgene, DNA vaccination could induce tolerance more easily than other vaccine technologies.

Currently, there is a single publication reporting the induction of tolerance with a specific malaria antigen in newborn mice (Mor et al., J Clin. Invest; 98, 2700-5, 1997). Since this concern has not been confirmed in any other models, this appears to be a theoretical issue with no practical impact for the melanoma vaccine development. This position is in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002.

In the case of melanoma, we consider development of tolerance to not be an issue due to the preponderance of target antigen in melanocytic cells associated with the tumor.

D.2. Th2: Allergic Reaction Induction Potential (activation of eosinophils and mast cells)
Although activation of eosinophils and mast cells following DNA vaccination has not been specifically assessed, there is no specific data to support the induction of allergic reactions following DNA vaccination in dogs.

Furthermore, any risk of protein related adverse effects (i.e. anaphylactic-like reactions) associated with the melanoma DNA vaccine is considered to be very low for the following reasons:
- the vaccine will be produced on medium
- the only proteins in the vaccine will be derived from the E. Coli host cells that contain no known allergens
- the production process will be free of
- the product will be a vaccine.

V. ADMINISTRATION

A. Proposed Mode of Administration

A.1. Characterization of Live Bacterial Carrier (if applicable)
Not applicable.

A.2. Injection Routes of Administration
It is proposed to aseptically administer the melanoma DNA vaccine via the transdermal route using a needle free device.
B. Target Host Species

B.1. Minimum Age and Indications for Use
This vaccine is recommended for the vaccination of dogs diagnosed with melanoma. There is no minimum age targeted because these cancers tend to appear in mature animals and are not expected in neonatal or juvenile animals.

B.2. Target Site of Administration
The vaccine is to be injected aseptically into the proximal half of the medial thigh caudal to the femur. This site of administration will target primary deposition into intramuscular tissues.

B.3. Shed and Spread of Plasmid

B.3.a. In Target Host Species Tissues
Once administered (parenterally) into vaccinated animals, plasmids have been shown to be transiently distributed throughout the body (likely through blood transport and not through specific uptake and tissue retention). They are subsequently lost from most tissues within several days, probably by degradation (endonucleases). However, around the injection site (in the corresponding lymph node and in muscular tissues), plasmid DNA can generally still be amplified by PCR after several weeks (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000).

Importantly, even though the plasmid DNA was found to persist in the muscle at the site of injection, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology indicative of an auto-immune disease was observed in the injected muscles. After a period of weeks to a few months, the plasmid will be cleared from the injection site area.

Distribution of plasmids to gonads has been inconsistent between studies. Although a transient migration into gonads cannot be ruled out, there is no substantive evidence to support any significant targeting and/or persistence in these organs.

B.3.b. In Environment
According to the literature, the risk of dissemination of plasmids in the environment from the vaccinated animal is exceedingly low because the plasmid cannot replicate within the vaccinated animal.

Furthermore, the accidental release of plasmids in nature should have no adverse effect because the risk that plasmids could be taken up by soil bacteria is very remote. Should this happen, the plasmid will not be maintained in the absence of [blank]. As this [blank] is not used in agricultural applications, the risk of having a plasmid disseminated by bacteria is exceedingly low. Minute amounts of plasmids, which could be accidentally ingested by animals will be degraded in their stomach and be of no consequence.
B.4. Duration of Detectable Expression
Long term expression (at least 1 month) of the transgene has been observed in the muscles of mice vaccinated intramuscularly with DNA vaccines (Hartikka et al, Human Gene Therapy, 7, 1205-17, 1996).

It is likely that the duration of detectable transgene expression is correlated to the persistence of plasmids in tissues. However, there are indications in the literature that the persistence/duration of expression in muscles is not directly related to the duration of immunity in DNA vaccinated animals (Hassett et al. J. Virol. 74: 2520-27, 2000).

Of note, plasmid DNA could be detected for more than 1 year in specific studies, as reported by Wolff et al. (Hum. Mol. Genet., 1; 363-369, 1992). The difference in the detectability of plasmid DNA across various studies using intramuscular injections may be due to the co-administration of chemicals that may alter plasmid biodistribution, persistence and expression.

B.5. Duration of Immunity
As discussed above, there is evidence that duration of immunity is not related to the persistence of plasmid in muscle. However, long lasting immunity (one year and more) has been described in different models of DNA vaccination.

Recently, the protection of monkeys 1 year after a single immunization with a rabies DNA vaccine has been reported, specifically demonstrating that DNA vaccine has the potential to trigger long-lasting immunity against rabies (Lodmell et al, Vaccine, 20; 838-44, 2002). Interestingly, since DNA vaccines trigger strong cell-mediated responses, the duration of immunity is frequently unrelated to the persistence of antibody titers.

B.6. Recommendations for Breeding Animals
B.6.a. Vertical Transfer to Germ Cells
Since, distribution to gonads of plasmids administered intramuscularly has been inconsistent between studies, the risk of plasmid reaching (and persisting in) gonads following intramuscular administration is very low. Accordingly, the risk of plasmid transfecting specific cells within the gonads is even lower and is likely to have no practical impact.

Canine melanoma patients usually represent the older segment of the pet population. These dogs are generally past prime breeding age. This further reduces any risk associated with exposure of germ cells to the plasmid DNA.

B.6.b. Vertical Transfer to Embryonic/Fetal Tissues or Reproductive Tissues
The risk of a plasmid becoming integrated into embryonic/fetal tissues or reproductive tissues has been theorized, but is believed to be without practical impact, as only a very small number of plasmid molecules enter into cells post injection.
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Furthermore, the probability of a DNA molecule to integrate into the chromosome of muscle cells after intramuscular injection is even lower [at least 3000x lower than the natural mutation rate in an organism [(Martin et al. Human gene Therapy, 10; 759-68, 1999; Ledwith et al., Intervirology, 43; 258-72, 2000)] The actual risk of integration into fetal tissues or reproductive tissues is believed to be exceedingly low.

Some experimental data indicates that, occasionally, when the plasmid was detected in gonads, it dissipated rapidly and was always extrachromosomal, confirming a low risk of germline transmission (Manam et al., Intervirology, 43; 273-281, 2000).

DNA vaccination in utero has been recently reported in the literature (Gerds et al., J. Immunol., 168; 1877-85, 2002). However, since this experiment is based on the administration of the plasmid into the oral cavity of lamb fetus during surgery, it is unrelated to the transmission of plasmid from a pregnant animal to its offspring and is not relevant to the issue of plasmid vertical transfer.

VI. IN VITRO EXPRESSION IN CELL LINE
(Potency Testing)

A. Characterization of Expression Cell Line
The qualitative expression assay for will be performed by an assay following transient transfection of cells. Since the cell line will only be used for QC there is no specific requirements as per 9CFR.

B. Methods and Protocols for Expression Characterization
A functionality assay is required to demonstrate that the plasmid has the potential to express . An IVGE assay will be established using an label following transient transfection of a cell line. Transient transfection of will be achieved using a reagent to facilitate the uptake of the DNA into tissue culture.

Following a incubation period, the recombinant will be labeled using a and detected by .

C. Stability and Duration of Master Seed Expression
The stability of the MSB will be established by a sequencing of the at both the and levels. The absence of mutations will demonstrate the stability of the seed lot system.
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The MSB will be stored frozen in the presence of a cryo-protectant. Under these conditions, the MSB is expected to remain viable. It is proposed to test its viability by [REDACTED].

D. Target Copy Number of Plasmid
It is proposed to determine the number of plasmid molecules per bacteria of the MSB by quantification of the plasmid after extraction. The extraction will be performed [REDACTED]. Quantification of plasmid will be derived from the quantification of [REDACTED].

This quantification technique is plasmid-specific and will not be biased by the presence of bacterial chromosomal DNA. The plasmid copy number will be calculated based on the plasmid molecular weight and on bacteria numeration.

VII. IN VIVO EXPRESSION (target host species and laboratory animal model)

A. Characterization of Expression

A.1. Localization of Expression Protein (target host cell membrane, excreted, etc.)
The [REDACTED] protein expressed by [REDACTED] is a membrane-anchored antigen. It is expected to remain associated to the membrane of the cell in which in vivo expression occurs.

A.2. Methods and Protocols for Expression Characterization
The in vivo expression of the [REDACTED] protein will be characterized indirectly by its ability to induce (1) [REDACTED] and (2) [REDACTED].

A.3. Range of Time Plasmid Expressed and Consequences in Target Host Species
As previously discussed in Section V.B.4., the actual duration of plasmid expression in vivo is poorly understood and may vary greatly depending on the tissue that is analyzed. Furthermore, as also previously discussed, the duration of plasmid expression is likely to be only loosely related to the duration of immunity.
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A.4. Shed and Spread of Expressed Protein

It is expected that the protein will remain associated with the membrane of the cell in which expression occurs. No active shedding of the antigen is expected. Passive spreading related to the lysis of transfected cells cannot be ruled out, but this is unlikely, as transfected cells will probably evolve towards apoptosis rather than toward lysis. It is anticipated that most of the protein will eventually be degraded within apoptotic bodies.

B. Characterization of Immunological Response

B.1. Targeted Response

B.1.a. Methods and Protocols for Assessment

DNA vaccination will trigger a broad immune response including both humoral (antibodies) and cellular components. Specific antibody can be measured.

Specific techniques to assess cell-mediated immunity in dogs include.

The therapeutic benefit provided by the vaccine will be confirmed in animal trials with dogs diagnosed with melanoma.

B.1.b. Onset and Duration of Immunity

Onset of immunity induced by DNA vaccination is poorly understood. Although the induction of specific antibodies (by DNA vaccines in general) may be slower than with conventional inactivated-adjuvanted vaccine, there are indications, in the literature, that cell-based responses could be in place even in the absence of antibody responses (Siegrist et al. Eur. J. Immunol. 28: 4138-48, 1998).

B.2. Other Beneficial Immune Responses

None are anticipated.

B.3. Undesirable Immune Responses

No specific undesirable immune response is expected, as per Section IV.D.
VIII. RISK ASSESSMENT

Risk assessment may be defined as the process of identifying the likelihood of an adverse event occurring and the consequences if that adverse event occurs. Adverse events are defined as safety hazards to animals, public health, or the environment. A safety hazard is defined as a danger, risk, or peril; absence of predictability associated with an event; or an expected or unpredicted event.

A. Procedure
The risk assessment is conducted based on safety characteristics of the vaccine. The safety characteristics are based on empirical data and established scientific facts. The completion of a risk assessment requires that the vaccine microorganism is properly characterized. This information is provided in Section III of this document.

B. Hazard Identification
Hazard identification consists of identifying all possible adverse events related to animal safety, public health safety, and environmental safety relative to the recommended use of the DNA vaccine.

B.1. Animal Safety

B.1.a. Summary
The DNA vaccine has been administered to dogs. No adverse events were reported from dogs in this study at any dose level. has administered the plasmid to approximately 85 dogs with melanoma. No significant toxicities were observed in these dogs; however, two dogs exhibited mild depigmentation (ear and chin).

It should be noted that the target population is adult dogs because cancer is not considered an issue in neonatal and juvenile animals. The comments in this section are made to address specific issues, which have been associated with DNA vaccination.

B.1.b. Target Animal Safety

B.1.b.1 Vaccination

B.1.b.1.a. Autoimmunity in General
The development of melanoma occurs due to failure of the immune system to recognize proteins expressed on the malignant cells as foreign to the host animal. Induction of an immune response against one or more of these proteins is desirable in order to induce stable disease or initiate tumor regression. This may involve induction of an immune response against a protein otherwise considered to be “self” by the host animal (an auto-immune response).
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It this case, it is desirable to target a protein that does not result in a major adverse systemic or physiologic impact on the host.

has approximately 85% homology with . The difference between the two proteins is sufficient to induce an immune response in the dog against protein, but similar enough that on cells will be targeted. In this respect, an auto-immune condition is induced to the benefit of the host. The side effect hypothesized would involve depigmentation of normal melanin laden tissues (refer to section III.B.2.c). In a trial with the vaccine in dogs with melanoma, this phenomena was not identified as an issue. has administered the plasmid to approximately 85 dogs with melanoma. No significant toxicities were observed in these dogs; however, two dogs exhibited mild depigmentation (ear and chin). If depigmentation does develop, the risk associated with this side effect is minimal in consideration of the short survival times and high mortality from melanoma.

B.1.b.1.b. Anti-DNA Antibodies
The possibility to induce anti-DNA antibodies through DNA vaccination was presented as a theoretical critical issue in the early days of the technology development. Although no evidence of anti-DNA antibodies has been obtained in most laboratory animals, a single publication reports a slight increase of anti-DNA antibody titers when multiple injections of plasmid DNA were performed on lupus-prone mice (Gilkeson et al, J. Clin. Invest., 95; 1398-1402, 1995). However, these limited antibody titers had no impact on the onset and/or on the severity of the disease in this mouse model. We consider that this observation is anecdotal and likely to be specifically related to the mouse model used in this published study.

In human medicine, anti-DNA antibodies have been monitored over the course of numerous clinical trials but no clear seroconversion has been observed. 

As such, Merial (in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSAC meeting held at NIBSC UK on June 21, 2002) considers that the induction of anti-DNA antibodies is a theoretical issue, without practical implications for the development of a canine melanoma vaccine.

B.1.b.1.c. Tolerance
Another safety issue related to autoimmunity, which has been raised for DNA vaccines, is tolerance. It has been hypothesized that due to the low but long term expression of the transgene, DNA vaccination could induce tolerance more easily than other vaccine technologies.
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Currently, there is a single publication reporting the induction of tolerance with a specific malaria antigen in newborn mice (Mor et al., J Clin. Invest; 98, 2700-5, 1997). Since this concern has not been confirmed in any other models, this appears to be a theoretical issue with no practical impact on the melanoma vaccine development. This position is in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002.

In the case of melanoma, we consider development of tolerance to not be an issue due to the preponderance of target antigen in melanocytic cells associated with the tumor.

B.1.b.1.d. Th2: Allergic Reaction Induction Potential (activation of eosinophils and mast cells)

Although activation of eosinophils and mast cells following DNA vaccination has not been specifically assessed, there is no specific data to support the induction of allergic reactions following DNA vaccination in dogs.

Furthermore, any risk of protein related adverse effects (i.e. anaphylactic-like reactions) associated to the melanoma DNA vaccine is considered to be very low for the following reasons:

- the vaccine will be produced on medium
- the only proteins in the vaccine will be derived from the E. Coli host cells that contain no known allergens
- the production process will be free of 
- the product will be a vaccine.

B.1.b.1.e. Tumorigenesis by insertional mutagenesis

Consequences of a recombinant event involving the chromosomal DNA are subject to speculation. Problems associated with such events have not been reported, likely due to the extremely low probability of occurrence. Safety evaluations of injection sites have been performed. Plasmid DNA was found to persist in the muscle at the site of injection; however, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology was observed in the injected muscles (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000). In addition, the does not contain known sequences of oncogenic potential and does not contain sequences of origin.

B.1.b.1.f. Risk for specific classes of target species

The melanoma DNA vaccine is unique compared to conventional vaccines in that the targeted class is dogs with melanoma. These animals generally represent older individuals in the canine population. Proulx, et.al., reported the median age for 140 dogs diagnosed with melanoma to be 11 years with a range of 2-19 years, (Veterinary Radiology & Ultrasound, 44(3):352-359, 2003). Therefore, neonatal and juvenile dogs are not the target class for this vaccine.
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With regard to vaccination of breeding animals, the risk of a plasmid becoming integrated into embryonic/fetal tissues or reproductive tissues has been theorized, but is believed to be without practical impact, as only a very small number of plasmid molecules enter into cells post injection. Furthermore, the probability of a DNA molecule to integrate into the chromosome of muscle cells after intramuscular injection is even lower [at least 3000x lower than the natural mutation rate in an organism [(Martin et al. Human gene Therapy, 10; 759-68, 1999; Ledwith et al., Intervirology, 43; 258-72, 2000)], and the actual risk of integration into fetal tissues or reproductive tissues is believed to be exceedingly low.

Some experimental data indicates that, occasionally, when the plasmid was detected in gonads, it dissipated rapidly and was always extrachromosomal, confirming a low risk of germline transmission (Manam et al, Intervirology, 43; 273-281, 2000).

DNA vaccination in utero has been recently reported in the literature (Gerds et al., J. Immunol., 168; 1877-85, 2002). However, since this experiment is based on the administration of the plasmid into the oral cavity of lamb fetus during surgery, it is unrelated to the transmission of plasmid from a pregnant animal to its offspring and is not relevant to the issue of plasmid vertical transfer.

B.1.b.2. Vaccination/Challenge

This vaccine is utilized as a biotherapeutic in animals that already exhibit the target disease; there is no challenge. The therapeutic benefit provided by the vaccine will be confirmed in animal trials with dogs diagnosed with melanoma.

B.1.b.3. Reversion to Virulence

Since the melanoma DNA vaccine is not infectious and cannot replicate in eukaryotic cells, Merial is of the opinion that reversion to virulence is not a relevant issue for the melanoma DNA vaccine.

B.1.b.4. Purity Testing

B.1.b.4.a. MSB Purity Testing

The purity of the host bacteria in the MSB has been established according to 9CFR 113.27(d).

The MSB was found to be pure when tested in accordance with the procedures described above.

B.1.b.4.b. Vaccine Testing

The melanoma DNA vaccine will be released based on highly purified and well-characterized plasmid to ensure consistency of vaccine batch quality.
### B.1.b.5. Identity Testing

Identity of the plasmid in the DNA vaccine is based upon...

For the MSB host cell, the genus and species identification (i.e. demonstrate that the MSB is based on *E. coli*) has been established according to 9CFR 113.100 using metabolic markers specific of *E. coli*. The Gram staining results obtained for purity testing also contribute to the identification of the MSB as *E. coli*.

### B.1.b.6. Effect of Gene Manipulation on Pathogenicity

Since the melanoma DNA vaccine plasmid is not infectious and not pathogenic, Merial is of the opinion that the analysis of the effect of gene manipulation on pathogenicity is not a relevant issue for the melanoma DNA vaccine.

### B.1.b.7. Genetic Stability

The nucleotide sequence of the plasmid will be confirmed (on both strands) on the and on thereby demonstrating genetic stability.
B.1.b.8. Phenotypic Stability

The phenotypic stability of the DNA vaccine will be demonstrated via [redacted]. As the plasmid is the active ingredient in the melanoma DNA vaccine, and as it will be a highly purified, well-defined plasmid, Merial proposes that the DNA vaccine can be assessed based on [redacted]. The intended parameters are:

B.1.b.9. Alteration in Tissue Tropism

Since the melanoma DNA vaccine is not infectious and cannot replicate in eukaryotic cells, Merial is of the opinion that alteration of tissue tropism is not a relevant issue for a melanoma DNA vaccine.

B.1.b.10. Effect of Overdosing

Merial will confirm safety in the target species as per 9 CFR 113.40 (2 dogs @10x dose).

B.1.c. Non-Target Animal Safety

B.1.c.1. Susceptible Non-Target Animals

Since the melanoma DNA vaccine is not infectious, Merial is of the opinion that susceptibility of non-target species is not a relevant issue for the melanoma DNA vaccine.

B.1.c.2. Virulence in Non-Target Animals

Since the melanoma DNA vaccine is not infectious, Merial is of the opinion that virulence in non-target species is not a relevant issue for the melanoma DNA vaccine.

B.1.c.3. Possible Outcome of Non-Target Animal Exposure

Since the melanoma DNA vaccine is not infectious, and does not replicate in the target species the probability of unexpected spread to non-target species is exceedingly low. Exposure of non-target species (including humans) is not an anticipated. However accidental exposure of non-target species may result in the development of an immune response to the protein but no clinical disease or spread would be expected.
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In addition, Merial (in agreement with the 2002 revised WHO Guidelines 
on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK 
on June 21, 2002) considers that the induction of anti-DNA antibodies is a 
thoretical issue, without practical implications for the development of the 
canine melanoma vaccine.

B.2. Public Health and Safety

B.2.a. Summary
Public health concerns associated with the use of the melanoma DNA 
vaccine are not expected.

B.2.b. Probability of Human Exposure
The probability of human exposure is low. The vaccine containing the 
plasmid is for animal use only (under the prescription and 
supervision of qualified veterinary personnel). Administration of the 
injection by a qualified veterinarian further reduces the likelihood of 
accidental spread. The most likely exposure is through accidental self-
injection and therefore limited to personnel administering the vaccine 
(veterinarian or veterinary technical personnel).

B.2.c. Pathogenicity of the Parent Microorganisms in Humans
The parental plasmid is not pathogenic in humans because it is not 
infectious and it contains no sequence known to be involved in human 
pathology.

B.2.d. Virulence of the Vaccine Microorganism in Humans
Since the melanoma DNA vaccine is not infectious and it contains no 
sequence known to be involved in human pathology, Merial is of the 
opinion that the virulence of the vaccine microorganism in humans is not 
a relevant issue for this vaccine.

B.2.e. Possible Outcome of Human Exposure
The potential outcome of human exposure is the development of an 
immune response to the protein which could theoretically lead 
to depigmentation of skin and hair. has enrolled 18 human patients with melanoma in a 
FDA approved trial. These patients received plasmid DNA vaccine 
expressing either . No significant adverse events have been reported for these patients

B.3. Environmental Safety

B.3.a Summary
According to the literature, the risk of dissemination of plasmids in the 
environment from the vaccinated animal is exceedingly low because the 
plasmid cannot replicate within the vaccinated animal. Furthermore, the 
accidental release of plasmids in nature should have no adverse effect 
because the risk that plasmids could be taken up by soil bacteria is very 
remote. Should this happen, the plasmid will not be maintained in the 
absence of.
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As this is not used in agricultural applications, the risk of having a plasmid disseminated by bacteria is exceedingly low. Minute amounts of plasmids, which could be accidentally ingested by animals will be degraded in their stomach and be of no consequence.

B.3.b. Environmental Distribution
The existence and extent of an environmental distribution of the melanoma DNA vaccine has not been established or reported.

B.3.c. Shed/Spread Capabilities
Merial is of the opinion that the melanoma DNA vaccine is associated with an exceedingly low risk of environmental release for the following reasons:

- The vaccine is to be administered by a trained veterinarian. The small dose volume will minimize any risk of spill over during the injection. Visual observation of the procedure will guarantee that no vaccine is left on the skin of animal.
- The vaccine cannot be amplified in the vaccinated animal because it is based on a highly purified plasmid that has no eukaryotic origin of replication.
- Amount of plasmid per dose is very low.
- Once administered (parenterally) into vaccinated animals, plasmids have been shown to be transiently distributed throughout the body. They are subsequently lost from most tissues within several days, but may persist around the injection site (in the corresponding lymph node and in muscular tissues), for several weeks. Importantly, even though the plasmid DNA was found to persist in the muscle at the site of injection, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology indicative of an auto-immune disease was observed in the injected muscles. After a period of weeks to a few months, the plasmid will be cleared from the injection site area (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al Intervirology, 43; 273-281, 2000).

Based on the above Merial is of the opinion that the risk of plasmid shed/spread from a vaccinated animal into the environment is exceedingly small.

In the event of an accidental spill, there is no risk for target or non-target animals because the plasmid is (a) not infectious, (b) unstable in the environment and (c) extremely inefficient at penetrating into an organism after mucosal (e.g., oral) contact. Minute amounts that might be ingested by animals would be degraded in the stomach and be of no consequence.

Horizontal gene transmission or recombination events would require that the plasmid survive in the environment. Merial is of the opinion that this risk is exceedingly low due to the remote likelihood that the plasmid will survive in the environment (Section VIII.B.3.g).
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B.3.e. Potential for Transmission to Invertebrates
The potential for transmission to invertebrates is negligible. The plasmid [redacted] is not shed from vaccinated animals, therefore the only potential source of transmission to invertebrates would be an accidental spill. The ability of invertebrates to acquire the organism and to subsequently act as reservoirs or vectors is improbable given that the vaccine will be used in an animal hospital, the instability of plasmid in the environment, and its lack of infectivity. Collectively, this should exclude the probability of transmission to invertebrates.

B.3.f. Host/Range Specificity
Since the melanoma DNA vaccine is not infectious, Merial is of the opinion that the host/range specificity is not a relevant issue for this vaccine.

B.3.g. Survivability of the Microorganism in the Environment
Possible establishment in the environment and specifically in soil bacteria surrounding the test site is highly unlikely because:
- Plasmid cannot replicate spontaneously in the environment,
- Bacteria don't efficiently take up plasmids without a prior in vitro chemical or physical treatment.

Furthermore, should such an event occur it would be without consequence because the plasmid will not be maintained in the absence of [redacted]. As this antibiotic is not used in agricultural applications, the risk of having a plasmid disseminated by environmental bacteria is exceedingly low.

B.3.h. Physical/Chemical Factors Affecting Environmental Dispersion
Only the simultaneous presence of a competent bacteria, [redacted] and the plasmid in one location could potentially enhance the dispersion of the melanoma DNA vaccine in the environment. However, this risk is considered to be exceedingly low since:
- [redacted] is not used in agricultural applications,
- the plasmid will be administered aseptically into the muscle of target animals by trained veterinarians.

B.3.i. Ecological Concerns
Due to the exceedingly low risk of survivability in the environment, Merial is of the opinion that the melanoma DNA vaccine poses no threat to any ecological system.
C. Release Assessment

C.1. Contained release
Not applicable

C.2. Environmental release

C.2.a. Summary
The [Redacted] has been subjected to environment release as a consequence of canine and human trials. Merial has administered the [Redacted] plasmid to approximately 85 dogs with melanoma. Merial has administered the [Redacted] plasmid expressing [Redacted] to 18 human melanoma patients in a FDA approved study.

Merial anticipates environmental release will occur in association with clinical trials. An efficacy trial will enroll client owned dogs diagnosed with melanoma.

C.2.b. Location of test site
For the efficacy study, veterinary clinics will be chosen based upon availability of canine melanoma cases and veterinary oncology specialists. Participating veterinary clinics will be identified in the 9 CFR 103.3 request to conduct the study and in the final study report.

C.2.c. Characteristics of Test Site
For the efficacy trial, veterinary clinics will serve as the focal point for each site and will be considered the “test sites” for this trial. Dogs diagnosed with melanoma enrolled in the studies are expected to come from typical households, located in the area of each participating test site. These animals will remain in the custody of their owners during the course of the study. Non-target species within the areas may include any other domestic animals located at each test animal's place of residence and the wildlife population endemic to each given locale.

C.2.d. Personnel
Personnel will consist of participating veterinarians, and participating Merial personnel. Only qualified personnel (based on their previous experience, training, and education) will handle the animals during vaccine administration. A list of participating veterinarians and Merial personnel and their qualifications will be provided to APHIS. Dogs enrolled in the efficacy study will remain in the custody of their owners.
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C.2.e. Experimental Design
Efficacy: it is anticipated that dogs diagnosed with melanoma will be required for demonstration of efficacy. These dogs will receive doses of vaccine containing approximately of plasmid DNA at approximately intervals. They will be evaluated periodically. Because the vaccine is a therapeutic for dogs previously diagnosed with melanoma, this trial will not include use of a challenge organism.

A detailed trial protocol will be submitted to CVB for approval prior to the conduct of the efficacy trial.

C.2.e.1. The Number of Animals:
Efficacy: it is anticipated that dogs diagnosed with melanoma are required to demonstrate efficacy of the vaccine.

C.2.e.2. Description of Animals:
Efficacy: client owned dogs diagnosed with melanoma.

C.2.e.3. Route of Administration:
The vaccine will be administered by the route in the medial thigh region using a needle free vaccination device.

C.2.e.4. The dose:
volume doses of vaccine, each containing approximately of plasmid DNA administered at intervals.

C.2.e.5. The total amount of test material:
Efficacy: With a maximum enrollment of dogs in the efficacy trial, doses of vaccine would be required.

C.2.e.6. Frequency and duration of exposure:
Each animal will receive doses of vaccine administered at approximately intervals.

C.2.e.7. Method of disposing waste:
Empty and used vaccine vials will be returned to Merial Ltd. Athens, GA for disposal by autoclaving. The needle free device disposables will be disposed of according to guidelines used in each veterinary clinic (i.e., autoclaving or incineration). All unused vials will be returned to Merial Ltd. Athens, GA., where they will be archived until licensure.

C.2.e.8. Decontamination of test site:
All materials used in the clinic that have had direct contact with vaccine fluid (needle free disposables, trays, etc.) should be discarded, autoclaved or disinfected with a disinfecting solution.
C.2.f. Potential Escape and Dispersal in the Environment
Merial is of the opinion that the melanoma DNA vaccine is associated with an exceedingly low risk of environmental release for various reasons:

- The vaccine is to be administered by a trained veterinarian. The small dose volume will minimize any risk of spill over during the injection. Visual observation of the procedure will guarantee that no vaccine is left on the skin of animal.
- The vaccine cannot be amplified in the vaccinated animal because it is based on a highly purified plasmid that has no eukaryotic origin of replication.
- Amount of injected plasmid is very low.
- Low likelihood of Shed/spread of DNA from vaccinated animals

Based on the above Merial is of the opinion that the risk of plasmid escape and dispersal from a vaccinated animal into the environment is exceedingly small.

In the situation where plasmid accidentally spilled from the container, there is no risk for target or non-target animals because plasmid is (a) not infectious, (b) unstable in the environment and (c) extremely inefficient for penetration into an organism after mucosal (e.g., oral) contact. Minute amounts that could be ingested by animals will be degraded in the stomach and be of no consequence.

Based on the above Merial is of the opinion that the risk of plasmid escape and dispersal from an accidental spill into the environment is exceedingly small.

C.2.g. Potential for establishment in the environment:
Possible establishment in the environment and specifically in soil bacteria surrounding the test site is highly unlikely because:

- Plasmid cannot replicate spontaneously in the environment.
- Bacteria don't efficiently take up plasmids without a prior in vitro chemical or physical treatment.

Furthermore, should such an event occur it would be without consequence because the plasmid will not be maintained in the absence of selection pressure. As this antibiotic is not used in agricultural applications, the risk of having a plasmid disseminated by environmental bacteria is exceedingly low.

C.2.g.1. The biological organisms found on the test site:
There will not be intentional introduction of known animal or human pathogens into the test sites. Non-pathogenic environmental organisms may be found and pathogenic organisms typical of veterinary clinics and places of residence of domestic animal species may be found.

C.2.g.2. Nutrient status:
There is no known nutrient(s) at the test site that will allow or promote the growth of plasmid DNA.
C.2.g.3. **Physicochemical factors:**
The plasmid is very sensitive to degradation and would rapidly be destroyed in the presence of nucleases, pH variations, and/or oxidation.

C.2.g.4. **The presence of toxic chemicals and metabolites:**
Toxic chemicals and metabolites should not be generated from the administration of the test article.

C.2.h. **Monitoring**
Adverse environmental events are not anticipated given the characteristics of the vaccine already described within this document. Based upon the low risk of shed and spread of the plasmid as described previously, Merial does not believe an environmental monitoring plan is necessary.

C.2.i. **Contingency plans in case of adverse event**
No adverse events are expected. Should some vaccine be accidentally spilled into the environment, Merial recommends exposing the plasmid to a mild acid solution (pH=6), which would almost immediately destroy the plasmid structure. There is a potential risk of accidental injection of the personnel administering the vaccine, or of skin contact with droplets of vaccine material leaking either from the injection site or the vial. In case of accidental human injection, medical professionals will be notified and an assessment requested.

In the unlikely circumstance that an adverse event is observed, the principle investigator will be notified immediately. Vaccination of the animals will be suspended and previous vaccinates will be examined carefully. Moreover, Merial will collect, analyze and evaluate the information and take appropriate actions to further mitigate the situation. If the decision is made that there is risk to personnel, animals, or the environment, APHIS will be notified with respect to planned actions to mitigate the potential impact of such an adverse event.

D. **Risk Characterization**

D.1. **Likelihood ratings**
Likelihood ratings are assigned for animal safety, public safety, and environmental safety based on the following criteria:

- **Low** = An adverse event is unlikely to occur
- **Medium** = An adverse event could possibly occur
- **High** = An adverse event will most probably occur
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D.2. Consequence ratings
Consequence rating are also assigned for animal safety, public health safety, and environmental safety based on following criteria:

Low = The consequences if the adverse event occurs will not be severe (the adverse event is self limiting and would have negligible impact).

Medium = The consequences if the adverse event occurs is moderately severe (the adverse event will have an impact, but it is not permanent, and can be treated).

High = The consequences if the adverse event occurs is severe (the adverse event will have an impact, is permanent, and can not be treated).

D.3. Degree of certainty ratings
Each likelihood and consequence rating is qualified by a degree of certainty rating that is based on following criteria:

Certain = The rating is supported by direct scientific evidence.

Moderately certain = The rating is supported by indirect scientific evidence.

Uncertain = The rating is not supported by scientific evidence.

D.4. Calculating the expected risk
Numerical values have been assigned to the likelihood, consequence, and degree of certainty ratings described above (Table 3). Each numerical value rating was derived from the importance placed on the rating of each category. The assigned numerical values are weighed to place emphasis on the severity of expected risk. These values reflect the professional judgment of the applicant (Merial Ltd.).

D.5. Risk Ratings
The risk ratings are based upon the likelihood, consequence, and degree of certainty ratings and expected risk for each category, as per guidance documentation provided by APHIS (Table 4). The total of 81 rating combinations are possible; e.g., Likelihood Low Moderately Certain, Consequence Low-Moderately Certain. Each combination has been assigned a risk rating of low, medium or high. The assigned ratings were weighed to place emphasis on the severity of the risk. Again, the severity of risk reflects the professional judgement of the applicant (Merial Ltd.). The low, medium, or high risk ratings are defined for the purpose of decision making as follows:

Low = Acceptable risk-very little concerns are associated with the proposal (does not justify denying the proposal)

Medium = Unacceptable risk-moderate concerns are associated with the proposal (either identify valid mitigative procedures or deny the proposal)

High = Unacceptable risk-major concerns are associated with the proposal (deny the proposal)
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D.5.a. Risk to Animal safety

Likelihood Rating: Low (LL)
Degree of Certainty Rating: Certain (C)
Consequence Rating: Low (CL)
Degree of Certainty Rating: Certain (C)
Expected Risk Rating: 1.0000
Risk Characterization: LL.C.CL.C
Risk Rating: Low

Conclusion and discussion of risk:
The risk to animal safety associated with the proposal to conduct efficacy and safety studies in dogs with the melanoma DNA vaccine, is low. The melanoma DNA vaccine cannot replicate in eukaryotic cells. It is also (a) not infectious, (b) highly purified and well characterized, and (c) extremely inefficient for penetration into an organism after mucosal (e.g., oral) contact. Minute amounts, which could be ingested by animals will be degraded in their stomach and be of no consequence.

D.5.b. Risk to Public Health and Safety

Likelihood Rating: Low (LL)
Degree of Certainty Rating: Certain (C)
Consequence Rating: Low (CL)
Degree of Certainty Rating: Certain (C)
Expected Risk Rating: 1.0000
Risk Characterization: LL.C.CL.C
Risk Rating: Low

Conclusion and discussion of risk:
There are no public health concerns associated with the testing of the melanoma DNA vaccine. Human exposure will be limited to the qualified personnel administering the vaccine and the risk of exposure to these individuals is considered to be low.

D.5.c. Risk to Environmental safety:

Likelihood Rating: Low (L)
Degree of Certainty Rating: Certain (C)
Consequence Rating: Low (CL)
Degree of Certainty Rating: Moderately Certain (MC)
Expected Risk Rating: 0.7500
Risk Characterization: LL.C.CL.MC
Risk Rating: Low

Conclusion and discussion of risk:

Justification for rating:
Conclusion and Discussion of risk:
The safety risks to environment are low. The vaccine will be kept in single
dose sealed labeled glass vials in the participating clinic(s) and only
veterinarian(s) and/or professional staff should handle it. In the case that
vaccine is released into the environment, the inability of the vaccine to
infect and replicate any eukaryotic species should eliminate any risk of
persistence in the environment.

IX. Risk Management

A. Procedure
Risk management uses the information from the risk assessment, as well as,
regulatory, social, and economic realities, to determine whether the proposed
release should be approved. Risk management also includes the design and
implementation of mitigative procedures to reduce or eliminate potential risks. If
the risks to animals, public health, and the environment are low, the proposed
study is approved. If the risk is high the request is denied. Requests to conduct
studies that have been rated with medium risks are also denied, unless proper
mitigation procedures are identified and implemented.

B. Recommendations
The safety risks to animals, public health, and the environment are low. A low risk
rating is an acceptable risk, with very little concern associated with the proposal.
**Table 3: Calculating the Expected Risk**

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<th>VALUE RATINGS</th>
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<td>Medium (M)</td>
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**Consequence (C)**

| Low (L)       | CL = 1.00      |
| Medium (M)    | CM = 0.10      |
| High (H)      | CH = 0.01      |

*If the Likelihood rating is Medium or High and the Consequence rating is also Medium or High use Degree of Certainty Ratings I; for all other combinations use Degree of Certainty Ratings II.*

**Degree of Certainty Ratings I**

| Certain (C)  | C = 0.50     |
| Moderately Certain (MC) | MC = 0.75 |
| Uncertain (U) | U = 1.00  |

**Degree of Certainty Ratings II**

| Certain (C)  | C = 1.00     |
| Moderately Certain (MC) | MC = 0.75 |
| Uncertain (U) | U = 0.50  |

**EXPECTED RISK**

\[
\text{Risk Rating} = [(\text{likelihood}) \times (\text{degree of certainty})] \times [(\text{consequence}) \times (\text{degree of certainty})]
\]
### Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for Melanoma DNA Vaccine

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#### Table 4: Risk Ratings

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