Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

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General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50400
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Testing of immunomodulating properties

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This project proposal can be divided in two different components:

1. Immunogenicity testing: The product is administered for a number of times (mostly 1 or 2 times) in order to activate the immune system and (immunological) read-outs will be performed to evaluate the immunogenicity of the product.
2. Testing of immunomodulating properties: The effect of the product on the immune response will be determined: A general immune response will be initiated by administration of a general activator (for example an unrelated protein) or by passive transfer of immune cells, then the medical product will be administered to determine whether the immune response can be modified by that product (animal models: mice, including immunodeficient mice).

This appendix describes the second component.

The primary readout for testing the immune modulatory capacities of medical products is to determine the effect of the product on the immune response, mainly focusing on the B and T cell responses in the blood and/or specific organs. Also clinical read-outs (e.g. ear thickness) can be applied.

The study design contains:

- Initiating a primary immune response, in which an immune response is actively induced using an antigen unrelated to the product that will be tested or by initiating an immune response by passively transferring immune cells.
- Administration of the product, in which the mice will be administered with the product before, during, or after a primary immune response is initiated.

- Interim read-outs, in which blood or feces, for example, will be collected in order to determine the immune response before (baseline response) or after administration of the product.
- Clinical observations and body weight, in which the mice are monitored for general health and to observe if any (unexpected) adverse effects occur.
- Euthanasia and necropsy, in which the mice are euthanized and any (immunological) organs can be collected to further determine the response of the mice's immune response to the product.

The study design of each individual study will be described in accordance with the code of good practice (Diehl et al, J. Appl. Toxicol. 21, 15–23 (2001); A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes) and the Handbook of Laboratory animal science of van Zutphen et al.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The animal studies are performed in our American Association for Accreditation of Laboratory Animal Care (AAALAC) certified animal facility. All study procedures will be performed by qualified animal care specialists, which have extensive training in all animal procedures performed in these studies. Procedures are performed according to standard operating procedures (SOPs).

A study can include control group (s) and different treatment groups, for example:

- Negative control group: As a negative control, the mice will receive the vehicle (carrier) of the product to be tested.
- Positive control group: Mice will receive a reference compound, of which it is known that it will modulate the immune response.
- Treatment groups: The different groups which are incorporated in each study will depend on the number of new products to be tested and / or the amount of doses to be tested.
- Additional groups: (sub)groups for PK (pharmaco-kinetic) analysis could be added if an additional research question is to obtain more PK information on the administered compounds.

Because the groups will mostly be compared to the control group (either a positive or negative control group), the size of this group will be chosen in such way that sufficient statistical power is obtained when this group is used multiple times in the statistical analysis. As a result, the power is increased while the total number of mice can be reduced (unequal group sizes).

<u>Initiating a primary immune response</u>

An immune response can be actively induced using an antigen unrelated to the product that will be tested (example 1) or by initiating an immune response by passively transferring immune cells (example 2).

Example 1 (Delayed type hypersensitivity study design):

In a delayed type hypersensitivity (DTH) study design, the mice will be immunized against a known antigen, e.g. Ovalbumin (OVA) or KLH. Hereto, on day 0 all study animals will be immunized with the antigen (if necessary an adjuvant will be included to obtain an immune response). After one or two immunizations with the primary antigen, the mice will be challenged intradermally in the ear with (a part of) the same antigen under anesthesia. In case of the OVA-DTH model, an Ovalbumin peptide will be used. As a read-out ear thickness will be measured: as the ear will swell in response to the peptide challenge, the immunomodulating properties of the product (in this case bacteria), can be tested: If less ear swelling is induced in the treatment group compared to the control group, the product is effective in modulating the immune response. In addition to ear swelling, also an antibody response (for example OVA-specific IgG levels) can be determined to test the immune modulating effect of the product that is tested. Depending on the main read-out (ear swelling, which is mostly T cell mediated or antibody responses, which are mostly B cell mediated) the timing of priming, boosting (if necessary) and challenge can vary.

Example 2 (Passively transferring immune cells):

An immune response can be passively induced by adoptively transferring human T cells into immunocompromised mice. The product will be administered before or after transfer of the cells (depending on the research question) and the effect of the product on the T cells can then be monitored by collecting blood at set time points, isolating these cells and performing ex vivo analysis (e.g. flow cytometry).

Administration of the product
Treatment will generally start within a time window of 6 weeks before or after initiating the immune response. The route of administration is mainly determined by the specific characteristics of the product to be tested (for example, mice can be immunized subcutaneously near draining lymph nodes to obtain an immune response as efficient as possible). Products tested in these models (e.g. small molecules) will be administered subcutaneously, intravenously, intradermally, or intraperitoneally as these are the most common desired routes to obtain an immune response to the product. Some products, for example products directly targeting immune cells in the respiratory or gastrointestinal tract, will be administered intranasally, orally, or sublingually. In a minor percentage of the studies under this project it can be necessary to test combinations of products, for example if an existing product combined with a new product, are tested, two routes can be applied.
This maximum time period is chosen based on the immune response of the mice (which is most optimal, and therefore most relevant) in young adult mice. The dosing and dose interval will always adhere to the code of practice for the immunization of laboratory animals (Keuringsdienst van Waren, August 2000) and depends on the type of product tested and the research question of the study. The proposed study design will be discussed with the project team, in which an expert scientist is included and will be supported (statistically), and with the AWB. Then it will be determined whether the proposal is scientifically sound and whether there are refinements possible regarding the 3R's.

Interim read-outs
If required, at certain points in time, blood will be drawn from selected groups in order to determine the response to treatment or in order to evaluate the PK/PD/ADME of the product that is tested. In addition, feces may be collected, which will generally occur when mice are already handled, for example during allocation of the mice to their corresponding treatment groups or during measurement of body weight.

Clinical observations and bodyweight
Clinical signs and body weight are monitored according to internal Standard Operating Procedures on clinical signs. All mice will be monitored for clinical signs at least once daily. All observations and any individual abnormality will be recorded. In most studies, individual body weight measurements will be performed weekly. If necessary, for example if a (minor) change in body weight is expected, there will be a smaller time interval between the measurements. Baseline body weights will be performed in all mice shortly before the start of the study.

Euthanasia and necropsy
The exact length of the study depends on the product that is tested. In most studies, the mice will be euthanized within 6-10 weeks, with a maximum of 26 weeks (in case duration of the immune response is one of the research questions, this is the maximum expected time period). In case a terminal blood sample is required, the mice will be anesthetized, followed by blood sampling and euthanization.
Organs (e.g. spleens, lymph nodes, lungs) can be collected afterwards for further immune phenotypic analysis. Possible readout parameters are: antibody titers, cytokines & chemokines, immune phenotyping (activation markers, FoxP3, intracellular cytokines, etc.), proliferation assays and/or immunohistochemistry.

Example 1 (DTH):
Beneficial commensal microbes, in particular commensal bacteria have been implicated in the development, homeostasis and function of the immune system. Some of these bacteria can have direct and indirect anti-inflammatory properties on host immune system resulting in health benefits (e.g. Lactobacillus strains).
The objective of these studies with commensal bacteria will be to determine whether treatment with different strains of probiotic bacteria, can suppress the immune response in a mouse model for delayed-type hypersensitivity (DTH). If the bacteria are able to enhance the regulatory T cell function in vivo, the OVA-specific immune response will be dampened in this model.

Example 1 (Delayed type hypersensitivity study design):
Table study design, example 1:

Group (n=10)	Immunization (200 µg OVA in CFA) s.c.	Peptide challenge i.d. [b]	Treatment (day 1 to day 9)	Route	Blood sampling	Ear thickness measurements	Sacrifice
1-X	4 sites, 50 µL per site	Day 8	Bacterial strain A-Z	p.o.	Day 10	Day 7, 9, 10	Day 10
Y	4 sites, 50 µL per site	Day 8	Dexamathasone	i.p.	Day 10	Day 7, 9, 10	Day 10
Z	4 sites, 50 µL per site	Day8	Vehicle	i.m	Day 10	Day 7, 9, 10	Day 10

Example 2 (Passively transferring immune cells):
An immune response can be passively induced by adoptively transferring human T cells into immunocompromised mice. The product will be administered before or after transfer of the cells (depending on the research question) and the effect of the product on the T cells can then be monitored by collecting blood at set time points, isolating these cells and performing ex vivo analysis (e.g. flow cytometry).

Group N=7	T cells (i.v.)	Treatment Day 0 (i.v.)	Blood sampling for T cell analysis (Day)	Sacrifice & terminal blood collection (Day)	Flow cytometry analysis Optional (Day)
1	Day 0	Product A	0 (two hours after treatment), 1, 3, 7	14	0,1,3,7,14
2	Day 0	Product B	0 (two hours after treatment), 1, 3, 7	14	0,1,3,7,14
3	Day 0	Product C	0 (two hours after treatment), 1, 3, 7	14	0,1,3,7,14
4	Day 0	Product D	0 (two hours after treatment), 1, 3, 7	14	0,1,3,7,14
5	Day 0	Reference	0 (two hours after treatment), 1, 3, 7	14	0,1,3,7,14

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The total number of mice per group will be based on historical data from previous studies performed at our facility, or historical data from other facilities (including literature and historical data from the sponsor) using equal or comparable animal models and/or treatments. Both the historical data from previous studies and/or results from literature will lead to a scientific and statistical backing of the study design (e.g. a sample size calculation).

The power analysis and samples size calculation will be executed by a statistical department using statistical software (e.g. Genstat, SAS) or by using other sample size calculations tools (e.g. the tool made available by the University of Boston (http://www.bu.edu/orccommittees/iacuc/policies-and-guidelines/sample-size-calculations/).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The choice of a certain animal model depends on read-outs, the dosage form, and the drug development trajectory of the pharmaceutical drug candidate (see general information). The eventual choice of the animal species, origin, estimated number, also depends on the availability of historical data from previous studies with similar compounds (if applicable), data in literature and the intended relevant disease model to test the efficacy of the pharmaceutical drug in the following phase.

For these studies, we plan to use mice. The benefits of murine over other animal models is not only the their small size and availability, but most importantly the availability of immune reagents (antibodies, cytokines/chemokines, ELISA kits, etc) and a large amount of historical data available. Unless otherwise stated in the plan of study (for example if the product needs to be tested in older mice, if the product will be used in elderly individuals), the mice used will be 6-8 weeks old at the start of the study. The exact strain will be addressed in the study plan and AWB ethical application, but will be healthy animals obtained from a certified breeder. Immunodeficient mice will only be used when necessary, otherwise immunocompetent mice will be used.
In the DTH-studies, we will use female mice. As further described in the publication by Ma et al (Ma et al 2007 Journal of Endocrinology, Local cytokine levels associated with delayed-type hypersensitivity responses: modulation by gender, ovariectomy, and estrogen replacement), female mice mount stronger immune responses than males mainly due to skewing of the immune response by hormones. As the induction of an immune response, i.e. ear swelling, is the main read-out of the study, in this case female mice are preferred as they provide us with a larger treatment window than male mice. The immune response of male and female mice may respond differently to immunization and to limit variation the use of one sexe is preferred. Since there is an increased probability that adult male mice will become aggressive during the course of these studies, which may lead to fight wounds (including ear damage). As ear thickness is one of the main read-outs in this model, any chance of damage to occur to the ears should be avoided (Allen, 2013, Delayed-type hypersensitivity models in mice, Methods of molecular biology).
For the passive transfer experiments, the choice of gender of the mice will mainly depend on the origin of the cells: For example, due to the expression of the Y chromosome in male cells, these cell lines can not be transferred to female mice as they will be recognized as foreign antigen and will induce an unwanted immunological reaction.
Both the historical data from previous studies and/or results from literature will lead to a scientific and statistical backing of the group size. Where applicable, unequal group sizes will be used.

Estimated numbers of animals
The total number of animals per study may vary and is dependent on the number of groups and the group size. Based on an expected number of approximately 12 studies per year with 35-120 mice (e.g. 5 groups of seven mice or 12 groups of ten mice), the maximum number of mice is estimated at up to 7200 mice over a period of 5 years (12 studies x max. 120 animals x 5 years).

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

The immune modulating compounds to be tested, are selected on the basis of in silico, in vitro (cell lines) and/ or ex vivo (tissues, primary cells) experiments. Alternatives to mimic an immune response, for example by means cell or tissue culture provide an incomplete picture of the efficacy of medical products

in a living organism. The immune system is a complex, dynamic network of various cells and mediators, therefore at the moment it is not possible to replace it completely using in vitro assays.

The optimal group size for these studies is calculated using statistics, resulting in a minimal number of mice to be used for each experiment. Both the historical data from previous studies and/or results from literature will be used to perform a sample calculation, and/or a statistical analysis plan. The animal studies will be performed according to a predetermined protocol and by trained staff. This allows a maximum quality in combination with stress and discomfort to the mice being kept as low as possible. If it appears that variation within groups reduces over time, further optimizing the number of mice that will be used. In addition, if there are historical data of control groups which can be used for an experiment, the group size of that control group in the experiment can be reduced.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Procedures may warrant anesthesia and/or analgesia, e.g. i.d. immunization in the ears. In those instances, species appropriate anesthesia and/or analgesia is used after consultation of the AWB or designated vet.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☒ No > Continue with question I.

☐ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

In general, treatment related clinical signs are not to be expected for these kind of studies. However, due to the nature of these types of preclinical studies, unanticipated adverse effects cannot be excluded and can occur sporadically. An example of such an adverse effect may be temporary stiffness of the legs after intramuscular immunization or temporary piloerection after treatment.
In some of these studies, adjuvants (e.g. Complete or incomplete Freunds Adjuvant) will be used either to initiate an immune response or sometimes as part of the product which is tested, which can cause local inflammation. In consultation with the veterinarian and the animal welfare body, animals with such an inflammation will be removed from the study. The expected incidence of such an inflammation is <0.5% of the mice.

Explain why these effects may emerge.

In some of these studies, adjuvants (e.g. Complete or incomplete Freunds Adjuvant), which can cause local inflammation.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

If adjuvants will be used, which are known to (incidentally) cause inflammation, the route and volume of administration will be chosen in such a way that the chance of inflammation to occur and/or severity of inflammation is minimized. The expected incidence of adverse effects such as local inflammation is less than 1% of the mice.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Clinical observation of the individual mice, to detect reaction to treatment as well as unexpected signs of ill-health are performed at least once daily. If clinical signs are observed, immediate action will be taken in consultation with the veterinarian and the AWB. The mice will be monitored closely if necessary, the mice will be euthanized. An example hereof is that due to administration of Complete or incomplete Freunds adjuvant, inflammation, e.g. redness of the skin, scratching and other signs such as body weight loss may occur incidentally. In consultation with the veterinarian and/or AWB, mice may be euthanized if the inflammation is considered to affect the welbeing of the animal in such a way that it is not ethical to keep the animal in the study.

Indicate the likely incidence.

Less than <0.5% of the mice.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Body weight determination: mild discomfort
Administration of the product: mild to moderate discomfort*
Blood sampling: mild discomfort

*As some of the products tested under this proposal are still in a preliminary phase, unexpected side effects may occur. Upon registration of these signs, immediate action will be taken either in the form of implementing the humane endpoints or by taking actions to minimize the discomfort of the mice. An example hereof is providing a heat lamp for animals of which the body temperature drops unexpectedly.

Based on previous experience with these models, we expect that none of the animals will have 'severe' or 'non-recovery' discomfort. The majority of the animals (>60%) will have 'mild' discomfort and less than 40% will have moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be killed for the collection of tissues (e.g. lymphoid organs) or blood.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes