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).

1

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: 1 Type of animal procedure: Immunogenicity testing

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 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, and 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 first component.

The primary readout for testing the immunogenicity of medical products is to determine the antigen specific immune response, mainly focusing on the B and T cell responses in the blood and/or specific organs.

Therefore, each study design will generally contain the following procedures:

- Administration of the product, in which the animals will be administered with the product in order to initiate an immune response.
- Interim read-outs, in which blood or feces, for example, will be collected in order to determine the immune response against the product.
- Clinical observations and body weight, in which the animal is monitored for general health and to observe if any (unexpected) adverse effects occur.

- Euthanasia and necropsy, in which the animal is sacrificed and any (immunological) organs can be collected to further determine the response of the animal's immune response to the product.

The study design of each individual study will be performed 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 animals will receive the vehicle (carrier) of the product to be tested.
- Positive control group: Animals will receive a reference compound, of which it is known that it will induce an 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 animals can be reduced (unequal group sizes).

<u>Administration of the product</u>

The route of administration is mainly determined by the specific characteristics of the product to be tested (for example, animals can be immunized subcutaneously near draining lymph nodes to obtain an immune response as efficient as possible). Vaccines are generally administered via intramuscular injection as this generally is the anticipated route in humans. Other types of products (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 if combinations of products, for example an existing product combined with a new product, are tested, then two routes can be applied in the same study.

The exact dose and dosage interval will depend on the availability of the medical product in the body (if PK/PD experiments have been performed at an earlier stage this information will be used to determine dose and/or dose interval) and/or on previous experience and/or historical data with similar products.

The dose interval of studies under this project, which mainly applies to vaccine studies, is an average number of two or three doses with a maximum of five doses in a time range of 0-12 weeks. The dosing and dose interval will always adhere to the code of practice for the immunization of laboratory animals (Keuringsdienst van Waren, August 2000). The immune response will be monitored until maximally 26 weeks after the first immunization. This maximum time period is chosen, based on the immune response of the animal (which is most optimal, and therefore most relevant) in young adult animals.

The proposed study design will be discussed with the project team, in which an expert scientist is included and will be supported (statistically).Then it will be determined whether the proposal is scientifically sound and whether there are refinements possible regarding the 3R's.

<u>Interim read-outs</u>

If required, at certain points, 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 animals are already handled, for example during allocation of the animals 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 animals 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 change in body weight is expected, there will be a smaller time interval between the measurements. Baseline body weight measurement will be performed in all animals 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 animals will be euthanized within 12 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 animals 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.

A schematic representation of a possible vaccine study (induction of immune response) is shown below. The final design will be specified in the study plan and discussed with the AWB.

Example (induction of immune response):

In order to induce an immune response, the product will be administered to the animal. If the product is indeed immunogenic, this will lead to the induction of an antibody or T cell response in the host. In case of an antibody-inducing product, the purpose of the experiment can be to determine whether or not the antibodies are capable of neutralizing a certain micro-organism. Therefore, the antibodies isolated from the serum of a vaccinated animal can be tested in in vitro assays for its neutralizing capacity. For potential T cell inducing products, it will be relevant to isolate the T cells following administration of the product and further characterize these cells ex vivo (for example using flow cytometry).
An immunization protocol, e.g. designed to induce antibodies to a certain antigen, generally includes at least two dosage moments as the immune system will respond more vigorously to the second contact with a given product than the first contact, and will often include different doses (for example a high and a low dose) in order to obtain a dose response curve. The second goal could be to analyze the relation between the first dose (priming) and the following dose(s) (booster injections) on the antibody titers and to determine the dose (prime-boost) regimen that maximizes the antibody titers.

Example of a study design:

Group	**Antigen**	**Immunization (Prime)**	**Immunization (boost)**	**Route**	**Blood sampling**	**Sacrifice**
1 (n=8)	V dose 1	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
2 (n=8)	V dose 2	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
3 (n=8)	W dose 1	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
4 (n=8)	W dose 2	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
5 (n=8)	X dose 1	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70

6 (n=8)	X dose 2	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
7 (n=8)	Y dose 1	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
8 (n=8)	Y dose 2	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
9 (n=8)	Positive control dose 1	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70
10 (n=12)	Negative vehicle control	Day 0	Day 21, 42	i.m	Day -1, 20, 41	Day 70

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 animals 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 sample 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.

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 stated otherwise in the study design (for example if the product needs to be tested in older mice, as the product is intended for 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 application, but will be obtained from a certified breeder.

Choice of gender (female, male or a combination) of the animal, independent of the animal model chosen, is based 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 example, if for a certain product it is known that male and female animals respond in a similar manner, both male and female mice can be used. However, it can also be the case that one sexe responds more vigorously to the product. As the main goal is to test immunogenicity and not to test efficacy of the product in both sexes, to keep the number of animals as low as possible (as high variation will lead to a high standard deviation and thus a higher number of animals needed per group), one sexe will often be selected. In addition, the choice of gender in these immunogenicity studies may also depend on the efficacy models in follow-up studies: For efficacy studies with hepatitis B virus, for example, if only male mice are used (as male mice have slightly higher viral titers and less variability in these titers compared to female due to hormonal changes in these female mice) than it will be preferable to use male mice in immunogenicity testing as well (Hamatake et al, 2004, Hepatitis B and D Protocols: Immunology, model systems, and clinical studies).

<u>Estimated numbers of animals</u>
The total number of animals per study may vary and is dependent on the number of groups and the group size. Based on the studies performed in previous years we have an expected number of approximately 6 studies per year with 80-120 animals (e.g. 10 groups of eight animals or 12 groups of ten animals). The maximum number of animals is estimated at up to 3600 animals over a period of 5 years (6 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 products 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 of 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 animals 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 a lowest amount of stress and discomfort as possible. If it appears that variation within groups reduces over time, further optimizing the number of animals 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.

If necessary, measures will be taken. For example, if immunization with a test substance causes a temporary decrease in body temperature, a heat lamp will be provided for the animals around the time of immunization.

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 studies, when the product is still in an early phase of development, 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, a temporary reduction of body temperature, or temporary piloerection after treatment.

Explain why these effects may emerge.

Not to be expected.

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

If unexpected adverse effects of the test substance do arise, the study director (article 9) will be informed immediately. Where possible, and together with the veterinarian and/or AWB, actions will be taken to minimise severity or prevent occurrence during follow-up experiments. For example by changing the dose of administration or by taking other actions to relieve adverse effects, such as providing extra heat when a drop in body temperature is observed. Based on studies performed in the last three years, the incidence is expected to be less than 5%.

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.

Indicate the likely incidence.

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 to minimize the discomfort of the animals. 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' discomfort or 'non-recovery'. The majority of the animals (>75%) will have 'mild' discomfort and less than 25% 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