1

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in* 40100
☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	Stichting DLO
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	9 0 9 8 1 0 4
Straat en huisnummer	Akkermaalsbos 12
Postbus	59
Postcode en plaats	6700AW Wageningen
IBAN	NL10RABO0397066465
Tenaamstelling van het rekeningnummer	Wageningen UR

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☑ Mw.
Functie	Wetenschappelijk medewerker
Afdeling	[redacted]
Telefoonnummer	
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Wetenschappelijk medewerker
Afdeling	[redacted]
Telefoonnummer	
E-mailadres	

1.6 *(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.

(Titel) Naam en voorletters		☐ Dhr. ☐ Mw.
Functie		
Afdeling		
Telefoonnummer		
E-mailadres		

1.7 Is er voor deze projectaanvraag een gemachtigde?

☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*

☑ Nee

2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

2.2 Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?

☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier

☐ Nee > Ga verder met vraag 3

2.3 Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?

☐ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?

Startdatum	01-04-2015
Einddatum	01-04-2020

3.2 Wat is de titel van het project?

Induction of antibody responses in llamas for isolation of specific antigen binding recomb

3.3 Wat is de titel van de niet-technische samenvatting?

Opwekken van een antigeen-specifieke immuunrespons in lama's om specifieke antilicha

3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?

Naam DEC	DEC-DLO
Postadres	[redacted]
E-mailadres	

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- ☑ Nieuwe aanvraag Projectvergunning € 741,00 Lege
- ☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- ☐ Via een eenmalige incasso
- ☑ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☒ ~~Melding Machtiging~~ DEC-advies
- ☒ 2 x Description animal procedures
- ✓ order WUR839093 tbv factuur

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	gemandateerd vergunninghouder
Plaats	Wageningen
Datum	4 - 3 - 2015
Handtekening	[redacted]

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.zbo-ccd.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'.	40100
1.2	Provide the name of the licenced establishment.	Stichting Dienst Landbouwkundig Onderzoek (DLO)
1.3	Provide the title of the project.	Induction of antibody responses in llamas for isolation of specific antigen binding recombinant antibody fragments derived from heavy-chain antibodies (nanobodies)

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☐ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or animal health or welfare
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries

☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Monoclonal antibodies (mAbs) are since long valuable tools in various diagnostic applications. The last two decades they are being used also for therapeutic applications in humans and animals. An example is the mAb Synagis® that is used to help prevent a serious lung disease caused by respiratory syncytial virus (RSV) in children at high risk for severe lung disease from RSV. Most mAbs are isolated by immunisation of mice and hybridoma technology. Biochemically mAbs exist of a heavy and a light antibody polypeptide chain that are both needed for binding to an antigen. Since 1993 it is known that camelids (camels, dromedaries and llamas) make a special class of antibodies that exist of only two heavy antibody chains. The N-terminal domains of these heavy-chain antibodies are called nanobodies®. They can be isolated from peripheral blood lymphocytes (PBLs) of immunized llamas using phage display technology. Phage display is a powerfull molecular biological technology where one can retrieve antigen binding nanobodies from large libraries of more than a billion clones. For this purpose RNA is extracted from PBLs, converted into cDNA and used for PCR amplification of nanobody genes. These genes are then inserted into a phage display vector. Nanobodies offer a number of advantages for biotechnological applications as compared to conventional mAbs or recombinant antibody fragments derived from conventional mAbs (Harmsen and De Haard, 2007; De Meyer et al., 2014):
1) high biophysical stability, enabling application in harsh environments.
2) efficient production in microorganisms, enabling more facile and cheap production.
3) great amenability for phage display selection due to coding by a single gene, resulting in high functional phage display libraries.
4) highly suitable for making genetic fusions to other nanobodies (multimers), enabling production of more potent nanobody multimers and bispecific nanobody multimers. Bispecific antibodies are especially important for therapy. One can for example make bispecific nanobodies against a tumor antigen and a cytotoxic T-cell for recruitment of cellular immunity to fight cancer. Note that conventional mAbs are always monospecific.
5) recognition of cryptic antigenic sites, often in cavities such as enzyme active sites, that are not recognized by conventional antibodies. This enables for example enzyme neutralization.
6) efficient tissue penetration, which can be important for example for neutralization of snake or scorpion toxins.
7) highly soluble.

[redacted]. Since that time [redacted] immunises llamas (Lama glama) because these animals are relatively easy to handle and house. [redacted] also does contract research for third parties, often companies, with the aim to isolate nanobodies against specific antigens that the client delivers. The products that we then generate are llama sera and RNA isolates from PBLs of immunized llamas that are suitable for cDNA synthesis and further construction of phage display libraries or other methods for isolation of specifically antigen binding nanobodies. These nanobodies are to be used in biotechnological applications. Due to the expiration of a number of basic patents on nanobodies there is a rising interest in nanobodies. In a recent review (De Meyer et al., 2014) an overview of nanobody applications is given.

References:
De Meyer, T., Muyldermans, S., Depicker, A., 2014. Nanobody-based products as research and diagnostic tools. Trends Biotechnol. 32, 263-70.
Harmsen, M.M., De Haard, H.J., 2007. Properties, production, and applications of camelid single-domain antibody fragments. Appl. Microbiol. Biotechnol. 77, 13-22.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The aim of the project is to isolate nanobodies that bind specifically to particular antigens. The nanobodies to be isolated will be applied in human or veterinary diagnostics or therapy. The research is therefore applied or translational. The following three objectives are likely to be pursued by [redacted] the coming years.

A) Use of nanobodies in diagnostic tests for human and animal diseases

For example in Research and Development (R&D) or Quality Control (QC) of veterinary vaccines, especially foot-and-mouth disease virus (FMDV) vaccine. For this purpose the nanobodies are used in laboratory tests to determine the concentration and quality of the active ingredients in vaccines during the production process. In case of FMDV we have found nanobodies that bind specifically to intact (146S) FMDV virions but not to their discrete (12S) degradation products. These rare nanobodies have proven valuable to determine the stability of FMDV vaccines, which led to the discovery that a preservative that is often used in such vaccines - thiomersal - is detrimental for vaccine stability (Harmsen et al., 2011a). Because nanobodies that are 146S specific are often also strain specific they must often be isolated again for novel strains. Note that novel FMDV strains emerge in the field and vaccine strains therefore need constant adjustment.

B) Use of nanobodies as medicine

For example a therapeutic objective is the use of nanobodies for protection of humans or animals against intoxication from toxins produced by snakes, scorpions (Hmila et al., 2010), spiders or bacteria (Hussack et al., 2011). Because such intoxication is often acute and deadly a treatment that confers immediate protection such as antibody application is required. Such therapeutic antibodies are often produced by immunisation of large animals such as horse, sheep or goats with inactivated toxins. Replacement of such therapeutic antibodies by a nanobody produced in microorganisms will eventually lead to reduced animal experimentation.

C) Use of nanobodies to purify medicines

Due to their high stability nanobodies can also be used for application in purification of biologicals by immunoaffinity chromatography (Verheesen et al., 2003). Here nanobodies are coupled to a column matrix and a crude product containing the biological of interest is led throught the column. The biological is retained on the column due to binding by nanobody while the contaminants flow through the column. Then the binding of nanobody and biological is broken using a suitable buffer to elute the biological from the column. Using such Capture Select technology biologicals can be purified to a high level of purity that is difficult to obtain with other methods. It is especially used for the purification of biologicals produced by fermentation of animal cells for use in human therapeutic applications. Important biologicals that can be purified with nanobodies already are human antibodies and fragments thereof, albumin, growth hormone and follicle stimulating hormone.

The achievability of each objective is strongly dependent on the type of antigen. When the antigen is highly immunogenic it is highly probable (>95% chance) that antigen binding nanobodies will be isolated. For relatively facile applications such as R&D and QC of veterinary vaccines the availability of such nanobodies will probably result in usefull tests. However, when more rare specificities of the nanobody are required, such as specific binding to intact virions, the chance of achieving the objective is less likely. When using nanobodies for more complex therapeutic applications the requirements for the nanobody are

often more stringent resulting in a relatively lower chance of achieving the project objective. Note that the inability to isolate a suitable nanobody against a particular antigen to achieve a specific objective does not necessarily mean that a llama was immunized in vain since llamas are immunized with a cocktail of antigens that serve several objectives and this llama has most likely yielded good nanobodies against another antigen.

References:
Harmsen, M.M., Fijten, H.P., Westra, D.F., Coco-Martin, J.M., 2011a. Effect of thiomersal on dissociation of intact (146S) foot-and-mouth disease virions into 12S particles as assessed by novel ELISAs specific for either 146S or 12S particles. Vaccine 29, 2682-2690.
Hmila, I., Saerens, D., Ben Abderrazek, R., Vincke, C., Abidi, N., Benlasfar, Z., Govaert, J., El Ayeb, M., Bouhaouala-Zahar, B., Muyldermans, S., 2010. A bispecific nanobody to provide full protection against lethal scorpion envenoming. Faseb J 24, 3479-3489.
Hussack, G., Arbabi-Ghahroudi, M., van Faassen, H., Songer, J.G., Ng, K.K., MacKenzie, R., Tanha, J., 2011. Neutralization of Clostridium difficile toxin A with single-domain antibodies targeting the cell receptor binding domain. J. Biol. Chem. 286, 8961-8976.
Verheesen, P., ten Haaft, M.R., Lindner, N., Verrips, C.T., de Haard, J.J., 2003. Beneficial properties of single-domain antibody fragments for application in immunoaffinity purification and immuno-perfusion chromatography. Biochim. Biophys. Acta 1624, 21-28.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

In general the novel nanobodies will result in improved diagnostics or therapy of diseases in animals or humans. Thus, it contributes to human and animal health. Human therapeutic applications are most likely cancer and inflammatory diseases. Animal therapeutic applications are most likely infectious diseases.
An important objective of this project is to isolate nanobodies for purification of biologicals with Capture Select technology. This will lead to a higher degree of purification of such biologicals resulting in safer human therapy. Sometimes such biologicals exist in two isoforms which differ in therapeutic efficacy or levels of side effects. Purification of the most active isoform will lead to a more effective therapy or less side effects.
Due to their advantages nanobodies can be isolated that perform better than conventional antibodies for particular applications. Examples are:
1) Improvement of FMDV vaccines using nanobodies that discriminate intact and degraded FMDV virions (Harmsen et al., 2011a)
2) Improved tumor diagnostics using imaging techniques with nanobodies (De Meyer et al., 2014)
3) Improved or novel human therapy against various human diseases using nanobodies by the belgian company [redacted]

References:
De Meyer, T., Muyldermans, S., Depicker, A., 2014. Nanobody-based products as research and diagnostic tools. Trends Biotechnol. 32, 263-70.
Harmsen, M.M., Fijten, H.P., Westra, D.F., Coco-Martin, J.M., 2011a. Effect of thiomersal on dissociation of intact (146S) foot-and-mouth disease virions into 12S particles as assessed by novel ELISAs specific for either 146S or 12S particles. Vaccine 29, 2682-2690.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

[redacted] is a service provider for llama immunization. Clients are primarily third parties and occasionally internal [redacted] projects. The client usually delivers the antigen. Occasionally [redacted] buys or makes the antigen. [redacted] discussed the llama immunization protocol with the client and informs the client that immunization of a llama with several antigens in parallel is advantageous both for the projects costs and to reduce animal use. [redacted] isolates sera and RNA from PBLs of each immunized llama for isolation of nanobodies either by the client or in [redacted] laboratories.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

A humoral immune response against the antigen of interest will be induced in a llama (Lama glama) by immunization. The llama will be immunized with several antigens in parallel to reduce animal use. As a rule of thumb we immunize with a maximum of ten antigens per llama. It is difficult to determine in advance how many antigens can be pooled for one llama immunization. This depends on several factors:
1) The immunogenicity of the antigen that is often known from immunizations in other mammals. Highly immunogenic antigens can be pooled more easily.
2) The requirements for the nanobody. For example a human therapeutic nanobody often has much more stringent requirements such as low immunogenicity in humans, safety, high potency than a nanobody for veterinary diagnostics.
3) The matrix effects of mixing antigens. For example a formalin inactivated toxin cannot be mixed with an antigen whose immunogenicity is destroyed by formalin.
Immunization can be done in several ways:
1) with a dead or inactivated antigen mixed with an adjuvant
2) by DNA immunization, often in combination with method 1). Here DNA immunization is used for priming immunization and dead antigen is used for one or two booster immunizations.
3) by vectored immunization. Here the antigen of interest is inserted into a virus or bacterium that shows limited replication in a llama without causing disease signs. During replication the antigen of interest is expressed in the llama. Examples of such an approach are immunization with Newcastle Disease Virus - vectored vaccines (Harmsen et al., 2011b) or with canarypox virus - vectored vaccines (Paoletti et al., 1994).
It is also possible that a llama is immunized by a combination of all three methods using three different antigens or antigen cocktails. During the immunization small samples of blood are collected for serum preparation. This can be used to monitor immune responses. After the llama has developed a sufficiently high antibody response against the antigen of interest a large blood sample is taken for isolation of peripheral blood lymphocytes (PBLs). This blood sample is larger than the serum samples to ensure that a diverse set of B-cells producing heavy-chain antibodies is isolated which is necessary to ensure nanobody library diversity. Two of such blood samples are collected at different moments during the immunisation procedure to ensure even more diverse nanobody libraries.

References:
Harmsen, M.M., Antonis, A., Moormann, R.J.M., Kortekaas, J., 2011b. Parenteral vaccination of mammalian livestock with Newcastle disease virus-based vector vaccines offers optimal efficacy and safety. Bioengineered Bugs 2, 1.
Paoletti, E., Tartaglia, J., Taylor, J., 1994. Safe and effective poxvirus vectors--NYVAC and ALVAC. Dev Biol Stand 82, 65-69.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

The coherence between the llama immunizations is that they all serve the isolation of nanobodies. The experiments differ in the nature of the antigen. Different antigens are combined into a cocktail for llama immunization. [redacted]vaits with llama immunization until several antigens, sometimes serving different objectives, can be simultaneously used for llama immunization. Llamas are preferably immunized consecutively, after a half year resting period, with another antigen cocktail. Two llamas are immunized with the same antigen cocktail since different llamas have very different antibody responses. Such immunization of two llamas is normally sufficient for retrieving nanobodies that are suitable for achieving the objective. It is difficult to determine in advance how many llamas are needed, especially since llamas are often immunized with cocktails of antigens. For example when llamas are immunized with ten antigens that are lowly immunogenic and which serve socially highly relevant human therapeutic objectives then even more than two llamas could be immunized.
We do not have a go-nogo between the different phases in llama immunization: the induction of an immune response and the collection of a large blood sample for isolation of RNA from PBLs, because:

1) The experience with llama immunization has shown that phage display selection is such a powerfull selection methodology that one can even isolate good nanobodies from animals that do not (yet) show a good antibody response in serum. In other words: phage display selection is a more sensitive technology than simple ELISA.
2) Serum also contains conventional antibodies composed of both heavy and light chains which are not suitable for nanobody isolation. These antibodies interfere in the detection of the heavy-chain antibody response from which nanobodies are retrieved. Therefore additional methods are needed to separate conventional and heavy-chain antibodies.
3) It is highly impractical to collect an additional blood sample for serum preparation to predict antibody responses for future large blood sample collection since one then has to do a very rapid test within a few days. Furthermore, this also requires the collection of an additional blood sample. In our experience the collection of an additional blood sample is more discomforting to the llama than the collection of a single larger blood sample.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Immunization of llamas housed in a meadow for retrieving nanobodies
2	Immunization of llamas housed in contained stables for retrieving nanobodies
3	
5	
6	
7	
8	
9	
10	

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.zbo-ccd.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'.	40100
1.2	Provide the name of the licenced establishment.	Stichting Dienst Landbouwkundig Onderzoek (DLO)
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: Immunization of llamas housed in a meadow for retrieving nanobodies

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.

Llamas (Lama glama) will be immunized to induce an antibody response against a specific antigen. This is done using dead antigen formulated with an adjuvant or live cells formulated with or without adjuvant. One week after the penultimate and the last immunization a large blood sample will be collected for isolation of peripheral blood lymphocytes. These will form the source for cloning of the polyclonal nanobody repertoire into vectors suitable for subsequent

isolation of nanobodies that bind to specific antigens used for llama immunization. Most commonly these vectors are phagemids suitable for phage display selection.

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

The basic llama immunization procedure consists of three immunizations of a llama with a dead antigen, usually proteinaceous, formulated with an adjuvant. Immunizations are given with 3 to 4 week intervals. The first two immunizations are preferably given with the oil-emulsion adjuvant Specol (Stimune, Life Technologies), which is proven effective in llamas but gives side reactions such as itching and pain that can last for several days (Boersma et al., 1992). Post mortem inspection at [redacted] of llamas immunized with Specol has revealed encapsulated granuloma at injection sites. Immunizations are normally given intramuscularly, in a maximum volume of 5 ml per injection, that is given at two injection sites in the hind thigh. The third (last) immunization is preferably given with the adjuvant Montanide IMS1312 (Seppic) since this adjuvant gives less adverse reactions. Immunization is again intramuscularly in a maximum volume of 5 ml. The IMS1312 adjuvant is a watery adjuvant with less depot function than Specol. However, for the last immunization such a depot function is much less important since the last blood sample is collected a week later and any immune response thereafter is completely irrelevant. Preferably 100 microgram of protein is used per immunization. In exceptional cases, when the antigen to be used for llama immunization cannot be purchased separately and is only available in a commercial (adjuvanted) vaccines registered for veterinary use, we immunize with such vaccines.
A variant on immunization with dead antigens is immunization with live cells as antigen. This is often done to isolate nanobodies against membrane proteins. Such llama immunizations have been performed with tumour cells (Baral et al. 2011) or HEK293 cells expressing the antigen of interest (Jahnichen et al., 2010) and can be done with or without adjuvant.
In principle (to reduce the number of animals to be used) antigen cocktails are used for llama immunization. Cocktails can be made with as many as ten different antigens. In such cocktails, in theory antigenic competition can decrease the immune response against particular antigens. This is often described when evaluating mixtures of antigens that protect against different diseases. However, since the aim of the experiments is not to induce high antibody titres but rather to induce sufficiently high antibody titres for nanobody retrieval, we consider antigenic competition of less importance here. There are several literature examples of successful nanobody retrieval after immunization with cocktails (Conway et al., 2010; Harmsen et al., 2013).
Since the aim of the induction of antibody responses is to retrieve antigen specific nanobodies by molecular biological techniques there is no need to induce extremely high antibody titres. This is opposed to the use of vaccines for protection of the animal against disease, where higher antibody titres are often better for protection. However, for nanobody retrieval purposes, it is preferred to induce a sufficiently high immune response for successful isolation of a diverse set of nanobodies. When giving many booster immunizations to a llama one can sometimes reach higher antibody titres, but this often is accompanied by amplification of only a subset of the nanobodies that bind to only one epitope with high affinity. Such nanobodies could not be suitable for the project objective while other, more suitable, nanobodies have lower titres after the last boosters. In such a case the last boosters were detrimental to reach the project objective. Thus, it is better to aim for a diverse antibody response that is sufficiently high for monoclonal nanobody isolation using a limited number of immunizations. In [redacted] experience three conventional immunizations with proteinaceous antigens and adjuvant or four DNA immunizations are sufficient for this purpose.
One week after the last immunization and often also 1 week after the penultimate immunization a large blood sample will be collected for isolation of peripheral blood lymphocytes for subsequent nanobody retrieval. For this purpose the llamas are fixated (without sedation) and 150 ml heparinized blood is collected from the vena jugularis.
During the immunization procedure small samples of at most 20 ml blood are collected from the vena jugularis for preparation of serum that can be used to evaluate the immunization procedure by measuring antibody titres. Serum samples are never collected more than once weekly.

References:
Baral, T.N., Murad, Y., Nguyen, T.D., Iqbal, U., Zhang, J., 2011. Isolation of functional single domain antibody by whole cell immunization: implications for cancer treatment. J Immunol Methods 371, 70-80.

Boersma, W.J., Bogaerts, W.J., Bianchi, A.T., Claassen, E., 1992. Adjuvant properties of stable water-in-oil emulsions: evaluation of the experience with Specol. Res. Immunol. 143, 503-512.
Conway, J.O., Sherwood, L.J., Collazo, M.T., Garza, J.A., Hayhurst, A., 2010. Llama single domain antibodies specific for the 7 botulinum neurotoxin serotypes as heptaplex immunoreagents. PLoS ONE 5, e8818.
Harmsen, M.M., Blokker, J.C., Pritz-Verschuren, S.B., Bartelink, W., Van der Burg, H., Koch, G., 2013. Isolation of panels of llama single-domain antibody fragments binding all nine neuraminidase subtypes of influenza A virus. Antibodies 2, 168-192.
Jahnichen, S., Blanchetot, C., Maussang, D., Gonzalez-Pajuelo, M., Chow, K.Y., Bosch, L., De Vrieze, S., Serruys, B., Ulrichts, H., Vandevelde, W., Saunders, M., De Haard, H.J., Schols, D., Leurs, R., Vanlandschoot, P., Verrips, T., Smit, M.J., 2010. CXCR4 nanobodies (VHH-based single variable domains) potently inhibit chemotaxis and HIV-1 replication and mobilize stem cells. Proc. Natl. Acad. Sci. 107, 20565-20570.

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

No statistical methods are used since this is not necessary. Two llamas are immunized with each antigen or mixture of antigens. Since llamas are outbred animals the diversity of the immune response is much higher when using different animals. More than 2 llamas are normally not needed (empirical evidence). Llamas are immunized with several different antigens, serving different purposes, in parallel (see previous section). This is an important way to minimise the number of animals.

B. The animals

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

Llamas (L. glama) are used since the methods for nanobody retrieval are well described for this species (Van der Linden et al., 2000). Llamas of at least one year old are used, since these have a sufficiently mature immune system and sufficient body weight (more than 50 kg) to assure that the blood samples taken do not cause anemia. Llamas are always housed in groups of at least two to prevent social stress. Groups of llamas are always of the same gender. Female llamas are used, since these are more amenable to handling by biotechnicians. In [redacted] 20-years of experience female llamas react with less stress when fixated for immunization or blood collection. Non-pregnant female llamas are purchased from a conventional llama supplier in The Netherlands since there are no registered llama suppliers in the Netherlands.
[redacted] is currently housing a group of six female llamas that are to be (re-)used for llama immunization. In addition to this group of six llamas, which we consider part of earlier llama immunizations, we intend to immunize a total of 20 llamas in a 5-year period.

References:
Van der Linden, R., De Geus, B., Stok, W., Bos, W., Van Wassenaar, D., Verrips, T., Frenken, L., 2000. Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama. J. Immunol. Methods 240, 185-195.

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.

Llamas that are re-used will get a 6 month rest period between successive immunization cycles. Re-use is done for a maximum of four immunization cycles, where each cycle consists of a series of immunizations with 3-4 week intervals.

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

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: Nanobodies can only be retrieved from camelids and llamas are a suitable species for nanobody retrieval due to their relative ease of handling and well described procedures for nanobody retrieval. Good (potent, high-affinity, stable, specific) nanobodies are best retrieved from llamas immunized with the antigen of interest. In principle it is possible to retrieve antigen binding nanobodies from non-immunized animals. This is accomplished by isolating PBLs from many (>10) llamas and creating very large nanobody phage display libraries from these PBLs, resulting in so-called naïve libaries. Sometimes, further diversity can be introduced in such libraries by molecular biological techniques, resulting in synthetic naïve libraries. In general the antigen binding nanobodies isolated from such libraries are of lesser quality (potency, affinity, stability, specificity) than those isolated from immune libraries. For this reason, such approaches are only used for antigens that can not be used for immunization because they are not immunogenic or too toxic (De Meyer et al. 2014). If feasible, nanobodies are therefore preferentially retrieved by immunization of camelids.
Nurse sharks also produce a special class of single-domain antibodies that have been termed IgNAR. These, however, have a vestigial complementarity determining region 2, resulting in less diversity of antibody responses as compared to nanobodies (Stanfield et al., 2004). Also because of the difficulty in handling sharks for immunization and the higher immunogenicity of IgNAR domains upon human therapeutic use, camelids are preferred over sharks for isolation of single-domain antibodies.
Reduction: An important way to reduce animal use is to immunize llamas with multiple antigens serving different purposes in parallel, and to do repeated immunizations of llamas with novel antigen cocktails after a half-year resting period.
Refinement: In the last immuniztion [redacted] uses an adjuvant that gives less severe side reactions - Montanide IMS1312 - to reduce the secondary inflammatory reactions that are sometimes induced by the adjuvant Specol, that is used for the first two immunizations.

References:
De Meyer, T., Muyldermans, S., Depicker, A., 2014. Nanobody-based products as research and diagnostic tools. Trends Biotechnol 32, 263-270.
Stanfield, R.L., Dooley, H., Flajnik, M.F., Wilson, I.A., 2004. Crystal structure of a shark single-domain antibody V region in complex with lysozyme. Science 305, 1770-1773.

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

Llamas are getting a rest period of two weeks after transport to [redacted] to get accustomed to the new environment and the biotechnicians before they are immunized. The biotechnicians will regularly (once a week) contact the llamas to reduce stress upon future llama handlings. Female llamas are used since these show lower stress symptoms upon llama handling.
[redacted] uses an adjuvant that gives less severe side reactions - Montanide IMS1312 - for the last immunization to reduce the secondary inflammatory reactions that are sometimes induced by the adjuvant Specol, that is used for the first two immunizations. The first two immunizations are done with Specol since this adjuvant has a stronger depot effect and is proven effective in llamas. Specol can induce severe secondary inflammatory reactions. However, the long experience at the [redacted] with Specol-adjuvanted llama immunizations suggests that the level of discomfort is less severe than described in the code of practice. This could be due to the fact that the code of practice is developed for laboratory animals and not for large farm animals.
The llamas are killed at the end of the last experiment since they still contain antigens used for llama immunization at the injection sites. Since llamas are occasionally used for human consumption such a precaution is necessary to prevent intoxication of humans with antigens used for 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

In most instances llamas have never been immunized with the antigen of interest earlier and good nanobodies are not available against the target antigen. An advantage of llama immunization is that phage display libraries can be stored in the lab for future nanobody retrieval. Thus, when novel nanobodies are required for a particular project and a phage display library of a llama that was immunized with a higly related antigen is available then a novel llama immunization is not necessary. Such is the case for example when isolating nanobodies against a novel strain of influenza or foot-and-mouth disease virus, for which libraries derived from llamas immunized with different strains of the same serotype exist. Often conventional monoclonal antibodies already exist against the target antigen. However, for many applications, especially therapeutic applications, nanobodies offer many advantages over conventional monoclonal antibodies (see Project proposal) to justify nanobody isolation.

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 1.

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

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

The intramuscular injection of vaccine and blood sampling from the jugular vein with a needle induces a short pain that does not require pain relieving methods. The immunization with Specol adjuvant can result in adverse secondary reactions that can include pain. Experience at the [redacted] with Specol-adjuvanted llama immunizations suggests that the level of discomfort is less severe than described in the code of practice, and that giving painkillers is not perse necessary.

☐ Yes

I. Other aspects compromising the welfare of the animals

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

The immunization with Specol adjuvant will result in adverse inflammatory reactions that can, beside pain, include itching, encapsulated granuloma and reduced appetite, that can last for several days. A total of about 380 ml blood will be collected in a 2 month period. The llamas used (at least 1-year-old) have sufficient body weight and circulating blood volume to cope with such blood sampling without developing anemia. When llamas are housed in a meadow it is possible that unforeseen diseases may develop.

Explain why these effects may emerge.

Due to housing of llamas in the field they can develop any disease that is occurring in the environment.

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

A veterinarian will be consulted when animals show signs of disease and a proper treatment plan will be adopted.

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').

The repeated immunization with Specol adjuvant is expected to cause most discomfort to the llamas. Specol can induce severe secondary inflammatory reactions. However, the long experience at [redacted] with Specol-adjuvanted llama immunizations suggests that the level of discomfort is less severe than described in the code of practice. This could be due to the fact that the code of practice is developed for laboratory animals and not for large farm animals. The overall level of discomfort is estimated as moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

The llamas are killed at the end of the experiment since they still contain antigens used for llama immunization at the injection sites. Since llamas are occasionally used for human consumption such a precaution is necessary to prevent intoxication of humans with antigens used for immunization.

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

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.zbo-ccd.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'.	40100
1.2	Provide the name of the licenced establishment.	Stichting Dienst Landbouwkundig Onderzoek (DLO)
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: Immunization of llamas housed in contained stables for retrieving nanobodies

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.

Llamas (Lama glama) will be immunized to induce an antibody response against a specific antigen. This is done using either DNA immunisation or using a genetically modified virus or bacterium that expresses a gene encoding the antigen of interest. One week after the penultimate and the last immunization a large blood sample will be collected for isolation of peripheral blood lymphocytes. These will form the source for cloning of the polyclonal nanobody repertoire

into vectors suitable for subsequent isolation of nanobodies that bind to specific antigens used for llama immunization. Most commonly these vectors are phagemids suitable for phage display selection.

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

The basic llama immunization procedure described here is immunization with DNA or with genetically modified microorganisms that express the antigen of interest.
Llamas are immunized by DNA immunization using plasmids commonly used for DNA immunization of mammals, such as pVR1012-derived plasmids. These encode the gene of interest controlled by the human CMV promoter/enhancer region and the bovine growth hormone polyadenylation signal. DNA immunization is done by administering 50 microgram of plasmid in 1 ml Tris-EDTA buffer intramuscularly into the neck at four spots (i.e. 0.25 ml per spot). DNA immunization is normally less effective in inducing high antibody titres, but has the advantage of being highly specific for the antigen encoded by the plasmid. DNA immunization is therefore sometimes combined with booster immunizations with proteinaceous antigens with adjuvant, which is described in animal procedure with serial number 1.
Immunization with vectored vaccines will be done with Newcastle Disease Virus (NDV) - vectored vaccines derived from the lentogenic LaSota strain (Harmsen et al., 2011) or with canarypox (ALVAC) virus (Paoletti et al., 1994). These vectors are proven effective in mammals and do not cause clinical disease signs in mammals. Both vectors do not spread in mammals and can only give some local limited replication. NDV vector vaccines are given intramuscularly in a volume of at most 5 ml without adjuvant. ALVAC vectors are given intramuscularly in a volume of at most 5 ml with Carbopol adjuvant.
For llama immunization antigen cocktails are used. This can be done with as many as ten different antigens. In theory antigenic competition can decrease the immune response against particular antigens. This is often described when evaluating mixtures of antigens that protect against different diseases. However, since the aim of our experiments is not to induce high antibody titres but rather to induce sufficiently high antibody titres for nanobody retrieval, we consider antigenic competition of less importance here. There are several literature examples of successful nanobody retrieval after immunization with cocktails of proteinaceous antigens with adjuvant (Conway et al., 2010; Harmsen et al., 2013).
Since the aim of the induction of antibody responses is to retrieve antigen specific nanobodies by molecular biological techniques there is no need to induce extremely high antibody titres. This is opposed to the use of vaccines for protection of the animal against disease, where higher antibody titres are often better for protection. However, for nanobody retrieval purposes, we prefer to induce a sufficiently high immune response for successful isolation of a diverse set of nanobodies. When giving many booster immunizations to a llama one can sometimes reach higher antibody titres, but this often is accompanied by amplification of only a subset of the nanobodies that bind to only one epitope with high affinity. Such nanobodies could not be suitable for the project objective while other, more suitable, nanobodies have lower titres after the last boosters. In such a case the last boosters were detrimental to reach the project objective. Thus, we aim for a diverse antibody response that is sufficiently high for monoclonal nanobody isolation using a limited number of immunizations. In our experience three immunizations with NDV or ALVAC-vectored vaccines or four DNA immunizations are sufficient for this purpose.
One week after the last immunization and often also 1 week after the penultimate immunization a large blood sample will be collected for isolation of peripheral blood lymphocytes for subsequent nanobody retrieval. For this purpose the llamas are fixated and 150 ml heparinized blood is collected from the vena jugularis without sedation.
During the immunization procedure small samples of at most 20 ml blood are collected from the vena jugularis for preparation of serum that can be used to evaluate the immunization procedure by measuring antibody titres. Serum samples are never collected more than once weekly.

References:
De Meyer, T., Muyldermans, S., Depicker, A., 2014. Nanobody-based products as research and diagnostic tools. Trends Biotechnol 32, 263-270.
Conway, J.O., Sherwood, L.J., Collazo, M.T., Garza, J.A., Hayhurst, A., 2010. Llama single domain antibodies specific for the 7 botulinum neurotoxin serotypes as heptaplex immunoreagents. PLoS ONE 5, e8818.
Harmsen, M.M., Antonis, A., Moormann, R.J.M., Kortekaas, J., 2011. Parenteral vaccination of mammalian livestock with Newcastle disease virus-based vector

vaccines offers optimal efficacy and safety. Bioengineered Bugs 2, 1.
Harmsen, M.M., Blokker, J.C., Pritz-Verschuren, S.B., Bartelink, W., Van der Burg, H., Koch, G., 2013. Isolation of panels of llama single-domain antibody fragments binding all nine neuraminidase subtypes of influenza A virus. Antibodies 2, 168-192.
Paoletti, E., Tartaglia, J., Taylor, J., 1994. Safe and effective poxvirus vectors--NYVAC and ALVAC. Dev Biol Stand 82, 65-69.

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

No statistical methods are used since this is not necessary. Two llamas are immunized with each antigen or mixture of antigens. Since llamas are outbred animals the diversity of the immune response is much higher when using different animals. Llamas are immunized with several different antigens, serving different purposes, in parallel (see previous section). This is an important way to minimise the number of animals.

B. The animals

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

Llamas (L. glama) are used since the methods for nanobody retrieval are well described for this species (Van der Linden et al., 2000). Llamas of at least one year old are used, since these have a sufficiently mature immune system and sufficient body weight (more than 50 kg) to assure that the blood samples taken do not cause anemia. Llamas are always housed in groups of at least two to prevent social stress. Groups of llamas are always of the same gender. Female llamas are used, since these are more amenable to handling by biotechnicians. In [redacted] 20-years of experience female llamas react with less stress when fixated for immunization or blood collection. Non-pregnant female llamas are purchased from a conventional llama supplier in The Netherlands since there are no registered llama suppliers in the Netherlands.
We intend to immunize a total of 4 llamas in a 5-year period.

References:
Van der Linden, R., De Geus, B., Stok, W., Bos, W., Van Wassenaar, D., Verrips, T., Frenken, L., 2000. Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama. J. Immunol. Methods 240, 185-195.

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

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement: Nanobodies can only be retrieved from camelids and llamas are a suitable species for nanobody retrieval due to their relative ease of handling and well described procedures for nanobody retrieval. Good (potent, high-affinity, stable, specific) nanobodies are best retrieved from llamas immunized with

the antigen of interest. In principle it is possible to retrieve antigen binding nanobodies from non-immunized animals. This is accomplished by isolating PBLs from many (>10) llamas and creating very large nanobody phage display libraries from these PBLs, resulting in so-called naïve libaries. Sometimes, further diversity can be introduced in such libraries by molecular biological techniques, resulting in synthetic naïve libraries. In general the antigen binding nanobodies isolated from such libraries are of lesser quality (potency, affinity, stability, specificity) than those isolated from immune libraries. For this reason, such approaches are only used for antigens that can not be used for immunization because they are not immunogenic or too toxic (De Meyer et al. 2014). If feasible, nanobodies are therefore preferentially retrieved by immunization of camelids.
Nurse sharks also produce a special class of single-domain antibodies that have been termed IgNAR. These, however, have a vestigial complementarity determining region 2, resulting in less diversity of antibody responses as compared to nanobodies (Stanfield et al., 2004). Also because of the difficulty in handling sharks for immunization and the higher immunogenicity of IgNAR domains upon human therapeutic use, camelids are preferred over sharks for isolation of single-domain antibodies.
Reduction: An important way to reduce animal use is to immunize llamas with multiple antigens serving different purposes in parallel.

References:
De Meyer, T., Muyldermans, S., Depicker, A., 2014. Nanobody-based products as research and diagnostic tools. Trends Biotechnol 32, 263-270.
Stanfield, R.L., Dooley, H., Flajnik, M.F., Wilson, I.A., 2004. Crystal structure of a shark single-domain antibody V region in complex with lysozyme. Science 305, 1770-1773.

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

Llamas are getting a rest period of two weeks after transport to [redacted] to get accustomed to the new environment and the biotechnicians before they are immunized. The biotechnicians will regularly (once a week) contact the llamas to reduce stress upon future llama handlings. Female llamas are used since these show lower stress symptoms upon llama handling.

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

Often conventional monoclonal antibodies already exist against the target antigen. However, for many applications, especially therapeutic applications, nanobodies offer many advantages over conventional monoclonal antibodies (see Projectvoorstel) to justify nanobody isolation.
In most instances llamas have never been immunized with the antigen of interest earlier and good nanobodies are not available against the target antigen. An advantage of llama immunization is that phage display libraries can be stored in the lab for future nanobody retrieval. Thus, when novel nanobodies are required for a particular project and a phage display library of a llama that was immunized with a higly related antigen is available then a novel llama immunization is not necessary. Such is the case for example when isolating nanobodies against a novel strain of influenza or foot-and-mouth disease virus, for which libraries derived from llamas immunized with different strains of the same serotype exist.

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 1.

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

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

The intramuscular injection of vaccine and blood sampling from the jugular vein with a needle induces a short pain that does not require pain relieving methods. The immunization with Carbopol adjuvant can induce pain.

☐ Yes

I. Other aspects compromising the welfare of the animals

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

The llamas are housed in contained facilities since they are immunized with plasmids or genetically modified organisms. The immunization with Carbopol adjuvant will result in adverse reactions: itching, pain and reduced appetite, that can last for several days. However, the effects of this adjuvant are not as severe as with oil emulsion adjuvant. A total of about 380 ml blood will be collected in a 2 month period. The llamas used (at least 1-year-old) have sufficient body weight and circulating blood volume to cope with such blood sampling without developing anemia.

Explain why these effects may emerge.

According to GMO regulation llamas must be housed in contained facilities to prevent spread of genetically modified organisms into the environment.

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

Llamas receive adequate housing facilities.

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').

The llamas are housed in contained facilities. As a result they have limited ability to walk around and cannot graze. Sometimes an adjuvant is used in vectored-immunization, which does not give as severe side reactions as oil-emulsion adjuvants. The overall level of discomfort is estimated as moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

The llamas are killed at the end of the experiment since they cannot be introduced into the environment after immunization to prevent spread of genetically modified organisms into the environment.

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

5

WAGENINGEN UR
For quality of life

[redacted]
Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Dierexperimenten
Commissie DLO

DATUM
3 maart 2015

ONDERWERP
aanvraag projectvergunning
AVD40100201545

UW KENMERK
AVD40100201545

POSTADRES
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BEZOEKADRES
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INTERNET
www.wageningenUR.nl

CONTACTPERSOON
[redacted]

TELEFOON
[redacted]

E-MAIL
[redacted]

Geachte heer, mevrouw,

Onderstaand het advies van de DEC aangaande het project "Induction of antibody responses in llamas for isolation of specific antigen binding recombinant antibody fragments derived from heavy-chain antibodies (nanobodies)",

A. Algemene gegevens over de procedure

1. Aanvraagnummer: **AVD40100201545**

2. Titel van het project: Induction of antibody responses in llamas for isolation of specific antigen binding recombinant antibody fragments derived from heavy-chain antibodies (nanobodies)

3. Titel van de NTS: Opwekken van een antigeen-specifieke immuunrespons in lama's om specifieke afweerstoffen te isoleren tegen antigenen
4.
5. Type aanvraag: nieuwe aanvraag projectvergunning

6. Contactgegevens DEC:
   DEC-DLO
   [redacted]
   Secretaris: [redacted]

7. Adviestraject
   Ontvangen door DEC: 19-02-2015
   In vergadering besproken : 20-02-2015

8. Correspondentie met de aanvrager
   Datum vragen: 23-02-2015
   Strekking van de vragen:
   - De DEC heeft een aantal suggesties gedaan voor tekstuele verbeteringen
   - De DEC heeft vragen gesteld over:
     - de begrenzing van het doel van het project
     - het maximum aantal keren dat een dier hergebruikt kan worden
     - het mogelijke gebruik van pijnstillers
     - ongerief door reactie op de immunisaties

   Datum antwoorden: 24-02-2015
   Strekking van de antwoorden:
   - Er zijn tekstuele wijzigingen doorgevoerd
   - op vragen van de DEC:
     - het doel van het project is beter afgebakend
     - op proefplan niveau zal uitgewerkt worden of het mogelijk is om het aantal keren hergebruik te vergroten zonder dat het

ongerief van de dieren substantieel toeneemt, maar waardoor het aantal te gebruiken dieren kan afnemen
- op proefplanniveau zal het gebruik van pijnstillers uitgewerkt worden
- ongerief door reactie op de immunisaties is verduidelijkt.

De antwoorden hebben geleid tot aanpassing van de aanvraag.

B. Beoordeling (adviesvraag en behandeling)

1. De DEC heeft vastgesteld dat het project vergunningplichtig is (dierproeven in de zin der wet)
2. De aanvraag is een nieuwe aanvraag
3. De DEC is competent om over de aanvraag te adviseren vanuit het oogpunt van onafhankelijkheid, onpartijdigheid en beschikbare expertises.

C. Beoordeling (inhoud)

1. De DEC heeft vastgesteld dat het project uit wetenschappelijk oogpunt en vanuit het oogpunt van productiedoeleinden verantwoord is.
2. De DEC heeft vastgesteld dat de in de aanvraag aangekruiste doelcategorie in overeenstemming is met de hoofddoelstelling.
3. Het reële belang van het project, te weten het verkrijgen van een specifiek type antilichamen (nanobodies) voor humane en veterinaire diagnostiek of therapie, wordt door de DEC onderschreven.
4. De DEC stelt vast dat de expertise van de onderzoekers, de voorzieningen waar de experimenten uitgevoerd worden en de onderzoeksstrategie kunnen leiden tot het behalen van de doelstelling van het project.
5. De proeven kunnen alleen uitgevoerd worden met lama's en die kunnen niet van een geregistreerde instelling worden betrokken. Bij een deel van de proeven (indien er gewerkt wordt met GGO's) moeten de dieren achter een barrière gehuisvest worden en is hergebruik niet mogelijk. Het gebruik van GGO's als vector is soms onderdeel van de ontwikkeling van vaccins. Er kan in sommige experimenten sprake zijn van hergebruik onder bepaalde voorwaarden. Ook zal gekeken worden of pijnbestrijding toegepast kan worden om tot verdere verfijning te komen.
6. De DEC stelt vast dat een cumulatieve inschatting van ongerief als "moderate" realistisch is ingeschat en geclassificeerd. Ongerief in de experimenten zal bestaan uit hanteren, inspuiten, reactie op de immunisatie en bloedafname. Bij een deel van de proeven (indien er gewerkt wordt met GGO's) kunnen de dieren niet op conventionele wijze gehuisvest worden. Om in het welzijn van de dieren tegemoet te komen wordt in de dierverblijven kooiverrijking aangeboden en wordt er naar gestreefd eventuele stress te verminderen door de dieren vertrouwd te maken met de dierverzorgers.
7. De DEC heeft vastgesteld dat er geen alternatieven zijn om de doelstelling van het project te realiseren. Synthetisch vervaardigde nanobodies geven veel minder bruikbare resultaten.
8. De DEC heeft vastgesteld dat er optimaal tegemoet gekomen wordt aan de vereiste van vermindering van dierproeven. Er ligt geen statistische onderbouwing ten grondslag aan het aantal van twee dieren per experiment. Omdat uit ervaring is gebleken dat 1 dier vaak onvoldoende is voor een gewenste diversiteit in de immuunreactie moet een experiment met 2 dieren uitgevoerd worden en meer dieren blijkt niet nodig. Het aantal dieren voor het gehele project is realistisch ingeschat op basis van ervaringen in het verleden en de te verwachten vraag van opdrachtgevers. De DEC heeft de onderzoeker meegegeven om te onderzoeken of hergebruik van dieren meer dan 3 keer in bepaalde gevallen wetenschappelijk en vanuit welzijnsoogpunt acceptabel is. Dit zou kunnen leiden tot een verdere vermindering van het aantal proefdieren. De aanvrager beschikt over voldoende expertise om te voorkomen dat eerder gedaan onderzoek herhaald wordt.

9. De DEC heeft vastgesteld dat het project in overeenstemming is met de vereiste van verfijning van dierproeven. Het dierenwelzijn wordt geborgd door dieren te huisvesten op een wijze die zo veel mogelijk tegemoet komt aan de natuurlijke behoeften van de dieren en door kooiverrijking indien plaatsing in een weide niet toegestaan is. De DEC is overtuigd dat de dierproeven zo humaan mogelijk worden uitgevoerd.
   De DEC heeft de onderzoeker gevraagd om te bestuderen of het gebruik van pijnstilling een verdere vermindering van het ongerief zou kunnen opleveren.
   Er is geen sprake van belangwekkende milieu effecten.
10. De NTS is naar het oordeel van de DEC een evenwichtige weergave van het project, begrijpelijk geformuleerd en voldoet aan de vereisten in de herziene Wod Art. 10.a.1.7.

D. Ethische afweging

- De DEC is unaniem van mening dat het doel van het project het gebruik van proefdieren en het ongerief dat de dieren wordt aangedaan rechtvaardigt. Dit project kan een bijdrage leveren aan de ontwikkeling van humane en veterinaire medicijnen en diagnostische testen. Voor dit project zijn geen alternatieven beschikbaar. De uitvoering is verder niet in strijd met andere ethische overwegingen m.b.t. het gebruik van proefdieren.

E. Advies

De DEC adviseert unaniem om de vergunning te verlenen.

Met vriendelijke groet

[redacted]

secretaris DEC

8

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting DLO
t.a.v. [redacted]
Akkermaalsbos 59
6700 AW Wageningen

Centrale Commissie Dierproeven

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2500 EK Den Haag
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T 0900-2800028 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD40100201545

Datum 13 april 2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Bijlagen
1

Geachte heer/mevrouw,

Op 13 maart 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Induction of antibody responses in llamas for isolation of specific antigen binding recombinant antibody fragments derived from heavy-chain antibodies (nanobodies)" met aanvraagnummer AVD40100201545. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed, onder de in de vergunning opgenomen voorwaarden, op grond van artikel 10a, lid 3, van de Wet op de dierproeven (hierna de wet). U kunt met uw project "Induction of antibody responses in llamas for isolation of specific antigen binding recombinant antibody fragments derived from heavy-chain antibodies (nanobodies)" starten. De vergunning wordt afgegeven van 13 april 2015 tot en met 1 april 2020.

Procedure
Bij uw aanvraag heeft u een advies van de dierexperimentencommissie DEC-DLO gevoegd. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige

voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Het Bestuur van de Centrale Commissie Dierproeven
namens deze:

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals het bestuur van de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend:
  - Projectvoorstel
  - Niet-technische samenvatting
  - DEC-advies

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: Stichting DLO
Adres: Akkermaalsbos 59
Postcode en woonplaats: 6700 AW Wageningen
Deelnemersnummer: AVD40100

deze projectvergunning voor het tijdvak 13 april 2015 tot en met 1 april 2020 voor het project "Induction of antibody responses in llamas for isolation of specific antigen binding recombinant antibody fragments derived from heavy-chain antibodies (nanobodies)" met aanvraagnummer AVD40100201545, volgens advies van Dierenexperimentencommissie DEC-DLO.
De verantwoordelijk onderzoeker is wetenschappelijk medewerker.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 13 maart 2015.
2. de bij het aanvraagformulier behorende bijlagen:
    a. Projectvoorstel, zoals ontvangen bij brief op 13 maart 2015;
    b. Niet-technische Samenvatting van het project, zoals ontvangen bij brief op 13 maart 2015.
    c. Advies van dierexperimentencommissie d.d. 3 maart 2015 ontvangen op 13 maart 2015.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst	Voorwaarden
Immunization of llamas housed in a meadow for retrieving nanobodies	Lama (Lama glama)	16	Matig	De humane eindpunten moeten worden vastgelegd in overleg met de Instantie voor Dierenwelzijn voordat het project gestart wordt.
Immunization of llamas housed in contained stables for retrieving nanobodies	Lama (Lama glama)	4	Matig	De humane eindpunten moeten worden vastgelegd in overleg met de Instantie voor Dierenwelzijn voordat het project gestart wordt.

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast

zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.