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Radboud universitair medisch centrum
Medische Microbiologie

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268
Ministerie van Infrastructuur en Milieu
p/a RIVM/VSP/Bureau GGO
T.a.v. Loket Gentherapie, I
Postbus I
3720 BA Bilthoven

Datum	Ons kenmerk	Pagina
23 juni 2015	U15032	1 van 2

Uw kenmerk	Contactpersoon

Onderwerp
nieuwe aanvraag Deel A Gentherapie

Geachte

Bijgaand stuur ik u het aanvraagformulier voor een gentherapie studie die binnen het Radboudumc te Nijmegen zal gaan plaatsvinden. Het project is getiteld: "Safety and protective efficacy of genetically *modified Plasmodium berghei (Pb(PfCS@UIS4))* malaria parasites in healthy volunteers".

Op de bijgevoegde CD's (duplicaat) vindt u het ingevulde B4 Aanvraagformulier als Word-file (niet ondertekend) en als pdf-file (ondertekende versie) en de literatuur referenties. Tevens staat op de CD de D2 Background Information waarnaar we verwijzen in deze aanvraag.

Separaat sturen wij u per e-mail het SNIF-B formulier toe.

Ik hoop u hiermee voldoende te hebben geïnformeerd zodat de vergunning verder zonder problemen kan worden afgegeven.

Met vriendelijke groeten,

Afdelingshoofd Medische Parasitologie

Kopie aan:

Application form

Assessment of clinical research involving gene therapeutics in the Netherlands

Version 22-06-2015

Application form
Assessment of clinical study involving gene therapeutics

Part A: Bio-safety aspects
Part A Appendix 1: Points to consider in the conclusion about the possible environmental effects
Part A Appendix 2: General information (confidential part)
Part B: Patient-related aspects

If you have any questions, please get in touch with the Gene Therapy Office (E-mail: rik.bleijs@rivm.nl, phone: +31-30-2747569).

November 2010

CONTENTS

Part A. Bio-safety aspects

This part of the application form provides the information needed for the Ministry of Infrastructure and Environment (IenM) to grant the necessary licences.

All information provided in this form and the accompanying documentation constitutes part of the decision to be made and for this reason is in principle open to inspection by the public; the information will also be available for such public inspection during the procedure.

The applicant may ask for parts of the information provided to be kept confidential. In that case, the applicant must give reasons why the information is of a confidential nature as well as a convincing explanation that the lifting of confidentiality will adversely affect the applicant's competitive position. A publicly available summary of confidential information must be given, containing enough information for a clear general understanding of the application and in order to assess the risk analysis as described in the application and the decision.

An application does not need to be limited to the specific clinical protocol that the applicant wishes to perform. If there are no consequences for the risk analysis, the application can be drawn up with a wider scope, such as for a larger number of patients, an alternative way of administering the GMO, possibly in combination with other non-GMOs. Take note: such additions CANNOT be made later to a decision already given!

The aim is to draw up the final decision in such a way that several clinical protocols can be performed under it, using the information described in this application. Naturally these activities must be covered by the description of the experiment and the risk analysis provided. Before submitting such a broader application, you are advised to contact the GMO office for an informal discussion of the options.

The term 'test subjects' as used in this form means patients or volunteers taking part in the study.

Specific contact details of the person responsible for the project (contact person) and the environmental safety officer must be supplied as indicated in Part A Appendix 2:General information. The submitted data in this appendix will be kept confidential and will thus not be made publicly available in accordance with the Personal Data Protection Act.

This Notification Form may contain some questions that are not relevant to your case. We would ask you NOT to answer in your notification any questions that are irrelevant to the activities for which you are applying.

Specific issues:

- Literature that is referred to has to be sent in together with the application form.
- Confidential information has to be marked as such and has to be sent in separately.
- A SNIF B (other GMO) form has to be completed and to be sent in as an electronic file in Word format.

A1 General application details

General information

NOTE: See Appendix 1 for list of abbreviations.

A1.1. Application title:

Please give a descriptive title that provides sufficient information on the objectives and aspects relating to the genetic modification. The title should state the type of vector(s) and insertion(s) used and the nature of the application(s).

Exposure of human volunteers to live genetically modified rodent *Plasmodium berghei (Pb)* parasites, administered by mosquito bites. The transgenic strain, *Pb(PfCS@UIS4)*, contains the *Plasmodium falciparum* circumsporozoite protein (PfCS) gene inserted in the 230p neutral locus of *P. berghei* under the control of the *Pb*UIS4 promoter and expresses both the PfCS and the endogenous PbCS.

A1.2. Describe briefly the contents of the application, the objective of the study being aimed for and the intended application of the results

Describe briefly the genetically modified organisms that are to be used, or which may be produced with the application, such as through the recombination of genetic information between organisms or through the integration of genetic material in a genome. Describe the expected action of the gene products of the transgenes and give an explanation of the biological mechanism. Also describe the scientific and public importance of the study, and state how the results of the study may be used in this context.
The information provided will be used as the basis for a brief description of the study in the decision.

Malaria is one of the major infectious diseases in the world with high morbidity and lethality and tremendous impact on the quality of life, significantly contributing to ongoing poverty in endemic countries. A licensed vaccine that provides a high degree of sustained protection is not available but badly needed[1]. Despite years of effort testing a large number of (recombinant) sub-unit vaccines, only modest protection has been achieved in humans. Consequently renewing a strong interest in whole organism malaria vaccine approaches[2,3,4].

Induction of complete protective immunity in humans has been achieved by immunization with live attenuated *Plasmodium* sporozoites that invade but then completely arrest in the liver before pathogenic blood-stage parasites in the circulation[5,6,2,7,8]. Whole sporozoite immunization approaches can generate high-level (>90%) protection against malaria in humans in the controlled human malaria infection model[9]. These approaches are conducted under stringent clinical conditions through i) immunization with sporozoite forms of the parasite attenuated by irradiation or targeted gene deletion by genetic modification, or ii) when sporozoites are administered together with a chemoprophylactic dose of chloroquine[2,10]. This project builds on an alternative attenuation method by using a rodent *Plasmodium* species, *P. berghei*, that is non-pathogenic for humans due to its inability to invade and multiply in human red blood cells.

The aim of this study is to expose subjects to the transgenic strain, *Pb(PfCS@UIS4)* to determine safety and immunogenicity. Immunization will be performed by exposure of volunteers to repeated sessions of *Pb(PfCS@UIS4)*-infected mosquitoes bites. Once post-immunization safety data have been reviewed and conditionally approved, subjects will be subsequently challenged according to the standard protocol of the controlled human malaria

infection in the subsequent study; immunized volunteers will be exposed to bites of *P. falciparum*-infected mosquitoes to determine protective efficacy achieved through *Pb(PfCS@UIS4)* immunization.

The proposed mechanism of action of *Pb(PfCS@UIS4)* is either induction of cross-species protective immune responses by i) rodent *P. berghei* sporozoites against *P. falciparum,* ii) the genetically modified *P. berghei* presenting the *Plasmodium falciparum* CS protein or iii) a combination of both. Protection may be presumably mediated by *Plasmodium* specific antibodies and/or cell immune effector mechanism.

A1.3. Describe briefly the intended work.

Give here a detailed account of the activities to be carried out with the genetically modified organisms. State in chronological order which types of procedures will be carried out, and for which a licence is being applied for (e.g. production, transport, storage and administration of the vector, observation of patients, sampling, transport, storage and processing of samples, waste treatment).

Production, preparation, storage and transport of *Pb(PfCS@UIS4)*

The *Pb(PfCS@UIS4)* parasite was produced by the Leiden Malaria Research Group at the Leiden University Medical Centre (LUMC), Leiden, the Netherlands, using the "Gene Insertion Marker Out" (GIMO) technology[11]. Briefly, Blood stage *Pb(PfCS@UIS4)* parasites were transported to Instituto de Medicina Molecular (IMM), Lisbon, Portugal, in dry ice and maintained at -80 ºC until use.

Pb(PfCS@UIS4) parasites underwent three rounds of propagation through Specific Pathogenen Free (SPF) mice and *Anopheles stephensi* mosquitoes, followed by a cloning step. Briefly, SPF mice were infected with frozen blood stage *Pb(PfCS@UIS4)* parasites through intraperitonial injection. Parasitemia was allowed to progress to approximately 4-5% of total peripheral erythrocytes and gametocytemia was recorded. Mosquitoes were subsequently allowed to feed directly on an anesthesized infected mouse. Infected mosquitoes were maintained in controlled temperature and humidity conditions for at least twenty-one days, after which mosquitoes were allowed to bite back on SPF mice. The cycle was repeated and parasites propagated another two times. Blood stage parasites of these cultures were genotyped using a diagnostic PCR for the correct genotype and absence of wild type genotype. Whole genome sequencing of the parasites was performed before and after these propagation rounds to evaluate genome stability and purity, and to determine the gene sequence of the final parasite stabilates intended for mosquito infection. Microbiological analyses of blood samples of the mice used for parasite propagation were performed to verify the absence of microbiological contaminations. The cloned stabilate was expanded by collection of infected red blood cells from 20 infected SPF mice and finally used to construct a Master Cell Bank for this study. Frozen stabilates of infected erythrocytes from the Master Cell Bank were transported in dry ice to the Parasitology Research Unit of RIMLS, Radboudumc and kept in liquid nitrogen until use on SPF mice to infect mosquitoes. The infected mosquitoes will be kept in the Central Animal Facility (CAF) of the Radboudumc. The infected mosquitoes are kept in CAF using standard procedures of maintaining Pf-infected mosquitoes as described in GMO permission IG97-018 and are kept in mosquito curtains surrounded cages to prevent contamination with other infected mosquitoes. A detailed description of the methods to prevent mosquito escape is described in A4 risk analysis.

Infection of mosquitoes with *Pb(PfCS@UIS4)*

Mosquitoes intended for immunization of human volunteers with *Pb(PfCS@UIS4)* will be infected by biting on SPF mice infected with the frozen blood stages of the parasite at the

Parasitology Research Unit of RIMLS, Radboudumc. The SPF mice are kept in a SPF unit in the Central Animal Facility (CAF) of the Radboudumc (DM-II protocol). Doors are always locked during proceedings. A detailed description of the methods to prevent SPF mice escape is described in A4 risk analysis.

Administration of *Pb(PfCS@UIS4)* to human volunteers
Inoculation of human volunteers by the bite of *Pb(PfCS@UIS4)*-infected mosquitoes are similar to procedures used for Controlled Human Malaria Infections (CHMI) studies previously carried out at Radboudumc with *P. falciparum* parasites[10,9,12] and is described in greater detail in the clinical trial protocol. All infection procedures and immediate monitoring of the infected test subjects 30-60 minutes after exposure will be carried out in the Radboudumc.
Briefly, mosquitoes will be used for inoculation of the parasite between 20 and 28 days after they have been infected into human volunteers. Inoculation of human volunteers will be performed by allowing mosquitoes to feed on the forearms of the volunteers through a small contained mosquito cage. Directly after the feed, a sample of the mosquitoes will be dissected by a technician of the mosquito unit. This will be done to ensure that the blood meal has taken place and that the salivary glands of the mosquitoes that took a blood meal contained sporozoites. If necessary, the procedure will be repeated until the required number of infective mosquito bites is reached. The mosquitoes are killed with carbon dioxide and discarded according to standard malaria unit procedures (ML-II). After inoculation, volunteers will be monitored as described in the clinical trial protocol and briefly described below.

Observation of patients, sampling and storage
Study subjects will be monitored as described in detail in the clinical trial protocol. Briefly, observation of the volunteers will take place on an outpatient basis, following a pre-defined schedule. Observation will be initiated immediately after the first immunization with *Pb(PfCS@UIS4).*

- Samples will be collected at pre-defined intervals, as detailed in the clinical trial protocol. Blood samples will be collected following standard hospital procedures and regulations.
- Safety measurements are performed at the licensed clinical hematology and clinical chemistry laboratory of the trial centre.
- Blood of *Pb(PfCS@UIS4)*-infected test subjects for further investigations including blood stage parasitemia by thick smear or PCR will be handled, processes and stored as performed in previous CHMI trials.

Waste treatment
For waste treatment refer to the procedure of medical waste collection and treatment in the facility. Discard as medical waste in a blue SZA-bin UN 3291 and transport to the ZAVIN for immediate incineration.

The SPF mice will be killed and discarded according to the DM-II protocol (containment level according to GMO regulations for genetically modified animals in association with genetically modified micro-organisms) after the mosquitoes have been fed on the mice.

The clinical studies with *Pb(PfCS@UIS4)* comprises two phases:
phase 1: To evaluate the safety profile of *Pb(PfCS@UIS4)* administered to test subjects by mosquito bites.

phase 2: To evaluate the ability of *Pb(PfCS@UIS4)* to generate protective immunity against controlled malaria infection. Test subjects will be immunized by sessions of exposure to *Pb(PfCS@UIS4)*-infected mosquito bites. Immunization will be followed by a controlled infection with WT *Pf* parasites/sporozoites, administered by mosquito bites according to well established protocols for Controlled Human Malaria Infection (CHMI)[9,12].

A1.4. Intended start and end date:

The decision must state a time period within which the procedures will be carried out, and so an end date must be given. The chosen end date will be included in the decision. It is possible to obtain an extension to the decision; please note, however, that any extension procedure must be completed before the decision end date has passed. Instead of the end date, a maximum number of test subjects may also be stated. In that case, the decision end date will correspond to the completion of the study with the last test subject.

A maximum of 30 test subjects will be recruited for a first safety study (**phase 1**). The design of subsequent immunization/challenge studies (**phase 2 studies**) will be based on the results of the safety trial. A maximum of 200 subjects will be exposed to *Pb(PfCS@UIS4)* in all trials.

A1.5. At which locations will the intended work take place?

Since the work applied for may only be carried out under the direct control of the licence holder, it is only possible to carry out work at several locations if the licence holder has full control of the way in which the work being applied for is carried out at all locations, in such a way that the licence conditions are complied with. In that case you must state for each location what work will be carried out at which address and in which building. To clarify: you must state for all activities at which location they will be carried out. Apart from the location for the clinical activities with the GMO, you must also state the location or locations of laboratories in which activities with the GMO are carried out under the terms of this licence application, such as procedures with patient samples.
In cases where central control is not possible, such as with a multi-centre study, a separate application must be submitted for each location.

The creation and storage of the Master Cell Bank of *Pb(PfCS@UIS4)* and the pre-clinical studies have been carried out in the Portugal Instituto de Medicina Molecular (IMM), Fac. Medicina, Universidade de Lisboa (BSL-2). The clinical trial will be carried out in the Radboud university medical center (Radboudumc).

- Storage of the Master Cell Bank (MCB); in certified facility of the IMM Biobank, Lisbon, Portugal (BSL-2)
- Transport of MCB aliquot in dry ice from IMM, Lisbon to Radboudumc, Nijmegen (UN 3373 is "BIOLOGICAL SUBSTANCE, CATEGORY B")

The safety and protective efficacy study will be carried out in the Radboudumc, Division of Research Medical Parasitology.

- Storage of the *Pb(PfCS@UIS4)*: in Central Animal Facility Radboudumc, ML-II classification
- Storage SPF mice: Animal Facility Radboudumc, ML-II classification
- Inoculation of SPF mice with *Pb(PfCS@UIS4)*: Central Animal Facility Radboudumc, ML-II classification
- Mosquito infection with *Pb(PfCS@UIS4)*: Central Animal Facility Radboudumc, ML-II classification

- Maintenance of infected mosquitoes: Central Animal Facility Radboudumc, ML-II classification

The following locations are identical to previously performed CHMI:

- Infection of test subjects: Centre for Clinical Malaria Studies, Radboudumc
- Monitoring and collection of blood from test subjects: Centre for Clinical Malaria Studies, Radboudumc
- Storage of blood samples of test subjects: Radboudumc, Department Research Medical Parasitology
- Analysis of blood samples test subjects for identification of parasites by thick smear analysis or PCR or for immunological purposes: Radboudumc, Division of Research Medical Parasitology

A.1.6. Do you want to keep other information confidential? If so, please specify in concrete terms how the release of the information would harm your competitive position.

Unless marked "Confidential", all the information contained in the notification and its appendices may enter the public domain when the notification is publicly processed and the decision is published.
For the sections marked "Confidential", you are requested to give a publishable summary that contains enough information to ensure a good general understanding of the notification. Furthermore, give a reason why certain information is marked "Confidential".

N.A.

Purpose of the introduction into the environment

A1.7. General purpose of the work being applied for:

Please state here the underlying (secondary) purpose of the work, such as the development of a new therapy to treat skin cancer.

Development of a safe and effective vaccine against malaria that consists of live, genetically modified parasites.

A1.8. Specific purpose of the work being applied for:

The 'primary purpose' of the project: e.g. phase 1 study, to find out how the GMO is tolerated in the test subjects receiving it.

The primary purposes of the **phase 1 safety study** are i) to investigate the tolerability of increasing doses of *Pb(PfCS@UIS4)* in test subjects and ii) confirm the absence of a blood stage parasitemia.

The primary purpose of the subsequent, **phase 2 immunization/efficacy studies**, is to analyze the protective efficacy of *Pb(PfCS@UIS4)* to subsequent Controlled Human Malaria Infection (CHMI).

Details of applicant

Only the legal entity that has final responsibility for the work to be carried out may act as the applicant. This means that the applicant will normally be the Board (management) of the hospital (institution) where the treatment will be given. The licence holder must be able to enforce compliance with the licence regulations when carrying out the work. In order to do so, it is necessary for the employees involved in the clinical procedures to come under the authority of the licence holder. For this reason, employees must be directly employed by the licence holder. In those cases where an employee does not come under the authority of the licence holder, such as where a treating doctor is part of a partnership that is independent of the licence holder, an employment contract must be arranged for carrying out work under the licence, such as through a zero-hours contract with the licence holder. A contract must be concluded with the party or parties carrying out this work for non-clinical procedures that are not carried out in the institution in question, in such a way that final responsibility continues to rest with the licence holder.

A1.9. Name of legal entity:
Radboud University Medical Center (Radboudumc)
Department of Medical Microbiology (route 268)

A1.10. Address:
Geert Grooteplein 28

A1.11. Postcode and town/city:
6525 GA Nijmegen, The Netherlands

A2 Bio-safety details

The preparation administered to the test subject may consist of a cellular organism, live or otherwise (e.g. bacteria, from a viral vector or from naked nucleic acid). Please answer the questions that relate to the preparation to be used.

State the composition of the genetically modified organism to be administered to the test subject.

Answer:

- O Viral vector (questions A2.1. to A2.14)
- O Bacterial strains (questions A2.15. to A2.27.)
- O Naked nucleic acid (questions A2.28. to A2.30.)
- **X** Other (Contact the GMO office)

LIVE Genetically modified parasites (rodent malaria parasite, *Plasmodium berghei): Pb(PfCS@UIS4)*

Parasite strains

Parasite strain from which the genetically modified organism is derived

A2.15. To which species of parasites does the strain belong that has been used to construct the GMO?

If applicable: give its full scientific name, its trivial name (e.g. the commercial name), the subspecies and collection numbers.

Plasmodium berghei ANKA, cl15cy1
A detailed description of the origin of *Plasmodium berghei* can be found in the Background information, Chapter 2: *Origin of Plasmodium berghei Parasites.*

A2.16. Is the parental strain a GMO?

If yes: give a detailed description of the genetic modification. If the strain was developed or used in the Netherlands, give the number or numbers of the licences under which the work took place. If the strain has not been used before in the Netherlands, the description must contain the same level of detail as asked for in questions A2.22-A2.27.

No.

A2.17. What is the natural niche of the parasite strain?

Describe in what niche the parental strain occurs naturally. For pathogenic or commensal bacteria, state the hosts in which they occur and, for pathogens, hosts that can act as carriers.

Plasmodium berghei is found in the forests of Central Africa, where its natural cyclic hosts are the thicket rat *(Grammomys surdaster)* and the mosquito *(Anopheles dureni).*

Host: rodents (mice and rats)
Vector: *Anopheles* mosquitoes.
Only female mosquitoes of the genus *Anopheles* can transmit *P. berghei*

A2.18. Give relevant details of pathogenicity and any attenuation and biological containment of the parental strain.

If a pathogenic strain is involved: what is the pathogenic category? Also describe the possible infection route(s).
If an opportunistic pathogen is involved: indicate the criteria by which the bacterium is classified as an opportunistic pathogen.
If an attenuated strain derived from a pathogen is involved, please describe by which criteria the strain is classified in a lower pathogenicity category.
If the organism is biologically contained in another manner, please give reasons for the biological containment.

Plasmodium berghei is a rodent-infective *Plasmodium* species that is **non pathogenic** for humans. It is the most widely used model malaria parasite species in research laboratories worldwide. In the laboratory the natural hosts have been replaced by a number of commercially available laboratory mouse strains, and various mosquito species, including *Anopheles stephensi*, which is easily reared and maintained under defined laboratory conditions.

Plasmodium berghei is stored and used under BSL1 or BSL2 containment conditions without any attenuation. Further, its safety in humans has been demonstrated in the pre-clinical studies (not yet published), by using a variety of laboratory models, including a blood-humanized mouse model to show that it is unable to develop in human erythrocytes. 'Personal communication' proves colleagues who have been working with *Plasmodium berghei* for years, are frequently stung with a *Pb*-infected mosquito, but never got sick.

A2.19. Give details of the multiplication and survival of the parasite strain in natural hosts.

Describe the generation time under natural circumstances, in the natural hosts, and any survival or spreading structures formed by the parental strain.

Figure 1 depicts the life cycle of *Plasmodium berghei* in its natural hosts. Parasites (sporozoites) are injected into their vertebrate host through the bite of an infected mosquito. In the rodent host, the cycle comprises an asymptomatic hepatic stage, where each parasite that infects a hepatocyte asexually replicates into 20.000-30.000 newly formed parasites (merozoites), and a symptomatic blood stage, where each parasite that invades a red blood cell asexually replicates into 20-30 parasites. Gametocytes formed during the blood stage of infection can be taken up by a mosquito upon a subsequent blood meal. Inside the mosquito midgut, the sexual replication of the parasite occurs, which generates hundreds of oocysts that undergo a maturation process that culminates in thousands of sporozoites migrating to the mosquito salivary glands, ready for the next transmission step.

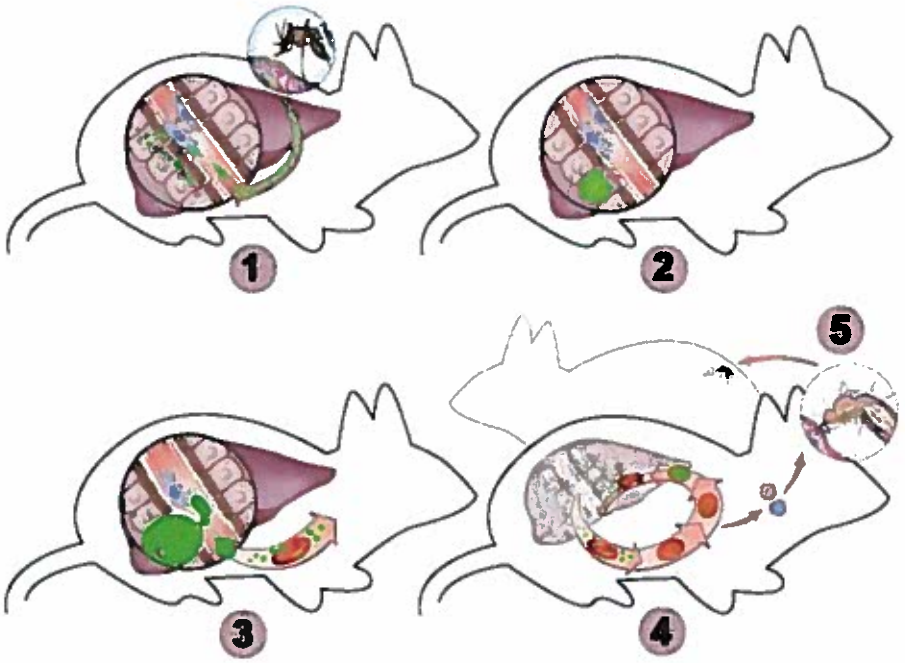


Figure 1 – Life cycle of *Plasmodium berghei*. Malaria infection is initiated when *Plasmodium* sporozoites enter the mammalian host through the bite of an infected female *Anopheles* mosquito. During a blood meal, sporozoites are deposited under the skin of the host, and migrate to the liver (**1**). There, sporozoites traverse a few hepatocytes and eventually productively invade one, with formation of a parasitophorous vacuole, initiating the **hepatic stage** of infection (**1**). Inside this vacuole, the parasites replicate extensively and develop into merozoites (**2**). Between 2 and 3 days later, thousands of merozoites per invading sporozoite are released into the bloodstream, initiating the **blood stage** of infection (**3**). Each merozoite will invade an erythrocyte, initiating a replication cycle of 24 hours that ends with the release of new merozoites from the mature infected erythrocyte (schizont), which go on to infect other erythrocytes (**4**). Malaria- associated pathology only occurs during the blood stage of infection. The *Plasmodium* life cycle continues when some merozoites develop into the sexual parasite stages, the male and female gametocytes, which can be taken up by mosquitoes during blood meals (**5**). During the **mosquito stage** of infection, gametocytes undergo fertilization and maturation in the mosquito midgut, forming an infective ookinete form that migrates through the mosquito midgut into the hemocele, developing into the oocyst in which sporozoites are formed. When fully matured, the oocysts burst and release sporozoites, which migrate into the mosquito's salivary glands, ready for the next transmission step (**5**).

A2.20. What are the possibilities for survival, multiplication and distribution in environmental circumstances other than in natural hosts?

State all observed and assumed spreading routes of the parental strain, describing the effectiveness of the spread and the role that the abovementioned distribution or survival structures play in this.

At no stage of their life cycle can *Plasmodium berghei* parasites be airborne nor can they be transmitted via contact between two vertebrate or between two invertebrate hosts.
Plasmodium berghei **sporozoites** can be collected from mosquito salivary glands but, once isolated from the mosquito, they are only viable for a few hours. Previously, a study has shown that *Plasmodium berghei* sporozoites can also be obtained *in vitro* under very specific culture conditions[13,14,15]. However follow up studies to confirm these results have not yet been performed.

Plasmodium berghei **merozoites** cannot survive or replicate outside their rodent host cells. They can be maintained in *in vitro* cultures in the laboratory only for the duration of one intra-erythrocytic cycle, as they cannot rupture and re-invade erythrocytes *in vitro*.

Plasmodium berghei-infected rodent erythrocytes can be frozen and maintained at -80°C or in liquid nitrogen. *Plasmodium berghei* blood stages can only initiate a new infection through a blood transfusion.
Plasmodium berghei **gametocytes** cannot survive outside the rodent or the mosquito hosts. They can originate sporozoites in mosquitoes or under specific *in vitro* conditions (see above).
Plasmodium berghei is **non pathogenic** to humans and we have further shown (in the, not yet published, preclinical studies) that it is unable to develop or to produce gametocytes (sexual stage parasites) in human erythrocytes, which are necessary for transmission through *Anopheles* mosquitoes Therefore a human cannot be a source for distribution in the population.

A2.21. Can the strain exchange genetic material with other organisms?

Give details of the self-transmissible elements, mobilisable plasmids, transposons or other sequences present in the strain that are involved in the distribution of DNA. Give details of the incompatibility category and the host range of these elements.

In *Plasmodium* no transposons or other transposable/transmissible genetic elements have been identified[16]. No genetic exchange has been reported between *Plasmodium* species or with genetic material from any other organism. Genetic exchange can only occur between *Plasmodium* parasites of the same species when gametes of different strains/isolates cross-fertilize. Fertilisation between gametes only occurs in the mosquito midgut.

The genetically modified parasite

A2.22. Has a vector been used in the genetic modification?

Is the vector fully or partially present in the GMO? Is the vector self-transmissible or mobilisable, or does it bear the sequences that are involved in the distribution of DNA?

The *Pb(PfCS@UIS4)* parasite (line 2266) has been generated into the background GIMO reference line 1596cl1 described in Lin et al. 2011[11] (transfection, selection and cloning of the transgenic mutant as described in this paper). Plasmid pL1988 (see section A2.23, below) has been used in the construction of parasite line 2266. This construct is not self-transmissible or mobilisable. The *Pb(PfCS@UIS4)* parasite (line 2266) has been genotyped by performing a Southern blot of separated chromosomes and showed the absence of the selectable marker cassette (GIMO transfections result in replacing the positive/negative selection cassette with the transgene). See Figure 2 for the Southern blot analysis of separated chromosomes of the 3 'independent' clones.

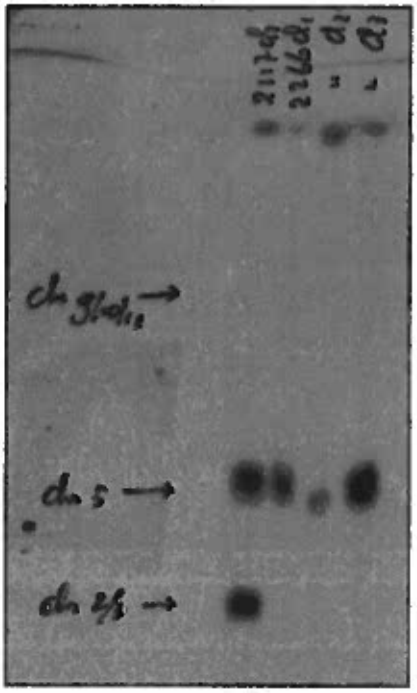


Figure 2 - Southern blot analysis of chromosomes (chrs) separated by pulsed-field gel electrophoresis (PFGE) to confirm integration of the DNA construct in the GIMO locus by showing the removal of the dhfr::yfcu SM cassette in cloned parasites. The Southern blot is hybridized with a mixture of two probes: one recognizing hdhfr and a control probe recognizing chr. 5. As a control (ctrl), parasite line 2117cl1 is used with the hdhfr::yfcu SM integrated into chr. 3. In summary, one transfection experiment (exp 2266) has been performed with one cloning experiment from the transfected and selected population (selected with negative selection). Three out of 10 mice became positive, thus 3 'independent' clones were generated.

NOTE: Please note that although no additional genotyping by Southern analysis of digested DNA or PCR was performed, the parasite will be fully sequenced before being used in humans.

A2.23. Describe in full the genetic material inserted in the parasite.

In your description please give details in particular of the following aspects:

- *Regulatory sequences, such as promoter, terminator and enhancer sequences;*
- *Structural genes;*
- *Function of the coded proteins in the donor organism (the organism from which the gene was originally isolated or where it naturally occurs is referred to as the donor organism);*
- *Whether the vector or the DNA inserted in the vector contains elements whose origin or function is unknown.*

The gene encoding *P. falciparum* circumsporozoite (CS) protein (PF3D7_0304600) as well as the *P. berghei* UIS4 (PBANKA_050120) promoter and 3'UTR regions were cloned in the pL1988 plasmid, flanked by the 230p targeting sequences (Figure 3). Plasmid pL1988 was used to insert the cloned sequences in the 230p neutral locus of the GIMO motherline PbANKA-230p;1596cl1 (Figure 4). A negative selection process was then used to obtain the genetically modified *Pb(PfCS@UIS4)* parasite (line 2266) (Figure 4). The primers used to clone the *P. berghei* UIS4 promoter and 3' UTR regions as well as the *P. falciparum* CS gene into the pL1988 plasmid are shown in Table 1. See Appendix 2 for Table 2 which lists the full sequences cloned in this plasmid.

Table 1 - Primers used to clone the *P. berghei* UIS4 promoter and 3' UTR regions as well as the *P. falciparum* CS gene into the pL1988 plasmid

Gene name	Gene product	Primers
Promoter regions		
uis4	PBANKA_050120	TATCCTGCAGGGTGATAGTGTAGATTTTTTTGTTTGAC/ ATAAGAATGCGGCCGCAGACGTAATAATTATGTGCTGAAAGG
3' UTR regions		
uis4	PBANKA_050120	CGGATATCTATAATTCATTATGAGTAGTGTAATTCAG/ GGCCGGTACCTTTCGCTTTAATGCTTGTCATC
P. falciparum transgene		
PfCSP	PF3D7_0304600	ATAAGAATGCGGCCGCCAATTCATGATGAGAAAATTAGC/ GTGTCACCGGCGAGATGTGTTCTTTATCTAATTAAGG

A2.24. Summarise the details under 2.22 and 2.23 in a diagram (map) of the genetically modified organism.

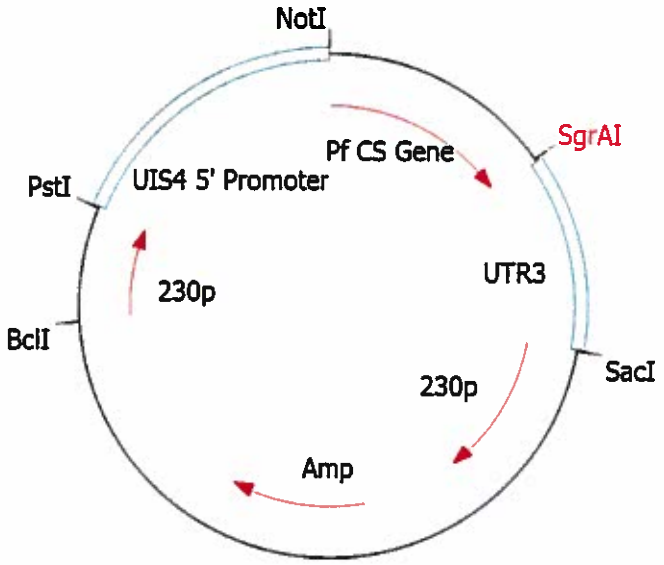


Figure 3 - The pL1988 plasmid (8067 bps)

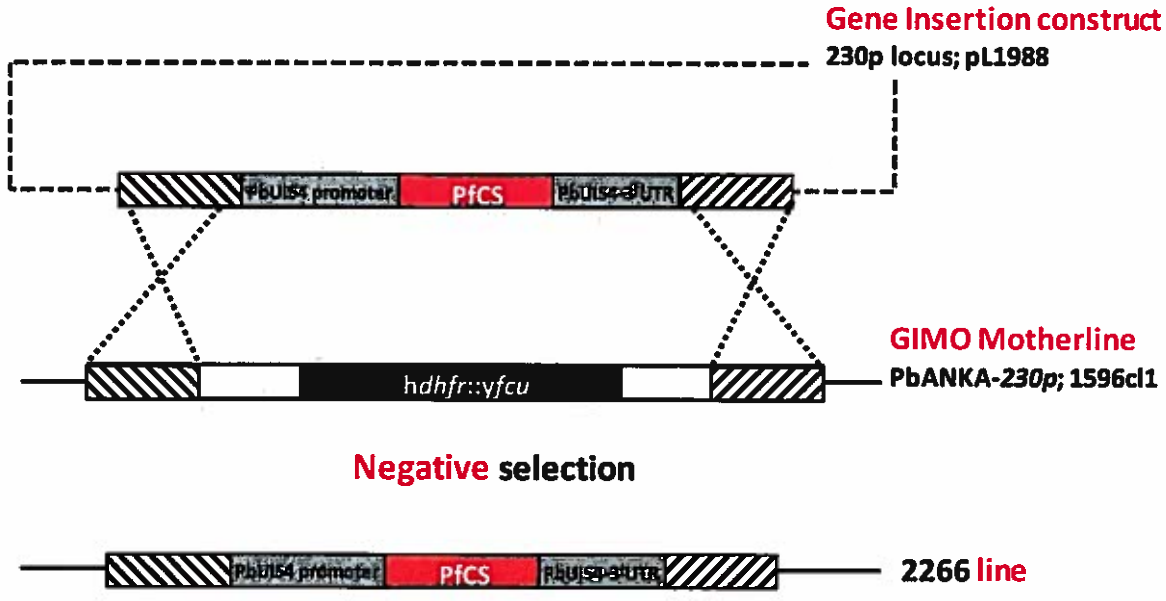


Figure 4 - Construction of the genetically modified *Pb(PfCS@UIS4)* parasite (line 2266) by the GIMO method.

A2.25. Which physiological (including pathogenic) effects can the genetically modified parasite cause; which treatments are available?

State which new physiological processes may occur through the use of the GMO in the host and to which phenotypes this may lead. Also describe to what extent the genetic modification affects the pathogenic characteristics of the GMO. As far as any pathogenic characteristics are concerned, please make a comparison with the pathogenic characteristics of the parental strain, taking into account pathogenic characteristics that may occur specifically through the action of the genetic modification.

The genetically modified *Pb(PfCS@UIS4)* parasite is expected to behave in a physiologically similar manner to the parental *Plasmodium berghei* strain and, like the latter, to be non-pathogenic to humans. The genetic modification introduced (insertion of the *Plasmodium falciparum* circumsporozoite protein gene in the 230p neutral locus of *Plasmodium berghei*, under the control of the *Plasmodium berghei* UIS4 promoter) is not expected to bear any influence on the non-pathogenicity of the resulting genetically modified parasite. Firstly, the circumsporozoite protein is normally not expressed in the potentially pathogenic *Plasmodium* blood stages and therefore plays no role in the invasion of erythrocytes by these parasites; secondly, the gene was inserted under the control of the *Plasmodium berghei* UIS4 promoter, which is only active during the hepatic stages of the parasite's life cycle; thirdly, the behavior of *Pb(PfCS@UIS4)* parasite towards human red blood cells was evaluated using a blood-humanized mouse model and, like the parental *Plasmodium berghei* strain, the *Pb(PfCS@UIS4)* parasite was found to be unable to develop inside human red blood cells. Thus, no new physiological processes are expected to occur through the use of the GMO in the host, as the genetic modification is not expected to alter the non-pathogenicity of the parental strain.

Treatments for blood stage *Plasmodium berghei* parasites and *Pb(PfCS@UIS4)* are available and include chloroquine, Malarone (atovaquone/proguanil), and artemisinin derivatives or artemisinin combination therapies such as Coartem (artemeter/lumefantrine) *(see Table 3).*

We intend to use Malarone (atovaquone/proguanil) or alternatively with chloroquine or Coartem (artemether/lumefantrine) as treatment for the genetic modified rodent *Plasmodium berghei* parasite in our first-in-human trial. Malarone, chloroquine and Coartem are widely used and very effective as both an anti-malarial treatment and prophylactic agent.

Both the parental *Pb* and *Pb(PfCS@UIS4)* are comparable and sensitive to the antimalarial drug concentrations with values in the nanomolar range. This is shown in the table below (table 3), where IC50 and IC90 represent the drug concentrations that are required for 50% and 90% inhibition *in vitro.* These inhibitory concentrations are comparable to *Plasmodium falciparum* strains that are clinically known to be sensitive to these antimalarial drugs[17,18,19,20,21].

To confirm the effectiveness of Malarone, chloroquine and Coartem against *Pb(PfCS@UIS4)*, in vivo clearance assay in mice is currently being tested.

Table 3 – *Results sensitivity for anti-malaria drugs*

	IC50 (nM)		IC90 (nM)	
	Cl15Cy1	***Pb(PfCS@UIS4)***	**Cl15Cy1**	***Pb(PfCS@UIS4)***
LUMEFANTRINE	73.6 ± 34.5	45.6 ± 6.5	98.1 ± 6.5	127.4
ARTEMISININ	23.7 ± 21.6	22.18 ± 15.5	122.5 ± 97.0	88.4 ± 21.9
CHLOROQUINE	38.6 ± 13.4	43.9 ± 28.3	85.88 ± 38.2	112
MEFLOQUINE	63.72 ± 11.6	54.5 ± 19.7	175.64	98.75
ATOVAQUONE	33.7 ± 6.5	21.2 ± 10.9	81.9	93.8

Inhibition assay : SYBR Green I-based fluorescence assay
Strain: *P. berghei* ANKA cl15cy1 and *P. berghei* ANKA CsPf@UIS4 (***Pb(PfCS@UIS4)***)
Determination: IC50/IC90 represent the drug concentrations that are required for 50%/90% inhibition *in vitro*.

A2.26. State to which extent the virulence of the genetically modified parasite has or may have been changed compared to the parental strain.

When answering this question, give an explanation of the virulence of the genetically modified bacterium compared to the parental strain. Also take into account any modifications made to arrive at the parental strain.

The virulence of the genetically modified parasite is not changed compared to the parental strain (please see A2.25, above, for more details).

A2.27. State via which routes the genetically modified parasites can spread.

When answering this question, make a comparison with the parental strain. Explain whether and how the genetic modification can affect the aspects described above concerning host range and spreading routes.

The life cycle of the *Pb(PfCS@UIS4)* parasite is similar to the one of the parental *Plasmodium berghei* strain, and is depicted in Figure 1.
Sporozoites can be collected from mosquito salivary glands but, once isolated from the mosquito, they are only viable for a few hours, unless they undergo a cryopreservation process. Previously a study has shown that *Plasmodium berghei* sporozoites can also be obtained *in vitro* under very specific culture conditions[13,14,15]. However follow up studies to confirm these results have not yet been performed.

Merozoites cannot survive or replicate outside their rodent host cells. They can be maintained in *in vitro* cultures in the laboratory only for the duration of one intra-erythrocytic cycle, as they cannot rupture and re-invade erythrocytes *in vitro*. *Plasmodium berghei*-infected rodent erythrocytes can be frozen and maintained at -80°C or in liquid nitrogen. *Plasmodium berghei* blood stages can only initiate a new infection through a blood transfusion.

Gametocytes cannot survive outside the rodent or the mosquito hosts. They can originate sporozoites in mosquitoes or under specific *in vitro* conditions (see above).
Thus, *Pb(PfCS@UIS4)* can only spread via their natural route of transmission, i.e., mosquitoes that have been infected with *Pb(PfCS@UIS4)* gametocytes by feeding on *Pb(PfCS@UIS4)*-infected laboratory mice, or by blood transfusion of *Pb(PfCS@UIS4)*-infected blood from one mouse to another. Like the parental *Plasmodium berghei* strain (see section A2.20), at no stage of their life cycle can *Pb(PfCS@UIS4)* parasites be airborne

nor can they be transmitted via contact between two vertebrate or between two invertebrate hosts. *Pb(PfCS@UIS4)* is **not pathogenic** to humans and we have further shown in pre-clinical studies, that it is unable to develop or to produce gametocytes in human erythrocytes.

In conclusion: *Pb(PfCS@UIS4)* can be spread by mosquitoes that are infected with gametocytes in rodents, in the laboratory. However, and in contrast to WT *Plasmodium falciparum* parasites, when humans are infected by a *Pb(PfCS@UIS4)*-infected mosquito we expect the parasite not to develop in human erythrocytes and therefore not to reach the infectious gametocyte stage. Therefore it is highly unlikely that *Pb(PfCS@UIS4)* produce the stages that could infect a mosquito (gametocytes) and as such it is highly unlikely that they would be able to infect another person.

A3 Other information

Environment-related information originating from earlier experiments

A3.1. Describe the results originating from earlier (pre-)clinical studies with the GMO, and which are important for the environmental risk assessment.

When answering this question, please describe the results achieved with an identical or comparable GMO, to the extent that these are relevant for the environmental risk analysis of the application in question, e.g. details of shedding, duration of latent presence of the vector/the GMO, spread of the vector/the GMO and potential interaction with other micro-organisms (including viruses) are important.

Transgenic *Plasmodium berghei* parasites have been used for many years and pose no greater environmental risks than the non-pathogenic, wild-type, parental strain. The life cycle of the *Pb(PfCS@UIS4)* parasite is similar to the one of the parental *Plasmodium berghei* strain (Figure 1). The genetically modified *Pb(PfCS@UIS4)* parasite is therefore expected to behave in a physiologically similar manner to the parental *Plasmodium berghei* strain and, like the latter, to be non-pathogenic to humans. The genetic modification introduced (insertion of the *Plasmodium falciparum* circumsporozoite protein gene in the 230p neutral locus of *Plasmodium berghei*, under the control of the *Plasmodium berghei* UIS4 promoter) is not expected to bear any influence on the non-pathogenicity of the resulting genetically modified parasite. Firstly, the circumsporozoite protein is normally not expressed in the potentially pathogenic *Plasmodium* blood stages and plays no role in the invasion of erythrocytes by these parasites. Secondly, the gene was inserted under the control of the *Plasmodium berghei* UIS4 promoter, which is only active during the asymptomatic hepatic stages of the parasite's life cycle. Thirdly, the behavior of *Pb(PfCS@UIS4)* parasite towards human red blood cells was evaluated using a blood-humanized mouse model and, like the parental *Plasmodium berghei* strain, the *Pb(PfCS@UIS4)* parasite was found to be unable to develop inside human red blood cells.

The *Pb(PfCS@UIS4)* parasite is also expected to behave similarly to its wild-type counterpart in the mosquito. The parasite infects the mosquito when the latter bites on an infected rodent. The parasite then undergoes a sporogonic development phase in the mosquito midgut that culminates in the invasion of the mosquito salivary glands by sporozoites. These will persist in the mosquito throughout the maximum duration of the mosquito's lifespan, i.e., approximately 6 weeks under optimal conditions. In the rodent host, the parasite exists in the liver for approximately 48 hours prior to initiating the blood stage of infection. The duration of the persistence of the parasites in the liver is unknown, but currently under investigation. No latent forms of the parasite exist at any point of its life cycle. No interactions between the *Pb(PfCS@UIS4)* parasite and other microorganisms have been described.

NOTE: Evaluation of the *Pb(PfCS@UIS4)* in the blood-humanized mouse model is expected to be carried out in June 2015

Production of the GMO or nucleic acid preparation

A3.2. State under whose responsibility the production of the GMO or nucleic acid is carried out.

Answer:

O Production will be under the responsibility of the applicant and forms part of this licence application.

O Production will be by and under the responsibility of the applicant but does not form part of this licence application:
O A separate application for production will be submitted for contained use
X Reference is made for the production to an existing licence for contained use: IG 97-018
O Production will be under the responsibility of third parties. If production is in the Netherlands, please state the number of the relevant GMO licence. Please state if production is outside the Netherlands.

A3.3. During which steps of the production process does quality control take place, which test methods are used and how are the tests carried out?

Give an overview of the production process of the GMO or nucleic acid preparation and describe the points in the production process at which quality control takes place. State which controls are carried out and which methods are used for the controls.

A quality control protocol of the sporozoites used for immunization in human trials is outlined in Figure 5. It includes (I) the creation of a Master Bank of parasites, with complete microbiological and genetic analysis of the samples stored there. (II) The use of SPF mice, subjected to complete microbiological analysis, that would be infected with Master Bank aliquot. (III) The collection of salivary gland sporozoites injected by infected mosquitoes, followed by genetic analysis to characterize the parasite-strain identity and exclude possible contamination. The whole cycle would be performed at least once before the actual trial and the analysis in (II) and (III) would be repeated on the actual batch to be used in the trial.

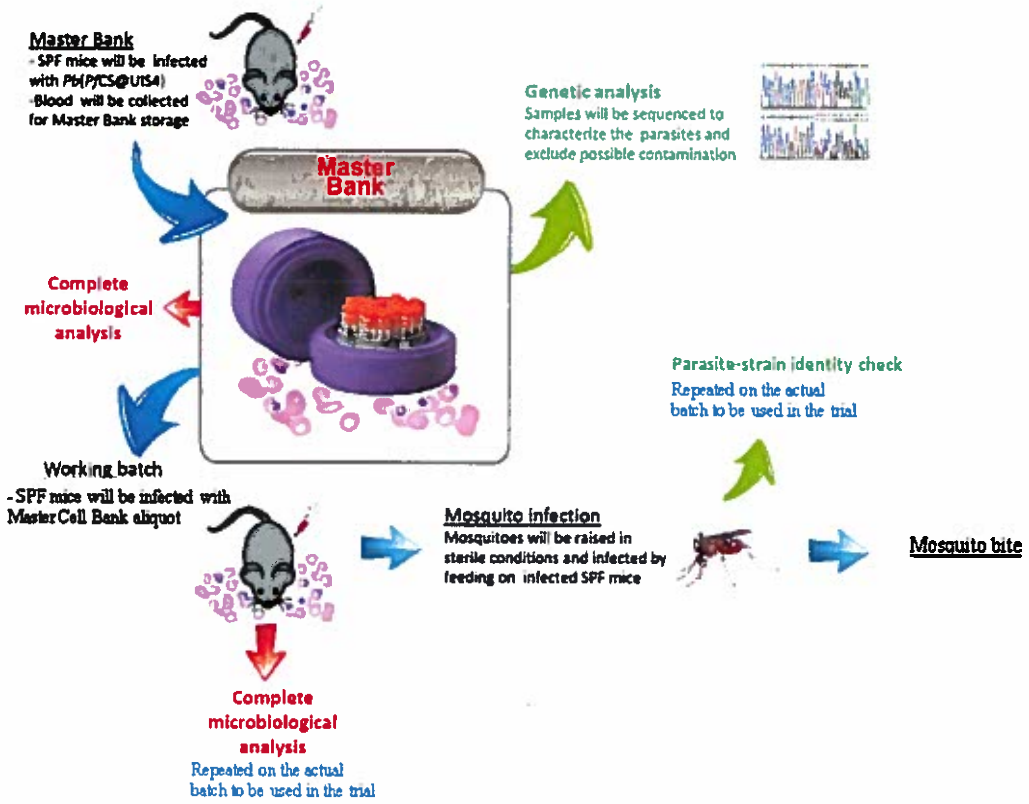


Figure 5 - Quality Control Plan for immunizing sporozoite material

Whole-genome sequencing will be performed in parasite samples prior and subsequent to cyclical propagation in SPF mice / mosquitoes. Sequencing will be carried out at the Sanger

Centre (UK).

qPCR analysis will be performed using a modified real time q-PCR analysis that we have previously developed to detect sub-microscopic levels of WT PfNF54 in the blood of test-subjects[22,23]. A similar qPCR is under development for *Pb(PfCS@UIS4)*.

Microbiological analyses will be outsourced to a certified CRO and include:

Animal quality control

SPF mice

- Import check of SPF mice (QM-diagnostics)
  (see specific pathogen free (SPF) status in Background Information; appendix 2)
- Serology for commonly blood transmitted human pathogens, would be repeated on the actual batch to be used in the trial.

Mosquitoes

- Parasite-strain identity check, would be repeated on the actual batch to be used in the trial.

Master parasite stocks control:

- Test for adventitious viruses 28 days with Vero, MRC-5, NIH-3T3, BHK-21 cells
- Bacteriostasis/Fungistasis + Sterility testing
- CLEAR PCR Comprehensive Human panel

Infection level of mosquitoes

Furthermore, the infection level of mosquitoes is assessed by counting oocysts in the midgut of mosquitoes (6-9 days) after infection of mosquitoes by feeding on SPF mice. From a pool of 10 mosquitoes, at least 40% should be infected. To subsequently assure that the mosquitoes are infectious to human test subjects, mosquito salivary glands are tested for the presence of sporozoites 20 to 28 days after feeding on mice for sporozoite production.

Pre-set criteria to determine the use of the batch are described in A3.4.

A3.4. Which criteria are imposed on a batch of the GMO before it is released for the application in question?

State which criteria are used to reject a batch.

A Master Cell Bank of the *Pb(PfCS@UIS4)* parasite was constructed and analyzed in terms of its genomic content and assayed for microbiological contamination. Whole-genome sequencing and full microbiological analyses need to demonstrate that the parasite to be used in the application in question is genetically homogeneous and that it is free from contamination with blood-borne pathogenic microorganisms (See also A3.3). SPF mice will receive complete microbiological analysis before the actual mosquito bites on the subjects. The infection level of mosquitoes is assessed by counting oocyst on the midgut in a sample of mosquitoes. The batch will be rejected if <40% of mosquitoes are infected. Rejection criteria are, therefore, the genetic heterogeneity of the parasite strain, microbiological contamination and mosquito infectivity.

NOTE: Master Cell Bank is generated and has been sequenced/analyzed for microbiological contamination. (See Background information; Appendix 4)

Aspects forming part of the study

A3.5. How many test subjects will take part in the study?

A maximum of 30 test subjects will be recruited for a first safety study. The subsequent studies will be based on the results of the safety trial. However, handing of the *Pb(PfCS@UIS4)* parasites and exposure/administration of the GMO to test subjects will be identical in all trials. A maximum of 200 subjects will be exposed in all trials.

A3.6. Which doses will be administered and at what times during the study will they be administered?

This will be a first-in-human randomized clinical trial of *Pb(PfCS@UIS4)* in healthy, malaria-naive adults. The study will consist of two phases. *(See **Figure 6** – Study Schedule)*

Phase 1: This will be a single-centre, dose-escalation trial.
A total of 18 healthy adult volunteers will be recruited over 3 groups. Three volunteers (Group1) will be exposed to bites of five *Pb(PfCS@UIS4)*-infected mosquito bites. Volunteers will be closely monitored for adverse events for a period of 28 days. When safe, a next group of three volunteers (group 2) will be exposed to 25 *Pb(PfCS@UIS4)*-infected mosquito bites. Finally, when considered safe, a group of 12 volunteers (group 3) will be exposed to 75 *Pb(PfCS@UIS4)*-infected mosquito bites. If one of the volunteers is not fit to participate in the study on day 0, an alternate included volunteer will replace him/her. For this purpose one additional volunteer will be screened for possible back up in group 1 and 2 and two additional volunteers will be screened for possible back up for group 3 respectively.

All exposed volunteers are subjected to close follow-up after exposure with regular visits to the clinical trial centre, periodic physical examinations, frequent blood sampling, and recording of adverse events in a diary. For all subjects, during this period all relevant investigations will be carried out on an outpatient basis.
All exposed volunteers will be treated with a curative regimen of Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), either at the time of detection of blood stage parasitemia by thick smear (or qPCR) or 28 days after exposure to *Pb(PfCS@UIS4)* infected mosquitoes for group 1 and 2. Volunteers of group 3 will subsequently start with phase 2, when phase 1 is considered safe, and when no blood stage parasitemia is detected during follow up. End of follow up will be 100 days after exposure to *Pb(PfCS@UIS4)* for group 1 and 2, respectively. End of follow up for group 3 will be 100 days after exposure to *P. falciparum* NF54 infection.

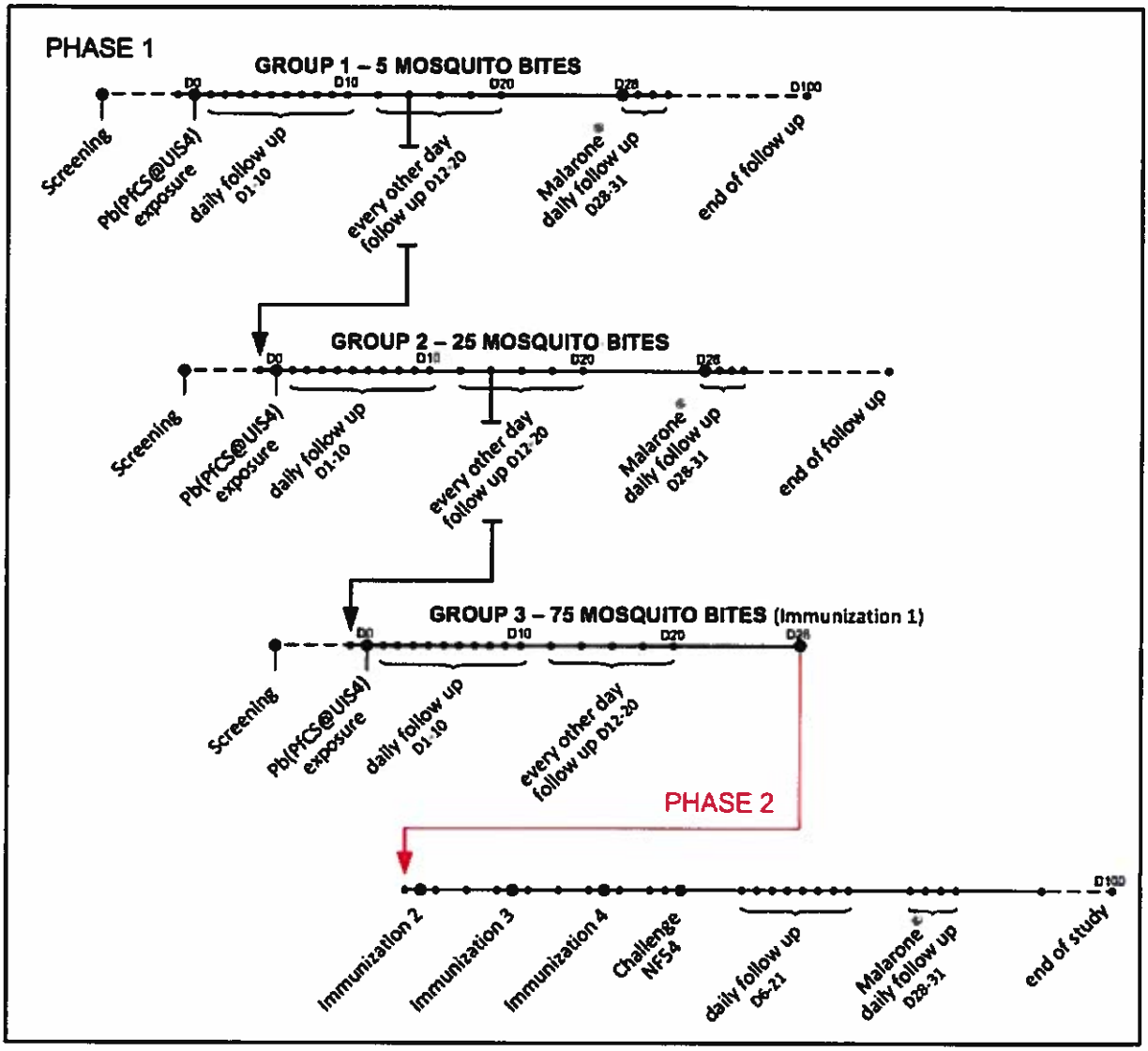
Phase 2: Design will be based on the results of the first safety trial. The trial objective is to evaluate the safety and protective efficacy of repeated exposure to *Pb(PfCS@UIS4)*-infected mosquito bites by subsequent controlled human malaria infection of test subjects. The same volunteer of group 3 will be exposed three more times to bites of 75 mosquitoes infected with *Pb(PfCS@UIS4)*, with a four to eight week interval. Three/Four weeks after the last exposure all volunteers will undergo a controlled human malaria infection with 5 *Plasmodium falciparum* (NF54)-infected mosquitoes. Six infectivity control subjects will be recruited to receive 5 infective mosquito bites.

All exposed volunteers are subjected to close follow-up after exposure with regular visits to the clinical trial centre, periodic physical examinations, frequent blood sampling and adverse events will be recorded in a diary. For all subjects, during this period all relevant investigations will be carried out on an outpatient basis, including frequent safety analyses.

Exposed volunteers will be treated with a curative regimen of Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), at the time of detection of blood stage parasitemia by thick smear during immunizations. During the challenge infection, all volunteers will receive a curative treatment, either at the

time of blood stage parasitemia by qPCR or 28 post controlled human malaria infection (CHMI). End of follow up will be 100 days after the last exposure to *Pb(PfCS@UIS4)*.
The exact number of sporozoites injected by a mosquito upon biting is unknown. However, estimations based on studies using rodent parasites, indicate that only 10-1000 (with an average of 50) are injected per mosquito bite, and the infection may double if mosquitoes are interrupted during feeding[24,25,26]. Therefore the range of sporozoite infection we predict from the bite of 75 mosquitoes that are interrupted during feeding, can range between 1500 (75 x 10 x 2) to 150 000 (75 x 1000 x 2) (= 1.5x10(3) to 1.5x10(5) sporozoites/immunization).

***Figure 6** – Study Schedule*



A3.7. How will the GMO preparation be administered to the test subject?

State via which route and how the GMO is administered. Also state what aspects may affect the safety of human beings and the environment.

The *Pb(PfCS@UIS4)* will be administered by mosquito bites using standard protocols (see Background Information, Chapter 7: Standard Operating Procedures) that are used in CHMI. The infections will be performed by placing one or three boxes containing mosquitoes on the forearm of the volunteer. Mosquito feeding will be allowed for 15 minutes. Volunteers will receive a local treatment (tripelennamine crème) for mosquito bites and will be observed for 15 minutes after the feed. Directly after the feed, a sample of the mosquitoes

will be dissected by a technician of the mosquito unit. This will be done to assure the presence of *Pb* sporozoites in the salivary glands of the mosquitoes. Exposure will be repeated until the predefined number of infected mosquito bites has been reached. The first group will be exposed to 5 mosquitoes, the second group to 20 mosquitoes and the third group will be exposed to 75 mosquito's.

A3.8. Are samples taken from the test subjects that do or may contain GMOs, and which tests are carried out with these samples?

Give an overview of the samples and state for which tests they are used. Describe how the sampling will take place. State whether GMO material is expected to be present in these samples.

Test subjects will be subjected to blood sampling at fixed time points (see **Figure 6** –*Study schedule*). Blood sampling will be performed according to the standard hospital procedures and will take place at the Clinical Research Center Nijmegen (Radboudumc).

Sample	Purpose	Laboratory	GMO expected
Blood	Safety	Clinical Haematology, clinical chemistry	Possible
Blood	Malaria diagnostics	Medical Microbiology Parasitology laboratory	Possible
Blood	Experimental procedures	Parasitology/Immunology research laboratories	Possible

It is possible that there will be GMO material present in the blood samples drawn from the test subjects. The genetically modified parasites *Pb(PfCS@UIS4)* can enter the erythrocytes but cannot develop or rupture an human erythrocyte. Our pre-clinical studies indicates that very few parasites might invade human red blood cells and degenerate into cryptic forms before 24 hours. It is therefore possible that GMO could be expected in very low quantity in blood samples taken from infected test-subjects. At any time after exposure to the genetically modified parasites *Pb(PfCS@UIS4)* blood is drawn, it will be checked for the presence of blood stage parasites.

A3.9. Will the test subject be admitted to hospital for the study and which criteria will be used for his/her discharge?

When answering this question, also state whether hospital admission is prescribed apart from medical reasons for the purpose of protection against potentially negative effects on human beings and the environment.

All test subjects will be followed on an out-patient basis as in previous CHMI studies. The anticipated adverse events, even the development of symptomatic malaria infection, are not expected to require hospitalization because of an intense out-patient follow up schedule. (In previous trials with *Plasmodium falciparum*, test subjects have been admitted to the Radboudumc only on rare occasions, when necessary for safety reasons such as monitoring after adverse events and vomiting while taking antimalarial drugs.) Should admission be necessary, subjects will always be admitted to the Radboudumc. Admission to either hospital is defined as a serious adverse event (SAE). The test subject will be discharged if deemed safe by the treating physician and treated with antimalarial treatment if needed. Test subjects are required to be reachable (24/7) by mobile telephone throughout the entire study period.

A4 Risk analysis information

Risk analysis

This is the most important aspect of the whole Notification!!

Give a detailed assessment of the expected effects of the GMO on human health and the environment on the basis of the answers to the above questions and in accordance with Appendix II of EU Directive No. 2001/18/EC and the corresponding guidance notes of the European Commission (2002/623/EC). Please take into account any direct, indirect, immediate and delayed effects of the GMP on human health and the environment.

A risk analysis should be carried out for each GMO included in this notification, as well as for combinations of the GMOs, if any.
The risk analysis must cover the effects of the GMOs that are due to interactions between the GMOs and the environment(s) where they are introduced or where they may end up under the present activities. The effects in question are those which are relevant to safety to human health and the environment.
The risk analysis should include at least the aspects mentioned in Annex 1 of this form. The risk analysis includes the following sections, which should be given in the same order as shown below (see questions A4.1 – A4.4):

1. *List of the likely adverse effects;*
2. *Estimate of the likelihood of these effects actually taking place;*
3. *Evaluation of the risks and an estimate of the severity of the effects, based on Items 1 and 2 above. The severity can be estimated by comparing it with the severity assigned to similar risks, such as for example the effects that occur with non-GMOs in similar situations ('baseline principle');*
4. *If you have concluded in Point 3 that the risk is high, you are requested to examine what measures can be used to mitigate the risk (e.g. by removing the flower-heads or consider isolation distances);*
5. *Final conclusion of the risk analysis, stating the risk management measures that will be employed, and a conclusion as to the acceptability of the risks when these measures are put into operation.*

A4.1. State according to which scenario the genetically modified organism and/or a derivate from the nucleic acid preparation can disperse from the test subject into the environment.

State in your answer the scenarios in which the GMO may disperse in the environment. Explain the level of risk that spread will actually occur. Also describe in your answer whether the number of test subjects and/or the dosage to be administered affects the risks to be identified. Please give further details in questions A4.2 to A4.3.

Pb is the wild type malaria parasite of the genetically modified *Pb(PfCS@UIS4)* and commonly used for experimental malaria infections in rodents in BSL2 laboratory. *Pb* infections can induce morbidity and lethality in rodents. The parasite cannot live freely in the environment and needs a rodent and/or mosquito host for survival. The natural mammal host for *Pb* is the thicket rat (Grammomys surdaster), Leggada bella, Praomys jacksoni and Thamnomys surdaster living in Central Africa. *Pb* does not circulate in rodent populations in the Netherlands. Pb parasites can mature and proliferate in the liver and red blood cells of rodents. Malaria parasites can be transmitted from one to a next animal by direct inoculation of Pb-infected blood or by malaria susceptible *Pb*-infected *Anopheles dureni, Anopheles stephensi* and *Anopheles gambiae* mosquitoes[27]. The *Anopheles stephensi* originates from Pakistan and is kept in a BSL2 lab for over 3 decades under

stringent laboratory conditions to prevent escape in the environment. *Anopheles stephensi, dureni* and *gambiae* are not present in natural conditions in the Netherlands. In conclusion *Pb* is not present in wild rodents or mosquitoes in the Netherlands.

Clinical *Pb* infections in human have not been reported. 'Personal communication' proves colleagues who have been working with *Plasmodium berghei* for years, are frequently stung with a *Pb*-infected mosquito, but never got sick. Preliminary in vitro and in vivo animal studies show that *Pb* can mature and develop in human liver but cannot propagate in human red blood cells. This study represents the first in human clinical trial involving infection with Pb. In *Pb(PfCS@UIS4)*, the genome of wild type *Pb* has been complemented with a *Pf* gene present in sporozoites and liver stages. Progression of the life cycle in rodents other than the liver stage and mosquitoes has not been compromised by this genetic modification.

No asexual blood stages and gametocytes will form in test subjects since *Pb(PfCS@UIS4)* cannot develop in human erythrocytes. Therefore *Pb(PfCS@UIS4)* cannot be dispersed from the test subject into the environment via blood transfusion or via *Anopheles* mosquitoes feeding on a infected test subject.

A Phase 1 safety study is planned to assess safety of human administration of *Pb(PfCS@UIS4)*, using infections with escalating numbers of bites of *Pb(PfCS@UIS4)*-infected mosquitoes. In the event of a breakthrough blood-infection the study will be terminated and will not proceed to phase 2.

Environmental Risks: In case of potential 'breakthrough blood infections', test subjects may theoretically disperse the genetically modified parasite in the environment by three routes:

A. Blood transfusion; Study subjects will not be allowed to donate blood and/or blood products (blood transfusion) during the study before standard Malarone (atovaquone/proguanil) treatment. Furthermore, study subjects are restricted to donate whole-blood after being challenged with malaria for a period of 3 years.

B. *Pb(PfCS@UIS4)*-infected *Anopheles* mosquitoes after a blood meal on a *Pb(PfCS@UIS4)* -infected study participant. The infected mosquito may infect a next person when taking a blood meal, only if infective gametocytes are presented, which is highly unlikely (See A4.1)

   1. In the Netherlands the number and density of *Anopheles* species that are susceptible to a *Plasmodium falciparum* (*Pf*) is extremely low and only present during the summer month. Indeed one possible mosquito subspecies for human malaria *(Pf)* has been identified in the Netherlands i.e. *Anopheles plumbeus* but its susceptibility to *Pb* has never been described. Therefore even if a test subject were to develop a blood infection and could develop gametocytes in their peripheral blood and will get bitten by an *A. Plumbeus* will remain extremely remote/negligible. The risk that the *Pb(PfCS@UIS4)* parasite is transmitted to a next human subject is negligible.

   2. In non-*Anopheles* mosquitoes (and other blood-sucking insects/animals) *Pb* parasites cannot survive and these mosquitoes are unable to spread the *Pb(PfCS@UIS4)*. Therefore there is no risk that this could be a route of *Pb(PfCS@UIS4)* dispersal into the environment.

   3. After mosquito bites, test subjects are monitored for the presence of asexual parasites in the blood by microscopy through thick smear analysis or by PCR. When a persistent increase of parasites is detected in the blood over a period of 48-hours, test subjects are removed from the trial, and immediately treated with Malarone

(atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), resulting in removal/killing of all parasites.

C. Needle-incident: Chances of getting a malaria infection after a needle stick incident depends on the density of parasites in the blood and the amount of blood transfused. The expected density of parasites in the blood is expected to be low. In the case needle-stick incidents occur, the risk of infection after the specific incident will be assessed by thick smear or PCR of the donor blood. In case the thick smear/PCR is positive, the victim will be advised, in consultation with the treating physician to either start pre-emptive treatment with anti-malarial drugs.

In CHMI in the Netherlands nearly 300 test subjects have been infected under the same conditions and dispersion of the WT Pf parasite from the test subjects into the environment has never occurred. (studies currently performed in this facility are NL48301.091.14 and NL48732.091.14). Further, CHMI experiments performed in >3000 subjects with the parental *Pf* strains worldwide (USA, UK[2]) have also not resulted in infections in people not enrolled in these studies.

Environmental Risks: Other risks of potential spread of *Pb(PfCS@UIS4)*:

- Infected mosquito escape: Mosquitoes are reared in a designated climate-controlled culture room. Parasite culture is located in a separate room. Infection of mosquitoes takes place in a separate climate-controlled chamber room which harbors infected mosquitoes only. Infected mosquitoes never leave this room, which is separated from the mosquito lab by a sluice. To prevent cross-contamination, different parasite strains/lines are cultured in separate rooms, mosquito-infections are performed sequentially, and mosquitoes are kept in the same cage. Cages are identified with a color label, up until their use for CHMI. Cages with mosquitoes infected with different strains are kept separately. To ensure that all procedures are performed appropriately, they are all performed by an experienced technician, and checked by a second technician present. The chamber where infectious mosquitoes are kept, is separated from the mosquito unit hallway by three doors, two mosquito nets and a wind curtain. All mosquitoes in this room are accounted for at any time they are handled. In case a mosquito escapes from its cage, emergency procedures are in place to ensure all doors are kept locked until the mosquito is caught or killed by insecticide *(see Background Information, Chapter 7: Standard Operating Procedures)*. In the event that a mosquito escapes, a red light is activated that alerts those outside the infectious unit not to enter. The mosquito is caught using a mosquito piston which is kept in the infectious mosquito unit as standard equipment. The mosquito is placed in a standard handling cage, always available within the infectious unit. Once the mosquito has been caught and placed in the cage, the red light can be deactivated. The mosquito is dissected and the midgut examined for oocysts and the salivary glands examined for sporozoites. In the case of a female mosquito that is negative for oocysts and sporozoites, these finding are noted in the calamity file and the laboratory supervisor is informed. In the case of a female mosquito that is positive for either oocysts in the midgut or sporozoites in the salivary glands, the findings are noted in the calamity file. In this case, the laboratory supervisor will inform the head of the department of medical parasitology to determine if further action is necessary.
- Radboudumc has 17 years of experience with Controlled Human Malaria Infections. In this timeframe, accidental infections have never taken place, infected mosquitoes have never escaped the culture units and mosquitoes have never been mistakenly exchanged.
- Infected rodent escape: The SPF mice are kept in a SPF unit in the Central Animal facility of the Radboudumc. Access is only possible for those who have received permission from the Biological Safety Officer (BSO) and are instructed by the

responsible employee (project staff with DM II entry, cleaning staff, caretakers). Doors are always locked during proceedings. Working in the SPF-unit of the Radboudumc is strictly followed by a DM-II protocol (Containment level according to GMO regulations for genetically modified animals in association with genetically modified micro-organisms), handling of GMO in a biological safety cabinet (Type Biowizard Koair BW 200 Silver and type Telstar, Euroflow). These methods are designed to prevent genetically modified material is distributed in the environment.

- No transfer of genetic elements of malaria parasites to other organisms have been reported.
- No transfer of genetic elements between one malaria species to another species has been reported.
- Genetic recombination/exchange between *Pb* isolates/lines can only occur after gametes which emerge from gametocytes after ingestion in a blood meal by a mosquito cross- fertilisation to form zygotes in the mosquito midgut. No exchange of genomic elements between blood-stages of different isolates have been reported in the blood of the human host or in culture.
- The parental species *Pb* (or other human parasites) is not present in either the mosquito or in the human host in the Netherlands, and therefore genetic recombination between the *Pb(PfCS@UIS4)* and wild type parasites is not possible.

In conclusion, the risk of spread of the genetically modified *Pb* parasites to the environment from either test subjects or from laboratory culture is extremely low.

A4.2. State which potentially harmful effects may be linked to exposure of human beings or the environment to the GMO.

Describe here the effects on human beings and the environment that could occur as a consequence of the use of the characteristics of the GMO described in earlier sections of this application. The aim here is identifying the hazards; the following questions deal with the chance that these hazards will actually occur.

Pb(PfCS@UIS4) infections can induce morbidity and lethality in rodents. But *Pb* and *Pb(PfCS@UIS4)* are expected non-pathogenic to humans because of their inability to propagate in the human red blood cell. Both parasites can invade the human liver but this has no clinical consequences as the symptomatic malaria starts only after invading the red blood cells.

The sporozoite and liver phase of *Pb* and *Pb(PfCS@UIS4)* development is non-pathogenic to humans, it is therefore expected that administration of the *Pb(PfCS@UIS4)* sporozoites will induce no serious adverse events or harmful effects.

There has never been a human study with *Pb* or *Pb(PfCS@UIS4)*. 'Personal communication' proves colleagues who have been working with *Plasmodium berghei* for years, are frequently stung with a *Pb*-infected mosquito, but never got sick.
In similar previous studies with *Plasmodium falciparum* (*Pf*) no severe or serious adverse events have been observed in humans that were infected with fully (radiation) attenuated sporozoites, inoculated either by intravenous injection or by mosquito bites[6,7,8].

In test subjects infected/immunized with *Pb* or *Pb(PfCS@UIS4)* no asexual blood stages and gametocytes are formed since the parasite cannot develop in human erythrocytes.

After mosquito bites test subjects are monitored for the presence of asexual parasites in the blood (thick smear analysis or PCR). When a persistent increase of parasites are detected in the blood over a period of 48-hours, test subjects are removed from the trial, and immediately treated with Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), resulting in removal/killing of all parasites. Adverse events are not expected to be aggravated due to the genetic attenuation. In the case of a break-through infection, adverse events are most likely similar as compared to previous CHMI studies.

Volunteer Risk: *Pb(PfCS@UIS4)* parasites will be administered by mosquito bites and no specific AEs are to be expected other than local reactivity due to the mosquito bites. Possible complications:

1. In case of a highly unlikely blood stage breakthrough infection, study subjects may present with aspecific symptoms of uncomplicated malaria; Blood will already be collected for qPCR (under development) according to protocol but extra samples will be obtained in case of suspicion of blood stage infection for diagnosis; when positive, patient will be treated with a curative standard regime of Malarone (atovaquone/ proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine). The risk will be remote and part of the primary study objective.
2. Persistence of *Pb(PfCS@UIS4)* parasites in the liver; These genetically modified parasites will arrest development in the liver. Intact wild type parasites disintegrate and disappear in rodent liver tissue over a short period of time. In human liver this is unknown. The duration and persistence of *Pb(PfCS@UIS4)* are not expected to be different but also unknown. All volunteers will be routinely treated with a curative dose of Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine).
3. Contamination of *Pb(PfCS@UIS4)* with other micro-organisms/infectious agents through SPF mice. The *Pb(PfCS@UIS4)* line to be used in this study has firstly undergone three consecutive cycles of propagation through *Anopheles* mosquitoes and SPF C57BL/6 mice in IMM, Lisbon, Portugal, to further reduce the risk of any contaminants. The *Pb(PfCS@UIS4)* line is completely sequenced prior to, and immediately following, this cyclical propagation process. The parasite resulting from this process is further analyzed for microbiological contamination (see Background information, Appendix 4) and used to construct a parasite Master Cell Bank, which is stored in the certified facilities of the IMM Biobank. Frozen stabilates of infected erythrocytes of the Master Cell Bank are then transported in dry ice to the Parasitology Research Unit of RIMLS, Radboudumc and kept in liquid nitrogen until inoculation of SPF mice for the infection of mosquitoes for this study.

Environmental Risks of potential spread of *Pb(PfCS@UIS4)*

(i.e. infection of a human by an infected mosquito):

No transfer of genetic elements of malaria parasites to other organisms have been reported. No transfer of genetic elements between one malaria species to another species has been reported.

Genetic recombination/exchange between *Pb* isolates/lines can only occur after gametes which emerge from gametocytes after ingestion in a blood meal by a mosquito) cross-fertilization to form zygotes in the mosquito midgut. No exchange of genomic elements between blood-stages of different isolates have been reported in the blood of the human host or in culture.

The parental species *Pb* (or other human parasites) is not present in either the mosquito or in the human host in the Netherlands, and therefore genetic recombination between the *Pb(PfCS@UIS4)* and wild type parasites is not possible.

A4.3. Give an estimate of the chance that the adverse effects described in A4.2 could actually occur.

Give a reasoned estimate of the chance of the aspects described in A4.1 and A4.2, also taking account of the number of test subjects and the dosage.

1) *The risk of Pb(PfCS@UIS4) producing a breakthrough blood-infection.*
All preclinical assessments performed both in animal models and with the *Pb(PfCS@UIS4)* in cultured human hepatocytes or mice engrafted with human liver tissue have demonstrated that the chance that *Pb(PfCS@UIS4)*-sporozoites produce a breakthrough blood infection is extremely low/negligible.

2) *The risk that a mosquito is infected by taking a blood meal on a test subject with a breakthrough blood-infection.*

a. After mosquito bites test subjects are monitored for the presence of asexual parasites in the blood (thick smear analysis or PCR). When a persistent increase of parasites are detected in the blood over a period of 48-hours, test subjects are removed from the trial, and immediately treated with Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), resulting in removal/killing of all parasites. Therefore the risk that mosquitoes take up *Pb(PfCS@UIS4*-gametocytes during a blood meal from a test subject is highly unlikely.
b. In addition the near absence of suitable mosquitoes (*Anopheles*) and conditions (temperature) for transmission in the Netherlands further diminish the chance that the *Pb(PfCS@UIS4)* will infect a mosquito. Please also refer to section A4.1.

3) *The risk that a Pb(PfCS@UIS4)-infected mosquito, infected by a blood meal on a test subject with a breakthrough blood infection, infects a new individual.*
In the Netherlands, the *Anopheles plumbeus* can transmit *Pf*[28]. It is unknown if the *Anopheles plumbeus* can transmit *Pb*. Nevertheless, transmission of *Pb* through humans (by mosquitoes bites) has never been described and is highly unlikely as humans are not a susceptible host.

4) *The risk that test subjects develop serious adverse effects resulting from, Pb(PfCS@UIS4)-sporozoites administered by mosquito bite (i.e. sporozoite and/or liver stage pathology).*
In case of a highly unlikely blood stage breakthrough infection, study subjects may present with aspecific symptoms of uncomplicated malaria; Blood will already be collected for thick smear analysis or qPCR according to protocol but extra samples will be obtained in case of suspicion of blood stage infection for diagnosis; when positive, patient will be treated with a curative standard regime of Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine). The risk will be remote and part of the primary study objective.
Persistence of *Pb(PfCS@UIS4)* parasites in the liver; These genetically modified parasites will arrest development in the liver. Intact wild type parasites disintegrate and disappear in rodent liver tissue over a short period of time. In human liver unknown. The duration and persistence of *Pb(PfCS@UIS4)* are not expected to be different but also unknown. All volunteers with be routinely treated with a curative dose of Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine).

In conclusion, no serious adverse effects are expected to occur and an effective treatment will ensure radical cure and no persistence of parasites in the liver.

5) *The risk that an individual not enrolled in the study is infected in the laboratory with Pb(PfCS@UIS4) parasites (through needle-stick incidents or by bite of an Pb(PfCS@UIS4)-infected mosquito).*
The risk of laboratory-infection with *Pb(PfCS@UIS4)* is similar or even reduced to the risk of infection with the parental strain WT *Pf* parasite (NF54 WCB) in CHMI, because of the inability to invade and develop in human erythrocytes. Nevertheless, to prevent laboratory-infection with *Pb(PfCS@UIS4)*, the same safety procedures will be applied as defined for CHMI. In the Netherlands nearly 300 test subjects have been infected in CHMI and no laboratory infections have been reported. Further, CHMI experiments in >3000 subjects performed with the parental WT *Pf* strains worldwide (USA, UK9) have also not resulted in infections in people not enrolled in these studies.

6) *The risk of contamination of the Pb(PfCS@UIS4)line.*
The *Pb(PfCS@UIS4)* line to be used in this study has firstly undergone three consecutive cycles of propagation through *Anopheles* mosquitoes and SPF C57BL/6 mice in IMM, Lisbon, Portugal, to further reduce the risk of any contaminants. The *Pb(PfCS@UIS4)* line is completely sequenced prior to, and immediately following, this cyclical propagation process. The parasite resulting from this process is further analyzed for microbiological contamination (see Background information, Appendix 4) and used to construct a parasite Master Cell Bank, which is stored in the certified facilities of the IMM Biobank. Frozen stabilates of infected erythrocytes of the Master Cell Bank are then transported in dry ice to the Parasitology Research Unit of RIMLS, Radboudumc and kept in liquid nitrogen until inoculation of SPF mice for the infection of mosquitoes for this study. Whole-genome sequencing will be performed in parasite samples prior and subsequent to cyclical propagation in SPF mice / mosquitoes. Sequencing will be carried out at the Sanger Centre (UK). A qPCR is under development for *Pb(PfCS@UIS4)*. Microbiological analyses will be outsourced to a certified CRO and include animal quality control and master parasite stock control (see A3.3 – Quality Control). The chance of contamination is therefore highly unlikely.

A4.4. Describe the risks that could occur as a consequence of the application of the GMO, taking into account the impact of any risk management measures taken.

Describe the risks in such a way that makes clear how the risks can be reduced through risk management.

1) *The risk that a mosquito is infected by taking a blood meal on a test subject with a breakthrough blood-infection.*
This could disperse the GMO in the environment. *Pb* infections can induce morbidity and lethality in rodents. Malaria parasites can be transmitted from one to a next animal by direct inoculation of Pb-infected blood or by malaria susceptible *Pb*-infected *Anopheles dureni, Anopheles stephensi* and *Anopheles gambiae* mosquitoes[27] which are not present in the Netherlands.
Risk management: Test subjects are extensively monitored for blood infections. When a persistent increase of parasites are detected in the blood over a period of 48-hours, test subjects are removed from the trial, and immediately treated with Malarone

(atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), resulting in removal/killing of all parasites.
The risk that *Pb(PfCS@UIS4)* can disperse through mosquitoes is highly unlikely/negligible.

2) *The risk that test subjects develop serious adverse effects resulting from, Pb(PfCS@UIS4)-sporozoites administered by mosquito bite.*
Risk management: As explained above this risk is highly unlikely/negligible as there are no indications from other studies that infection of humans with sporozoites, either through mosquito bite or needle injection, induces serious adverse effects. Subjects are thoroughly monitored for symptoms and/or breakthrough infections and will receive curative treatment if such symptoms or a breakthrough infection would occur.

3) The risk that an individual not enrolled in the study is infected in the laboratory with Pb(PfCS@UIS4) parasites (through needle-stick incidents or by bite of an Pb-infected mosquito).
This could disperse the GMO in the environment. *Pb* infections can induce morbidity and lethality in rodents. *Pb* is non-pathogenic in humans.
Risk management: To prevent laboratory-infection with *Pb(PfCS@UIS4)* the same safety procedures (See Background information, Chapter 7: Standard Operating Procedures,) will be applied as defined for CHMI. This risk for laboratory infection of individuals is highly unlikely/negligible. Please refer also to section A.4.3.5

The mosquito- and parasite laboratories are located in separate rooms. The mosquito lab is equipped with net curtains and insect killers to prevent mosquitoes from escaping. Infected mosquitoes never leave the 'infectious-unit', which is separated from the rest of the mosquito lab by a sluice.

4) *The risk of contamination of the Pb(PfCS@UIS4)line.*
This could lead to the inadvertent infection of volunteers with an unknown infectious pathogen.
Risk management: The *Pb(PfCS@UIS4)* line to be used in this study has firstly undergone three consecutive cycles of propagation through *Anopheles* mosquitoes and SPF C57BL/6 mice in IMM, Lisbon, Portugal, to further reduce the risk of any contaminants. The *Pb(PfCS@UIS4)* line is completely sequenced prior to, and immediately following, this cyclical propagation process. The parasite resulting from this process is further analyzed for microbiological contamination (see Background information, Appendix 4) and used to construct a parasite Master Cell Bank, which is stored in the certified facilities of the IMM Biobank. Frozen stabilates of infected erythrocytes of the Master Cell Bank are then transported in dry ice to the Parasitology Research Unit of RIMLS, Radboudumc and kept in liquid nitrogen until inoculation of SPF mice for the infection of mosquitoes for this study.
Whole-genome sequencing will be performed in parasite samples prior and subsequent to cyclical propagation in SPF mice / mosquitoes. Sequencing will be carried out at the Sanger Centre (UK). A qPCR is under development for *Pb(PfCS@UIS4).* Microbiological analyses will be outsourced to a certified CRO and include animal quality control and master parasite stock control (see A3.3 – Quality Control).

Risk management

A4.5. Which inclusion and exclusion criteria are adopted in the selection of test subjects and what is the effect of these criteria on environmental safety?

Give an overview of the inclusion and exclusion criteria that are necessary for the protection of the environment or which criteria might possibly have an effect on safety for human beings and the environment.

Inclusion and exclusion criteria are based on those applied in CHMI.

Inclusion criteria (relevant for environmental safety)

1. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
2. Subject will remain within the Netherlands during challenge period and is reachable (24/7) by mobile telephone throughout the entire study period.
3. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to current Sanquin guidelines.
4. For female subjects: subject agrees to use adequate contraception and not to breastfeed for the duration of study.

Exclusion criteria (relevant for environmental safety)

1. For female subjects: positive urine pregnancy test at screening or prior to infection.
2. Known hypersensitivity to or contra-indications (including co-medication) for use of chloroquine and primaquine, atovaquone, proguanil or artemether-lumefantrine.
3. Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.

A4.6. What limitation to the scope of the study, in relation to the number of test subjects and the dosages to be used, is used as part of risk management measures?

If you have already answered that the number of test subjects, the dosage of the GMO or nucleic acid preparation affect the risks to human beings and the environment, please state whether and, if so, which measures will be taken to manage the risks relating to these aspects.

N.A.

A4.7. Describe which measures are provided for in respect of the hospitalisation of the test subject.

When answering this question, please emphasise those aspects that are important in preventing spread in the environment of the test subject. Also state which discharge criteria are to be adopted.

Test subjects are not routinely hospitalized, because they will be followed on an out-patient basis as in previous CHMI studies. The anticipated adverse events, even the development of symptomatic malaria infection, are not expected to require hospitalization because of an intense out-patient follow up schedule. Should admission be necessary, tests subjects will be checked for the presence of blood-stage parasites and treated with antimalarial drugs if necessary.

A4.8. Describe which measures will be taken to prevent the spread of the GMO to third parties (including medical personnel).

For example, give an overview of relevant (hospital hygiene) measures that will be taken.

To prevent laboratory-infection with *Pb(PfCS@UIS4)*, standard laboratory hygiene measures

will be applied (see **A4.15**). This risk for laboratory infection of individuals is highly unlikely/negligible.
Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to current Sanquin guidelines.
Test subjects are extensively monitored for blood infections. When a persistent increase of parasites are detected in the blood over a period of 48-hours, test subjects are removed from the trial, and immediately treated with Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine), resulting in removal/killing of all parasites.
The risk that *Pb(PfCS@UIS4)* can disperse through mosquitoes is highly unlikely/negligible. Please refer also to Section A.4.3.5.

A4.9. Describe what aftercare will be given if a test subject ends his/her participation in the study prematurely.

Also state to what extent the aftercare deviates from the aftercare for test subjects who have completed the entire study.

If a test subject fails to appear for a follow-up examination, extensive effort (i.e. documented phone calls and certified mail) will be undertaken to locate or recall the test subject, or at least to determine his/her health status. These efforts will be documented in the subject's CRF and source documents. Any test subject withdrawing from the study will be followed by means of an adapted check-up schedule, which will withdraw test subjects from study procedures, but not safety check-ups. All test subjects will receive a curative regimen of Malarone (atovaquone/proguanil) or alternatively chloroquine or Coartem (artemether/lumefantrine). In total nearly 300 test subjects have been included in CHMI trials in The Netherlands; so far, none have missed a safety visit.

A4.10. Describe the procedures to be followed if changes in the risk management are required for medical reasons.

Consider situations in this respect where a test subject needs to be taken out of isolation, e.g. because treatment in intensive care is required, or where unforeseen effects are observed.

N.A.

A4.11. Describe which samples can be expected to contain GMOs, and for these samples state how sampling will take place and how the samples will be processed further.

When answering the question, also state how the spread of the GMO during sampling and testing will be prevented. State which physical containment will be used with the further processing. If the activities concerned do not form part of the present licence application, you are referred to a licence for work under Contained Use.

1) *Samples during production of Pb(PfCS@UIS4), Pb(PfCS@UIS4)-infected mosquitoes and Pb(PfCS@UIS4) –infected SPF mice*
All procedures of parasite sampling during *Pb(PfCS@UIS4)* production and production of *Pb(PfCS@UIS4)*-infected mosquitoes are similar to procedures used for CHMI studies in Nijmegen using WT *Pf* parasites (NF54 WCB).

2) *Collecting of blood samples of test subjects after infection by bite of Pb(PfCS@UIS4)-infected mosquitoes*

All blood samples taken from test subjects after exposure and before antimalarial treatment can possibly contain genetically modified parasites. When drawing blood, standard hygienic hospital measures will be taken to prevent blood contact and thus spread of the GMO. In the hospital's clinical haematology and chemistry labs, standard hospital measures to prevent blood contact will be respected.

Monitoring and waste processing

A4.12. How is the GMO preparation detected after being administered?

State, if applicable, when GMO components are detected during or after administration and why detection at that particular moment of the test is regarded as important. Describe the nature of the samples that are tested, the method used and the detection limit that can be achieved.

Test subjects will be followed on an out-patient basis, according to a tight schedule as described in the Clinical Trial Protocol. All blood samples taken after exposure and before antimalarial treatment will be tested for the presence of parasites by thick smear analysis or PCR as described in section **A.3.3.**

A4.13. Describe how the monitoring will be set up to identify any spread of the GMO.

In answering this question, pay attention to the method followed, but also to the period during which any positive result can be expected in connection with the applicable scenario. Also state in this context during which period monitoring will take place.

Test subjects will be followed on an out-patient basis, according to a tight schedule. All blood samples taken after exposure and before antimalarial treatment will be tested for the presence of parasites by thick smear analysis or PCR as described in section **A.3.3.**
Test subject will be monitored for a period of 35 days after exposure to the bite of *Pb(PfCS@UIS4)*-infected mosquitoes.

A4.14. Give an overview of the nature and quantity of the waste produced and describe how the waste will be disposed of.

State which waste flows can be distinguished. State which waste flows may potentially contain the GMO and how the GMO is prevented from being released into the environment via the waste flows.

Waste flow: *Production of Pb(PfCS@UIS4)-infected mosquitoes and infection of test subjects by bite of Pb(PfCS@UIS4)-infected mosquitoes*
All procedures for infection of mosquitoes with *Pb(PfCS@UIS4)* and associated waste disposal are similar to procedures used for CHMI studies in Nijmegen using WT Pf parasite (NF54 WCB). The SPF mice will be killed and discarded according to the DM-II protocol (containment level according to GMO regulations (I97-018) for genetically modified animals in association with genetically modified micro-organisms) after the mosquitoes have been fed on the mice.

Waste flow: Blood samples of test subjects.
Handling of blood samples will be performed according to standard hospital procedures and licensed regulations:

- 000000 – Prevention of blood transmissible diseases
- 025473 – Inactivation of GMO-waste and contaminated materials.
- 023832 - GMO, manufacturing and use of – and the use of ML-I en ML-II classified facilities
- 020123 ImmGen: code of conduct

A4.15. Describe the hospital hygiene measures used to prevent the spread of the genetically modified organism.

If existing guidelines are followed, please state which ones. Additional or deviating measures should also be described.

All procedures of parasite sampling during *Pb(PfCS@UIS4)* production of *Pb(PfCS@UIS4)*-infected mosquitoes are similar to procedures used for CHMI studies in Nijmegen using WT *Pf* parasite (NF54 WCB) (studies currently performed in this facility are NL34273.091.10, NL33904.091.10, NL37563.058.11).
Venapunctures in exposed test subjects will be performed according to routine hospital procedures:

- 000000 – Prevention of blood transmissible diseases
- 025473 – Inactivation of GMO-waste and contaminated materials.
- 023832 - GMO, manufacturing and use of – and the use of ML-I en ML-II classified facilities
- 020123 ImmGen: code of conduct

In the Central Animal Facility GMO material is processed and disposed according to GMO regulations of Radboudumc and DM-II regulations.

Part A Appendix 1: Points to consider in the conclusion about the possible environmental effects

Directive 2001/18/EC Annex II under Point D.1 gives a number of aspects that should be used whenever applicable as the basis of the conclusions about the possible environmental effects of the introduction of the GMP into the environment. All these points should be taken into account when drafting the conclusions of the risk analysis.

1. **Likelihood of the GMO to become persistent and invasive in natural habitats under the conditions of the proposed release(s).**
   Since *Pb(PfCS@UIS4)* is expected not to produce blood stages or gametocytes in the test subjects and absence of the natural mosquito host in our environment, the risk that it becomes persistent and invasive in natural habits seems highly unlikely.

2. **Any selective advantage or disadvantage conferred to the GMO and the likelihood of this becoming realized under the conditions of the proposed release(s).**
   The genetic modification introduced in the WT *Pb* is not expected to give any selective advantage nor disadvantage in context of possible environmental effects. The modification (PfCS@UIS4) has only been introduced to induce the immunity and likelihood of better protection against *Plasmodium falciparum* (*Pf*). (Please refer also to the Background information, section 3).
   Like the parental *Plasmodium berghei* strain, the *Pb(PfCS@UIS4)* parasite was found to be unable to develop inside human red blood cells.
   The *Pb(PfCS@UIS4)* parasite is also expected to behave similarly to its wild-type counterpart in the mosquito vector.

3. **Potential for gene transfer to other species under conditions of the proposed release of the GMO and any selective advantage or disadvantage conferred to those species.**

   Transfer of genetic elements of malaria parasites to other organisms have not been reported and transfer of genetic elements between one malaria species to another species have also not been reported. Since the *Pb(PfCS@UIS4)* is expected not to produce blood stages or gametocytes in the test subjects, the potential for gene transfer is unlikely.

4. **Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO and non-target organisms (if applicable).**
   Due to the absence of interactions of the *Pb(PfCS@UIS4)* with other organisms the potential of immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO and non-target organisms is highly unlikely.

5. **Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GMO and persons working with, coming into contact with or in the vicinity of the GMO release(s).**
   Due to the absence of interactions of the *Pb(PfCS@UIS4)* with other subjects apart from the test subjects, there is no potential immediate and/or delayed effect.

6. **Possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from consumption of the GMO and any product derived from it, if it is intended to be used as animal feed.**
   N.A.

7. **Possible change in the current medical practice**
   None.

APPENDIX 1: LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

AE	Adverse Event
BSL	Biosafety Level
CAF	Central Animal Facility
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CHMI	Controlled Human Malaria Infection
CRF	Case Report Form
CRO	Contract Research Organization
CS	Circumsporozite
CS(P)	Circumsporozoite (Protein)
EU	European Union
GAP	genetically attenuated Parasites
GIMO	gene insertion/marker out
GMO	Genetically Modified Organisms
IV	Intravenous
NF54	Nijmegen *falciparum* strain 54
P.	*Plasmodium*
Pb	*Plasmodium berghei*
Pb(CSPk@UIS4)	Transgenic *Plasmodium berghei* parasite expressing P. knowlesi CS under the control of the *P. berghei* UIS4 promoter
Pb(PfCS)	Transgenic *Plasmodium berghei* parasite with replacement of the endogenous CS protein by *P. falciparum* CS
Pb(PfCS@UIS4)	Transgenic *Plasmodium berghei* parasite expressing *P. falciparum* CS under the control of the *P. berghei* UIS4 promoter
PbCS	*Plasmodium berghei* Circumsporozoite
PbCSP	*Plasmodium berghei* Circumsporozoite (Protein)
PbSPZ	*Plasmodium berghei* sporozoites
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PFCS	*Plasmodium falciparum* Circumsporozoite
PfSPZ	*Plasmodium falciparum* sporozoites
qPCR	Real-time Quantitative Polymerase Chain Reaction
Radboudumc	Radboud university medical center
RIMLS	Radboudumc Institute of Molecular Life Science
SAE	Serious Adverse Event
SOP	Standard Operating Procedure
SPF	Specific-pathogenen-free
Sponsor	The Sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the Sponsor, but referred to as a subsidising party.
SPZ	Sporozoites
WT	Wild type
ZAVIN	Ziekenhuis Afval Verwerkings Installatie Nederland (Hospital Waste Disposal company)

APPENDIX 2: Table 2 – Sequences cloned in the PL1988 plasmid

Element	Position	Sequence
Pf CS Gene	7-1200	ATGATGAGAAAATTAGCTATTTTATCTGTTTCTTCCTTTTTATTTGTTGAGGCCTTATTCCA
		GGAATACCAGTGCTATGGAAGTTCGTCAAACACAAGGGTTCTAAATGAATTAAATTATGA
		TAATGCAGGCACTAATTTATATAATGAATTAGAAATGAATTATTATGGGAAACAGGAAAA
		TTGGTATAGTCTTAAAAAAAATAGTAGATCACTTGGAGAAAATGATGATGGAAATAACG
		AAGACAACGAGAAATTAAGGAAACCAAAACATAAAAAATTAAAGCAACCAGCGGATGGT
		AATCCTGATCCAAATGCAAACCCAAATGTAGATCCCAATGCCAACCCAAATGTAGATCCA
		AATGCAAACCCAAATGTAGATCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA
		AATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA
		AATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA
		AATGCAAACCCCAATGCAAATCCTAATGCAAACCCAAATGCAAACCCAAACGTAGATCCT
		AATGCAAATCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATGCAAACCCC
		AATGCAAATCCTAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATGCAAACCCA
		AACGCAAACCCCAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATGCAAACCCA
		AATGCAAACCCAAATGCAAACCCCAATGCAAATCCTAATAAAAACAATCAAGGTAATGGA
		CAAGGTCACAATATGCCAAATGACCCAAACCGAAATGTAGATGAAAATGCTAATGCCAAC
		AGTGCTGTAAAAAATAATAATAACGAAGAACCAAGTGATAAGCACATAAAAGAATATTTA
		AACAAAATACAAAATTCTCTTTCAACTGAATGGTCCCCATGTAGTGTAACTTGTGGAAAT
		GGTATTCAAGTTAGAATAAAGCCTGGCTCTGCTAATAAACCTAAAGACGAATTAGATTAT
		GCAAATGATATTGAAAAAAAAAATTTGTAAAATGGAAAAATGTTCCAGTGTGTTTAATGTC
		GTAAATAGTTCAATAGGATTAATAATGGTATTATCCTTCTTGTTCCTTAATTAG
UTR3	1231-2238	TATAATTCATTATGAGTAGTGTAATTCAGAAAGAGAACAAATTGTTTTATGATAAATATA
		TTTATAAAATAAAAAGTAAGAGAAAAGGAAAAATAATGTTATAGAGAAAAAAAGGGAAA
		ACGTTATAAAAAAAATAGCATCTTAACTATTATTATTTTACTTTATGTGTGTATAAAACTTT
		TACAATATATTTTTTTATTTTGAACGTATATAAAAATATTTCCTTTAATTTGTAGTGTGC
		AAATAATTTATAAAAATAATTTTTTTAAATATAAATATATTGTCTCGAAAAGAAATGAAA
		ATGGATTCTAGCAAATAAACAAGTCTGGCTTCGAAAAGAAATATAAGGCAAAAATTATAT
		ATAATATATTTGAAAATTATATTTGTGAGAGTGTGTATTATTTTTGTTTGAAAGATAATT
		TTATGGGTTTTTGAGAGTGATTTTTAGTGTTTTATTTGTTTTTTTTTAATAATTTGTAAG
		TTTTGTCAATGTAGAGAGAATTATACTTCATAACGTATTATTTGAACGATTAAAAAATAT
		TTTTTTTAAACAGTGAAATATAAATATGAATGGAAGCAGCCGTATATGCAAACTTTTTTA
		CATATGTTGTATGCATAACATGCGTTTTTATTTTTTACGAAGTATACATAATTCCCTTTA
		AAATATAACTTTATATAATTTGTTTTTATTGTAATGGATATAGTATTGTATAAATTTTAT
		ATTTATTTCCTAAAGACTTTTTCATAAAAATTGTTGGTGTTCCAGATATATTATTTTAA
		AAGTCAATATATCAAGTTGTTATATTGTCTTAGAGTTCAAAAGTTTGTCCTAATTGAATT
		TTTTATAACTTTATAATATTTATTGTTTTACTTTGGACCATTAAGTATATATAAGTAGTA
		ACTTATTAAAAATAATAAATTATATGTATCATAATTCATGGCTTTATAGATTCTGCATTAA
		CCTTAAATATGAAAAACACAAAAATGATGACAAGCATTAAAGCGAAAG

Element	Position	Sequence
230p	5993-6547	TTCTCTTGAGCCCGTTAATGAAATAGATACAATTCATTCATGTTATATACATCTAGAACA
		TAATCTGAATATGGTTCAAGTTAAATGTCCAAAAATTATAAAAAGTGATGATATTTTTGA
		TGGTAATACCATAATAGACACCAAGGTAACATCACGAAGTAGTCAACAAAATAATTTTTA
		TTTAGAAAATACAGATGTTGAACCAGAAGAAATAGAGAAATATAAAAATATAGAATACAT
		ACCAGAAAACGATGAAGTAATGCATCTAGACAAAAAAGAAAAGCTAGATGATATATTAC
		CAGGTGTTATCATATTAGATAAAAATAAAATGTTCAAAGAAAAAGGACATTTCACTTTTGT
		TACTCCATTAATTGTAGAAAAGGTATTAATATTAAAAATATATTGTGATAATACTAAAAC
		AATAATTAATAATATGAAAGGGAAAAAAGGTATTACAGTAATAAGGATTTCTCAAAATAC
		AACAAAAAATAAATTTTATGGATGTGACTTTTCAGGTAATTCTAAAAAAACATTTTACTA
		TTCCAATGTTTATGATTTAGAAAAAAAAAATGAGTTTTGTGAAATAGAATTAAAAGAAAA
		TATAGTAGTTAGCTTAAATTGTCCAACTGGTAAAATTAATCCAAAAAATTGTTTTAGAAA
		TGTATATATAAAAAGTAATATGAATGAACAAACAACCGAAAATATAGAAAATATATTTAA
		CGAAATAAAAGTTATAGATGCAGATTATTTTATAAATAATTCATCAACCTTTTTGATGAT
		TTCCAAAATTACAAAAAAAGAGTTTGATTTTTATTGTACATGTGAAGATTATAAAACCAA
		AAATATAGGAACAATATATATTAAAAATTATGAATATCTAGATTCAAAACCTAAATATAA
		AAATAAACAAATTTCCTAT
Amp	3806-4666	ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTT
		TTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACG
		AGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGA
		AGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGT
		ATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTT
		GAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATG
		CAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGG
		AGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGA
		TCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATG
		CCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCT
		TCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCG
		CTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTC
		TCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTA
		CACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGT
		GCCTCACTGATTAAGCATTGGTAA
230p	5993-6547	ATGACATCATTTATAAATCATGGATCATATCCACTAACAATAGAATGTGGTGTAACAAAT
		GGTGGAACTAGTTATAAAAGAGCAATTATTTTATTGCATGTTCGAACTGATTTAAAAGAT
		AGACCAGTTTCATTTTGTGATTTTCGAAAAGGAGAATTATATAATTATTTGAATGCTTAT
		ACTGAAGGGGATGTATGCATAATAATTTCCAAATCAAATACAAGTTTTGGTTTTAGATGC
		CCAGTAAATACAAAAAAAATGCCAAAAAATTGTTTTACGCAAGTATATGAAAAAGGGTAT
		CTAAATGACGCCAATAAAATTAATACTAAAAATGTTATTAACTATTCATTTGAAAATCCA
		GAATATGCGCTAGCTGGTTYTAATTATACATTAACAAAATCGTATCAATTTGAATGTCAT
		TGTGTAGATAAAGAAACAGAACAAATTGTAAAAACGGTTTTAGTCAAATATGTAAATGAA
		GATGAAATATATGATTATAATGATTTTCCAATGGTGAATCACAAACCTATTATTGCACAT
		CCAAATAAAACACAT
UIS4 5'promoter	6568-8060	GTGATAGTGTAGATTTTTTTGTTTGACCATTGCATCATTGTCTTTTATTCATCGATTTTA
		TTATGTTATATATATTTTTTTAAATATCATAATATTTTTTCAATATAGCTTCTTTGAGCA
		AATACTGAACAATAAGGAATGCTTCTATGCATCCGTGGATGTATATGTACGTAATAAAAT
		AATTTTTTGTCCATAAAAATATCTTTAACAGCTGTTATACAAAAGCATTGAATTGGCTGA
		TACATTTTTTGTCGTAAATAAAATAAATGAATTAATGGTACAAAATTACAATAAAAAGGAA
		GCGAATTTATTGCATTTTGTTTAATTTATGTCCTTTCCATTTTATTAATACAATTATGGT
		ACTAGTAATTCGTATTAAACATATATTTTGCATGTACCAAATGTTTGGGAAATAAGCATA
		TGGATCCCACTAAATTTAATAATATTCTGCGATTTTTCTTATATTTACTATTAAATATAA
		TGGATTCATTTTTTGATGCATGCAATTTTTTCTTTTAATGTATTAATTAGTGTGACAATT
		TTGAAAAACTTTACTCATTTATTTATTTTCCTTATTATATTGGATTTATAATACCAGTAT

		TATTATATAAACCACATTAAATAATTTGTAATTTATTCGAGGGTATCAAAAAAAACATAT
		ACAAAATGCATATATCCACATATGGCTCATTATAGGGTGAATAAAAATGGGAACAGCAGA
		TTAATTATTATATCATGAAAGTAATGAAAAAATTTAAATTATATGGATATATACATATAT
		AATATGTTAAAAAATAATAATAAATAAATAAAATGTATTATAAATCTTATAAAATAAGTT
		TTGTTGTGAATAATCCACGAAATATGCCATAAATAGACACTGAACAAATTAGTGGTTCTT
		AATATTATTTTGGATACATGCGGATATTATCATTGTCAGATGATTTATTTTTTGTTATTT
		TTAAATTATAAATATTCATATTTTATAGTCCTCAAAAAATAGGATGTTTTATTTTTTTAT
		AGCTATATTTTATGGTTGATCCTTTCCTTTTATGGTGTTTCATAAAAATTTTATTGAGCT
		ATATACAATCCAATAAAAAAAGGCAATTGAATTTTGAAATATATTAAACTATTTTTATAA
		TAAAATAAATATAATTTTTAAATAATGTTATATTATATATAATTTTTATCTTTACACAGA
		AATTTTTTTTTAGAGTCCAATATATATAATTAGTTATATATATAGAGATATACACTACAT
		ACACCACCATAAATAATTATAAGGAAAACCAGTTATTTTAATTTTAACTGAAGAAATTAA
		ATAAATATATAAAAAAGAAAAGAACAAAAAATAAAACGACAGCAACCTTAAAATTTTATT
		ATTATTACATATTTATACATAAAAAAAATAAAATACTTAGACAAAAAATAATAAATATTA
		TAGATCGATATTAGCATACATATATACCTTTCAGCACATAATTATTACGTCTG
Complete	1-8067	CAATTCATGATGAGAAAATTAGCTATTTTATCTGTTTCTTCCTTTTTATTTGTTGAGGCC
		TTATTCCAGGAATACCAGTGCTATGGAAGTTCGTCAAACACAAGGGTTCTAAATGAATTA
		AATTATGATAATGCAGGCACTAATTTATATAATGAATTAGAAATGAATTATTATGGGAAA
		CAGGAAAATTGGTATAGTCTTAAAAAAAATAGTAGATCACTTGGAGAAAATGATGATGG
		AAATAACGAAGACAACGAGAAATTAAGGAAACCAAAACATAAAAAATTAAAGCAACCAG
		CGGATGGTAATCCTGATCCAAATGCAAACCCAAATGTAGATCCCAATGCCAACCCAAATG
		TAGATCCAAATGCAAACCCAAATGTAGATCCAAATGCAAACCCAAATGCAAACCCAAATG
		CAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATG
		CAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATG
		CAAACCCAAATGCAAACCCCAATGCAAATCCTAATGCAAACCCAAATGCAAACCCAAACG
		TAGATCCTAATGCAAATCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATG
		CAAACCCCAATGCAAATCCTAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATGC
		AAACCCAAACGCAAACCCCAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATGC
		AAACCCAAATGCAAACCCAAATGCAAACCCCAATGCAAATCCTAATAAAAACAATCAAGG
		TAATGGACAAGGTCACAATATGCCAAATGACCCAAACCGAAATGTAGATGAAAATGCTA
		ATGCCAACAGTGCTGTAAAAAATAATAATAACGAAGAACCAAGTGATAAGCACATAAAA
		GAATATTTAAACAAAATACAAAATTCTCTTTCAACTGAATGGTCCCCATGTAGTGTAACTT
		GTGGAAATGGTATTCAAGTTAGAATAAAGCCTGGCTCTGCTAATAAACCTAAAGACGAAT
		TAGATTATGCAAATGATATTGAAAAAAAAATTTGTAAAATGGAAAAATGTTCCAGTGTGT
		TTAATGTCGTAAATAGTTCAATAGGATTAATAATGGTATTATCCTTCTTGTTCCTTAATTAG
		ATAAAGAACACATCTCGCCGGTGACACATCTATAATTCATTATGAGTAGTGTAATTCAGA
		AAGAGAACAAATTGTTTTATGATAAATATATTTATAAAATAAAAAGTAAGAGAAAAGGAA
		AAATAATGTTATAGAGAAAAAAAGGGAAAACGTTATAAAAAAAATAGCATCTTAACTATTA
		TTATTTTACTTTATGTGTGTATAAAACTTTTACAATATATTTTTTTTATTTTGAACGTATA
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		ATAAATATATTGTCTCGAAAAGAAATGAAAATGGATTCTAGCAAATAAACAAGTCTGGCT
		TCGAAAAGAAATATAAGGCAAAAATTATATATAATATATTTGAAAATTATATTTGTGAGA
		GTGTGTATTATTTTTGTTTGAAAGATAATTTTATGGGTTTTTGAGAGTGATTTTTAGTGT
		TTTATTTGTTTTTTTTTAATAATTTGTAAGTTTTGTCAATGTAGAGAGAATTATACTTCA
		TAACGTATTATTTGAACGATTAAAAAATATTTTTTTTAAACAGTGAAATATAAATATGAA
		TGGAAGCAGCCGTATATGCAAACTTTTTTACATATGTTGTATGCATAACATGCGTTTTTA
		TTTTTTACGAAGTATACATAATTCCCTTTAAAATATAACTTTATATAATTTGTTTTTATT
		GTAATGGATATAGTATTGTATAAATTTTATATTTATTTCCTAAAGACTTTTTCATAAAAA
		TTGTTGGTGTTCCAGATATATTATTTTTAAAAGTCAATATATCAAGTTGTTATATTGTCT
		TAGAGTTCAAAAGTTTGTCCTAATTGAATTTTTTATAACTTTATAATATTTATTGTTTTA
		CTTTGGACCATTAAGTATATATAAGTAGTAACTTATTAAAATAATAAATTATATGTATCA
		TAATTCATGGCTTTATAGATTCTGCATTAACCTTAAATATGAAAAACACAAAAATGATGA
		CAAGCATTAAAGCGAAAGGTACCGAGCTCGAATTCTCTTGAGCCCGTTAATGAAATAGAT

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GGTATTACAGTAATAAGGATTTCTCAAAATACAACAAAAAATAAATTTTATGGATGTGAC

TTTTCAGGTAATTCTAAAAAAACATTTTACTATTCCAATGTTTATGATTTAGAAAAAAAA

AATGAGTTTTGTGAAATAGAATTAAAAGAAAATATAGTAGTTAGCTTAAATTGTCCAACT

GGTAAAATTAATCCAAAAAATTGTTTTAGAAATGTATATATAAAAAGTAATATGAATGAA

CAAACAACCGAAAATATAGAAAATATATTTAACGAAATAAAAGTTATAGATGCAGATTAT

TTTATAAATAATTCATCAACCTTTTTGATGATTTCCAAAATTACAAAAAAAGAGTTTGAT

TTTTATTGTACATGTGAAGATTATAAAACCAAAAATATAGGAACAATATATATTAAAAAT

TATGAATATCTAGATTCAAAACCTAAATATAAAAATAAACAAATTTCCTATATAGATGTA

GTTCCATACCCGCGGGGAAAGGGCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTG

GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTG

GCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATG

GCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCAT

ATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCC

GCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACA

AGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACG

CGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAAT

GGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTT

ATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCT

TCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCC

CTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAA

AGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCG

GTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGT

TCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCG

CATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTAC

GGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTG

CGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACA

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TGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTAC

ATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT

TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACG

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ACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTAT

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GATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAG

CGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCC

CGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGG

GCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTAC

ACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG

GAAACAGCTATGACCATGATTACGCCAAGCTTCCGCGGGTATATGGTAAAGAACCTACTA

ACACAATAAAATATTTAAATAATGTATTTCCTATAAATAAATTTACAGATTTATTTTTTAAT

ACAAAAGATATAGATATACCAGAAATAAATGATCAGTTTAAAGGTTTTAAATTCTTTATGA

CATCATTTATAAATCATGGATCATATCCACTAACAATAGAATGTGGTGTAACAAATGGTG

GAACTAGTTATAAAAGAGCAATTATTTTATTGCATGTTCGAACTGATTTAAAAGATAGACC

AGTTTCATTTTGTGATTTTCGAAAAGGAGAATTATATAATTATTTGAATGCTTATACTGAA

GGGGATGTATGCATAATAATTTCCAAATCAAATACAAGTTTTGGTTTTAGATGCCCAGTAA

ATACAAAAAAAAATGCCAAAAAATTGTTTTACGCAAGTATATGAAAAAGGGTATCTAAATG

ACGCCAATAAAATTAATACTAAAAATGTTATTAACTATTCATTTGAAAATCCAGAATATGC

GCTAGCTGGTTYTAATTATACATTAACAAAATCGTATCAATTTGAATGTCATTGTGTAGA

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AACACATCAAGCTTGCATGCCTGCAGGGTGATAGTGTAGATTTTTTTGTTTGACCATTGC

ATCATTGTCTTTTATTCATCGATTTTATTATGTTATATATATTTTTTTAAATATCATAAT

ATTTTTTCAATATAGCTTCTTTGAGCAAATACTGAACAATAAGGAATGCTTCTATGCATC

CGTGGATGTATATGTACGTAATAAAATAATTTTTTGTCCATAAAAATATCTTTAACAGCT

GTTATACAAAAGCATTGAATTGGCTGATACATTTTTGTCGTAAATAAAATAAATGAATTA

ATGGTACAAAATTACAATAAAAAGGAAGCGAATTTATTGCATTTTGTTTAATTTATGTCC

TTTCCATTTTATTAATACAATTATGGTACTAGTAATTCGTATTAAACATATATTTTGCAT

GTACCAAATGTTTGGGAAATAAGCATATGGATCCCACTAAATTTAATAATATTCTGCGAT

TTTTCTTATATTTACTATTAAATATAATGGATTCATTTTTTGATGCATGCAATTTTTTCT

TTTAATGTATTAATTAGTGTGACAATTTTGAAAAACTTTACTCATTTATTTATTTTCCTT

ATTATATTGGATTTATAATACCAGTATTATTATATAAACCACATTAAATAATTTGTAATT

TATTCGAGGGTATCAAAAAAAACATATACAAAATGCATATATCCACATATGGCTCATTAT

AGGGTGAATAAAAATGGGAACAGCAGATTAATTATTATATCATGAAAGTAATGAAAAAA

TTTAAATTATATGGATATATACATATATAATATGTTAAAAAATAATAATAAATAAATAAAA

TGTATTATAAATCTTATAAAATAAGTTTTGTTGTAATAATCCACGAAATATGCCATAAA

TAGACACTGAACAAATTAGTGGTTCTTAATATTATTTTGGATACATGCGGATATTATCAT

TGTCAGATGATTTATTTTTTGTTATTTTTAAATTATAAATATTCATATTTTATAGTCCTC

AAAAAATAGGATGTTTTATTTTTTTATAGCTATATTTTATGGTTGATCCTTTCCTTTTAT

GGTGTTTCATAAAAATTTTATTGAGCTATATACAATCCAATAAAAAAAGGCAATTGAATT

TTGAAATATATTAAACTATTTTTATAATAAAATAAATATAATTTTTAAATAATGTTATAT

TATATATAATTTTTATCTTTACACAGAAATTTTTTTTTTAGAGTCCAATATATATAATTAG

TTATATATATAGAGATATACACTACATACACCACCATAAATAATTATAAGGAAAACCAGT

TATTTTAATTTTAACTGAAGAAATTAAATAAATATATAAAAAAGAAAAGAACAAAAAATA

AAACGACAGCAACCTTAAAATTTTATTATTATTACATATTTATACATAAAAAAAATAAAA

TACTTAGACAAAAAATAATAAATATTATAGATCGATATTAGCATACATATATACCTTTCA

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