Application form

Assessment of clinical research involving gene therapeutics in the Netherlands

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Viral vectors

June 2016

Application form
Assessment of clinical study involving genetically modified viruses

Part A: Bio-safety aspects

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

June 2016

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Consent form

The undersigned:

Hereby gives his/her consent to the gene therapy office to send on the application forms and the accompanying file to the relevant assessment authorities.

Gives his/her consent to the assessment authorities to use the fully completed file in making its assessment.

Gives his/her consent to the assessment authorities to inform each other and the gene therapy office about the status of the submitted application.

Signed:

Name

Date

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 licenses.

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. Confidential information must be included in a separate attachment marked 'confidential'.

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.

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 personal details of the contact person and the environmental safety officer are to be submitted by entering the required information under Section A7, General (personal) 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.

List of abbreviations

AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukemia
ATMP	Advanced therapy medicinal products
bp	Base pairs
CAR	Chimeric antigen receptor
CHOP	Children's hospital of Philadelphia
R-CHOP	Rituximab + chemotherapy cocktail treatment
COGEM	Committee genetic modification
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus promotor
CSF	Cerebrospinal fluid
CRS	Cytokine release syndrome
CTF	Cell-Therapy Facility
CVPF	Cell Vaccine Production Facility
DLBCL	Diffuse large B-cell Lymphoma
EOP	End of production
EOPC	End of production cells
ESO	Environmental Safety Officer
GMO	Genetic modified organism
GMP	Good manufacturing practice
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEK293	Human Embryonic Kidney 293 cells
HIV	Human Immunodeficiency Virus
HSCT	Hematopoietic stem cell transplantation
HTLV	Human T-cell lymphotropic viruses
LOD/Q	Level of detection/quantification
LTR	Long terminal repeat
MCB	Mast cell bank
MHC /HLA	Major Histocompatibility complex/ Human Leukocyte Antigen
MOI	Multiplicity of Infection
NC	Nucleo capsid
NHL	Non-Hodgkin lymphoma
ORF	Open reading frame
PBS	Primer binding site
PERT	Product Enhanced Reverse Transcriptase
PMC	Princess Maxima Center
PPM	Parts per million
PTT	Polypurine tract
RCL	Replication competent lentivirus
rdLVV	Replication-deficient lentiviral vector
RNA	Ribonucleic acid
(r/r)	relapsed / refractory
RRE	Rev response element
RT	Reverse transcriptase
SCT	Stem cell transplantation
SIN	Self-inactivating
TLS	Tumor lysis syndrome
TU	Transducing units
UMCU	University Medical Center Utrecht
UPenn	University of Pennsylvania
WIP	Work group infection prevention
WCB	Working cell bank
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
VSV-G	Vesicular stomatitis virus glycoprotein

A1. General application details

General information

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 GMO(s) and insertion(s) used and the nature of the application(s).

Testing CTL019 in children with B-cell malignancies.
CTL019 (tisagenlecleucel) is a novel adoptive cancer immunotherapy whereby autologous T-cells are ex-vivo genetically modified/transduced by a replication-deficient lentiviral vector to express anti-CD19 antibody based receptors on the surface of T-cells (the GMO) to target CD19 antigens on the surface of malignant B-cells.

A1.2. The objective of the study for which the application is submitted:

Provide a short description of the study's objective. For example: 'The objective of this Phase I clinical trial is to study the safety of the oncolytic adenovirus for the purpose of developing a new therapy to treat skin cancer.'

The goal of the clinical trials is to assess the safety and efficacy of CTL019 (ex-vivo genetically modified autologous T-cells expressing a transmembrane chimeric anti-CD19 receptor) for the purpose of developing a novel therapy against B-cell malignancies.

A1.3. Describe briefly the contents of the application, the objective of the study being aimed for and the intended application of the results

Provide a short description – of no more than half a page – of the genetically modified organisms (GMOs) applied, or of the GMOs that may thus be created, such as via recombination of genetic information between organisms or by integration of genetic material into 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.

GMO:
CTL019 is a novel, investigational, adoptive cancer immunotherapy agent consisting of autologous T-cells that have been ***ex-vivo*** genetically modified to express a transmembrane chimeric antigen receptor (CAR) to target CD19 on the cell surface of malignant B-cells. The active substance CTL019 is comprised primarily of CD4+ and CD8+ T-cells that have undergone *ex-vivo* T-cell activation, gene transfer by replication-deficient lentiviral vector (RDLVV) transduction, expansion and formulation in an infusible cryomedium. Please note that viral transduction of autologous T-cells is done outside the Netherlands. The shipped CTL019 cell product (*ex vivo* transduced T-cells) does not contain viral particles (for rationale see A2.10).

Mechanism of action:
T-cells expressing the chimeric antigen receptors specifically bind the CD19 antigens present on B-cells with the variable regions of a CD19 specific antibody coupled to the intracellular zeta chain of a CD3 molecule and a costimulatory molecule, thus allowing the immune system to specifically target and destroy these B-cells in an MHC/HLA independent manner (Kalos and June, 2013). CTL019 may represent an effective treatment modality for patients diagnosed with B-cell malignancies.

CD19, a 95kDa transmembrane glycoprotein, is a component of a cell surface complex that regulates signal transduction through the B-cell receptor (Stamenkovic 1988, Ledbetter 1988, Fearon 2000). It is expressed on B-cells from early development until differentiation into plasma cells but is not present on pluripotent blood stem cells and most normal tissues other than B-cells (Freedman 1987, Uckun 1988, Fearon 2000). The restricted expression of CD19 on B-cells and their precursors makes CD19 a relatively safe target for therapeutic intervention in B-cell neoplasms such as B-cell acute lymphoblastic leukaemia or diffuse large B-cell lymphoma and consequently, the B-cell marker CD19 has emerged as a therapeutic target in the past years (Sadelain 2003, Brentjens 2010, Porter 2011).

Results from ongoing clinical trials of CTL019 at Novartis and earlier at the University of Pennsylvania in r/r CLL, NHL and B-cell ALL have shown anti-tumour efficacy (Porter et al 2011 and 2013, Grupp et al 2013, Maude et al 2014). CTL019 may offer a therapeutic alternative for patients with (r/r) B-cell malignancies who are either stem cell transplantation (SCT) ineligible or have relapsed after SCT. CTL019 may offer a greater durability of response than current salvage therapies.

Rationale:
With current treatment regimens, approximately 80-85% of children with precursor-B-cell acute lymphoblastic leukemia (ALL), can be cured. Treatment of refractory and high-risk ALL patients includes intensive chemotherapy treatment. In this group of patients (approximately 5-8% of patients, approximately 8 patients/year in The Netherlands) the role of CAR-T cells will be studied in future protocols.
In approximately 15-20% of patients, relapsed ALL occurs. The treatment and prognosis of children with relapsed precursor-B-cell ALL depends on the duration of first complete remission, localization of the relapse (marrow or extra-medullary) and the response on relapse-treatment. Survival of so-called high-risk relapsed ALL patients (in The Netherlands approximately 5 patients/year) is only 40%, despite aggressive treatment, often including allogeneic stem cell transplantation. These children are candidates for treatment with CAR-T cells.
CD19 is also expressed on tumor cells of children with non-Hodgkin lymphoma. The majority of these children can be cured with current, aggressive chemotherapy regimens. Patients not-responding to these chemotherapy regimens, are eligible for treatment with CAR-T cells .

A1.4. Describe briefly the intended work.

State in chronological order which types of procedures will be carried out, and for which a license 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). Where applicable, also indicate for which part of the study a Contained Use (IG) permit applies, and provide the number of the GMO permit concerned.

Production, transport and storage
The patients T-cells are harvested by leukapheresis at the Princess Máxima Center, transported to the Cell-Therapy Facility (CTF) at the University Medical Center Utrecht (UMCU) for cryopreservation and shipped to the designated tisagenlecleucel manufacturing site (either the Novartis site in Morris Plains USA or the Fraunhofer Institut für Immunologie und Zelltherapie in Leipzig, Germany) where production is taking place in compliance with current Good Manufacturing Practices.
After production, tisagenelcleucel (consisting of autologous T-cells that have been transduced *ex-vivo* and do not contain viral particles, the GMO) is transported to the Fraunhofer Institute in Leipzig, Germany where the cells are temporarily stored pending EU QP release.
After release CTL019 is transported to the CTF at the UMCU, where the storage and handling of the product is performed according to GMP.

When the patient is admitted to the Princess Máxima Center, the product is released and physically transported from CTF at the UMCU to the patient ward. The UMCU and the Princess Máxima Center are on the same campus, and connected by a tunnel (appr. 500 meters).

Administration of the CTL019 product and observation of the patients
At the patient ward the cryobag containing the CTL019 T-cells will be thawed according to local hospital guidelines and directly coupled to the intravenous line of the patient and the CTL019 T-cells will be infused. Thawing will take place in a location with restricted access with surfaces suitable for disinfection in the unlikely event that a spill would occur, with a door notice stating ‘Only entrance for

authorized personnel' with attending personnel all registered as project member for this specific project. Administration will be done in accordance with the hospital guideline on infusion of cellular products.
The patient will be observed to monitor for any potential immediate adverse events. The duration of admission depends on the clinical course of the patient (in the previous phase I/II trials the range of the number of admission days was 1-22 days, median: 9 days).

Sampling and sample analysis
Sampling of blood, urine, bone marrow aspirates and, if indicted, lymph node biopsy will be performed for study purposes and/or routine patient care purposes. Sampling, transportation of samples within the hospital, storage and processing will be done according to standard hospital procedures as they do not contain viral particles and any genetically modified cells present in the samples do not represent a specific safety concern (see A5.2-A5.5).
Although CTL019 is not infectious and does not contain RCL, and, therefore, the risk to the environment is considered negligible, RCL lab assessments are performed in patient blood samples after the hospitalization (see A.2.13).

Waste treatment and disposal of unused material.
Following handling and administration of CTL019 to the patient, any unused residual compound remaining in the cryobag and all used materials that have been in contact with CTL019 (like gloves and tubing) will be disposed of according to the guidelines as described in appendix 9 of the GMO regulations 2013.

A1.5. 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.

Start date: 01 November 2017
Stop date: 31 December 2047

In the upcoming years different clinical trials with CTL019 will be started in children with B cell malignancies (e.g. high-risk ALL, high-risk relapsed ALL and refractory/relapsing non-Hodgkin lymphoma).

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".

The specifications including acceptance criteria used for release of CTL019 are proprietary confidential information. No such product has been approved yet and publishing the specifications would give an advantage to competing companies.
The specifications are provided as Appendix I CTL019 testing (confidential), and a summary is provided in A3.2.

The plasmid maps and detailed sequence information for the 4 plasmids used to manufacture the lentiviral vector used as starting material for CTL019 are proprietary confidential information, and publishing the annotated plasmid maps could give an advantage to competing companies. The plasmid maps are provided as Appendix III (confidential) and a summary is provided in A2.8 and A2.11.

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 license holder must be able to enforce compliance with the license 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 license holder. For this reason, employees must be directly employed by the license holder. In those cases where an employee does not come under the authority of the license holder, such as where a treating doctor is part of a partnership that is independent of the license holder, an employment contract must be arranged for carrying out work under the license, such as through a zero-hours contract with the license 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 license holder.

A1.7. Name of legal entity:
Princess Maxima Center for Pediatric Oncology

A1.8. Chamber of Commerce (KvK) number:
54327946

A1.9. Visiting address of legal entity:
Uppsalalaan 8, 3584CT Utrecht

A1.10. Postal code and town/city of location of legal entity:

Uppsalalaan 8, 3584CT Utrecht

A1.11. 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 license holder, it is only possible to carry out work at several locations if the license 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 license 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 license application, such as procedures with patient samples.
In cases where central control is not possible, such as with a multi-center study, a separate application must be submitted for each location.

Leukapheresis will be performed in the in-patient rooms of the Prinses Máxima Center.
Infusion of the GMO will be performed in the in-patient rooms of the Prinses Máxima Center.
Patients will be observed in the in- and out-patient rooms of the Prinses Máxima Center, where also the blood sampling will be performed.
Bone marrow punctures will be performed in the in- or out-patient rooms of the the Prinses Máxima Center, or in the operation rooms (if performed under anesthesia) of the Wilhelmina Children Hospital.
If the patient needs intensive care treatment, this will be provided in the Wilhelmina Children Hospital.
Laboratory tests will be performed at the laboratories of the UMCU (routine investigations) or Dutch Childhood Oncology Group in The Hague (ALL-specific investigations).

Site:
Pediatric Oncology Department
Princess Maxima Center for Pediatric Oncology
Lundlaan 6

3584EA Utrecht
The Netherlands

The manufacturing and genetic modification will be performed in the Novartis manufacturing site in Morris Plains in the USA or in the Fraunhofer Institute in Leipzig Germany.

A2. Construction and composition of the GMO

Virus from which the genetically modified vector was derived

A2.1. Which virus was used as the original virus in the construction of the GMO?

Where applicable, supply the full scientific name, a trivial name (e.g. the commercial name), the strain and the isolate used.

The viral vector used in the manufacturing of the CTL019 GMO product (manufactured outside of the Netherlands, only the genetically modified T-cells are entering The Netherlands) is a a self-inactivating (SIN), non-replicating, recombinant lentiviral vector derived from the Human Immunodeficiency 1 Virus (HIV-1) lentiviral genome (family Retroviridae; it belongs to the Group VI (ssRNA-RT) according to the Baltimore classification). The vector is designed to prevent emergence of replication competent lentivirus (RCL) by distributing the transgene and the viral proteins required for packaging, reverse transcription and integration into the host DNA on four different plasmids.

The development of the replication-defective minimal lentiviral vector system from the wild type HIV-1 is described in A2.8.

A2.2. Describe how the identity of the original virus was determined.

The GMO to be administered is often based on a strain that was derived, sometimes by genetic modification, from the original virus. The properties of the original organism form the basis for the environmental risk assessment, which is why confirmation of the identity is important. Provide a description of the characteristics that determine the identity of the original virus. This may be based on bio-information analyses, such as sequence analysis, alignments or phylogenetic analysis. Information on the origins of the original virus, such as a micro-organism originating from the American Type Culture Collection (ATCC), may also be submitted.

The basal virus for the current lentiviral constructs used to prepare the GMO is the species HIV from the genus lentivirus.
The majority (approximately 85%) of the native HIV-1 sequence has been removed to produce a replication defective minimal lentiviral vector system.
The wild type HIV-1 genome (Figure 1a, question A2.8) has nine distinct open reading frames (ORFs) encoding;

- Gag (structural polyprotein),
- Pol (polyprotein comprising the enzymes protease, reverse transcriptase, RNaseH and integrase),
- Env (envelope glycoprotein that interacts with CD4 cell surface expression on target cells thereby facilitating cellular entry of viral particles),
- Tat (transcriptional transactivator that is specific for viral enhancers contained within the long terminal repeats (LTR),
- Rev (an RNA binding protein involved in RNA export to the cytoplasm),
- Vif (disables cellular antiviral factor APOBEC3G),
- Vpr (induces G2 arrest resulting in increased production of viral particles),
- Vpu (enhances release of viral particles)
- Nef (induces down-regulation of CD4 and major histocompatibility complex molecules thereby preventing superinfection and destruction of the infected cell respectively).

There are also cis-acting sequences on the viral nucleic acid that are involved in the HIV-1 viral replication cycle and control of gene expression. The long terminal repeats (LTR) contain R regions (i.e., repeated sequences at the extreme ends of the RNA required for reverse transcription and which also interact with Tat and contribute to gene expression); the U3 region (i.e., region located at the 3' end of the viral RNA preceding R that contains an attachment (att) target recognition site for integration, as well as viral enhancers and promoters); and the U5 region (i.e., short region at the 5' end of the viral RNA just downstream of R that contains a partial polyadenylation (pA) site that only functions in the integrated DNA copy, and an att site for integration).

The primer binding site (PBS) is located immediately downstream of U5, and is required to initiate the process of reverse transcription via binding to a cellular tRNA. Close to the PBS is the major splice donor site.

The packaging signal (Ψ) is an RNA structural feature with a complex stem-loop structure that is located at the 5' end of the RNA genome and is contained within the first 400 nucleotides. It interacts with the nucleocapsid (NC) region of Gag in a process referred to as packaging, resulting in the incorporation of viral RNA genome within particles as they assemble within the cytoplasm.

The polypurine tract (PPT) is a region towards the 3' end of the genome that is required for second strand DNA synthesis. The central polypurine tract (cPPT) is conserved among lentiviruses but its precise function is not well characterized.

The Rev Response element (RRE) is recognized by Rev protein, resulting in the export of full-length unspliced or partially spliced RNA's to the cytoplasm from the nucleus, a complex process that results in a degree of control of viral gene expression via inhibition of splicing (fully unspliced RNA corresponds to the full-length viral genome sequence which contains the Ψ), and is therefore packaged within viral particles to generate progeny infectious viral particles from the integrated pro-viral sequence.

A2.3. What is the host range of the original virus?

Describe the hosts in which the original virus naturally occurs, also including hosts that serve as a reservoir. For each possible host, indicate the tissue and cell tropism.

The host range of wild type HIV-1 is limited to humans and chimpanzees.
Wild type HIV-1 can infect many cell types, mainly lymphocytes, but also macrophages, and microglia in the brain, and other neurological cells (Kallings 2008).

A2.4. Provide relevant data on pathogenicity and possible attenuation and biological restrictions of the original virus.

What is the class of pathogenicity of the original virus? If this concerns an attenuated virus, the basis for attenuation must be described. If the organism is biologically restricted in another way, the grounds for the biological restriction must be substantiated.

Wild-type HIV-1 is classified in pathogenicity class 3 (COGEM).

The wild-type HIV virus has been modified by removing approximately 85% of the virus sequences. Thus 85% of the wild-type HIV-1 virus is not present in the synthetically generated lentiviral plasmid system. For details see A2.8.

A2.5. What are the pathogenic properties of the original virus and what are the available treatment methods?

Name the symptoms that are known or assumed to be caused by the original virus. Also indicate the treatment methods that are available to treat such an infection and provide an indication of the effectiveness of these treatments.

Wild type HIV-1 virus can cause HIV infection which can result in AIDS.
HIV infection is generally controlled by using multiple combination antiretroviral treatment. Often multiple drugs that act on different viral targets are used. HIV treatment is at this moment so

successful that in many parts of the world HIV infection has become a chronic condition in which progression to AIDS has become increasingly rare. In case of progression to AIDS patients are threatened by opportunistic infections, which can be treated with antibiotics and other antimicrobial drugs and by secondary malignancies, which can be treated with chemotherapy.

A2.6. What are the transmission routes of the original virus?

State all the observed and assumed transmission routes of the virus, and indicate the effectiveness of the transmission.

Wild type HIV-1 is transmitted primarily via unprotected sexual intercourse, contaminated blood transfusions, hypodermic needles, and from mother to child during pregnancy, delivery, or breastfeeding. Depending on the type of sexual intercourse the probability of HIV-infection probability varies between 1:100 to 1:2500. Receiving contaminated blood transfusion results in a much higher infection probability.

A2.7. How could the original virus survive outside the host?

State all survival options and the survival time of the original virus under optimal environmental conditions, and describe the factors that may be of influence.

Survival depends on which body fluid it is in, volume of the body fluid, concentration of the virus within it, temperature, acidity, and exposure to sunlight and humidity. HIV-1 can remain viable in blood in syringes at room temperature for 42 days, and in blood and cerebrospinal fluid from autopsies for up to 11 days (Abdala 2000 and Ball 1991). Although drying in the environment is known to cause a reduction in HIV concentration, under experimental conditions, cell-free HIV-1 dried onto a glass coverslip in 10% serum can survive for longer than 7 days, depending on the initial titre (Van Bueren 1994).

The genetically modified viral vector

A2.8. Describe the 'original vector or vectors'.

Describe the 'original' vector, and indicate – with or without the help of bio-information analysis – in what way the original vector deviates from the original virus. For the application of a viral system (e.g. lentiviral particles), in addition to the viral (transfer) vector, you must also describe for example packaging and pseudotyping plasmids. Provide a description of the characteristics that determine the identity of these original vectors. This can be done on the basis of bio-information analysis, such as sequence analysis, alignments or phylogenetic analysis.

Please note that the end-product are the genetically modified cells and thus no viral vector is actually administered to the patients. The lentivirus is only used to transduce the T-cells of the patient *ex vivo*. T-cells are extracted from the patient, shipped to the manufacturing site (outside the Netherlands) and transduced *ex vivo* (see A2.10). The transduced cells (CTL019) are then shipped back to the Netherlands and administered to the patients. The cell product does not contain vector/viral particles anymore (for rationale see A2.10).

Wild type HIV-1 virus genome

The wild type HIV-1 genome (Figure 1a) is described in Question A2.2.

Lentiviral vector system

To generate a safe vector system all the dispensable HIV-1 ORFs (Tat, Vif, Vpr, Vpu, Env,and Nef) have been removed. In addition, the essential components of the vector system are split among four plasmid constructs (Figure 1b) in order to minimize the potential for homologous recombination, thereby preventing the generation of a replication-competent virus.

The transfer plasmid encodes the vector genome, which is incorporated into the viral particles in the form of single-stranded RNA. The vector genome contains the cis-acting sequences that are required for vector packaging, reverse transcription and integration of the vector genome into the host cell genome (no viral genes are expressed).

The necessary structural and functional viral proteins required for vector packaging, reverse transcription and integration are expressed from three separate packaging plasmids; the Gag/Pol packaging plasmid (pRKHSYNGP), the envelope packaging plasmid (pRKHG) and the Rev packaging plasmid (pRKHREV). Regions of the packaging components that share sequence homology with wild-type HIV-1, or with each other, are indicated with dotted lines in Figure 1. . The plasmid maps with description of regulatory and coding sequences are provided as Appendix III Plasmid maps (confidential).

Gag/ Pol packaging component

The Gag/Pol packaging component (expressed from pRKHSYNGP) contains (in order from 5' to 3') a Cytomegalovirus promoter (CMVp) and an ORF for Gag/Pol that has been codon engineered to retain the primary amino acid sequence but to exploit the preferential codon usage of human cells (Kotsopoulou 2000). This ORF is linked by a non-codon optimised ribosome frame shift region which is required to maintain the correct stoichiometry of Gag and Gag/Pol polyproteins at a ratio of approximately 20 Gag polyprotein molecules to 1 Gag/Pol polyprotein molecule, followed by a heterologous pA site.

When cleaved by the viral protease, the Gag polyprotein comprises structural proteins Matrix (MA), Capsid (CA; p24) and Nucleocapsid (NC). The Gag/Pol polyprotein, after proteasedirected cleavage, produces the enzymes protease, reverse transcripase and integrase. Reverse transcriptase is an enzyme that converts the vector RNA sequence into DNA, and this DNA is then integrated into the chromosomal DNA of target cells via integrase activity.

In addition, the plasmid backbone also contains a kanamycin resistance gene and a bacterial origin of replication (ori). A plasmid map is provided in Appendix III (confidential information).

Envelope packaging component

The envelope packaging component (expressed from pRKHG) contains (in order from 5' to 3') a CMVp, an ORF for vesicular stomatitis virus glycoprotein (VSV-G) which facilitates entry of vector particles into target cells, followed by a heterologous pA site. VSV-G is the envelope glycoprotein from Vesicular Stomatitis Virus (VSV). VSV-G is commonly used to pseudotype lentiviral vectors as it conveys a broad tropism and because of the increased stability of the viral particles.

In addition, the plasmid backbone also contains a kanamycin resistance gene and a bacterial origin of replication (ori). A plasmid map is provided in Appendix III (confidential information).

Rev packaging component

The Rev packaging component (expressed from pRKHREV) contains (in order from 5' to 3') a CMVp, an ORF for Rev and a heterologous pA site.

In addition, the plasmid backbone also contains a kanamycin resistance gene and a bacterial origin of replication (ori). A plasmid map is provided in Appendix III (confidential information).

CTL019 vector genome

The CTL019 vector genome component (expressed from pRKHVmuEC19) (see Figure 1c) contains (in order from 5' to 3') the following sequences:

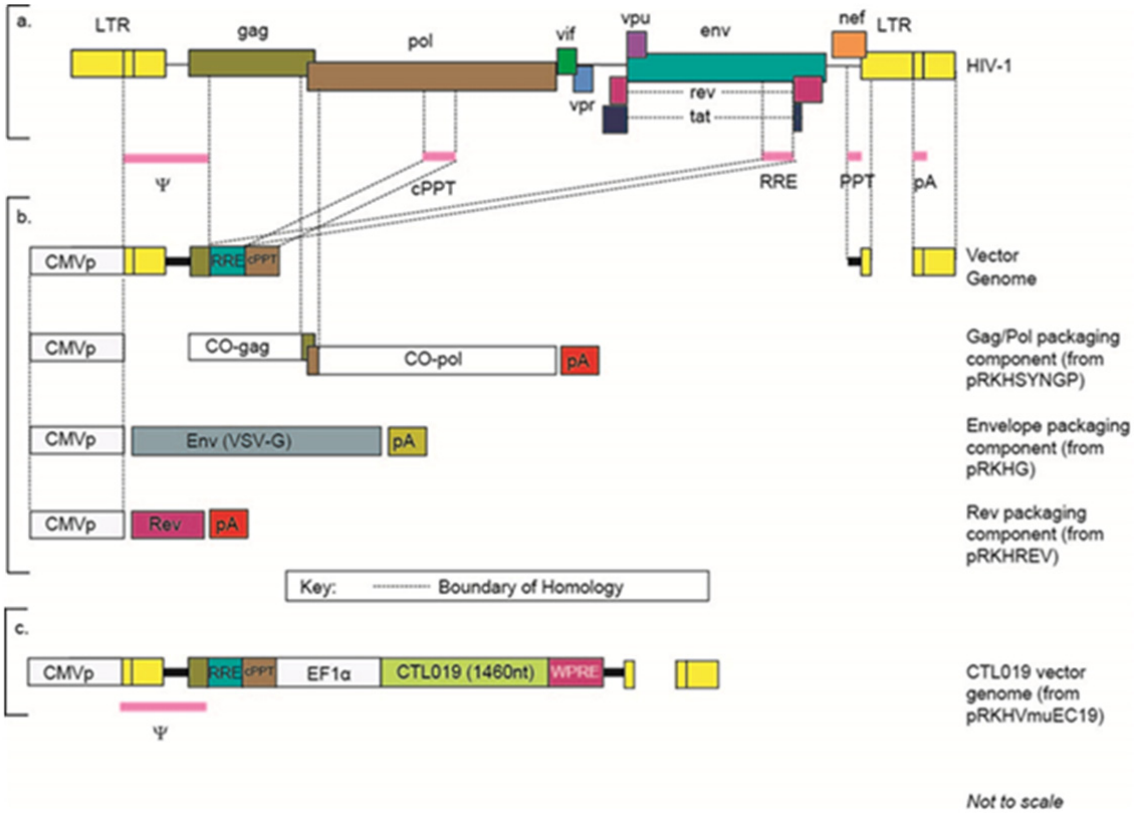
- CMVp-R-U5 region
- Leader-packaging (Ψ) signal
- RRE containing the tat2/rev2 splice acceptor
- cPPT
- Internal EF1α promoter
- ORF encoding CAR-19
- Modified woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)
- PPT (this overlaps with Nef therefore a 59 nt partial fragment of Nef is present in the genome)
- Self-inactivating (SIN) LTR

In addition, the plasmid backbone also contains a kanamycin resistance gene and a bacterial origin of replication (ori). These are not part of the vector genome and will not be integrated into the T-cell genome. A plasmid map is provided in Appendix III (confidential information).

The total vector system used for preparation of the genetically modified T-cells is an rdLVV based on the 3rd generation of lentiviral vectors. The four plasmids used for the rdLVV generation and described above are:

- pRKHG (VSV [Vesicular stomatitis virus]G envelope),
- pRKHSYNGP (HIV-1 Gag-Pol),
- pRKHREV (HIV-1 REV),
- pRKHVmuEC19 (HIV-1 Murine CTL019).

Figure 1.Schematic diagram showing the genetic structure of a. HIV-1 wild-type virus, b. the HIV-1 vector system and c. CTL019 vector genome plasmid



A2.9. Regarding the pathogenicity of the original virus, have certain properties of the 'original vector' been altered that would determine the pathogenicity of the original vector?

Here, an elaboration could be given of the modifications made to the 'original vector', such as replication deficiency, which cause attenuation of the pathogenic properties.

The majority (approximately 85%) of the native HIV-1 sequence has been removed to produce a replication-defective minimal lentiviral vector system (see A2.8).

Changes in binding and entry compared to parental virus

The pseudotyped rdLVV envelope derived from Vesicular stomatitis virus (VSV) has been added which can transduce cells of many species since it interacts with non-dividing cells including T-cell .

Changes in assembly compared to parental virus

The vector design prevents emergence of RCL by distributing the transgene and the viral proteins required for packaging, reverse transcription, and integration into the host DNA on four different plasmids. The transgene bearing plasmid itself contains a Cytomegalovirus (CMV) promoter instead of the U3 region of the 5' LTR and is modified to a self-inactivating (SIN) type by deleting about 400 base pairs (bp) of 3' long term repeat (LTR).

The minimal vector system design, in combination with the use of a codon-optimised Gag/Pol construct and a heterologous envelope glycoprotein, means that sequence homology between vector components has been minimized. This strategy was employed to further reduce the chance of replication competent lentivirus generation through homologous recombination, thereby enhancing safety of the vector system. The safety features of the vector system are summarised in the Table 2 below.

Table 2.Vector system safety features

Feature	Benefit
Viral components segregated between four separate plasmids	The chance of a replication competent lentivirus being generated is highly reduced as multiple recombination events would be necessary for acquisition of all components.
SIN LTR	Expression in transduced cells is confined to the therapeutic transgene (no full length viral genome transcribed).
Codon-engineered Gag/Pol	Reduced homology between Gag/pol and viral genome components reduces the likelihood of recombination
ORFs of non-essential accessory genes removed	No expression of any pathogenic HIV-1 proteins in producer or transduced cells. Even although two small fragments as detailed in the text are present, generation of wild type HIV-1 via recombination is not possible because the essential genes are not present.
Heterologous envelope	Generation of wild type HIV-1 via recombination is not possible.
Modified WPRE used	Expression of the X-protein and peptides derived from it has been ablated

Consequently, based on the information and rationale provided, the vector cannot be assembled in the final host since the gag gene cannot be transcribed due to the lack of the plasmid that codes for the gag (Figure 1), in addition the pol and all accessory elements are absent from this vector. Therefore, compared to the parental virus, the rdLVV in the proviral form cannot be assembled to form new viral particles once integrated into its final host cell genome unless complementation/recombination with endogenous retroviral sequences of the human host occurs.

Changes in replication compared to parental virus

The vector is replication incompetent by design. The viral sequences necessary for packaging are segregated into three different plasmids: one for gag and pol, one for Rev, and one for envelope (env). The vector genome is encoded by a forth plasmid. This approach allows for a reduction of the risk of RCL production since three independent recombination events are required for the generation of a functional RCL.

HIV-1 possesses regulatory functions encoded by the *tat* and *rev* genes as well as accessory genes that include *vif*, *vpr*, *vpu*, and *nef*. The accessory genes of HIV-1 are not required for virus replication in appropriate cell culture systems *in vitro*. Therefore they are not included in the vector production system. HIV-1 wild-type open reading frames (ORFs) of *tat*, *vif*, *vpr*, *vpu*, *env* and *nef* have been removed. The lack of these genes in HIV-1 based vector production systems further increase the safety in terms of generation of RCL. Recombination of vector packaging plasmids and cellular DNA leading to RCL has not been reported with the current viral vector systems in the literature (Cornetta 2011).

Based on the discussion and rationale provided in this section there is no risk for replication competency of CTL019 integrated in the patient's T-cell T-cell genome or the generation of RCL.

Changes in cell to cell spreading compared to parental virus

The viral vector is integrated in proviral form in the final cell host at the end of the transduction process. Definite copies of viral vector are integrated as provirus in the genome of the target cells. The gag/pol, envelope and rev packaging component sequences are not present in the target cells; therefore new vector particles cannot be formed. Therefore compared to the parental type, the provirus cannot further spread to other cells once integrated into the final host cell.
Please refer to the schematic diagram showing the genetic structure a. HIV-1 wild-type virus, b. the HIV-1 vector system and c. CTL019 vector genome plasmid in Figure 1.

A2.10. Describe the method of production of the clinical viral vector from the 'original vector or vectors'.

Answer this question preferably by using a diagram that describes the various production steps.

The investigational medicinal product contains the genetically modified T-cells and does not contain free viral vector particles. The vector is a starting material for the manufacturing of CTL019 final product.

Manufacture of the viral vector (starting material):
The rdLVV for transduction of CTL019 is produced in Human Embryonic Kidney (HEK) 293 cells using transient transfection of transfer and packaging plasmids (for information on the plasmids, see question A2.8). After harvest, the vector is purified, sterilized, concentrated, and filled in vials. The manufacture of the vector is carried out under GMP conditions.

Release of vector for use in CTL019 manufacture is done against predefined specifications (see A3.2).

Manufacture of the CTL019 cell product (the actual GMO):

CTL019 is produced under GMP conditions using cryopreserved autologous leukapheresis as source material.

Thawing and preparation of cell starting material (day 0):
After thawing of the cryopreserved leukapheresis source material (i.e., starting material #1) at the manufacturing site, a T cell enrichment step is performed by binding the cells to beads in a flow-through system.

Lentiviral transduction:
The vector (starting material #2) is added to the cell culture in two steps. The first vector addition takes place on day 0 and the second vector addition takes place on day 1.
The volume of vector required to transduce the cells is calculated based on the titre of the vector lot (in Transducing Unit (TU)/mL) as well as the vector Multiplicity of Infection (MOI) necessary to achieve a target of 20% transduction efficiency in T-cells. Typically, $1.8x10^{8}$-$3.9x10^{8}$ TU, corresponding to $8.8x10^{10}$-$2.3x10^{12}$ vector RNA copies, are used to manufacture one batch of CTL019 cell product.
The indicated maximum vector RNA copy number of 8.8x1010-2.3x1012 is the combined maximum number from the two transduction steps taken together.
The number of lentiviral particles is not quantified directly but can be estimated from the number of RNA copies. Wildtype lentivirus particles typically contain two viral genomes. As a worst case, each lentiviral vector particle can be considered to contain one vector RNA copy. "Empty" lentiviral particles without RNA copies are also potentially present, however these particles are not considered relevant for the risk assessment since they do not contain RNA. For the risk assessment, the worst-case number of lentiviral particles is therefore considered to be equal to the number of vector RNA copies and hence the maximum number of lentiviral particles used per batch of CTL019 product is $2.3x10^{12}$.

Vector wash:

Cells are washed to remove residual unintegrated vector on Day 3. Two washes are performed followed by resuspension in culture media (i.e. cells are centrifuged and resuspended 3 times).

Cell expansion:
Cells are expanded for a minimum of 8 days after addition of the vector.

Harvest:
The harvest comprises a first bead removal step, a cell wash step, and a second bead removal step. The cell wash step consists of two washes followed by resuspension (i.e. cells are centrifuged and re-suspended 3 times).

Final product formulation:
The cells are centrifuged, supernatant is removed and cells are resuspended in formulation buffer with Cryopreservation Medium, filled in primary container and cryopreserved.

Manufacture of both the lentiviral vector and the CTL019 cell product takes place outside of the Netherlands. CTL019 final product is shipped via Germany to the Netherlands where it will be infused (see A4.2 and A4.3).

Vector inactivation, removal and dilution during the CTL019 manufacturing process

Three aspects contribute to the clearance of residual lentiviral vector during the CTL019 T-cell manufacturing process:

1. VSV-G pseudotyped lentiviral vectors (such as the lentiviral vector used to manufacture CTL019), have been reported to lose about 90% activity when cultured at 37°C for up to 48 hours (Higashikawa 2001), while the CTL019 manufacturing process includes after the second vector addition a cell culture step of at least 8 days at 37°C.
2. VSV-G pseudotyped lentiviral vectors are inactivated by the complement in human serum. Incubation in 80% human serum at 37°C for one hour has been reported to inactivate 98% of lentiviral particles (DePolo at el., 2000). The culture media used during the CTL019 cell expansion (minimum 8 days at 37°C) contains 2% human serum.
3. Another aspect is the theoretical removal/dilution factor during the manufacturing process. Cells are washed and expanded various times: two wash steps during the vector wash, two wash steps during cell harvest, dilution during the cell expansion steps, and a centrifugation step during final product formulation. In total, the cells are centrifuged, supernatant is removed and cells are resuspended in new media/buffer 7 times during the T-cell manufacturing process (see above).

The COGEM Advice CGM/090331-03 recommends the following formula for calculating a reduction ratio of lentiviral vector particles in transduced cell cultures:

$$\text{Reductieratio} = (20^{W} \times 200^{I} \times 2^{2,4T})/C_i$$

W = number of wash steps
I = number of inactivating wash steps with trypsin or human serum
T = number of days in culture
Ci = number of viral particles added

Considering the 7 centrifugation/resuspension steps as wash steps, no inactivating wash steps, 8 days of culture, and 2.3×10^{12} vector particles as worst-case, the calculated reduction ratio is 339. This calculation however does not take into account the exposure to 2% human serum for 8 days, which is expected to result in additional inactivation of the vector.

The COGEM Advice CGM/090331-03 recommends that cells transduced with 3rd generation lentiviral vector with SIN can be safely handled under ML-1 or under open conditions when the reduction ratio is >100.

In conclusion, the viral vector starting material is effectively deactivated and/or removed during the CTL019 manufacturing process. The amount of infectious vector particles potentially remaining in the CTL019 cell product is considered to be zero.

A2.11. Describe the coding genes and the regulatory sequences present in the vector and in the DNA inserted into the vector.

A full description must be provided of the inserted or deleted genetic material, also discussing the functions of the sequences, for example:

- *regulatory sequences, such as promoter, terminator, and enhancer sequences;*
- *structural genes;*
- *in case of insertion of a transgene: the function of the coded proteins in the donor organism (the donor organism is the organism from which the gene originally was isolated or in which it occurs naturally) and the expected function in the test subject;*
- *in case of deletion: the function of the deleted genetic material in the original organism;*
- *in case of point mutation(s): the effect of the point mutation or mutations on the function of the modified genetic material in the original organism and the expected function in the test subject;*
- *whether the vector or the DNA inserted into the vector contains elements of which the origin or function is unknown.*

The sequences present in the lentiviral vector genome and the transfer plasmid pRKHVmuEC19 are described below. For the sequences present in the packaging plasmids, see A2.8.
The CTL019 vector genome on the transfer plasmid pRKHVmuEC19 contains a CMV promoter, minimal lentiviral sequences, and the transgene expression cassette (EF1α promoter, CAR transgene and a modified WPRE) (see Figure 1c for a schematic drawing).

In addition, the transfer plasmid pRKHVmuEC19 also contains a kanamycin resistance gene and a bacterial origin of replication (ori) in the plasmid backbone (not part of the vector genome and not integrated into T-cell genome). A plasmid maps is provided in Appendix III (confidential information).

CMV promoter

A Cytomegalovirus (CMV) promoter is used in the transfer plasmid pRKHVmuEC19 to transcribe the vector genome in the HEK293T cells during production of the lentiviral vector. However, the CMV promoter itself is not part of the single stranded mRNA vector genome that is transcribed from the transfer plasmid and incorporated into lentiviral particles. Furthermore, during the process of transgene integration into T cells, the 3' self-inactivating LTR of the lentiviral vector is duplicated, replacing the 5' LTR, such that integrated vector sequence is flanked at both ends by a copy of the self-inactivating 3' LTR. Therefore, even if a small portion of the CMV promoter would be present at the 5' end of the vector genome in the vector particles, it would not get integrated into the T-cell genome upon transduction.

Lentiviral sequences

The vector genome contains the minimal cis-acting viral sequences that are required for vector packaging, reverse transcription and integration of the vector genome into the host cell genome.
The LTRs contain repeated sequences at the extreme ends of the RNA required for reverse transcription, and attachment (att) target recognition sites for integration. The LTR have been modified to self-inactivating (SIN) LTRs, which mean that no full length vector genome is transcribed.
The packaging signal (Ψ) overlaps the 5'LTR and a small portion of the gag gene. This is an RNA structural feature with a complex stem-loop structure required for packaging, i.e. incorporation of viral RNA genome within particles as they assemble within the cytoplasm.
The Rev Response element (RRE) is a structural element recognised by the Rev protein, resulting in the export of the RNA from the nucleus to the cytoplasm where the lentiviral particles are assembled. The RRE is part of the Env gene.
The polypurine tract (PPT) is a region towards the 3' end of the genome that is required for second strand DNA synthesis. The central polypurine tract (cPPT) is conserved among lentiviruses and needed to generate lentiviral vector, but its precise function is not well characterized.

EF-1α promoter

Transgene transcription is under control of the human elongation factor 1α (EF-1α) promoter. The CAR-19 transgene was combined with the human EF-1α promoter in the lentiviral transfer plasmid.

The CAR-19 transgene

The transgene is a chimeric antigen receptor targeted against the CD19 antigen (CAR-19). It consists of a murine anti-CD19 scFv, a human CD8α hinge and transmembrane domain, and human 4-1BB (CD137) and CD3ζ (T-cell receptor ζ) intracellular signalling domains (Figure 2 and Table 3). The anti-CD19 scFv was originally derived from the mouse hybridoma cell line FMC63, as described in Nicholson et al. (1997). The leader peptide, CD8α hinge and transmembrane region and the intracellular domains of 4-1BB and CD3ζ were obtained by PCR sub-cloning using a human spleen cDNA library (Imai 2004). The confirmed sequences were combined at the University of Pennsylvania (UPenn) in the USA by standard molecular cloning to construct the CAR-19 transgene (Imai 2004).

Figure 2. Structure of the CTL019 CAR

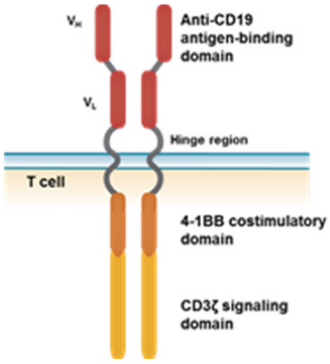


Table 3.Origin and Source of Transgene elements

Transgene	Element	Origin of the sequence Source
Murine single chain antibody fragment (scFv) specific for CD19	Murine hybridoma cell line FMC63 (Nicholson 1997)	Synthetically generated
CD8 transmembrane region	Human spleen cDNA library (Imai 2004)	Synthetically generated
CD3zeta signalling chain	Human spleen cDNA library (Imai 2004)	Synthetically generated
4-1BB intracellular signalling domain	Human spleen cDNA library (Imai 2004)	Synthetically generated

WPRE

The expression cassette includes the modified Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The WPRE enhances expression from the transgene cassette (Zufferey et al 1999). Expression of the X-protein and peptides derived from it has been ablated by mutations in the promoter and at the translation start site (Kingsman et al 2005). The sequence was originally isolated from the Woodchuck hepatitis B virus (WHV8) DNA (Donello et al 1998). The modified WPRE contained within the CAR-19 expression cassette was generated synthetically.

A2.12. Provide a molecular characterization of the genetically modified viral vector.

A sequence analysis must be supplied for all inserted or deleted sequences, so that the precise location of the modification and, in case of insertion, the number of copies present and the orientation of the insertion cassette can be determined. The sequence to be submitted must contain the region of the intended modification as well as the recombination sequences used, also including the flanking sequences of the genome of the original organism. In the case of biological unrestricted GMOs, newly created fusion ORFs must be described and subjected to a bio-information analysis.

The complete genome of the viral vector must be characterized on a molecular level. This may be done according to a sequence analysis or a Southern blot analysis. During the production of a GMO, naturally occurring processes may lead to unintended modifications in the genome. These modifications may affect the fitness of the ultimate GMO and, thus, the results of the environmental risk assessment. Observed anomalies in the genome compared with the expected sequences, such as unexpected deletions, mutations or recombinations, must be described and interpreted. Phenotypic data may be used to support the data on the molecular characterization.

The sequence of the complete genome does not need to be submitted. A bio-information analysis must be carried out for the sequences and the annotated results must be presented. For more information, please refer to the COGEM advisory report CGM/130227-05.

Sequencing pf plasmids

The transgene plasmid and the 3 packaging plasmids are all fully sequence-characterized before use. Only plasmid batches with a sequence corresponding 100% to the expected sequence is used to manufacture lentiviral vector.

Sequencing of transduced CTL019 cells

After integration into human T-cell genomic DNA, the vector sequence was verified by sequencing the entire vector genome, from long terminal repeat (LTR) to LTR. The experimentally determined sequence was identical to the expected sequence based on the transfer plasmid pRKHVmuEC19. These data support the conclusion that the CTL019 rdLVV performed as expected and successfully integrated the vector backbone elements and the CAR-19 transgene transcriptional unit into the T-cell genomic DNA with full fidelity.

A2.13. Describe the origins of the cells/cell lines in which the original viral vector is cultivated. Also indicate which of the genetic components of the cell could possibly cause complementation or recombination.

When answering this question, elaborate on the characteristics of all cell lines to be used. Describe which cell types this concerns as well as their origins (e.g. human kidney epithelial cells).Also discuss the possibility of the genetic material in the cells/cell lines causing a certain interaction with the original vector, such as by complementation or recombination.

The lentiviral vector is manufactured in the Human Embryonic Kidney (HEK) 293 cell line.
The cell line has been extensively tested for adventitious virus and is free from lentivirus that could complement or recombine with the lentiviral vector.

Reversion of vector to replication competency theoretically might also occur as a consequence of recombination between vector and human endogenous retroviral sequences (HERVs). HERV sequences are known to be present in the human genome (Brodsky 1993, Leib-Mosch 1990, Lower 1981, Seifarth 2005, Wilkinson 1993) HERVs are retrotransposons consisting of retrovirus-like DNA sequences such as LTRs and *gag-pol* and *env* open reading frames (ORFs). HERVs entered the human genome as exogenous retroviruses which later became endogenised. HERVs account for 8% of the human genome (Landers 2001,) but all known HERVs are replication incompetent due to the accumulation of inactivating mutations and deletions over hundreds of thousands or millions of years. The most complete HERVs belong to the HERV-K group and are present as full length proviruses in the human genome (Turner 2001,). The majority of HERVs are related to beta-retroviruses but sequences related to gamma-retroviruses have also been identified by sequence homology searches (Dewannieux 2013, and Villesen 2004). Sequence homology would be necessary between the vector and HERVs. The vector only retains a limited portion of the lentiviral genome, the LTRs and the extended ψ packaging signal. This limited portion of lentiviral sequences makes recombination with HERVs unlikely.

The starting material, lentiviral vector is prepared via a transient transfection system in which the HEK293T cell line is transfected with the pRKHmuEC19 transfer plasmid and the three packaging plasmids, as described in A2.10.

The produced virus is used to transduce autologous T-cells *ex vivo* generating CTL019 (see A2.10).

To generate a safe vector system all the dispensable HIV-1 ORFs (Tat, Vif, Vpr, Vpu, Env, and Nef), representing 85% of the original genome have been removed . In addition, the essential components of the vector system are split among four plasmid constructs (Figure 1) and the packaging plasmid that expresses the Gag and Pol polyproteins from nucleotides sequences that are not identical to wild type HIV-1 Gag and Pol gene sequences (the same amino acids, but codons are divergent from wild type HIV DNA sequences) in order to minimize the potential for homologous recombination, thereby preventing the generation of a replication-competent virus.

Recombination of vector packaging plasmids and cellular DNA leading to RCL has not been reported with the current viral vector systems in the literature (Cornetta 2011).

For details see A2.9.

Moreover, viral vector system, vector product and CTL019 cell product are tested to ensure the absence of RCL (for details see A3.2).

Conclusion

In conclusion, the emergence of RCL in the CTL019 cell product is considered extremely unlikely and thus is mainly a theoretical risk with no data in support of that risk. Taking into account the safety features in the design of the lentiviral system, the rigorous testing of the vector lots to be used in the manufacturing of CTL019 for the presence of RCL and the VSV-G testing of the final CTL019 cell product as a surrogate test for RCL, the risks related to RCL formation are negligible.

A2.14. Summarize the data in a diagram ('map') of the genetically modified organism. Also indicate any relevant helper sequences that may possibly be present.

Present clear maps of the genetically modified organism, such as plasmid maps, showing all the constituent parts of the vector. In this schematic depiction, the construction of the GMO must be clearly indicated.

See question 2.8 and 2.11.

A2.15. Indicate the degree to which the host range of the genetically modified viral vector has been or may be altered, relative to the original virus.

When answering this question, provide an argument that elaborates on the host range, host specificity and the tissue and cell tropism of the genetically modified viral vector, relative to

the original virus. Also consider any modifications that were made in order to create the original vector.

Host range of wild type HIV-1 is limited to humans and chimpanzees.
Wild type HIV-1 can infect many cell types, mainly lymphocytes, but also macrophages, and microglia in the brain, and other neurological cells (Kallings 2008)

The genetically modified viral vector used to transduce the T-cells to produce CTL019 has an increased host range and tropism compared to the wild type HIV-1 by using the heterologous coat protein VSV-G in place of the native HIV-1 envelope protein. VSV-G pseudotyped lentiviral vectors have a very broad host range and infect most mammalian and also non-mammalian cells (i.e pantropic).

Please note that the viral transduction is done ex-vivo. The transduced cell suspension that is infused does not contain virus particles anymore (see A2.10)

A2.16. What physiological (including pathogenic) effects may be caused by the genetically modified viral vector; and what are the available treatments?

Indicate which physiological processes may occur following the application of the GMO in the host.
A comparison must be made between its possible pathogenic properties and those of the original virus. In particular, the pathogenic properties that may be created specifically by the GMO should be considered.

Immunogenicity. It is possible that the transduced CTL019 cells may be immunogenic, and that the patients will have an immune response directed against the scFv; this has not had clinical consequences in previous CTL019 trials. If an immune response to the cells occurs, it is possible that the cells will be rejected.

Immunoglobulin depletion. Transient or permanent host B-cell depletion is also a potential risk with CTL019 cells, since normal B-cells also express CD19. This is expected to resolve when the CTL019 cells are cleared. Patients that develop CTL019 related B-cell aplasia are at increased risk of infections and may require periodic infusions with immunoglobulin to prevent infections.

Transformation/insertional mutagenesis. There is a risk that people who receive gene transfer may develop new tumours derived from their genetically modified cells. This risk is primarily associated with viral gene transfer vectors that integrate into the cellular DNA where they may dysregulate genes controlling proliferation. Transformation has not been observed following adoptive T-cell transfer in hundreds of cancer and HIV patients receiving gammaretroviral modified T-cells treated on multiple protocols at many academic centers.

Theoretically, the insertion of the DNA sequence of the rdLVV into the T-cells is non-directional and therefore, bears the risk of insertional mutagenesis. However, three factors prevent such insertion events from being directly carcinogenic:

1. First, retrovirus vector insertion is almost uniformly monoallelic.
2. Second, some gene insertions may trigger differentiation or apoptosis, or otherwise reduce the survival probability of the affected cell clone.
3. Third, a single insertional mutation is, to the current knowledge, not sufficient to develop a malignant phenotype (Baum 2003).

Classical life time studies in rodents are not appropriate for genetically modified cell therapy products. However, in B-ALL xenografted mice treatment with CTL019 did not lead to derailment of T-cell growth in mice kept for 6 to 7 months. (The follow-up of these immunocompromised mice is limited to 6-7 months due to the shorter life expectation in comparison to wild type mice).

Furthermore, no cases have been described in the literature in which malignant transformation of a mature CAR genetically modified T-cell has been demonstrated. Additionally, UPenn analysed the malignant potential of CAR modified mature CD4 T-cells and followed up more than 500 patient-years

after introducing gamma-retroviral vector-engineered T-cells and did not find any evidence of vector-induced immortalization of T-cells (Scholler 2012).

A lentivirus insertion site analysis (LISA) of tisagenlecleucel samples manufactured for 6 paediatric ALL (B2202) and 6 DLBCL (C2201) patients as well as tisagenlecleucel manufactured with cells from 2 healthy volunteers showed a highly polyclonal product and a near-to-random distribution of insertion sites, as expected for a lentiviral vector.

There is no risk of insertion into the germline as mature CD4+ and CD8+ T-cells will be used as starting material.

If a malignant transformation would occur, this could be treated using chemotherapy and/or radiation therapy.

Uncontrolled T-cell proliferation. CTL019 cells could proliferate without control of normal homeostatic mechanisms. In pre-clinical studies, CTL019 cells have only proliferated in response to physiologic signals or upon exposure to CD19. In the context of this protocol it is possible that the T-cells will proliferate in response to signals from the malignant tumour or normal B-cells. This could be beneficial or harmful depending on the extent of proliferation. If any subject develops excessive CTL019 cell accumulation, corticosteroids will be administered to eradicate the infused cells. This has not been observed to date with CTL019.

Risk of tumour lysis syndrome (TLS) related to cytoreductive chemotherapy or CAR T-cells. The risk of TLS is dependent on the disease and burden of disease, but in most cases, this risk will be low. Patients will be closely monitored both before and after chemotherapy and CTL019 infusions including blood tests for potassium, phosphate, LDH and uric acid. Patients will receive hydration and allopurinol or rasburicase to minimize any toxicity should signs of significant acute tumor lysis begin to occur.

Cytokine Release Syndrome (CRS) / Macrophage Activation Syndrome (MAS) CRS has been observed in patients after treatment with CTL019. Patients with clinical responses exhibited some level of CRS that ranged from mild to severe consisting of fever, hypotension, capillary leak, hypoxia or other symptoms.
Cytokine production is required for the activation, expansion and cytolytic function of T-cells and for CTL019 T-cells. Therefore some degree of CRS may be a desired clinical outcome. Premature or early intervention with anti-cytokine therapy may therefore abrogate the anti-tumor efficacy of CTL019. However, in case of severe CRS requiring treatment, tocilizumab (an anti-IL6-receptor antibody) therapy has been utilized (described below) with effective management of CRS and in these cases successful CTL019 T-cell expansion was still seen.

A2.17. Indicate the possible transmission routes of the genetically modified viral vector.

Provide all the observed and assumed routes of transmission. Indicate the degree to which the transmission of the GMO could be made easier by helper functions, or by the presence of replication-competent virus. Furthermore, indicate the degree of impact on transmission due to the modification and the cells in which the GMO was cultivated or those infected with the GMO. Make a comparison with the original virus.

Viral vector

The viral transduction is performed outside of the Netherlands. The final cell suspension product (CTL019) does not contain infectious viral particles anymore (for rationale, see A2.10).

Even if a very low number of infectious viral particles would be present in the CTL019 cell product, the vector is replication-deficient and could only integrate once and no further spreading is possible. In addition, any remaining vector will be inactivated by the complement component of human serum after administration to the patient or accidentally to an unintended recipient.

CTL019 (Transduced T-cells)

The predicted habitat of the genetically modified cells (the CTL019 cell product) is only the patient from which the cells were derived. Factors that may allow release of CTL019 cells outside the restricted host are blood tests and accidental injury. If these occur, there is no risk to the environment as CTL019 does not survive in the environment, and is rapidly inactivated by standard means of disinfection.

Any released CTL019 cells cannot be transmitted by air, but could be transferred through accidental injection or contact with broken skin. Medical personnel (or other individuals), if exposed to CTL019 through accidental injection of minimal numbers of CTL019 cells, would eliminate the cells via their immune system and not experience adverse effects beyond a normal immune reaction. Thus no lasting negative consequences are expected in this case.

A3. Production of the GMO

A3.1. State under whose responsibility the production of the GMO is carried out.

Answer:

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

O Production will be by and under the responsibility of the applicant but does not form part of this license application:

- O A separate application for production will be submitted for contained use
- O Reference is made for the production to an existing license for contained use: (State here the number of the relevant GMO license)
- X Production will be under the responsibility of third parties. If production is in the Netherlands, please state the number of the relevant GMO license. Please state if production is outside the Netherlands.

Production of CTL019 will take place outside of the Netherlands.

A3.2. 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 and describe the points in the production process at which quality control takes place. State which controls are carried out, the sensitivity of the tests and which methods are used for the controls.

Quality control of the Viral Vector (rdLVV)

The lentiviral vector (starting material for CTL019) is produced in HEK293 cells using transient transfection of transfer and packaging plasmids. After harvest, the vector is purified and then sterilized, concentrated and filled in vials.
The quality of the vector is verified through in process testing and release testing that must meet predefined acceptance criteria:

- The vector harvest is tested for absence of adventitious agents (viral contaminants and mycoplasma).
- Final filled vector as well as end-of-vector-production cells are tested for the absence of RCL (for more details on RCL tests see below).
- Levels of residual plasmid DNA are controlled in the final filled vector
- Vector quantity is determined by RT-qPCR, and expressed as vector RNA copies/mL
- Biological activity of the vector is determined as the efficiency the vector can transduce HEK293 cells. This is quantified by a qPCR-based genome integration assay, and results expressed as transducing units per mL
- Additional release tests of purified and final filled vector include tests for identity, sterility, endotoxin, host cell DNA

Quality control of CTL019 cell product

The CTL019 manufacturing process is described in A2.10, In process control (IPC) and process monitoring tests performed during manufacture include:

- Cell number and viability is controlled at various steps throughout the manufacturing process
- Transduced cells are quantified by flow cytometry for the CAR transgene during the cell expansion phase and pre-harvest
- qPCR for VSV-G DNA to confirm absence of RCL is performed pre-harvest

Release testing of the final CTL019 cell product includes:

- Identity by qPCR for the CAR transgene
- Total cell count, number of viable cells and viability
- Percentage of T-cells and B-cells
- Potency is determined as percentage of cells expression the CAR transgene (by flow cytometry), and by measuring cytokine release in response to CD19-expressing target cells
- Endotoxin, sterility and mycoplasma
- qPCR for VSV-G DNA to confirm absence of RCL (for details on the RCL test see A3.2).

RCL testing

RCL testing on the viral vector

During the manufacture and release testing of the rdLVV, a cell-based RCL assay is performed. The absence of RCL in the vector is confirmed by a C8166 cell-based RCL test with PERT detection This test is performed on the final vector preparation as well as on end-of-production cells used to manufacture the vector. No RCL has been detected in any of the vector lots manufactured,

The assay is capable of detecting a single infectious unit of the positive control virus per test vessel in the presence of test article vector (LOD = 1 RCL / test vessel). The level of confidence of detecting a hypothetical RCL present at extremely low concentrations is dependent on the sample volume tested, and the only way to definitively demonstrate the absence of RCL in a batch is to test the entire batch which is not feasible. Therefore, a statistical approach based on FDA guidance (*Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*) is used: a sufficient volume of the final vector is tested to allow 95% confidence of detecting RCL if present at a concentration of 1 RCL/100 mL in crude vector material. This corresponds to roughly 1 RCL / $6x10^7$ transducing units (TU). The average amount of vector used for transduction is $3x10^8$ TU. Therefore, it could be considered that there is 95% confidence of detecting hypothetical RCL if present at a level of 5 RCL per vector transduction “dose”.
It should be noted that there are no reported events of RCL generation from a 3rd generation lentiviral vector system, and the vector system used for CTL019 has added safety features wherein all meaningful homology between the separated vector components has been removed from the system (see A2.8, A2.9 and A5.3). Therefore, RCL is considered only a hypothetical possibility and the actual theoretical number of RCL per transduction is close to zero. No RCL has been detected in any of the vector lots manufactured.

Since the cells are expanded for at least 8 days after transduction, it is not possible to calculate how many RCL could be theoretically present and administered per infusion. Any hypothetical RCL added during transduction would likely be inactivated/removed during the cell process similar to potential free vector particles (see A2.10), but could also potentially multiply by replication; in the latter case it would likely be detected by the RCL testing of the final product (see below).

RCL testing on the final CTL019 cell product and during CTL019 manufacture: qPCR for VSV-G DNA

To confirm absence of RCL in the CTL019 cell product, a quantitative PCR (qPCR) assay has been applied. The assay specifically detects VSV-G sequence in genomic DNA using primers and a probe that hybridize to VSV-G sequences. The limit of quantification (LOQ) for this assay has been determined to be at 10 copies/unit DNA (=50 copies/µg DNA). Detection of VSV-G DNA is used as marker for RCL, therefore the acceptance criterion is "no detection of VSV-G DNA" as a marker for RCL, i.e. less than the LOQ of the test method.

The VSV-G qPCR assay is performed on both the pre-harvest and on the final CTL019 cell product.

The limit of quantification (LOQ) for this assay has been determined to be at 10 copies/unit DNA (=50 copies/µg DNA, corresponding to 1 copy / 3 044 cells using a theoretical mass of 6.57 pg DNA per cell). The maximum total number of cells administered per patient is 2.5x1010. Please note that the main RCL testing is done during release testing of the vector starting material (see above) and the VSV-G qPCR on the cells is only performed to re-confirm the absence of actively replicating RCL. No events of recombination leading to RCL formation has not been reported with the current lentiviral vector systems.

RCL testing on the final CTL019 cell product: cell based assay

A cell-based RCL testing is not performed routinely as part of at the release testing of the final CTL019 cell product, since this is controlled at the vector.

One lot of CTL019 cell product which was manufactured at the Novartis cell processing facility in Morris Plains, New Jersey, US, using healthy donor apheresis starting material was tested for RCL based on the cell based assay and RCL was confirmed negative (<LOD). In addition, the first 5 patient clinical lots will be subjected to RCL testing with indicator cells (co-cultivation with C8166 cells).

RCL testing in patients

In addition, peripheral blood is collected from patients in the study and long term follow-up (Pre-treatment, 3, 6, 12, 24, 36, 48 and 60 months, after 60 months once per year until 15 years) to further substantiate the absence of RCL by VSV-G using qPCR.

A3.3. 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.

CTL019 final product testing

The investigational CTL019 cell product is quality controlled according to specific SOPs. The full release specifications are confidential and provided in Appendix I –*CTL019 testing* (confidential). A summary of the release testing is provided in A3.2.

Summary of criteria used for release of the final CTL019 cell product:

- Absence of RCL is confirmed by qPCR for VSV-G DNA; the acceptance criterion is "no detection of VSV-G DNA" i.e. less than LOQ (<50 copies/µg DNA)
- Identity has to be confirmed (by qPCR detection of the transgene)
- Transduction efficiency, percentage of T-cells, total cell count, number of viable cells and viability are above the specification limits set
- Functionality of the genetically modified cells has to be confirmed (by measurement of biological functionality in response to CD19-expressing target cells)
- Impurity levels of residual beads and B-cells are below specification limits
- Sterility and absence of mycoplasma has to be confirmed
- Endotoxin levels are below specification limits

A4. Description of the research

Administration

A4.1. How many test subjects will take part in the study?

Here, the maximum number of test subjects to be treated should be indicated. This number can be higher than the intended number of test subjects. Please be aware of the fact that the number you enter limits the permit. For example, if you indicate that there will be 50 test subjects, then no more than 50 can be included in the study.

B cell malignancies comprise a heterogeneous group of neoplasms. Different trials with CTL019 will be set up for these different B-cell malignancies. In total, in the Netherlands a maximum of 200 patients with different B-cell malignancies will be treated in clinical trials with CTL019.

A4.2. Describe how the batch containing the GMO will be transported and prepared for administering to the test subjects.

Describe how, after delivery, the GMO preparation will be transported to the hospital pharmacy for example. Also describe the way in which the GMO preparation will be handled, as well as the conditions under which they will be handled, in order to get the preparation ready to be administered. If this handling will be carried out under an existing permit for Contained Use (IG), the number of the GMO permit concerned must be stated. In addition, describe the way in which the GMO preparation will be transported to the test subject.

CTL019 cell product will be transported to the Cell Therapy Facility (CTF) of the UMCU where it will be stored until use. From there, the frozen CTL019 product will be transported to the Prinses Máxima Center according to GMO guidelines. At the Prinses Máxima Center ward the cryobag is examined for any breaks or crack before being thawed in a water bath at 37°C. The CTL019 cell bag must be placed in a secondary overwrap bag to avoid direct contact between the primary container and the water. In the event of leakage from the bag, the secondary overwrap bag prevents contamination of the water bath. The secondary overwrap bag should be kept open at the top. The cryobag within the overwrap bag is placed in the pre-heated water bath; the ports of the bag should not be immersed below the water surface. When the contents of the cryobag become slushy, the bag is removed from the 37 ºC water bath and once the cell suspension is completely thawed, the outside of the bag is wiped with sterile gauze.

CTL019 cells will be administered through IV infusion by directly spiking the cryobag with the infusion line and then hanging the product bag. CTL019 will be administered to the patient through the IV infusion by gravity.

A4.3. 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.

Experienced nurses will administer the CTL019 cells intravenously using standard medical precautions for immunosuppressed patients. Patient protective isolation is according to institutional standards and policies.

Even though CTL019 has not been associated with disease, is not pathogenic and does not replicate, exposure of people other than the patients will be avoided. CTL019 is not transmitted by air but could be transferred through accidental injection or contact with broken skin. The personnel handling the product bags containing CTL019 cells will follow the appropriate guidelines for infusion of cellular products.

A4.4. Which doses will be administered and at what time points during the study will they be administered?

Indicate which doses of the GMO will be administered. Also provide an administration diagram, showing at what times they will be administered.

CTL019 drug product is formulated as a single-dose cell suspension cryobag for infusion. The dosing depends on the study and can vary from one single infusion to three infusions spread over 3 days. Each cryobag will contain between 2 x 10^6 and 5 x10^8 CTL019 transduced viable T-cells per dose.

A4.5. What other medication will be administered to the test subject that may possibly affect the GMO to be administered?

Any medication that would affect the GMO and possibly also the environmental risk assessment should be named here. If, for example, a vaccination study is conducted, this may also include challenge with the wild-type virus.

No other medication which could influence the product will be administered during CTL019 infusion. Only in case of severe toxicity concomitant medications can be used for best supportive care.

Sampling

A4.6. Describe which of the samples taken from the test subject may contain GMOs.

Provide an overview of the samples and indicate whether GMO material is expected to be present in them.

The patients' blood, bone marrow or lymph node biopsy samples can contain the patients modified / transduced T-cells, but CTL019 T-cells are not pathogenic, do not replicate or survive outside the patient. See also A5.2 and A5.3.
Patient samples cannot contain free viral vector: The CTL019 cell product contains no or negligibly low levels of free viral vector (see A2.10), and any potential remaining vector particles in the product would be inactivated by the complement component of human serum after administration to the patient.

A4.7. Describe the method of sampling and how the samples will be subsequently processed.

In answering this question, also indicate how transmission of the GMO during sampling and testing will be prevented. For the subsequent processing, indicate what physical restrictions apply. In case the work concerned will be carried out in the Netherlands but is not part of the current permit application, then refer to the permit for work under Contained Use and provide the number of the GMO permit this concerns.

Sampling of blood and bone marrow will be performed according to local procedures by experienced laboratory personnel and nursing staff. To avoid possible spread of the blood the blood draw will be performed with a closed tube system and the used materials (i.e. needle) will be disposed in a sealed container for contaminated material.
On several time points after CTL019 T-cell infusion approximately 50 mL will be drawn from the patients to perform the local and central laboratory assessments.

Samples will either be determined directly, stored for future use or transported to the external laboratories.
The local laboratory is classified as Beheersingsniveau 1 as described by the Arbeidsomstandigheden besluit/ARBO informatie 09 'Biologische agentia'

CTL019 transduced T-cells, like human T-cells, are easily inactivated outside the host by inappropriate media, or exposure to low pH, higher temperatures (>50°C), pasteurization (60°C for 10 h), and microwave.

Cells present in the CTL019 product are easily killed by lipid solvents, alcohol and disinfectants.

Vesicular stomatitis virus G (VSV-G) pseudotyped lentivirus vectors are inactivated by human serum complement and sensitive to any standard detergent, desiccation, non-physiological pH conditions or above room temperature.

At site 70% Ethanol will be used for regular disinfection on used surfaces. In case of a spill, the surface will be treated with 1000 ppm Chloride.

Sampling, transportation of samples within the hospital, storage and processing will be done according to standard hospital procedures as the samples do not contain viral particles (see A2.10 and A4.6) and any genetically modified cells present in the samples do not represent a specific safety concern (see A2.17 and A5.2-A5.5). They will be treated as regular hospital samples and are considered to be putatively infectious human diagnostic/study samples at all stages of sampling, storage and transport.

A4.8. How is the GMO preparation detected after being administered?

State, if applicable, when GMO components or 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.

According to current understanding, complete and long-lasting remission/response in B cell malignancies likely requires CAR expressing effector T-cells able to kill CD19 expressing target cells efficiently, robust expansion of the infused CAR expressing T-cells, and some level of persistence of CAR+ memory cells in the patient.

After administration of CTL019 the patients' blood is tested for the presence of CTL019 cells presence by q-PCR and flow cytometry during most patient visits.

- Q-PCR assay is used to detect CAR+ cells (transgene copies/microgram DNA) in whole blood, bone marrow and other tissues/fluids, such as cerebrospinal fluid (CSF)(if obtained for other purposes).
- Flow cytometric analysis is used to detect CAR expressing cells in whole blood, bone marrow and other tissues (if feasible). Mononuclear cells are isolated from peripheral blood and bone marrow samples using a standard methodology and examined for the frequency of CD19 antigen-specific chimeric antigen receptor (CAR)-expressing T cells using flow cytometry.

The patients will also be monitored to ensure absence of RCL, for details see A2.13.

Based on previous experience with similar clinical trials in which RCL was never detected and the fact that CTL019 is not an infectious agent, a monitoring plan for unintended recipients is not implemented. However, any potential or known unintended exposure will be reported to the ESO, the State Secretary and Novartis within 24 hours of the investigator's knowledge of the event of exposure. Novartis will seek to follow-up with the exposed individual, if necessary, to collect more information about the exposed individual contact with clinical trial subject, signs and/or symptoms related to the exposure, medical history, and/or outcome of the exposure. If the exposed individual is reporting signs or symptoms suspected to be related to CTL019 exposure, the exposed individual may be asked to have a blood test to evaluate the presence of CAR-19.

Waste management

A4.9. 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 prior to, during and after administration of CTL019

The original cryo bag that contained the GMO T-cells, infusion tubing and other materials that have been in contact with the product before administration to the patient will be disposed in according to the local guidelines for GMO waste, following the GMO regulation 2013.

All solid and liquid waste will be processed according to the standard hospital guidelines.

Patient room: After use the room will be cleaned using standard hospital cleaning and disinfection procedures with a hydrogen peroxide solution (2% Aseptix or equivalent).

A5. Environmental risk analysis

Environment-related information originating from earlier experiments

A5.1. Describe the results originating from earlier (pre-) clinical studies with the GMO and which are important for the environmental risk assessment.

In answering this question, you should elaborate on results that were achieved using an identical or similar GMO, if these are relevant for the environmental risk assessment of the present application. Important data include data on shedding, duration of latent presence of the vector/GMO, transmission of the vector/GMO, and possible interaction with other micro-organisms (including viruses). For each of these results, describe the trial set up (e.g. doses used, method of administration, detection test and detection limit) and the relevance to the work in the present permit application.

In addition to testing each vector lot before use in the CTL019 manufacturing, the first 5 patient clinical lots were subjected to RCL testing with indicator cells (co-cultivation with C8166 cells) and all returned negative for RCL (see A3.2 for RCL testing details). More than 250 patients with r/r B-cell malignancies for whom no other effective therapeutic options were available have been treated so far and no RCL has been detected.

The risks of abnormal cell-toxic behaviour, derailed cell growth control and abnormal biodistribution in vivo have been adequately addressed in an immunocompromised mouse model xenografted with human ALL tumour. No tisagenlecleucel-related toxicities as well as no hyperproliferative disorders indicating a vector related tumour risk were identified in this study. Unscheduled animal deaths were attributed to graft vs. host disease (GVHD) and B-ALL tumour progression. Furthermore, no immortalization of transduced T cells was observed in vitro.

Various Phase 1 and 2 studies in relapsed/refractory (r/r) B-cell malignancies are ongoing under University of Pennsylvania sponsorship at Penn (adult patients) and at the Children's Hospital of Philadelphia CHOP (paediatric patients) as well as under Novartis sponsorship at various study sites worldwide.

Shedding of the vector and/or formation of RCL is not expected in patients of this trial.
Irrespective of the expected safety outcome of the GMO, some considerations will be implemented in the unlikely event of RCL detection:

i. Intensive follow-up of the patient in consultation with gene therapy experts, study investigators, and Health Authorities;
ii. Inform local and country specific public health officials;
iii. Identify sexual partners and provide appropriate counseling and intervention.

For further details on RCL testing see A2.13

Risk analysis

This is the most important aspect of the whole application!

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 GMO 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. Section A6 of this form describes those aspects which must at least be taken into consideration.

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 A5.2 – A5.5):

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;*
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.*

A5.2. 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.

Possible adverse effects related to the CTL019 cell product (genetically modified T-cells)

The genetically modified T-cells are patient-specific and do not survive outside the patient. The cells are not pathogenic and do not persist or replicate in the environment. Therefore, no adverse effects on the environment are expected.

In the unlikely event of the provision of CTL019 cells to an unintended human recipient (through accidental injection), the CTL019 cells would be recognized as 'foreign' cells (due to the mismatching of human leukocyte antigens) and therefore eliminated through the individual's innate (i.e. complement mediated lysis and phagocytic cells) and adaptive immune system (i.e. secreted antibodies and B- and T-cells). Adverse effects would be limited to a normal immune reaction to foreign cells, and no specific adverse effect related to the genetic modification of the cells is expected.

There is a theoretical possibility that the CTL019 cells could persist if transmitted to an immunocompromised individual. In this highly unlikely case, the theoretical adverse effects would be the same as the possible adverse effects in patients which are described in A2.16 (immunogenicity, immunoglobulin depletion, insertional mutagenesis, uncontrolled T-cell proliferation, cytokine release syndrome).

Possible adverse effects related to free vector particles

The level of free vector particles in the CTL019 cell product is considered to be practically zero (see A2.10). After administration to the patient, any potential remaining viral particles would be inactivated by the complement component of human serum.

In the event of accidental injection of CTL019 cell product into unintended recipients, any theoretical remaining infectious viral particles would likely be inactivated by the complement in human serum. Even if an infectious particle would survive long enough to infect a cell, the vector is replication-deficient and could only integrate once, and no further spreading is possible. No adverse effects would be expected due to a single or very few integration events.

Possible adverse effects related to the theoretical formation of RCL

In the highly unlikely event of RCL being present in the CTL019 cell product or formed in the patients, a recombinant virus encoding the CAR19 transgene could be spread from cell to cell and also potentially to other individuals. In case of such an event, the viral particles would not survive in the environment outside of the body, as described for the parental virus.

A5.3. State according to which scenario the GMO 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.

Scenarios that can lead to spread of the CTL019 cell product (genetically modified T-cells)

The likelihood of release of CTL019 from the patient is minimal except in case of accidental injury.

Since the CTL019 cell product consists of genetically modified autologous T-cells and the genetic modifications introduced do not affect the host range, the habitat of the cells is restricted to the patient from which the cells were derived.

In the case of accidental release of the CTL019,cells the transduced cells cannot persist outside the patient for long periods and maintain viability (except for in appropriate cell culture conditions in the laboratory). Since the cells are sensitive to even moderately harsh conditions, it is considered highly unlikely that spread would occur in the environment and the CTL019 cells would quickly be rendered non-viable by the prevailing conditions.

The possible routes for any released CTL019 cells to be transmitted to unintended recipients is through accidental injection or contact with broken skin, or via exposure to the mucous membranes of the eyes, nose or mouth. However in this unlikely case, the CTL019 cells would be eliminated through the person's immune system. Only in immunocompromised individual is there a theoretical risk that the CTL019 cells could persist, and in this case they would not be expected to spread further. The risk of widespread infection is considered negligible.

There is clear evidence that the transduced T-cells cannot be a source for further transduction, since replication-defective rdLVV integrate their genetic cargo into the T-cell genome without transferring virus-derived coding sequences (Baum 2003).

Scenarios that could lead to spread of free vector particles

As described in A5.2, the level of free vector particles in the CTL019 cell product is considered to be practically zero (see A2.10). The purity of the product is confirmed as part of product release a and no monocytes or macrophages or dendritic cells have been detected within the final product so far. CTL019 is typically composed of >99% T-cells, which is assessed as part of product release testing.

After administration to the patient, any potential remaining viral particles would be inactivated by the complement component of human serum. The risk of shedding or release of infectious viral particles from the patient is therefore considered negligible.

Scenarios that could lead to formation of RCL

Formation of RCL during vector manufacture

RCL particles could theoretically be formed during vector production through recombination of viral sequences from the 4 plasmids. Several safety features in the design of the lentiviral system used in vector production are implemented to prevent this risk. The essential components of the vector system are split among four plasmid constructs (Figure 1) and therefore, 3 separate recombinant events would be necessary to create a RCL. All the dispensable HIV-1 ORFs (Tat, Vif, Vpr, Vpu, Env,and Nef) representing over 85% of the original genome have been removed. The minimal vector system design, in combination with the use of a codon-optimised Gag/Pol construct and a heterologous envelope glycoprotein, means that sequence homology between vector components has been minimized limiting the possibility of homologous recombination (for more details on the safety features, see A2.8 and A2.9). To date there are no known cases where this type of vector (3rd generation lentiviral vector with SIN produced by four plasmids) has produced RCLs. The risk of formation of RCL during vector production is considered negligible.

Furthermore, the vector lots to be used in the manufacturing of CTL019 as well as the final CTL019 cell product are tested to confirm absence of RCL (see A3.2).

Formation of RCL due to recombination with retrovirus

Theoretically, formation of a RCL could occur through recombination of the integrated vector with a retrovirus infection in the transduced cells. The inclusion/exclusion criteria provide strict standards that exclude retroviral infections (i.e., HIV, HTLV, HBV, and HCV). Treated patients are not likely to become infected with HIV post-administration due to general precautionary measures. However, even if this would occur, homologous sequences need to be present in the T-cells and in the viral sequences for recombination to occur. Deletions and changes of viral sequences in rdLVV, specifically the LTRs, have caused a reduction of sequences homologous to wild type HIV that do not allow for homologous recombination anymore. Therefore, homologous recombination between the rdLVV provirus inserted in the CTL019 and HIV is not expected. The likelihood of formation of RCL through recombination with a retrovirus is therefore considered negligible.

Formation of RCL through mobilization of human endogenous retroviral sequences (HERVs)

Reversion of vector to replication competency theoretically might also occur as a consequence of recombination between the vector and HERV sequences present in the human genome (Brodsky 1993, Leib-Mosch 1990, Lower 1981, Seifarth 2005, Wilkinson 1993). HERVs are retrotransposons consisting of retrovirus-like DNA sequences such as LTRs and *gag-pol* and *env* open reading frames (ORFs). HERVs entered the human genome as exogenous retroviruses which later became endogenised. HERVs account for 8% of the human genome (Landers 2001,) but all known HERVs are replication incompetent due to the accumulation of inactivating mutations and deletions over hundreds of thousands or millions of years. The most complete HERVs belong to the HERV-K group and are present as full length proviruses in the human genome (Turner 2001,). The majority of HERVs are related to beta-retroviruses but sequences related to gamma-retroviruses have also been identified by sequence homology searches (Dewannieux 2013, and Villesen 2004).
Sequence homology would be necessary between the vector and HERVs for recombination to occur. The vector only retains a limited portion of the lentiviral genome, the LTRs and the extended ψ packaging signal. This limited portion of lentiviral sequences makes recombination with HERVs very unlikely. And since all known HERVs are replication incompetent due to inactivating mutations and deletions as described above, even if a recombination event would occur this is unlikely to create a replication-complement virus.

Patients treated with CTL019 have been systematically monitored to determine the risk of RCL generation after administration of the product. No events have occurred within the ongoing studies or in any of the previous studies conducted with CTL019. RCL testing in these studies include a cell-based assay on the vector before use for production of CTL019, a cell based and/or a qPCR assay for VSV-G on the cell product before release, and a monitoring of the patient by qPCR for VSV-G post-infusion. All RCL test results conducted were negative.

A5.4. Give an estimate of the chance that the adverse effects described in A5.2 could actually occur.

Give a reasoned estimate of the chance (likelihood) of the aspects described in A5.2 and A5.3, also taking account of the number of test subjects and the dosage.

Likelihood of adverse effects related to the CTL019 cell product (genetically modified T-cells)

The likelihood that adverse effects will occur with CTL019 T-cells is very low because the autologous CTL019 T-cells are not pathogenic, and do not replicate or survive outside the patient, as described in A5.2 and A5.3.

Only if accidentally injected into immunocompromised individual, a theoretical risk exists that the CTL019 cells could persist. The likelihood of this is considered very low. In this case, adverse effects of CTL019 cannot completely be ruled out. However, the number of CTL019 cells injected would be much lower than in patients, which further reduces the likelihood of adverse effects.

Likelihood of adverse effects due to formation of RCL

As described in A5.3, the risk of formation of RCL during either vector production or through recombination in patient cells is negligible. Therefore, the likelihood of adverse effects related to RCL formation is also negligible.

Likelihood of adverse effects related to free vector particles

As described in A5.2 and A5.3, the amount of vector particles is CTL019 cell product is practically zero and no free vector can persist in the patient. Therefore, the likelihood of free vector particles spreading to the environment or other individuals is very low.

In the highly unlikely case of accidental injection of CTL019 cell product containing a very low number of free vector particles into unintended human recipients, the viral particles would likely be inactivated by the complement in human serum. Even if an infectious particle would survive long enough to infect a cell, the vector is replication-deficient and could only integrate once and further spreading is not possible. The likelihood of adverse effects due to a single vector integration is considered extremely low.

A5.5. 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. If risk management measures are necessary in order to limit the risks, these should be specified further in the questions below.

The habitat of the CTL019 cells is restricted to the patient from which the cells were derived. CTL019 does not have the capacity to disseminate vertically or horizontally in the ecosystem. Accidental injury of the patient and/or loss of blood or blood testing will not lead to dissemination of CTL019 into the environment, as cells will undergo rapid inactivation outside the patient.

Dissemination of CTL019 cells could therefore only occur between human beings. However, persons, other than the patients from whom the cells have been derived, would eliminate the cells as a result of their immune response.

Even though CTL019 has not been associated with disease, is not pathogenic and does not replicate or survive outside its host, exposure of people other than the patients will be avoided. Patients will be instructed not to donate blood, organs, tissues and cells for transplantation (applicable for the lifetime of the patient).

Personnel who participate in the administration of blood components must be trained in transfusion procedures, in recognition and management of adverse reactions in the patients and safe handling of genetically modified organisms.

Following administration of CTL019 cells at the study site, materials used during infusion (e.g., gloves, gauze) will be disposed of in accordance with the institutional requirements for GMO hospital waste

Only in immunocompromised individual is there a theoretical risk that the CTL019 cells could persist and give rise to adverse effects similar to the possible adverse effects in patients (A2.16). Thus, immunocompromised health care professionals should be advised of the risk prior to engaging in work with CTL019 cells.

Double packaging for internal transport and safe administration procedures are supposed to prevent accidental spills of CTL019 cell product. However, if a spill occurs despite of these conditions, spill will be done according to the UMC Utrecht GMO spill procedure (Appendix). Following these procedures, accidental release into the environment can be excluded.

Accidental loss of CTL019 cell product is unlikely to occur due to controlled procedures of transportation, delivery and use, including all necessary documentation.

In the unlikely event of the transmission of CTL019 cells to an unintended human recipient, if clinically indicated to alleviate any symptoms of primary infection (if deemed necessary), physicians will take all necessary measures to assure safety. Further spread from the individual can be mitigated by educational materials to increase awareness of the infection and preventative measures which can be taken to prevent transmission to close contacts.

The amount of free vector particles in the CTL019 cell product is expected to be negligible (zero or practically zero (A2.10)). After administration to the patient, any potential remaining viral particles would be inactivated by the complement component of human serum. Therefore, the vector cannot be transmitted to the environment or other individuals from the patient. Theoretically, if a low number of free vector particles were present in the CTL019 cell product, they could enter a medical health care professional by accidental injection, however this cannot lead to further spreading and is not expected to cause adverse effects (see A5.4).

As described in A5.3, the risk of formation of RCL is negligible. As part of the patient monitoring, patients are tested for RCL. In the very unlikely event of RCL detection, the following mitigation will be implemented:

i. Intensive follow-up of the patient in consultation with gene therapy experts, study investigators, and Health Authorities;
ii. Inform local and country specific public health officials;
iii. Identify sexual partners and provide appropriate counseling and intervention.

In conclusion, the risks to the environment related to the genetically modified cells (CTL019 cell product), and the possibility of free vector particles and/or RCL formation are negligible.

Risk management

A5.6. 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 <u>only</u> necessary for the protection of the environment or which criteria might possibly have an effect on safety for human beings and the environment.

The question of whether replication-competent LV could be generated upon recombination within the CTL019 after administration has been considered. For recombination to occur, homologous sequences need to be present in the T-cells and in the viral sequences. The inclusion/exclusion criteria provided strict standards that exclude retroviral infections (i.e., HIV HBV, and HCV). In addition, the quality control standards support absence of RCL from CTL019 prior to infusion to patients or the likelihood for the formation of replicative progeny post treatment

Exclusion criteria:
Uncontrolled active or latent hepatitis B or active hepatitis C
HIV infection

Human T-cell lymphotropic viruses (HTLV) infection

Moreover, patients will be instructed not to donate blood, organs, tissues and cells for transplantation after CTL019 transfusion (applicable for the lifetime of the patient).

A5.7. Describe which measures are provided for in respect of the hospitalization of the test subject.

When answering this question, please emphasize those aspects that are important in preventing spread in the environment of the test subject. Also indicate if, apart from medical reasons, hospitalization is prescribed as a way of protecting against possible effects for humans and the environment.

Patients are only hospitalized for medical reasons; no hospitalisation is required for protection of the environment because the amount of infectious lentiviral particles within the transduced T-cells (CTL019) is zero/below detectable limit and the original DNA vectors used to construct the viral particles are completely absent from the medicinal product. Moreover CTL019 T-cells are not pathogenic and does not replicate. The medicinal product itself therefore does not infer a risk for the environment.

A5.8. If the test subject is to be hospitalized, what are the criteria for his or her release from hospital?

Describe the criteria on the basis of which the test subject will be released from hospital.

Patients will be monitored for adverse events such as fever, chills and shortness of breath. Patients who are clinically stable and do not have any signs or symptoms can be released from the hospital and followed up at the outpatient department. Hospitalization is only necessary for medical reasons and not because of possible contamination risk to the environment. Discharge criteria used for patients treated with CTL019 are therefore no different from discharge criteria for other, similar, patients.

A5.9. 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. In case existing guidelines will be used, please indicate what they are (such guidelines must be attached to this application). Additional or deviating measures also must be described.

Prior to and during administration the GMO is contained; there will be no activities where third parties including medical personnel come into direct contact with CTL019.
In case of spill we will follow the UMC Utrecht guidelines for GMO waste, following the GMO regulation 2013.

Procedure in case of unexpected situations and serious incidences

A5.10. Describe the procedures to be followed if changes in the risk management are required for medical reasons.

This may concern situations in which a test subject needs to be removed from isolation; for example because he or she requires intensive care, or in situations when unexpected effects are being observed, including Suspected Unexpected Serious Adverse Reactions (SUSARS) or Serious Adverse Events (SAEs). Also consider the situation of a test subject having died and an autopsy being required.
All unexpected situations and serious incidences must be reported immediately. See the 'Procedure for unwanted incidences' (www.loketgentherapie.nl)

Since there is no isolation of the patients and the risk of the GMO spreading in the environment is negligible, the same measures apply in all cases.

A5.11. 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 patient discontinues the study prematurely, the total monitoring of 15 years will be maintained. If a patient is lost to follow-up after receiving the treatment. no further specific measure will be applied

Monitoring

A5.12. 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.

The amount of infectious lentiviral particles within the transduced T-cells (CTL019) is zero/below detectable limit and the original DNA vectors used to construct the viral particles are completely absent from the medicinal product. Moreover CTL019 T-cells are not pathogenic, do not replicate and do not survive outside the patient. The medicinal product itself therefore does not infer a risk for the environment. Therefore monitoring of the environment is considered not necessary

A6. Conclusions of 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).

The predicted habitat of CTL019 is exclusively the treated patients.
The use of CTL019 will be limited to medical facilities clinics, where controls are in place to limit use and spread beyond the defined patient treatment regimen. Even if residual rdLVV is present in the preparation, the vector is self-limiting and thus the expansion or survival of rdLVV in the environment is impossible.
There is no risk concerning the contamination of the environment with genetically modified T-cells. T-cells are not able to survive outside their human environment unless they are specifically cultured in cell culture or in an adequate live model. Furthermore, genetically modified T-cells cultured ex-vivo will not survive more than a few days in culture. Direct measurements of circulating T-cell kinetics in normal (healthy) humans, CD4+ and CD8+ T-cells had half-lives of 87 days and 77 days, respectively, with absolute production rates of 10 CD4+ T-cells/µl per day and 6 CD8+ T-cells/µl per day (Hellerstein 1999). Another study has shown that transduced T-cells do not survive more than 8-12 days in culture conditions (Schuberth 2013).
CTL019 cells can persist in the patient for long time periods as memory cells. Persistence of CTL019 cells can been detected by flow cytometry at low levels at least 6 months after CTL019 infusions in responding patients and remain detectable by quantitative polymerase chain reaction (qPCR) in patients with sustained remissions.
As to the transducing vector, rdLVV: In the present protocol, there is no in-vivo administration of the viral vector, but ex-vivo genetically modified T-cells are administered. The chance that free viral particle are associated with the cells at administration approximates zero. Even in the unlikely case that some free viral particles are present; these have extremely low fitness when exposed to environmental conditions.

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).**

In the proposed clinical protocol the cells used are transduced CD4+ and CD8+ T lymphocytes, engineered to target malignant B-cells. Once infused the transduced T-cells do not, likely, possess any selective growth advantage *in-vivo*, their half-life time is similar to or lower than the non-transduced T-cells.

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.**

There is no evidence on the possibility of gene transfer from CTL019 to other species under the conditions of proposed administration. CTL019 administration is performed in strict aseptic controlled conditions; therefore the transmission to other species under the conditions of the proposed administration is not possible.
Finally, in case of accidental transmission of the autologous medicinal product to an allogeneic, non-target human subjects, the cells will be recognized as MHC mismatched and therefore be cleared by the immune system of a non-target individual.

4. **Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO and non-target organisms (if applicable).**

None known or predicted

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).**

The likelihood of post-administration shifts in biological interactions or host range is negligible.

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.**

The product is not intended for animal feed or as a veterinary product neither any subproduct/metabolites are intended for this scope. Therefore this point is not applicable.

7. **Possible immediate and/or delayed effects on biogeochemical processes caused by possible direct or indirect interaction between the GMO and the target and non-target organisms in the vicinity of the GMO introduction(s).**

None known or applicable

8. **Possible change in the current medical practice.**

Not known

A7. General (personal) information (confidential part)

Responsible employees (RE)
Should the work described so require, a distinction will be made between an RE (contact person) for work other than the clinical application (RE-1, such as a pharmacist or researcher) and an RE (contact person) for the clinical application (RE-II, such as the treating doctor).

Responsible Employee for work other than the clinical application of the GMO (RE-I)

A7.1. Title, initial, prefix, surname:

A7.2. Institution/company:

A7.3. Department/section:

A7.4. Correspondence address:

A7.5. Postal code and town/city:

A7.6. Telephone and fax number:

A7.7. E-mail address:

Responsible Employee for the clinical application of the GMO (RE-II)

A7.8. Title, initial, prefix, surname: :

A7.9. Institution/company: Prinses Máxima Centrum voor kinderoncologie

A1.10. Department/section: Dept. of pediatric Oncology

A7.11. Correspondence address: PO Box 113

A7.12. Postal code and town/city: 3720 AC Bilthoven

A7.13. Telephone and fax number:

A7.14. E-mail address:

Environmental Safety Officer (ESO)

A7.15. Title, initial, prefix, surname:

A7.16. Institution/company: p/a University medical Center Utrecht

A7.17. Department/section: Afd. Laboratorium en Apotheek

A7.18. Correspondence address: heidelberglaan 100

A7.19. Postal code and town/city: 3584 CX Utrecht

A7.20. Telephone and fax number:

A7.21. E-mail address:

Signature

On behalf of the legal entity a person must sign who is registered as an authorized signatory in the Dutch Business Register (NHR). A person may also be mandated to sign on behalf of public legal entities. This mandate showing the authority to sign must be submitted together with the application.

On behalf of the legal entity date
Name: Professor

REII (clinical application) date
Name: Professor

ESO date
Name: Dr.

Appendices:

I. CTL019 testing dd. 01-nov-2017 [CONFIDENTIAL]
II. SNIF for CTL019
III. Plasmid maps [CONFIDENTIAL]
IV. Safety regulation for gene therapy PMC dd. 12 September 2017

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