AMT-061
AAV5-hFIXco-Padua (serotype 5 adeno-associated viral vector containing a codon optimised human factor IX Padua gene)

**Belgium**

# Technical Dossier
# for Deliberate Release into the Environment of Genetically Modified organisms in accordance with Annex II of Directive 2001/18/EC of the European Parliament

**Version 1.1**

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**Sponsor**
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## 1. LIST OF ABBREVIATIONS

| | |
|---|---|
| AAV | Adeno-associated viral vector |
| AAV-n | Adeno-associated viral vector serotype number (1, 2, etc) |
| AAV5-hFIXco-Padua | Serotype 5 adeno-associated viral vector containing a codon optimised human factor IX gene; the gene is codon-optimised to enhance expression and the product is a hyperactive variant of factor IX carrying an R338L mutation (referred to as “FIX-Padua”) |
| BEVS | Baculovirus expression vector system |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FIX | Factor IX |
| gc | Genome copy |
| GMO | Genetically modified organisms |
| hAAT | Human alpha-1-antitrypsin |
| HCR | Hepatic control region |
| hFIX | human Factor IX |
| hFIXco | Codon optimised human Factor IX |
| IMP | Investigational Medicinal Product |
| ITR’s | Inverted Terminal Repeats |
| kb | kilobytes |
| Kg | Kilogram |
| L | Litre |
| LOD | Limit of detection |
| LOQ | Limit of quantitation |
| µg | Microgram |
| µL | Microlitre |
| mL | Millilitre |
| ng | Nanogram |
| ORF | Open reading frame |
| PCR | Polymerase Chain Reaction |
| ppm | Parts per million |
| QC | Quality control |
| qPCR | Quantitative Polymerase Chain Reaction |
| rAAV | Recombinant adeno-associated viral vector |
| rcAAV | Replication competent adeno-associated viral vector |
| SV40 | Simian virus 40 |
| wt | Wild type |

## 2. GENERAL INFORMATION

### A. Name and address of the notifier

uniQure Biopharma BV
Paasheuvelweg 25A
1105 BP Amsterdam
Netherlands

### B. Title of the project

Open label, single dose, multi centre trial investigating an adeno associated viral vector containing a codon optimized Padua derivative of human factor IX gene (AAV5-hFIXco-Padua, AMT-061) administered to adult patients with severe or moderately severe hemophilia B.

## 3. INFORMATION RELATING TO THE GMO

### A. Characteristics of (a) the donor, (b) the recipient or (c) (where appropriate) parental organism(s)

#### 1. Scientific name

Recombinant adeno associated viral vector containing the wild type modified to express the Padua derivative of human coagulation factor IX cDNA.

#### 2. Taxonomy

Serotype 5 adeno-associated viral vector.

#### 3. Other names (usual name, strain name, etc.)

AAV5-hFIXco-Padua, AMT-061.

#### 4. Phenotypic and genetic markers

The Sponsor has developed two gene therapy vectors to treat severe or moderately severe hemophilia B. The AMT-060 and AMT-061 based gene therapy vectors are identical except for a two-nucleotide substitution resulting in a single codon change (AGG to CTG) in the coding sequence for FIX, corresponding to an Arginine to Leucine substitution in the transgenic protein. The 5' and 3' terminal regions of the vector genome are known as Inverted Terminal Repeats (ITRs). These sequences, cloned from the viral genome of wild type AAV-2 (Samulski et al., 1987), contain self-complementary sequences forming hairpin structures. During production of the GMO, the ITRs are required for packaging of the viral genome into the particles. After transduction of the target cells, the ITRs are required for stabilization of the viral genome. The ITRs initiate complementation of the (unstable) single stranded genome DNA into (stable) double stranded DNA by host cell polymerases. Alternatively, because the ITRs are palindromic, multiple viral genomes can assemble ITR to ITR to form larger double stranded DNA structures known as concatemers. These concatemers remain transcriptionally active and stable episomal structures (Schnepp et al., 2005). The ITRs do not contain any open reading frames for protein expression.

The ITRs directly flank the expression cassette, consisting of the LP1 enhancer/promoter, the SV40 intron, the codon optimized hFIX-Padua therapeutic transgene and a polyA sequence.

The LP1 enhancer/promoter consists of consecutive segments of the human apolipoprotein hepatic control region (HCR) and the human alpha-1-antitrypsin (hAAT) gene promoter. The enhancer/promoter is followed by a modified SV40 small t antigen intron, to enhance expression (Nathwani et al., 2006). The LP1 enhancer/promoter mediates robust and liver-specific expression of the therapeutic transgene (Nathwani et al., 2011).

The codon optimized hFIX-Padua sequence encodes for the normal human clotting factor IX protein. The nucleotide sequence was codon optimized to enhance protein expression. The codon-optimized hFIX-Padua sequence was designed based on a naturally occurring FIX-Padua variant.

Codon optimization was performed by replacing the codons in the natural FIX-Padua sequence which are less frequently found in highly expressed human genes with the (synonymous) codons that are most frequently found in highly expressed eukaryotic genes, using a previously described algorithm (Haas et al. Current Biology. 1996(6):315–324). The optimized sequence was synthesized as oligonucleotides, and subsequently assembled by ligation of these oligonucleotides. This strategy of codon optimization does not affect the amino acid sequence of the protein. The protein translated from codon optimized messenger RNA is therefore the same as the naturally occurring protein. By consequence it does not have any effect on the normal functioning of the protein and the immune response.

Expression of this transgene in liver cells yields functional human clotting factor IX-Padua which is secreted into the circulation. Hence, the Padua Factor IX transgene is the therapeutic payload of AMT-061.

The SV40 polyA sequence serves to stabilize the messenger RNA.

The nucleotide sequences between the abovementioned functional sequences mainly consist of sequences that enable(d) molecular engineering of the construct (e.g. multiple cloning sites). The vector genome contains 5 cloning/joining sites. These sites are extremely short in length, at 18, 4, 12, 5, and 23 nucleotides. Because of this, the possibility that coding or functional sequences in the cloning/joining sites can reasonably be excluded.

**5. Degree of relatedness between parental organisms**

**Parental Organism**

The parental virus is adeno-associated virus (AAV). Adeno associated viruses are small (approximately 25 nm in diameter) non-enveloped, non-pathogenic parvoviruses. AAV infect cells through a receptor mediated process, after which the viral DNA is transported to the nucleus. AAV needs a helper virus, such as adenovirus or herpes virus, to replicate.

Wild-type AAV have a linear single-stranded DNA genome approximately 4.7 kilobase (kb) long. The genome consists of two coding elements: the replicase (rep) gene (encoding rep78, rep68, rep52 and rep40) required for AAV replication and packaging, and the capsid (cap) gene encoding the capsid proteins (VP1, VP2, and VP3). These elements are flanked by two inverted terminal

repeats (ITRs), which serve as substrates for the Rep proteins during replication and packaging of the vector genome. The vector genome may consist of a positive (+) or a negative (-) strand.

The parental virus consists of a single-stranded DNA vector genome (derived from AAV2) which is encapsidated by an icosahedral protein capsid (derived from AAV5). Both parental AAV's, AAV-2 and AAV-5, are AAV serotypes that naturally occur in the human population. The vector genome used contains two DNA sequences (inverted terminal repeats: ITRs) that are derived from the viral genome of AAV serotype 2 (AAV-2). The capsid proteins are derived from AAV serotype 5 (AAV-5).

**Parental Organism Host Range**

Dependoviruses with similarity to AAV have been identified from other species; the AAV serotypes found in humans seem to be restricted to primates (Arbetman et al, J. Virol., 2005). Both humans and primates may show pre-existing (neutralising) antibody titres against AAV. Infections with AAV occur frequently, and are world-wide. The prevalence of neutralising antibodies against AAV is found in the adult European population to be for AAV5 (3.2%) followed by AAV8 (19%) and is highest for AAV2 (59%) and AAV1 (50.5%) (Boutin et al., 2010; Calcedo et al., 2009). AAV infections are non-pathogenic, i.e. not associated with disease manifestations.

**Table 1: Classification of Adeno-Associated Virus**

| | |
|---|---|
| **Baltimore classification** | Group II |
| **Family** | Parvoviridae |
| **Genus** | Dependovirus |
| **Species** | Adeno-Associated Virus |

Tissue/cell tropism is determined by serotype, i.e. the capsid moiety. In the case of AMT-061, the capsid is AAV-5 derived. Preclinical studies in non-human primates (including uniQure' s own studies in non-human primates) have shown that, following administration, AAV-5 displays strong liver-directed tropism, with vector DNA sequence also detected in the spleen and adrenal glands (Nathwani et al., 2011).
The vector genome is packaged into capsids composed of the three viral proteins, VP1, VP2, and VP3. Each capsid consists of 60 VP proteins in total, which are arranged in icosahedral symmetry.

## 6. Description of identification and detection techniques

The Rep sequences used to produce AMT-061 was cloned from the vector genome of wild type AAV-2.

The cap gene used to produce AMT-061 was cloned from the vector genome of wild type AAV-5. The resulting baculovirus construct was designed so that it encodes all three Cap proteins of the

wild type AAV-5 serotype. These proteins, originating from overlapping reading frames, together form the dense icosahedral capsids of AAV-5.

The ITRs that are present in the vector genome of the GMO AAV5-hFIX_Padua were cloned from the viral genome of wild type AAV-2 (Samulski et al., 1987). The Baculovirus Expression Vector System exploits the fact that for the production of recombinant AAV particles, Rep and cap can be provided *in trans*, i.e. the vector genome does not need to contain these sequences as long as the proteins they encode are provided alongside it. As such, the genome of a recombinant AAV-based vector can be gutted to contain little more than the transgene expression cassette encoding the therapeutic protein of interest. The resulting baculovirus construct thus contains the two AAV-2-derived ITR's, which flank the transgene expression cassette. The gutting of the vector genome renders the resulting recombinant AAV-based vector completely replication defective, i.e. even in the presence of a helper virus, because the rep and cap genes are missing.

In conclusion, the three components necessary for production of AMT-061 in insect cells are offered in the form of three recombinant baculoviruses, containing a Rep gene, a cap gene, and the AAV vector genome (ITRs + transgene expression cassette). These three sequences were each derived (cloned) from wild type (parental) AAV. The DNA sequences were generated *in vitro* using molecular biological techniques. As such, the DNA sequences used to produce AMT-061 are synthetically derived and they do not have a true physical origin in AAV.

An (early) batch of AMT-061 was assigned to serve as primary reference standard. The primary reference standard was subjected to massively parallel sequencing. Full coverage was returned and alignment to the reference standard was demonstrated (see also confidential information). These results demonstrated correct sequence identity of the vector.

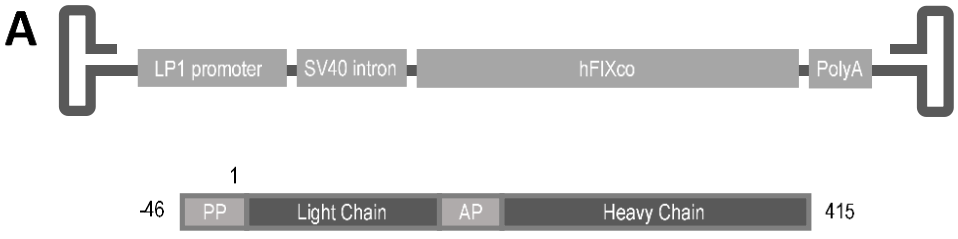
**Origins of the cells/cell lines in which the original viral vector is cultivated**
As outlined, AMT-061 is produced in an insect cell-based production platform in which the three sequences required to produce AMT-061 particles are offered by three different recombinant baculoviruses.

The platform utilises a proprietary cell line (expresSF+), which is derived from the insect *S. frugiperda*. The baculovirus backbones used to generate the recombinant baculoviruses are part of the same proprietary system.

Regarding potential for interactions between cellular DNA and parental (baculovirus) vectors: high throughput sequencing data of the GMO AMT-061 vector preparations have shown that more than 99% of the DNA present in AMT-061 represents the anticipated vector genome. Small amounts (0.2% of total) of baculovirus derived DNA and traces of insect DNA were detected. These DNA impurities are controlled for during quality testing. For previous vectors produced using the Baculovirus Expression system it was shown that these fragments do not harbor coding sequences.

The traces of insect DNA detected in AMT-061 represented short sequences randomly scattered across the insect genome. These results strongly suggest that the baculovirus- and insect-derived DNA impurities present in AMT-061 are not the result of recombination events but due to promiscuity of the Rep packaging protein.

AMT-061 does not contain the Rep - or cap sequences necessary for replication of wild type AAV. However, these sequences are provided *in trans* during the production process. A quality control assay for the presence of replication-competent AAV particles in AMT-061 preparations is in place. To date, no replication-competent particles have been observed in any of the GMO AMT-061 preparations tested, confirming that the formation of replication competent particles is a hypothetical risk and not a commonly occurring phenomenon.



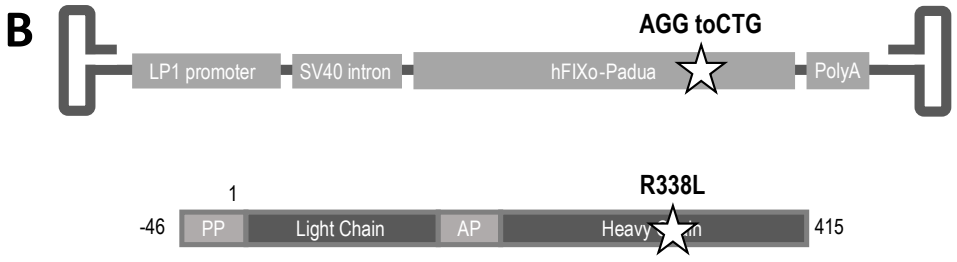



**Figure 1: Structure of Vector Genomes and Corresponding Expressed FIX Protein, for AMT-060 (A) and AMT-061 (B).**
**A: Structure of AMT-060 (AAV5-hFIXco) vector genome** and derived wild type human FIX protein. The hFIXco expression cassette is flanked by two ITRs (hairpin structures) and consists of the LP1 promoter, SV40 intron, hFIXco coding sequence, and polyA signal, in that order. Below the vector genome a schematic representation of the translated protein is provided (PP: pre-pro-peptide, AP: activation peptide). **B: Structure of AMT-061 (AAV5-hFIXco-Padua) vector genome** and derived human FIX-Padua protein. The AMT-061 vector genome is identical to the AMT-060 vector genome except for a two-nucleotide substitution (AGG to CTG as indicated). This substitution results in an Arginine to Leucine substitution in the translated protein, at position 338 (R338L).

**Changed host range of the genetically modified viral vector relative to the original virus**
The host range, tissue specificity, and tropism of AAV particles are determined by the capsid. The capsid of AMT-061 is composed of the exact same proteins that make up wild type AAV-5. The host range and tropism of AMT-061 and wild type AAV-5 are therefore not different.

**Changed physiological / pathogenic effects caused by the genetically modified viral vector**
Infection with wild type AAV is asymptomatic; AAV is not known to cause any noticeable pathology. Given that the structure and thus the potential to infect will not have changed, it is expected that the safety profile of the parental wild type AAV and the GMO AMT-061 are similar. In addition, it should be considered that AMT-061 lacks the Rep- and Cap genes present in wild type AAV2 or wild type AAV5 (i.e. the parental strains). Due to the lack of these two genes, the vector is replication-defective. Even in the presence of helper virus, the vector genome of AMT-061 will therefore not be replicated, nor will capsids be formed. As such, two processes associated with wild type AAV infection (i.e. replication and capsid formation) will not take place after infection with AMT-061. In a strict sense, the safety profile of AMT-061 is therefore theoretically more favorable as compared to the safety profile of wild type AAV.

Following administration in humans, the GMO AMT-061 is expected to home to the liver, where it will infect and transduce liver tissue. Such GMO administration will elicit an immune response very similar to natural infection with (wild type) AAV.
Following liver transduction delivery of the GMO-derived DNA to the nucleus, the therapeutic transgene is transcribed and translated, and human Factor IX protein is produced by the liver cells. The liver is a natural source of Factor IX protein, i.e. the transgene is expressed in a natural environment. Local responses to GMO-mediated Factor IX expression are therefore not expected.

The GMO-derived Factor IX protein is a human protein to which eligible patients have extensively been exposed in the form of protein replacement therapy, therefore immunological responses against this Factor IX protein are not expected to occur. The codon optimized Padua-FIX sequence resembles a naturally occurring FIX protein with normal function of the protein although a significantly higher specific activity.

Altogether as infection with wild type AAV already does not result in any noticeable pathology and Factor IX is expressed in its natural environment (the liver), in practice, the safety profiles of AMT-061 and the parental strains will be similar, in that neither will mediate any noticeable pathological effects.

Infection with wild type AAV is asymptomatic and AAV is not known to cause any noticeable pathology. Thus, treatment methods against the GMO are therefore not considered relevant.

**Possible transmission routes of the genetically modified viral vector**

GMO derived DNA is expected to shed through blood, urine, saliva, faeces and semen. The GMO-derived vector DNA which is known to be shed in body fluids or excrements is assumed not to represent infectious GMO.

The GMO AMT-061 is replication-deficient even in the presence of a helper virus infection. Replication-competent AAV (rcAAV) can theoretically be formed during production of the GMO. Presence of rcAAV is monitored as part of routine quality control for the GMO. To date, no rcAAV has ever been detected (detection limit: 10 rcAAV per $2\times10^{10}$ gc of GMO). In the unlikely event that rcAAV levels would have increased in a specific batch, this batch would fail rcAAV acceptance criteria and consequently this batch would not be administered to a patient. Altogether this scenario is considered unlikely to occur.

Ultimately, all the above scenarios are likely without consequence: in the remote chance that wild-type/replication-competent AAV is formed, this does not result in pathology/disease symptoms as infection with AAV is asymptomatic. Therefore, the GMO or sequence derived from the GMO, are not likely to replicate within the recipient following administration and therefore the absolute amount of GMO that could theoretically be spread cannot exceed the dose administered. Spread of the GMO is not facilitated through helper functions, presence of rcAAV, or recombination events between the GMO and wild type AAV.

## 7. Sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques

The quality tests relevant for environmental risk analysis are summarized in Table . Test parameters were relevant for environmental risk assessment when they (1) were representative of a GMO other than the intended vector, or (2) were representative of a nucleic acid sequence derived of such a GMO. The tests are discussed in detail below. All other quality testing is performed on test parameters not relevant for environmental risk assessment such as concentration or purity of the AMT-061 vector itself, or chemical or other non-GMO related impurities.

**Table 2: Quality tests assessing environmental risk-related parameters**

| Test | Method | Acceptance criteria [1] | Method Sensitivity |
|---|---|---|---|
| Replication competent-AAV (rcAAV) | Bio-assay | < 10 rcAAV per $2\times10^{10}$ gc [2] | 10 rcAAV per 2 $\times10^{10}$ gc[2](LOD [2]) |
| Residual infectious baculovirus | Bio-assay | < 6.8 iu [4]/mL | 6.8 iu/mL (LOD) |
| Residual baculovirus DNA | QPCR | ≤ $8\times10^{-9}$ geq / 1.0 x $10^{13}$ gc | $5.0\text{x}10^{-10}$ geq/mL (LOQ[5]) |
| Rep full-length sequences | QPCR | ≤ $9\times10^{8}$ copies/1.0 x $10^{13}$ gc | $2.2\times10^{7}$ copies/mL (LOQ) |

[1]Acceptance criteria are in place on the active substance. [2](AAV5-hFIX-) genome copies, Limit of Detection. [3]Limit of Detection. [4]Infectious units. [5]Limit of Quantitation.

**8. Description of the geographic distribution and of the natural habitat of the organism including information on natural predators, preys, parasites and competitors, symbionts and hosts**

As described in Section 3.A.5 Degree of relatedness between parental organisms , Adeno-Associated Virus naturally occur in the human population. The AAV serotypes found in humans seem to be restricted to primates despite the similarity of certain dependoviruses to AAV (Arbetman et al, J. Virol., 2005).

**Pathogenicity, attenuation and biological restrictions of the original virus**

Wild type AAV-2 and AAV-5, elements of which form the basis of the GMO, are non-pathogenic. Regarding attenuation: wild type AAV per se is dependent on the presence of a helper virus (adenovirus or herpes virus) to allow successful replication (Berns K & Parrish CR, 2007). Wild type AAV can infect host cells and release its genome to the host cell nucleus. However, in the absence of a helper virus, it remains dormant.

**9. Organisms with which transfer of genetic material is known to occur under natural conditions,**

Infection with wild type AAV is asymptomatic and AAV is not known to cause any noticeable pathology (Berns, 2005). Treatment methods are therefore not considered relevant. Wild type AAV likely spreads via the respiratory or gastro-intestinal route (Berns K & Parrish CR, 2007). Infections with wild type AAV occur frequently, and are world-wide. For example, up to 59% of the human population is seropositive for AAV-2 (Boutin et al, Hum Gen Ther 2010). Wild type AAV is dependent on a helper virus for replication, e.g. adenovirus or herpes virus. During an

active helper virus infection, newly formed wild type AAV particles can be spread together with the helper virus. The spread of wild type AAV therefore will depend on whether a helper virus is present, and whether it is present in the same compartment, i.e. at the site of active infection (Berns 2005).

**10. Verification of the genetic stability of the organisms and factors affecting it,**

Wild type AAV is a small, non-enveloped virus with a very stable capsid. Exposure to heat, UV radiation, or extreme pH can inactivate (recombinant) AAV.

**11. Pathological, ecological and physiological traits**

AAV infections are non-pathogenic, i.e. not associated with disease manifestations.

**12. Nature of indigenous vectors**

As mentioned earlier, wild type AAV per se is dependent on the presence of a helper virus (adenovirus or herpes virus) to allow successful replication (Berns K & Parrish CR, 2007). Wild type AAV can infect host cells and release its genome to the host cell nucleus. However, in the absence of a helper virus, it remains dormant. It should be noted that the GMO AMT-061 itself is an attenuated (recombinant) AAV: by design it does not contain any of the AAV genes that are essential for replication of viral DNA, the formation of viral particles, or the packaging of viral DNA into these particles.

**13. History of previous genetic modifications.**

Please see Section 3.C.1.h.

**B. Characteristics of the vector**

**1. Nature and source of the vector**

The GMO is not constructed from a parental virus or vector in the classical sense. Rather, AMT-061 is assembled from individual molecular components which are expressed using an insect cell-based Baculovirus Expression Vector System (BEVS).

The BEVS for recombinant AAV is based on the fact that the three elements required for the generation of functional AAV particles, being the Rep protein(s), the Cap protein(s) and the vector genome, can be provided *in trans*. In practice, this means that each can be provided by a different Baculovirus vector. Intracellular expression of Rep and Cap, in the presence of the AAV vector genome, results in (1) replication of the AAV vector genome by Rep protein, (2) assembly of capsid proteins to capsid particles, and (3) packaging of vector genome DNA into the capsid particles by Rep, in this case resulting in AMT-061. The three recombinant baculoviruses used for the production of AMT-061 are 1) Bac-Rep, 2) Bac-Cap and 3) Bac-hFIXco-Padua.

The system to generate these recombinant baculoviruses entails two elements, being (1) the Baculovirus genome ('backbone DNA') and (2) the pPSC10 donor plasmid. The Baculovirus DNA is linearized, using Bsu36I. This restriction enzyme cuts the Baculovirus DNA at two sites; one flanking the ORF 603 open reading frame and one within the essential ORF1629 open reading frame. This digestion excises the polyhedrin gene (Figure 2A). The pPSC10 donor plasmid is a

plasmid that can be conventionally propagated in bacteria. It contains a cloning site that is flanked by sequences homologous to the remaining ORF603- and ORF1629 sequences in the Baculovirus backbone, and can be conventionally engineered to contain an insert of choice, e.g. the Rep gene, the Cap gene or the vector genome (Figure 2B).

When Sf9 insect cells are transduced with linearized Baculovirus DNA and with pPSC10 containing the insert, homologous recombination occurs between the ORF sequences in the linearized Baculovirus DNA and in the ORF sequences present in the pPSC10 plasmid. Recombination results in exchange of the insert and restoration of ORF603 and ORF1629, generating circular, recombinant Baculovirus DNA (Figure 2C). The recombinant Baculovirus DNA is polyhedrin-deleted but otherwise replication competent, hence it propagates in Sf9 cells and positive clones can be selected by plaque forming assay. Clones are selected for genomic integrity and stability.

To survive outside the host, wild type Baculoviruses organize into so called occlusion bodies, to which the Polyhedrin protein is an essential component (Rohrmann 2013). Due to the absence of the Polyhedrin gene, and the resultant absence of polyhedron protein, recombinant Baculovirus cannot form occlusion bodies and are not stable outside of the culturing system.

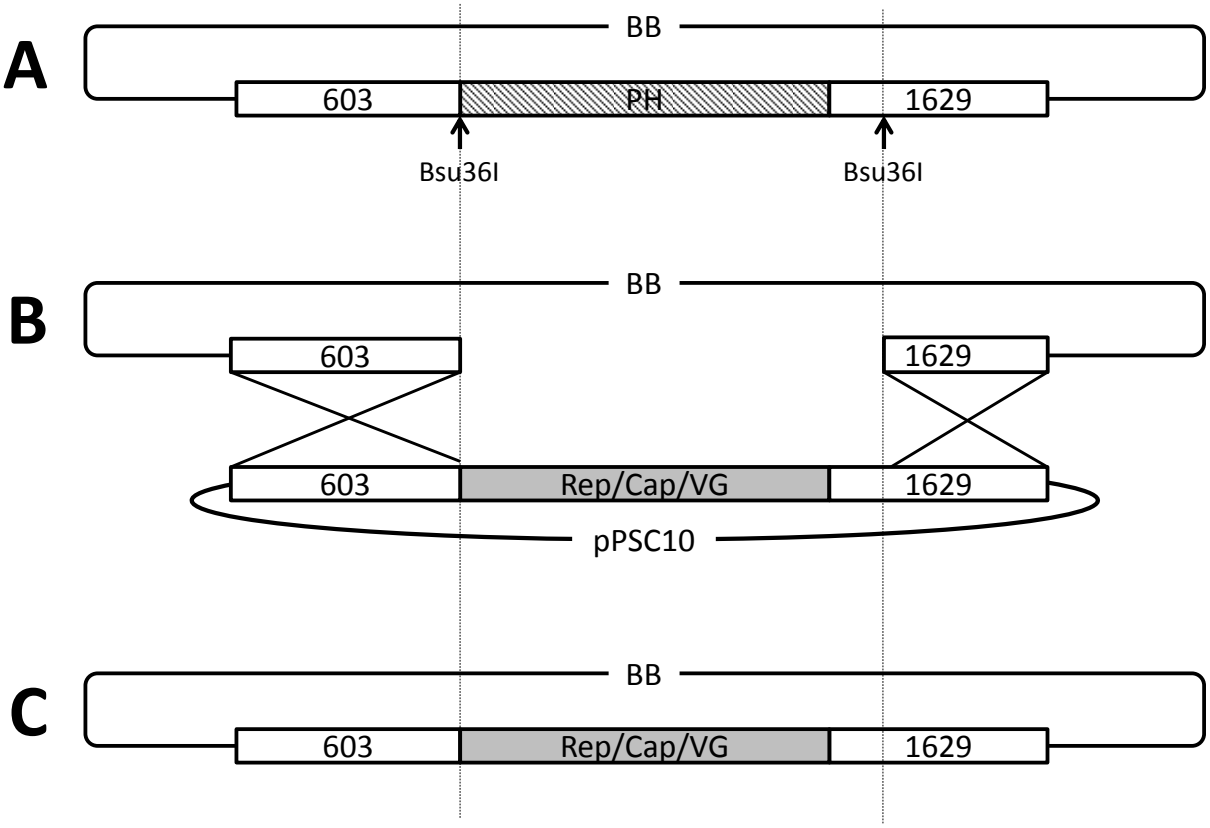
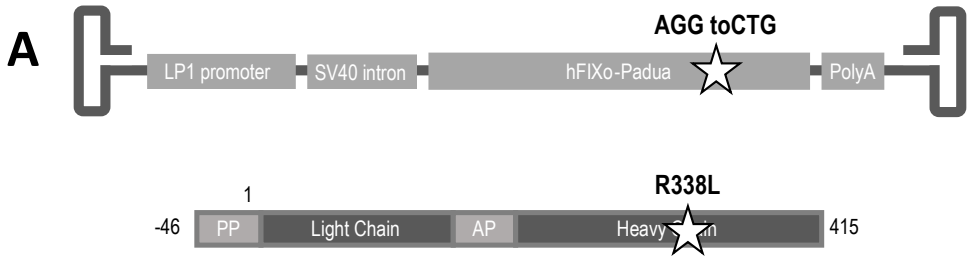


**Figure 2: Principle of recombinant Baculovirus generation.**
**A:** The Baculovirus backbone is generated from circular Baculovirus DNA by means of a digest with Bsu36I. Resultantly, the entire polyhedrin gene and part of the flanking essential ORF1629 sequence are excised. **B:** Upon co-transfection of Sf9 cells with linearized Baculovirus backbone and pPSC10 containing the insert of choice recombination takes place, resulting in exchange of the insert and restoration of ORF1629. **C:** As a result of recombination, circular recombinant Baculovirus DNA is generated. This Baculovirus can be propagated in insect cell culture but does not form occlusion bodies due to absence of the polyhedrin gene. Legend: BB; Backbone, PH; Polyhedrin, VG; Vector genome

## 2. Sequence of transposons, vectors and other non-coding genetic segments used to construct the GMO and to make the introduced vector and insert function in the GMO

The Rep sequences used for the production of AMT-061 were cloned from the vector genome of wild type AAV-2. The Cap gene used for the production of AMT-061 was cloned from the vector genome of wild type AAV-5. The three components necessary for production of AMT-061 in insect cells are offered in the form of three recombinant baculoviruses, containing a Rep gene, a Cap gene, and the AAV vector genome (ITRs + transgene expression cassette). These three sequences were each derived (cloned) from wild type (parental) AAV. The DNA sequences were generated *in vitro* using molecular biological techniques. As such, the DNA sequences used to produce AMT-061 are synthetically derived and they do not have a true physical origin in AAV.
The FIX coding sequence is under the control of the LP1 promoter (Nathwani et al., 2006). Between the LP1 promoter and the FIX coding sequence is a SV40 intron. The entire expression cassette is flanked by intact inverted terminal repeats (ITRs) from AAV2 (Samulski et al., 1987).

During production of the GMO, the ITRs are required for packaging of the viral genome into the particles. After transduction of the target cells, the ITRs are required for stabilisation of the viral genome. The ITRs initiate complementation of the (unstable) single stranded genome DNA into (stable) double stranded DNA by host cell polymerases. Alternatively, because the ITRs are palindromic, multiple viral genomes can assemble ITR to ITR to form larger double stranded DNA structures known as concatemers. These concatemers remain transcriptionally active and stable episomal structures (Schnepp et al., 2005). The ITRs do not contain any open reading frames for protein expression.



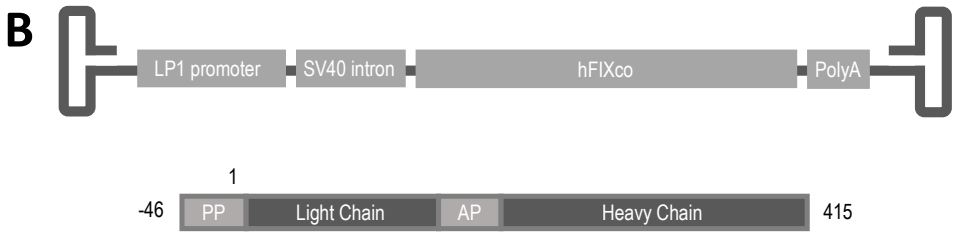



**Figure 1A:** Structure of AMT-061. Compared with the previous generation product (AMT-060; see Figure 1B) AMT-061 has a two-nucleotide substitution resulting in a single codon change (AGG to CTG as indicated) in the sequence for FIX. This substitution results in an arginine to leucine substitution in the translated protein, at position 338 (R338L). The hFIXco expression cassette is flanked by two ITRs (hairpin structures) and consists of the LP1 promoter, SV40 intron, hFIXco coding sequence, and polyA signal, in that order. A schematic representation of the translated protein is also provided where PP is the pre-pro-peptide and AP is the activation peptide
**Figure 1B:** Structure of the previous generation AMT-060 vector genome and derived wild type human FIX protein.

## 3. Frequency of mobilisation of inserted vector and/or genetic transfer capabilities and methods of determination

Wild type AAV likely spreads via the respiratory or gastro-intestinal route (Berns K & Parrish CR, 2007).

As noted above, infections with wild type AAV occur frequently, and are world-wide. For example, up to 59% of the human population is seropositive for AAV-2 (Boutin et al, Hum Gen Ther 2010).

As wild type AAV depends on a helper virus for replication, e.g. adenovirus or herpes virus, the spread of wild type AAV will depend on whether a helper virus is present, and whether it is present in the same compartment, i.e. at the site of active infection (Berns 2005).

GMO derived DNA is expected to shed through blood, urine, saliva, faeces and semen. The GMO-derived vector DNA which is known to be shed in body fluids or excrements is assumed not to represent infectious GMO.

The GMO AMT-061 is replication-deficient even in the presence of a helper virus infection. Replication-competent AAV (rcAAV) can theoretically be formed during production of the GMO. Presence of rcAAV is monitored as part of routine quality control for the GMO. To date, no rcAAV has ever been detected (detection limit: 10 rcAAV per $2\times10^{10}$ gc of GMO). In the unlikely event that rcAAV levels would have increased in a specific batch, this batch would fail rcAAV acceptance criteria and consequently this batch would not be administered to a patient. Altogether this scenario is considered unlikely to occur.

Ultimately, all the above scenarios are likely without consequence: in the remote chance that wild-type/replication-competent AAV is formed, this does not result in pathology/disease symptoms as infection with AAV is asymptomatic. Therefore, the GMO or sequence derived from the GMO, are

not likely to replicate within the recipient following administration and therefore the absolute amount of GMO that could theoretically be spread cannot exceed the dose administered. Spread of the GMO is not facilitated through helper functions, presence of rcAAV, or recombination events between the GMO and wild type AAV.

The samples taken will be analysed for the presence of vector DNA using a validated QPCR-based method as this is the most sensitive technique available to date for this specific purpose.

**4. Information on the degree to which the vector is limited to the DNA required to perform the intended function.**

The vector components of AAV5-hFIX (The AAV5 capsid and the wild type hFIX gene cassette) have been used separately in previous clinical trials (in patients with acute intermittent porphyria (http://www.aipgene.org) and haemophilia B patients (uniQure AMT-060 study, Nathwani et al., 2011), respectively). The principle of using AAV vector approaches for treatment of haemophilia B has already been established in academic studies (Cancio et al., 2013). AAV2 and AAV8, containing human FIX expression cassettes have been used in previous trials of haemophilia B. AAV-based gene therapy vectors have been used in more than 100 patients with various diseases (Ginn et al., 2013), and recently the first AAV based gene therapy product (Glybera; uniQure) has been approved for treating patients with lipoprotein lipase deficiency (LPLD) in Europe (EMA/CHMP/474664/2012 and EMEA/H/C/002145). The clinical experience with liver directed AAV gene therapy includes more than 35 patients with severe or moderately severe haemophilia B. Several clinical trials in haemophilia B patients using liver directed AAV gene therapy approaches are registered on www.clinicaltrials.gov and are known to be actively recruiting patients.

Clinical studies in humans do not include in the study design assessment of biodistribution of the gene therapy in the various organs as it would require taking biopsies. However, biodistribution is frequently studied by measuring the presence of vector sequences in various bodily fluids such as plasma, semen, saliva, nasal mucus and urine, and in faeces.

The clinical results to date with regards to AAV5-hFIX in hemophilia B (AMT-060) demonstrate that shedding occurs in (1) urine (2), saliva, faeces, nasal secretions (3) semen, and (4) blood. However, following the risk assessment as described in this document for AMT-061, the likelihood that infectious vector particles are present in biosamples is extremely low.

## C. Characteristics of the modified organism

**1. Information relating to the genetic modification:**

**(a) Methods used for the modification**

As described in Section B.1, a baculovirus system was used to manufacture AMT-061.

**Host for GMO Expression - Baculovirus Expression Vector System**

Replication-competent AAV are frequently detected in AAV preparations that were produced using conventional, mammalian cell-based production platforms; as an example, Allay *et al*. reported approximately 1 rcAAV per million particles (Allay *et al*., 2011). The preference for the

expression system chosen includes the increased safety profile of baculovirus system-produced AAV resides in the fact that the Rep and Cap genes are under control of an insect promoter and hence, in humans, do not mediate functional expression of these genes.

The GMO AMT-061, is not constructed from a parental virus or vector in the classical sense. Rather, AMT-061 is assembled from individual molecular components expressed using a baculovirus expression vector system (BEVS). BEVS is characterised by its excellent safety characteristics. First, baculoviruses are insect-specific viruses that are not capable of replicating in vertebrates (Airenne *et al*., 2013). Second, they can easily be cleared from recombinant AAV preparations, because in contrast to AAV they are large enveloped viruses which are susceptible to inactivation by surfactants and to removal by nano-filtration.

The BEVS for recombinant AAV is based on the fact that the three required elements for the generation of functional AAV particles, being the Rep protein(s), the Cap protein(s) and the vector genome, can be provided in trans, i.e. the vector genome does not need to contain these sequences as long as the proteins they encode are provided alongside it. In practice this means that each can be provided by a different baculovirus vector. As such, the genome of a recombinant AAV-based vector can be gutted to contain little more than the transgene expression cassette encoding the therapeutic protein of interest. The resulting baculovirus construct thus contains the two AAV-2-derived Inverted Terminal Repeats (ITR's), which flank the transgene expression cassette. The ITRs that are present in the vector genome of the GMO AMT-061 were cloned from the viral genome of wild type AAV-2 (Samulski *et al*., 1987). The gutting of the vector genome renders the resulting recombinant AAV-based vector completely replication defective, i.e. even in the presence of a helper virus, because the Rep and Cap genes are missing. Intracellular expression of Rep and Cap, in the presence of the AAV vector genome, therefore results in (1) replication of the AAV vector genome by Rep protein, (2) assembly of capsid proteins to capsid particles, and (3) packaging of vector genome DNA into the capsid particles by Rep, in this case resulting in AMT-061.

To survive outside the host, wild type baculoviruses organise into so called occlusion bodies, to which the polyhedrin protein is an essential component (Rohrmann, 2013). Due to the absence of the polyhedrin gene, and the resultant absence of polyhedron protein, recombinant baculovirus cannot form occlusion bodies and are not stable outside of the culturing system.
Baculoviruses are incapable of replication in mammalian cells. They have a limited range of hosts and are typically restricted to a range of closely related insect species (Airenne *et al*., 2013).

The parental (baculovirus) vectors used in the manufacture of the GMO AMT-061 are able to infect and replicate in the insect cell line used for the production.
Regarding pathogenicity, baculoviruses are not harmful to humans (Berns K & Parrish CR., 2007).

**(b) Methods used to construct and introduce the insert(s) into the recipient or to delete a sequence**

Please see Section C.1.c below.

**(c) Description of the insert and/or vector construction**

The vector construction is already described in Section 4.B.2.

The nucleotide sequence in AMT-061 was codon optimised to enhance FIX protein expression. The codon optimised hFIX-Padua sequence was designed based on a naturally occurring FIX-Padua variant.

Codon optimisation was performed by replacing the codons in the natural FIX-Padua sequence which are less frequently found in highly expressed human genes with the (synonymous) codons that are most frequently found in highly expressed eukaryotic genes, using a previously described algorithm (Haas et al., 1996). The optimised sequence was synthesised as oligonucleotides and subsequently assembled by ligation of these oligonucleotides. This strategy of codon optimisation does not affect the amino acid sequence of the protein. The protein translated from codon optimised messenger RNA is therefore the same as the naturally occurring protein. By consequence it does not have any effect on the normal functioning of the protein and the immune response.

Expression of this transgene in liver cells yields functional human clotting FIX-Padua which is secreted into the circulation. Hence, the FIX transgene is the therapeutic payload of AMT-061.

**(d) Purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function**

The insert has not been taken from an unknown sequence. In addition, inserts cloned in the baculoviruses used for the construction of the AAV vector were sequenced using massively parallel sequencing methodology.

An (early) batch of AMT-061 was assigned to serve as primary reference standard. The primary reference standard was subjected to massively parallel sequencing. Full coverage was returned and alignment to the reference standard was demonstrated. These results demonstrated correct sequence identity of the vector.

**(e) Methods and criteria used for selection**

Baculovirus vectors used were selected through plaque assay. Details on genomic stability of the baculovirus system are addressed in Section 5.A.3.B.3.b.5.

**(f) Sequence, functional identity and location of the altered/inserted/deleted nucleic acid segment(s) in question with particular reference to any known harmful sequence.**

The constructs are discussed in Section 4.B.1, 4.B.2 and 4.C.1.c.

A subset of the QC tests are indicative of correct DNA sequence identity, being (1) Vector DNA identity, (2) Vector DNA composition, (3) Potency, (4) Infectious vector titer, and (5) Genome copy concentration.

An (early) batch of AMT-061 was assigned to serve as primary reference standard. The primary reference standard was subjected to massively parallel sequencing.

There is no known harmful sequence identified.

**2. Information on the final GMO:**

**(a) Description of genetic trait(s) or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed**

**Changed host range of the genetically modified viral vector relative to the original virus**

The host range, tissue specificity, and tropism of AAV particles are determined by the capsid. The capsid of AMT-061 is composed of the exact same proteins that make up wild type AAV-5. The host range and tropism of AMT-061 and wild type AAV-5 are therefore not different.

**Changed physiological / pathogenic effects caused by the genetically modified viral vector**

Infection with wild type AAV is asymptomatic; AAV is not known to cause any noticeable pathology. Given that the structure and thus the potential to infect will not have changed, it is expected that the safety profile of the parental wild type AAV and the GMO AMT-061 are similar. In addition, it should be considered that AMT-061 lacks the Rep- and Cap genes present in wild type AAV2 or wild type AAV5 (i.e. the parental strains). Due to the lack of these two genes, the vector is replication-defective. Even in the presence of helper virus, the vector genome of AMT-061 will therefore not be replicated, nor will capsids be formed. As such, two processes associated with wild type AAV infection (i.e. replication and capsid formation) will not take place after infection with AMT-061. In a strict sense, the safety profile of AMT-061 is therefore theoretically more favorable as compared to the safety profile of wild type AAV.

As AMT-061 is expected to home to the liver, it likely will elicit an immune response very similar to natural infection with (wild type) AAV.

Following liver transduction delivery of the GMO-derived DNA to the nucleus, the therapeutic transgene is transcribed and translated, and human Factor IX protein is produced by the liver cells. The liver is a natural source of Factor IX protein, i.e. the transgene is expressed in a natural environment. Local responses to GMO-mediated Factor IX expression are therefore not expected. The GMO-derived Factor IX protein is a human protein to which eligible patients have extensively been exposed in the form of protein replacement therapy, therefore immunological responses against this Factor IX protein are not expected to occur. The codon optimized Padua-FIX sequence resembles a naturally occurring FIX protein with normal function of the protein although a significantly higher specific activity.

Altogether as infection with wild type AAV already does not result in any noticeable pathology and Factor IX is expressed in its natural environment (the liver), in practice, the safety profiles of AMT-

061 and the parental strains will be similar, in that neither will mediate any noticeable pathological effects.

Infection with wild type AAV is asymptomatic and AAV is not known to cause any noticeable pathology. Thus, treatment methods against the GMO are therefore not considered relevant.

**(b) Structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified organism**

Low levels of baculovirus DNA are a known DNA impurity in AMT-061. Short baculovirus DNA sequences, particularly those located close to the vector genome insert, have been detected in baculovirus-produced rAAV. It is assumed that these sequences are co-packed with the vector genome into vector particles due to some degree of promiscuity of the packaging Rep protein. Resultantly, these DNA impurities are co-purified with the vector particles. As AMT-061 is constructed in insect cells, low levels of DNA originating from these cells are a second known DNA impurity of AMT-061. However, these sequences are short and random, and not integrated into the AMT-061 vector genome.

Several AAV gene therapy vectors manufactured in the baculovirus-based manufacturing platform have been administered to patients, i.e. Glybera (approved marketing authorization in 2012) and AMT-060 (applied to patients in Phase I/II clinical trials). For both products no harmful effects that could possibly be related to either of these DNA impurities have ever been observed. This justifies the conclusion that a risk related to residual insect cell DNA and residual baculovirus DNA can be considered negligible.

**(c) Stability of the organism in terms of genetic traits**

AMT-061 itself is replication deficient. Nonetheless, scenarios in which it would replicate *in vivo*, or even revert to a replication-competent AAV, are theoretically conceivable. In any case, such scenarios would require Rep and Cap sequences to be present in the same cell, and hence would require co-infection with wild type AAV. Presence of wild type AAV would however still not circumvent the dependence on a helper virus infection. Hence, replication or revertance of the GMO would require simultaneous infection of one-and-the-same cell with (1) AAV5-hFIX, (2) wild type AAV, and (3) a helper virus. This scenario of triple infection is in itself unlikely. Nonetheless, the conceivable scenarios upon triple infection are outlined below.

Without recombination taking place, triple infection will result in replication of the AAV5-hFIX vector genome by the Rep proteins provided by wild type AAV. Consequently, the triple-infected cell would in this case produce replication-deficient AAV particles of the wild type serotype containing the AAV5-hFIX vector genome, as well as wild type AAV particles. In any case, no replication-competent AAV5-hFIX particles would be formed regardless of the presence or absence of helper virus. Consequently, it can be concluded that the presence of helper virus in patients does not impact the risk profile of the product and its intended use. Therefore, helper virus positive patients can safely be included in the proposed study.

Homologous recombination would only involve the ITRs present in the AAV5-hFIX vector genome and the ITRs present in the wild type AAV, as only these sequences may share homology. Essentially, homologous recombination could yield the AAV5-hFIX expression cassette flanked by wild type AAV-derived ITRs, and the wild type AAV-derived genome flanked by AAV5-hFIX-derived ITRs. The likelihood of homologous recombination would depend on the degree of homology between the AAV5-hFIX-derived ITRs (derived from wild type AAV2) and the wild type AAV-derived ITRs, which logically depends on the serotype of the wild type AAV in question. In the case of triple infection, both recombinants would be subject to intracellular replication by the Rep protein provided by wild type AAV. As a result, the triple-infected cell would in this case produce replication-defective AAV particles containing the AAV5-hFIX vector genome, wild type AAV particles, and AAV particles containing either of the recombined genomes. Also in this scenario, no replication-competent AAV5-hFIX particles would be formed regardless of the presence or absence of helper virus.

Non-homologous recombination could theoretically produce a hybrid sequence containing wild type AAV-derived Rep and Cap sequences as well as the hFIX expression cassette. The likelihood of such recombination events is intrinsically much smaller than the likelihood of homologous recombination. Depending on its primary structure (i.e. depending on whether it is flanked by ITR sequences or not) such hybrid genomes may or may not be valid substrates for Rep protein. In the case of triple infection, hybrids representing valid Rep substrates would be subject to intracellular replication by the Rep protein provided by wild type AAV. Because of their expected size however, such hybrids cannot be packaged into the wild type AAV capsids. The maximum packaging capacity of AAV capsids is approximately 5 kb, and rep- and cap- and ITR sequences already make up for 4.7 kb (Daya et al. Clin Microbiol Rev (2008):583-593). The triple-infected cell would in this case only produce AAV particles containing the AAV5-hFIX vector genome and particles containing the wild type AAV genome, as outlined in the no-recombination scenario described above. Replication-competent AAV5-hFIX particles could however not be formed or released by the infected cell.

Most importantly, in any of the scenarios described above, immunological responses will be mounted against rAAV- and/or wild type AAV-derived Cap proteins. Therefore, adaptive immunity would silence any extracellular manifestation that could result from triple infection, just as it would in the case of a naturally occurring co-infection with wild type AAV and helper virus. The risk of spread to other cells (which would, also in these cells, require the event of triple infection) is therefore extremely small. It should be noted that cells that are infected with AAV5-hFIX only will not be affected by these immune responses as they do not express AAV-derived proteins. Hence, none of these responses would compromise therapeutic efficacy.

Taken together, the likelihood of recombination is small as it requires concurrence of a constellation of events at a singular moment in time. Replication-competent AAV5-hFIX would not be formed in any case. Recombinant replication-deficient particles could theoretically be formed but none of these would have different characteristics as compared to the vector or wild type AAV, respectively. In any case these particles would not have any significant systemic consequences, as they would be neutralized by the immune system as soon as they leave the cellular compartment. None of these events would affect cells transduced with the therapeutic vector alone. The risk associated with recombination is therefore considered to be negligible.

Even in the very unlikely event of recombination, the products of the outlined recombination events would not increase the likelihood of shedding and transmission based on the shedding and transmission scenarios outlined above. It is therefore concluded that the environmental impact will not be affected by any of the theoretical and unlikely recombination events.

**Potential post-dosing recombination events – SV40 sequences**
The AAV5-hFIX vector contains an SV40 intron and an SV40 polyA sequence. The possible recombination of these viral sequences with wild-type viruses is discussed below.

Recombination of the SV40-derived sequences present in the AAV5-hFIX vector genome with other virus-derived sequences depends on two likelihoods, being (1) the likelihood that homologous sequences are present, and (2) the likelihood that in such case recombination occurs (the likelihood of non-homologous recombination was taken to be negligible).

The simian SV40 virus can infect humans, as approximately 1 in every 5 individuals may be seropositive for this virus (Taronna 2013), (Corallini 2012), (Mazzoni 2014). Infection in any case remains subclinical (Garcea 2003). Persistence of SV40 DNA in the normal population is a matter of debate. A review article summarized that half of the available screening studies suggested SV40 is not present in the normal population, while the other half suggested that it is, at frequencies of 5 to 25% (Paracchini 2006). It is not unthinkable that SV40 sequences are present in patients during AMT-061 administration but the likelihood is low. Using BLAST search engines, no human viruses were found that displayed significant homology with the SV40 sequences present in the AAV5-hFIX vector genome [http://blast.ncbi.nlm.nih.gov], suggesting that, in humans, presence of virus-derived sequences (SV40 or other) with significant homology is not a likely event.

In the hypothetical case that homologous sequences are present, the likelihood of homologous recombination depends on the extent of homology. In a study on this exact relation, the recombination frequency of SV40 DNA in monkey cells sharply dropped when the length of the homologous sequences was less than 200 base pairs (Rubnitz 1984). The SV40 intron in the AAV5-hFIX vector genome is 94 base pairs in length, the SV40 polyA signal sequence is 133 bp in length. As such, even in the presence of homologous sequences, recombination is an unlikely event.

**(d) Rate and level of expression of the new genetic material. Method and sensitivity of measurement**
In healthy individuals, FIX levels may range from 50% to 200% of the population mean (Khachidze 2006). As a transgene for gene therapy, FIX has a broad therapeutic window. In Hemophiliacs, levels as low as 2% are expected to result in therapeutic benefit. Only extreme overexpression is associated with risk of thrombosis. Extreme overexpression of hFIX as the result of AAV gene transfer has been established in uniQure's pivotal safety study in mice, where infusion of $2.3\times10^{14}$ gc/kg (more than 10 times the high dose of the clinical Phase I/II study) resulted in 70-fold overexpression, i.e. 70 times the level found in the normal human population. No adverse effects were associated with this immense overexpression. The absence of adverse events was

not due to impaired or lacking functionality of hFIX, as hFIX expressed in mice displays normal functionality and was shown to revert the clotting deficiency in FIX-deficient mice (Nathwani 2006). These preclinical results suggest that overexpression of hFIX is not associated with adverse effects.
In non-human primates, infusion of AMT-060 at the intended clinical dose resulted in 1% to 10% of normal human levels. This intended clinical dose corresponds to approximately 25 to 100 mL of vector preparation per 50 kg body weight, infused intravenously to reach the liver.

In uniQure's Phase I/II study on AMT-060, circulating FIX activity levels reached up to 12% of normal human levels, demonstrating that, at the intended doses, the scenario of achieving extreme overexpression was not realistic.

The Padua modification (AMT-061) was introduced to achieve higher levels of circulating FIX activity at the same dose. The modification entails the replacement of two adjacent nucleotides in the wild type FIX coding sequence. The modification results in a non-synonymous codon change which translates to an Arginine to Leucine substitution in the protein, yielding the so called Padua FIX variant. Relative to the wild type FIX protein encoded by AMT-060, the Padua FIX protein encoded by AMT-061 is expected to display a six-to eightfold increased specific activity. Relative to AMT-060, AMT-061 is therefore expected to mediate increased efficacy at the same dose and the same protein expression levels.

The modifications defining AMT-061 are restricted to the FIX coding sequence. Other than potency, all quality attributes of AMT-060 and AMT-061 are expected to return similar, and AMT-061 is expected to mediate identical FIX protein expression levels as compared to AMT-060. The modification is therefore expected to return the same the safety profile as AMT-060.

The toxicity study with AMT-061 confirmed that a single intravenous infusion at an equal dose of $5 \times 10^{12}$ gc/kg, AMT-060 and AMT-061 returned with a similar circulating FIX protein levels. The study also confirmed that dosing in the dose range of $5 \times 10^{11}$ to $9 \times 10^{13}$ gc/kg was well tolerated in non-human primates. No adverse findings were reported, although at a dose of $9 \times 10^{13}$ gc/kg the overall clotting cascade was affected as shown by prolonged PT and shortened APTT. These effects of AMT-061 on the clotting cascade are likely a consequence of the supra-physiologic FIX activity levels that were reached after infusion of AMT-061 at the high dose (reaching up to 500% of normal, at the dose of $9 \times 10^{13}$ gc/kg which is ~5x the intended clinical dose). Plasma thrombin-antithrombin complex and D-dimer levels were however not affected, suggesting that also at supra-physiological FIX (-Padua) activity levels, the overall clotting cascade was functioning within normal physiological boundaries. Nonetheless, the pharmacodynamic effect on the clotting cascade observed at this dose should be taken into consideration when considering doses higher than the planned clinical dose. The no-observed-adverse-effect level for AMT-061 based on the study in non-human primates is set at $9 \times 10^{13}$ gc/kg.

A potential risk by introducing a Padua-FIX is the onset of an immune response to the neotransgene product. The risk on immunogenicity of Padua-FIX has been investigated in hemophilia B dogs treated by AAV gene therapy (Finn 2012). These authors report the absence of formation of inhibitory antibodies to the Padua-FIX protein demonstrated on multiple challenges

with wild type FIX protein (even > 1 year after stopping immunosuppression). These observations were supported by the lack of IFN-ɣ secretion by T-cells after exposure to wild type FIX protein peptides spanning the 338 residue with either the wild type FIX or Padua FIX amino acid sequence. Finn et al concluded that no detectable immunogenicity to Padua-FIX could be observed (Finn 2012). These conclusions are aligned with the result of in-silico analysis performed by UniQure. The full length wild type human FIX sequence as well as the Padua mutation were evaluated for their immunogenic potential by use of an in-silico platform for epitope identification and prediction (EpiMatrix system developed by Epivax, Inc) for both Class I (all nucleated cells) and Class II (antigen presenting cells) HLA. The accuracy of the EpiMatrix system has been thoroughly documented (Koren 2007). The Padua mutation does not result in a significant change in EpiMatrix hits restricted by Class I or Class II HLA, with minimal observed changes in EpiMatrix score. Altogether it is concluded that the immunogenic difference between the wild type factor IX and the Padua Factor IX is insignificant.

In conclusion, the only risk associated with the Padua-FIX modification would relate to unintended achievement of supraphysiological levels of circulating FIX activity, either as the result of intended or unintended exposure. It has been reported that only in patients with these supraphysiological levels of Padua-FIX (>700% of normal) thrombosis may be observed (Simioni 2009).

In case of intended exposure, i.e. in patients, the scenario of reaching extreme levels of circulating FIX activity is highly unlikely. Exposure to a dose 5x higher than the intended clinical dose needs to occur to reach supra-physiological levels, as shown in the non-human primates. It is therefore concluded that the risk of thrombosis following intended exposure to AMT-061 is negligible.

The probability of unintended exposure to significant amounts of AMT-061, in such a way that the vector will be able to transduce hepatocytes and mediate detectable FIX expression is extremely low. It would entail unintended intravenous infusion of 25 mL vector preparation or more. In addition, the probability that such unintended exposure would result in overexpression of FIX expression levels is extremely low, as the target levels for intended exposure are close to 5% of normal. Finally, the probability that overexpression of hFIX would have any clinical consequence for a third party is low, as already in the normal population there is considerable ‘over’ expression in otherwise healthy individuals, and non-clinical studies suggest that even extreme overexpression holds negligible biological consequence. The overall risk that overexpression of hFIX in third parties due to unintentional exposure will result in observable effects is therefore negligible.

**(e) Activity of the expressed protein(s)**

See section (d) Rate and level of expression of the new genetic material. Method and sensitivity of measurement.

**(f) Description of identification and detection techniques including techniques for the identification and detection of the inserted sequence and vector**

**Absence of residual infectious baculovirus**

Substantial amounts of recombinant baculovirus are used during the production process. As baculovirus is an enveloped virus of a much larger size than AAV particles, it is susceptible to inactivation by detergents and can be cleared by virus filtration. Furthermore, many process steps have been designed to maximise baculovirus clearance. In the final product, absence of residual infectious baculovirus is confirmed by bio-assay monitoring utilising baculovirus-permissive insect cells. The assay can detect single infectious particles and the sensitivity is only restricted by the tested volume. The bioassay has a detection limit of 6.8 infectious units (i.e. infectious particles) per mL. No residual infectious baculovirus has ever been detected in any of the batches of AMT-061 that were thus far produced, nor in any of the other recombinant AAV-based product that were thus far produced. These results confirm that the viral clearance capacity of the process suffices for complete clearance of recombinant baculoviruses used in the production of AMT-061.

**Residual baculovirus DNA**

AMT-061 is also assessed for residual baculovirus DNA by means of a QPCR-based method. The test was qualified for use with a limit of quantitation well below the levels detected in the IMP.

**Absence of replication competent-AAV (rcAAV)**

The co-existence of Rep and Cap sequences with recombinant vector genome during fermentation could theoretically result in recombination events that yield replication-competent AAV (rcAAV). AMT-061 is routinely assessed for the presence of rcAAV by a sensitive limit test. In this test, vector preparations are plated onto AAV- and adenovirus permissive cells, in the presence of adenovirus. Thus far, no replication-competent AAV has ever been detected in any batch of AMT-061, nor in any of the other recombinant AAV-based product that have thus far been produced. The absence of detectable amounts of replication-competent AAV confirms that recombination of sequences yielding infectious, replication-competent particles is extremely unlikely.

Replication competent AAV is defined as any AAV which is capable of replicating in the presence of helper virus (as stated elsewhere, AMT-061 is not able to replicate, even in the presence of helper virus). Generation of rcAAV during manufacturing is a theoretical possibility with an extremely low likelihood. AMT-061 is controlled for the presence of rcAAV by bio-assay able to detect 10 rcAAV amidst 2 x $10^{10}$ genome-containing AMT-061 particles. To date, no rcAAV has been found in any of the batches produced in the baculovirus production system.

**Rep full-length sequences**

AMT-061 is assessed for full-length Rep levels by means of long-amplicon QPCR. The associated drug substance release criteria are a ratio of Rep full-length sequences relative to genome copies which is well above the levels detected in AMT-061.

**DNA impurities**

Regarding the potential for interactions between cellular DNA and parental (baculovirus) vectors: high throughput sequencing of the GMO AMT-061 vector preparations have shown that more than 99% of the DNA present in the IMP represents the anticipated vector genome. Small

amounts (0.2% of total) of baculovirus derived DNA and traces of insect DNA were detected. These DNA impurities are controlled during quality testing as described above. For previous vectors produced using the baculovirus expression system it was shown that these fragments do not harbour coding sequences.

The traces of insect DNA detected in AMT-061 represented short sequences randomly scattered across the insect genome. These results strongly suggest that the baculovirus and insect derived DNA impurities present in AMT-061 are not the result of recombination events but due to promiscuity of the Rep packaging protein.

Since the Rep and Cap sequences are provided *in trans* during the AMT-061 production process, theoretically, recombination could occur resulting in replication-competent (albeit still helper-virus dependent) vector particles. Therefore, a quality control assay for the presence of replication-competent AAV particles in AMT-061 preparations is in place. To date, no replication-competent particles have been observed in any of the AMT-061 preparations tested, confirming that the formation of replication competent particles is a hypothetical risk and not a commonly occurring phenomenon.

**(g) sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques**

Quality control tests are performed to confirm the quality of the final investigational medicinal product. The quality tests relevant for environmental risk analysis are summarised in Table .

**(h) History of previous releases or uses of the GMO**

**Brief history of the development program**

AMT-060 has been evaluated in a Phase I/II clinical trial to investigate its safety and efficacy in adults with severe or moderately-severe hemophilia B (FIX ≤ 2%). To date, all 10 subjects received a single IV dose of AMT-060 in one of two escalating dose cohorts: $5 \times 10^{12}$ gc/kg (N=5) or $2 \times 10^{13}$ gc/kg (N=5). Hemophilia B patients treated with AMT-060 shifted from the severe to the moderate/mild phenotype, and prophylaxis was discontinued with AMT-060 derived circulating FIX activity levels ranging from 3% to 13%. Four out of 5 patients in Cohort 1 and 4 out of 4 patients in Cohort 2 (one patient in Cohort 2 was on on-demand therapy) discontinued prophylactic therapy and the risk of spontaneous bleedings was remarkably reduced. Safety assessments included treatment-related adverse events, as well as immunological assessments, including T-cell activation assays (ELISpot) against AAV5 capsid antigens and assessment of total antibody and inhibitory antibody titers against the viral capsid and FIX, respectively; vector/DNA shedding was also assessed from blood, saliva, nasal secretions, urine, faeces and semen. Efficacy assessments included endogenous FIX activity, total exogenous FIX usage and spontaneous annualized bleeding rates.

In an attempt to achieve even higher circulating FIX activity levels with the goal of alleviating the need for all exogenous therapy. To achieve this goal the Sponsor introduced a design modification by modifying the wild type FIX coding sequence within the AMT-060 vector genome to encode

the naturally occurring Padua FIX gain-of-function variant, resulting in AAV5-hFIXco-Padua (AMT-061).

**AMT-061 (hFIXco-Padua), versus AMT-060 (hFIXco)**
The AMT-060 and AMT-061 based gene therapy vectors are identical except for a two-nucleotide substitution (AGG to CTG) in the coding sequence for FIX, corresponding to an Arginine to Leucine substitution in the transgenic protein (Figure 1**).** The modified transgenic protein displays increased specific activity as compared to wild type FIX.

The R338L substitution represents a gain-of-function FIX variant, which was reported to result in an increased specific activity-to-protein ratio of up to 8.6 (Simioni et al., 2009). This augmentation is thought to be largely caused by increased affinity of the activated protein to activated clotting factor VIII (FVIIIa; Kao et al., 2013). A number of preclinical studies in hemophilic mice and dogs demonstrated the potential utility of this variant for hemophillia B gene therapy, as it enables higher FIX activity levels without the need for a higher administered vector dose to increase transgene expression (Crudele et al., 2015; Cantore et al., 2012; Finn et al., 2012; Suwanmanee et al., 2014; Monahan et al., 2015; Kao et al., 2013).
The critical quality attributes of both vectors are expected to be comparable, which will be confirmed through comparability/bridging studies. A similar infectious vector titer will result in equal transduction of the liver cells, with a similar level of FIX protein expressed and secreted per unit of vector dosed. In the case of AMT-061, the 2-nucleotide substitution in the FIX coding sequence and resulting Arginine to Leucine substitution in the mature protein ultimately results in a higher FIX activity per unit of FIX protein expressed. Therefore, the design modification to AMT-061 would enable achievement of higher circulating FIX activity, without altering any of the previously established *in vivo* correlations between vector dose, circulating FIX protein levels, and safety.

**(i) Considerations for human health and animal health, as well as plant health**
**Environmental exposure to AMT-061**
Overall the exposure of the environment to AMT-061 is considered limited. The most likely groups being exposed to the GMO are:

- Healthcare professionals involved in preparation and administration of the GMO and obtaining clinical samples.
- Laboratory professionals involved in sample preparation and analysis.
- Close contacts of the patients who potentially may be exposed to shed vector.

The likelihood for exposure of these groups will be different and depending on different scenarios. Administration will be performed at the hospital and is restricted to the treatment room. Transport to and from the hospital is carried out under transport conditions applicable for GMOs. Due to the well-controlled administration conditions, the potential contact of the product with healthcare professionals at the administration site environment is extremely limited. The only possibilities for a contact of the administration site environment with the product would be accidental spillage of the product solution to surfaces; accidental self-administration (needle-stick injury), (mucous) skin contact with the product or inhalation of aerosolized product by the administering health care

professional. As the vector is replication deficient, the only effect could be a marginal increase in FIX levels if any and an immune response to the AAV5 capsid.
Laboratory professionals working with clinical samples may also be exposed to the GMO. However, analysis of patient samples are not within the scope of the current application as these activities will be performed within laboratories which are covered through different licenses.
To evaluate the scenarios of exposure of close contacts shedding of the GMO is to be evaluated. Shed vector DNA does not equal infectious vector particles. It has been described that infectious particles can only be found in blood during the first 3 days after vector infusion (Favre et al., 2001). After this period, all infectious particles are likely to have infected test subject cells or to have been rendered non-infectious through other mechanisms (e.g. degradation by test subject effector mechanisms). The level of risk that spread will occur is therefore dependent of the scenario, i.e. fluid or excrement type:

Because of the above, spread of infectious GMO into the environment through nasal secretions, saliva, urine or faeces is considered negligible. Spread of infectious GMO through blood is conceivable, as blood samples are drawn during the first 3 days after administration of the GMO. However, following the worst-case scenario's described above, even the risk that AAV5-hFIX will spread into the environment through blood and thus leads to a GMO related risk is considered negligible.

It is not known whether recombinant AAV shed in semen is infectious or, like AAV shed in the other fluids, represents non-infectious vector DNA. In either case, the risk of horizontal or vertical transmission cannot be excluded. Although it is not anticipated that this will relate to a risk it is considered an unwanted effect as such any potential risk is addressed by requiring the use of a condom during the trial in the period from administration of the AAV5-hFIX until the AAV5 vector has been cleared from semen, as evidenced by negative analysis results for AAV5 vector for at least three consecutively collected semen samples.

Assessment of vector DNA in semen will be performed using a validated method. The likelihood that the GMO will spread through semen is low and controlled.

**Exposure through shedding**
Shedding in non-clinical studies was assessed using a QPCR based method. Serum, saliva, urine and faeces were collected at several time points after dosing. Shedding of AMT-060 was assessed in cynomolgous macaque. Clearance curves in saliva and urine mainly followed the clearance from the serum with the vector DNA concentrations bout 2 – 4 logs lower. Serum cleared between weeks 12 and 26. Saliva was cleared between weeks 8 – 12. Vector DNA levels in urine were low and reached the limit of detection around week 8. The shed material will mainly include DNA fragments tested positive using the QPCR method. A high level of shedding of infectious vector particles has not been observed.
Non-clinical biodistribution and shedding studies with AMT-061 confirmed the earlier observations with a highly similar AMT-060 vector and demonstrated distribution and shedding in plasma urine and tissues. For urine AMT-061 vector DNA was not detectable 3 months after administration of the highest dose of $9 \times 10^{13}$ gc/kg.

Shedding of AMT-060 (which in essence resembles AMT-061), was evaluated in clinical Phase I/II studies. Samples of whole blood, saliva, nasal secretions, urine, semen and faeces were tested. Vector DNA disappeared from whole blood at week 27 for 1 patient in cohort 1, however remained to be detectable for all other cohort 1 patients as well as all patients in cohort 2 until last assessment (Cohort 1, 78 weeks; Cohort 2, 52 weeks). Vector DNA disappeared from faeces between week 6-16 in Cohort 1 and week 16-20 in cohort 2 (2 patients still positive at last assessment at week 52), from nasal secretions between week 5-18 in Cohort 1 and week 7-12 in Cohort 2 (2 patients still positive at last assessment at week 52), from saliva between week 6-20 in Cohort 1 and between week 9-16 in Cohort 2 (2 patients still positive at last assessment at week 52), from semen between week 9-48 in Cohort 1 (1 patient still positive at last assessment at week 78) and in week 12-22 in Cohort 2 (3 patients still positive at last assessment at week 52).

As already mentioned, a detected copy of vector DNA does not necessarily represent presence of infectious vector particles. More likely, it represents DNA from a degraded vector particle, a particle that has been taken up by a cell, or a cell which has been transduced by the vector (e.g. leukocytes or epithelial cells of the bladder). In preclinical studies on recombinant AAV, it has been shown that urine containing AAV vector DNA does not contain infectious particles (Favre et al., 2001), and that infectious vector is restricted to the plasma compartment and cleared from circulation within 48 to 72 hours after infusion.

In a scenario where infectious AAV is shed into the environment the amount of shed infectious particles is likely to be extremely low. This is based on observations that in most cases only vector DNA material could be observed through PCR, however, infectious particles are restricted to the plasma compartment and cleared from circulation within 48 to 72 hours after infusion (Favre et al., 2001). In the unlikely event that infectious particles will be shed, these will still be replication-deficient. The amount of shed infectious particles will be extremely low and the material shed will be replication deficient based on the vector design and manufacturing strategy. Shedding of vector material may lead to exposure of third parties which theoretically may result in transmission to these third parties. However, as shedding of infectious vector particles is already considered unlikely, the likelihood of transmission to and infection of third parties should also be considered a highly unlikely event. In conclusion, spreading of the vector through shedding and transmission to third parties is considered a theoretical scenario for dispersion of the AMT-060 and AMT-061 from the test subject into third parties.

**Risk estimation**

There are no significant risks associated with the application of the vector.

The overall risk of the GMO has been evaluated by summing the potential risks and consequences with respect the likelihood, as detailed in Table .

**Table 3: Estimation of the risk posed by each identified characteristics of the GMO**

| Adverse effect | Type of exposure* | Magnitude | Likelihood | Risk |
|---|---|---|---|---|
| Toxic effects to humans | self-inoculation | negligible | low | negligible |
| | exposure | negligible | negligible | negligible |
| Pathogenicity to humans | self-inoculation | negligible | low | negligible |
| | exposure | negligible | negligible | negligible |
| Immunogenicity to Padua FIX | self-inoculation | negligible | negligible | negligible |
| | exposure | negligible | negligible | negligible |
| Tumorigenicity to humans | self-inoculation | negligible | low | negligible |
| | exposure | negligible | negligible | negligible |
| Thrombosis following supraphysiological Padua FIX activity | self-inoculation | negligible | low | negligible |
| | exposure | negligible | negligible | negligible |
| Germ-line transmission | self-inoculation | low | negligible | negligible |
| | exposure | negligible | negligible | negligible |
| Genome integration in humans | self-inoculation | low | negligible | negligible |
| | exposure | negligible | negligible | negligible |
| Disease or any other adverse effect to animals or plants | exposure | negligible | negligible | negligible |
| Population dynamics and genetic diversity of populations | exposure | negligible | negligible | negligible |
| Facilitating the dissemination of infectious diseases | exposure | negligible | negligible | negligible |
| Compromising prophylactic or therapeutic treatment | exposure | negligible | negligible | negligible |
| Disturbance of environmental biogeochemistry | exposure | negligible | negligible | negligible |

* Accidental self-inoculation by a healthcare professional, exposure = due to incidental spillage or shedding.

The overall risk of AMT-061 to people and the environment can be considered negligible.

Therefore, no specific risk management measures are deemed necessary. Nevertheless, standard biosafety measures (outlined below) are implemented which relate to general hospital procedures which by no means relate to any risk management to reduce the already negligible risk related to the gene therapy with AMT-061.

**(i) toxic or allergenic effects of the GMOs and/or their metabolic products**

Not applicable

**(ii) comparison of the modified organism to the donor, recipient or (where appropriate) parental organism regarding pathogenicity**

Not applicable

**(iii) capacity for colonization**

Not applicable

**(iv) if the organism is pathogenic to humans who are immunocompetent:**

**. diseases caused and mechanism of pathogenicity including invasiveness and virulence,**
**. communicability,**
**. infective dose,**
**. host range, possibility of alteration,**
**. possibility of survival outside of human host,**
**. presence of vectors or means of dissemination,**
**. biological stability,**
**. antibiotic resistance patterns,**
**. allergenicity,**
**. availability of appropriate therapies.**

Not applicable:

AAV, infections are asymptomatic and AAV is not known to cause any noticeable pathology. Similarly, dose-dependent administration of AAV/based GMO´s to humans has been shown not to cause any vector related pathogenicity and can be considered safe. A dose-dependent immune response does occur in a recipient but will be without clinical consequence.

**(v) other product hazards.**

Not applicable.

## 4. INFORMATION RELATING TO THE CONDITIONS OF RELEASE AND THE RECEIVING ENVIRONMENT

### A. Information on the release

**1. Description of the proposed deliberate release, including the purpose(s) and foreseen products**

The Sponsor proposes to conduct clinical studies to evaluate the safety and efficacy of AMT-061 in patients with severe or moderately severe hemophilia B.

Clinical study proposed: Open label, single dose, multi centre trial investigating an adeno associated viral vector containing a codon optimized Padua derivative of human factor IX gene (AAV5-hFIXco-Padua, AMT-061) administered to adult patients with severe or moderately severe hemophilia B.

**2. Foreseen dates of the release and time planning of the experiment including frequency and duration of releases**

AMT-061 will be administered intravenously to haemophilia B patients in a few hospital centres as a single treatment with proposed period of release from Q1-2019 until Q4-2021.

The complete administration procedure including preparation of the infusion system is expected to take less than 24h.

**3. Preparation of the site previous to the release**

Treatment of patients will occur in a hospital environment. The AMT-061 infusion bag will be transported to the treatment room in accordance with GMO requirements and/or local hospital procedures.

The hospital centres are expected to have adequately trained health care professionals involved in the study in the safe handling of GMOs and to have best biosafety practices implemented in order to minimise any accidental exposure to the product, be it personnel, contact persons or the environment.

**4. Size of the site**

AMT-061 is not released in the environment but will be administered to patients in a controlled area (clinical site). Planned participating sites are located in Brussels and Leuven.

**5. Method(s) to be used for the release**

The hospital pharmacy, or equivalent, will receive and store AMT-061 in accordance with the requirements laid down in the GMO requirements and/or local hospital procedures. In the hospital pharmacy, or equivalent, AMT-061 will be pulled from the supplied glass vials into an infusion bag in a Class II Biological Safety Cabinet. The infusion bag will be connected to a pre-filled tubing containing a sterile saline solution. The pre-filled tubing of the infusion bag will be connected to the main infusion tubing, which will have been primed with sterile saline. The AMT-061 infusion bag will be transported to the treatment room in accordance with GMO requirements and/or local hospital procedures.

Treatment of patients will occur in a hospital environment. Administration and monitoring of the patient occurs at the ward, which could also be multiple patient rooms. During infusion the patient will remain in one location. The vector will be administered to the patient as an intravenous infusion.

**6. Quantities of GMOs to be released**

Patients will be treated with a single intravenous infusion in a range of $5 \times 10^{12}$ to $1 \times 10^{14}$ gc/kg AMT-061. Up to 10 patients will be treated in Belgium over the planned clinical trial period.

**7. Disturbance on the site (type and method of cultivation, mining, irrigation, or other activities)**

Not applicable. AMT-061 will be administered to patients in a controlled area (clinical site).

**8. Worker protection measures taken during the release**

The hospital centres are expected to have adequately trained health care professionals involved in the study in the safe handling of GMOs and to have best biosafety practices implemented in order to minimise any accidental exposure to the product, be it personnel, contact persons or the environment.

Accidental exposure and/or spilling of AMT-061 are mitigated by the following the standard precautions:

- Personnel administering AMT-061 will wear protective clothing and additional protective measures like safety glasses, gloves and mouth-nose mask.
- The hospital pharmacy will provide the infusion bag connected to pre-filled tubing with sterile diluent. Should spillage and/or aerosol formation occur, this will be only 0.9% sodium chloride.
- The pre-filled tubing of the infusion bag will be connected to the main infusion tubing, which has also been primed with sterile 0.9% sodium chloride. When removing the intravenous catheter after completion of the infusion, there is an increased risk of spillage and/or aerosol formation. This risk is mitigated by flushing the infusion tubing with 0.9% sodium chloride, before removing the intravenous catheter.
- The catheter, tubing, infusion bag and other ancillary items used are all disposables and are disposed of as biohazard waste in accordance with the local hospital procedures and GMO requirements. Non-disposable materials (tools, devices) are cleaned with a disinfectant with viricidal activity, e.g. a chlorine releasing disinfectant lyke hypochlorite containing 0.1% available chlorine (1000 ppm) after usage and then autoclaved, if possible. Contact surfaces are disinfected with a similar disinfectant.

**9. Post-release treatment of the site**

The infusion administration line will be flushed with sterile saline and the intravenous catheter removed. The nature of the waste generated includes (partially) used vials, ancillary components used for preparation and administration (e.g. tubing, needles, syringes, infusion bag, personal

protective equipment, etc.) and components used for collecting body fluids samples after administration. Following handling and administration of AMT-061 the unused vector remaining in the vials, and ancillary materials, will be disposed of per GMO legislation and/or local hospital procedures.
After administration, the treatment room will be decontaminated with a disinfectant. A 250 ppm chlorine solution will be used for regular disinfection on used surfaces. In case of a spill, the surface will be treated with 1000 ppm chlorine solution.

**10. techniques foreseen for elimination or inactivation of the GMOs at the end of the experiment**
As AMT-061 is not infectious after being shed and therefore the risk to the environment is considered negligible, standard hospital hygiene measures are sufficient and no additional measures are required for the period after administration of AMT-061.

**11. information on, and results of, previous releases of the GMOs, especially at different scales and in different ecosystems.**

## B. Information on the environment (both on the site and in the wider environment):

**1. Geographical location and grid reference of the site(s) (in case of notifications under part C the site(s) of release will be the foreseen areas of use of the product)**
AMT-061 is not released in the environment but will be administered to patients in a controlled area (clinical site). Planned participating sites are located in Brussels and Leuven.

**2. Physical or biological proximity to humans and other significant biota**
Please see section 5.1.

**3. Proximity to significant biotopes, protected areas, or drinking water supplies**
Please see section 5.1. The receiving environment for the shed vector particles is most likely waste water.

**4. Climatic characteristics of the region(s) likely to be affected**
A hospital treatment room and ambient indoor conditions will be used for administration to clinical trial subjects. The receiving environment for the shed vector particles is most likely waste water and ambient temperature. Planned participating sites are located in Brussels and Leuven.

**5. Geographical, geological and pedological characteristics**
Please see section 5.1.

**6. Flora and fauna, including crops, livestock and migratory species**
Please see section 5.1.

**7. Description of target and non-target ecosystems likely to be affected**

The parental virus is Adeno-Associated Virus (AAV). AAV needs a helper virus, such as adenovirus or herpes virus, to replicate. Wild type AAV is a small, non-enveloped virus with a very stable capsid. Little is known about the stability of wild type AAV. Exposure to heat, UV radiation, or extreme pH can inactivate (recombinant) AAV (non-published data). Dependoviruses with similarity to AAV have been identified from other species; the AAV serotypes found in humans seem to be restricted to primates (Arbetman et al, J. Virol., 2005).

**8. A comparison of the natural habitat of the recipient organism with the proposed site(s) of release**

Please see section 5.1.

**9. Any known planned developments or changes in land use in the region which could influence the environmental impact of the release.**

Not applicable. AMT-061 will be administered to patients in a controlled area (clinical site).

## 5. INFORMATION RELATING TO THE INTERACTIONS BETWEEN THE GMOs AND THE ENVIRONMENT

### A. Characteristics affecting survival, multiplication and dissemination

**1. Biological features which affect survival, multiplication and dispersal**

To survive outside the host, wild type Baculoviruses organise into so called occlusion bodies, to which the Polyhedrin protein is an essential component (Rohrmann, Baculovirus Molecular Biology, 2013). Due to the absence of the Polyhedrin gene, and the resultant absence of polyhedron protein, recombinant Baculovirus cannot form occlusion bodies and are not stable outside of the culturing system.

Baculoviruses are incapable of replication in mammalian cells. They have a limited range of hosts, and are typically restricted to a range of closely related insect species. Regarding pathogenicity, baculoviruses are not harmful to humans.

The parental (baculovirus) vectors used in the manufacture of the GMO AMT-061 can infect and replicate in the insect cell line used for the production (production cells: derived from the insect *S. frugiperda*.

It should be noted that a number of steps in the down-stream production process of AMT-061 are designed to clear the baculovirus vectors from the crude GMO AMT-061 bulk by means of inactivation or physical removal. The final GMO AMT-061 preparation is tested for residual infectious baculovirus to confirm such inactivation/removal. The test entails a bioassay with a detection limit of 6.8 infectious units (i.e. infectious particles) per mL.

Wild type AAV can only replicate in the presence of a helper virus. The wild type AAV genome carries only two genes; the Cap gene, which encodes the proteins forming the capsid, and the Rep gene, which encodes for proteins that replicate the viral genome and package it into the capsids.

The AMT-061 vector genome can be considered a gutted wild type genome, as it does not contain the Rep and Cap genes. Thus, AMT-061 is replication-defective, even in the presence of a helper virus.

As a result, AMT-061 is expected to harbor even lower pathogenicity as compared to the parental AAV2 and AAV5 viruses that were used for its construction, where wild type AAV are already considered to be non-pathogenic. The pathogenic potential of AMT-061 in relation to the parental viruses is therefore considered negligible.

The GMO could spread from the recipient into natural habitats via blood, urine, faeces, saliva, nasal secretions and semen.

AAV-based GMO found in body fluids is not infectious (Favre et al., 2001), except for the blood compartment where infectious AAV-based GMO was found briefly after administration. Therefore, persistence through infection is unlikely when the GMO spreads into natural habitats via body fluids, since true shedding will only occur through body fluids such as urine, faeces, and saliva.

Persistence through replication and/or integration is dependent on infection of a (natural) host. As stated above infection of a host following spread via body fluids of the recipient is unlikely. In addition, the GMO has been rendered replication defective, by removal of Rep and Cap sequence from the genome. Integration occurs at a low frequency and at random even when administered IV at a high dose (Paneda et al., 2013).

Taken together, the likelihood of persistence of the GMO into natural habitats is considered extremely unlikely.

No selective advantage has been conferred to the GMO. In contrast, the GMO has been rendered replication-defective through omission of Rep and Cap sequences. Therefore, a selective disadvantage has been conferred to the GMO (vs for example wild type AAV), which is likely to be realized under the conditions of the proposed release (by IV injection). In addition, spread of infectious GMO following release is limited by the fact that the GMO shows poor potential for infection once shed via body fluids as shed material will predominantly contain only DNA fragments of the GMO and is unlikely to contain infectious particles.

Wild type AAV dissemination is mainly through the airway, although sexual transmission has been hypothesized.

As the likelihood that infectious vector particles are present in biosamples is extremely low, and the environmental risk of spread of infectious GMO into the environment through nasal secretions, saliva, urine or faeces is considered negligible, these matrixes are not sampled for studies with AMT-061.

Spread of infectious GMO through blood is conceivable, as blood samples are drawn during the first 3 days after administration of the GMO. However, even the risk that AMT-061 will spread into the environment through blood and thus leads to a GMO related risk is considered negligible.
Blood samples will be assessed for determination of vector DNA levels, particularly as blood samples have shown the longest persistence for AMT-060 related sequences.

It is not known whether recombinant AAV shed in semen is infectious or, like AAV shed in the other fluids, represents non-infectious vector DNA. In either case, the risk of horizontal or vertical transmission cannot be excluded. Although it is not anticipated that this will relate to a risk it is considered an unwanted effect as such any potential risk is addressed by requiring the use of a condom during the trial in the period from administration of the AMT-061 until the AAV5 vector has been cleared from semen, as evidenced by negative analysis results for AAV5 vector for at least three consecutively collected semen samples.

Shedding in non-clinical studies was assessed using a QPCR based method. Serum, saliva, urine and faeces were collected at several time points after dosing. Shedding of the Sponsor's predecessor product, AMT-060 (which in essence resembles AMT-061) was assessed in cynomolgous macaque. Clearance curves in saliva and urine mainly followed the clearance from the serum with the vector DNA concentrations about 2 – 4 logs lower. Serum cleared between weeks 12 and 26. Saliva was cleared between weeks 8 – 12. Vector DNA levels in urine were low and reached the limit of detection around week 8. The shed material will mainly include DNA fragments tested positive using the QPCR method. A high level of shedding of infectious vector particles has not been observed.

Non-clinical biodistribution and shedding studies with AMT-061 confirmed the earlier observations with the highly similar AMT-060 vector and demonstrated distribution and shedding in plasma urine and tissues.

No vector DNA was detected in faeces at any time point in any animal. The study with AMT-061 revealed that shedding of vector DNA expressed as plasma half live was around 29 hours for both AMT-061 and AMT-060. Complete clearance from urine was confirmed for AMT-061 at week 13.

**2. Known or predicted environmental conditions which may affect survival, multiplication and dissemination (wind, water, soil, temperature, pH, etc.)**
Wild type AAV is a small, non-enveloped virus with a very stable capsid. Little is known about the stability of wild type AAV. Exposure to heat, UV radiation, or extreme pH can inactivate (recombinant) AAV (non-published data).

**3. Sensitivity to specific agents.**
Please see section 5.2

**B. Interactions with the environment**

**1. Predicted habitat of the GMOs**
AAVs are frequently found in humans and animals, but they are not pathogenic, virulent, allergenic, or a carrier (vector) of a pathogen. AAV is not known to be associated to plants. The known host range includes humans and non-human primates. In natural conditions, wild type AAV is found to transmit to humans in the presence of a helper virus. It does not activate latent virus and is not able to colonise other organisms.

**2. Studies of the behaviour and characteristics of the GMOs and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms, greenhouses**
AMT-060 has been evaluated in a Phase I/II clinical trial to investigate its safety and efficacy in adults with severe or moderately severe hemophilia B (FIX ≤ 2%) as mentioned before (Section 4.C.2.h.1).

**3. Genetic transfer capability**

**(a) Postrelease transfer of genetic material from GMOs into organisms in affected ecosystems;**
Due to the low numbers of vector DNA copies potentially released into the environment through shedding, horizontal gene transfer is highly unlikely. Even if horizontal gene transfer occurred, the sequences would not confer a selective advantage to other organisms such as bacteria since AMT-061 does not contain any prokaryotic promoters, any antibiotic or other types of resistance genes or any genes, which would enhance or constrain their growth. Therefore, it is unlikely that AMT-061 would interfere with the control of pathogenic microorganisms or that it would have an effect on the natural dynamics of microbial populations or the biogeochemical cycles at any given site in the environment.

**(b) Postrelease transfer of genetic material from indigenous organisms to the GMOs;**
AMT-061 contains the ITR-sequences of AAV2, there is a (remote) possibility of homologous recombination of the vector with wild type AAV2 in case of a co-infection in exposed persons. The result of such a recombination would be that AMT-061 would gain functional genes of the AAV2 required for replication and encapsidation, but in turn would lose the transgene. Hence, recombination would lead to the formation of viruses that are identical to the starting material and replication incompetent.

**4. Likelihood of postrelease selection leading to the expression of unexpected and/or undesirable traits in the modified organism**

The genetic material from the Rep and Cap genes together with the transgene would be too large in size to be packed in an AAV capsid. Thus, it is highly unlikely that the recombination would result in a replication-competent vector containing transgenes. Any recombination would result in the expression of PADUA hFIX by infected cells.
Neither wild type AAV nor AAV5-hFIX are pathogenic to humans or the environment.

**5. Measures employed to ensure and to verify genetic stability. Description of genetic traits which may prevent or minimise dispersal of genetic material. Methods to verify genetic stability**

Genomic stability of each baculovirus is assessed by passaging it beyond the maximum number of passages that are used for the production of AMT-061. For genomic stability assessment, each passage is analysed for (1) the relative frequency of specific regions spaced across the recombinant baculoviral genome (i.e. the insert versus baculoviral genomic regions) and by (2) assessing the integrity of the entire expression cassette. The first is generally assessed using QPCR-based methodology, the latter by end-point PCR or western blotting). For each of the three baculoviruses used for the production of AMT-061, both were confirmed, demonstrating that the baculoviruses are genetically stable within the number of virus passages that is relevant for the production process.

AMT-061 is replication incompetent. In the absence of an intrinsic mechanism for genetic variation or instability and based on the known genetic stability of wild type AAV, the genetic traits of the organism are expected to be stable.

**6. Routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact, burrowing, etc.**

Wild type AAV dissemination is mainly through the airway, although sexual transmission has been hypothesised. The GMO cannot enter an infectious cycle even in the presence of helper function.

**7. Description of ecosystems to which the GMOs could be disseminated**

AAVs are frequently found in humans and animals, but they are not pathogenic, virulent, allergenic, or a carrier (vector) of a pathogen. AAV is not known to be associated to plants. The known host range includes humans and non-human primates. In natural conditions, wild type AAV is found to transmit to humans in the presence of a helper virus. It does not activate latent virus and is not able to colonise other organisms.

The vector is not capable of replication and does not code for sequences which would provide a detrimental environmental advantage to microorganisms or plants.

**8. Potential for excessive population increase in the environment**

Reproduction of wild-type AAV is dependent on co-infection with helper virus (Adenovirus or Herpesvirus). AMT-061 is an attenuated (recombinant) AAV: genes essential for DNA replication and DNA packaging into an AAV particle have been removed.

**9. Competitive advantage of the GMOs in relation to the unmodified recipient or parental organism(s)**

No selective advantage has been conferred to the GMO. In contrast, the GMO has been rendered replication-defective through omission of Rep and Cap sequences. Therefore, a selective disadvantage has been conferred to the GMO (vs for example wild type AAV), which is likely to be realized under the conditions of the proposed release (by IV injection). In addition, spread of infectious GMO following release is limited by the fact that the GMO shows poor potential for infection once shed via body fluids as shed material will predominantly contain only DNA fragments of the GMO and is unlikely to contain infectious particles.

**10. Identification and description of the target organisms if applicable**

(Human) male patients suffering from haemophilia B.

**11. Anticipated mechanism and result of interaction between the released GMOs and the target organism(s) if applicable**

AMT-061 has been designed to deliver the human coagulation FIX gene to the liver of patients suffering from haemophilia B enabling the restoration of stable expression of coagulation FIX thus ameliorating the bleeding phenotype and improving the quality of life of these patients.

**12. Identification and description of non-target organisms which may be adversely affected by the release of the GMO, and the anticipated mechanisms of any identified adverse interaction**

The only possibilities for a contact of the administration site environment with the product would be accidental spillage of the product solution to surfaces; accidental self-administration (needle-stick injury), (mucous) skin contact with the product or inhalation of aerosolised product by the administering health care professional. As the vector is replication deficient, the only effect could be a marginal increase in FIX levels if any and an immune response to the AAV5 capsid.

The probability of unintended exposure to significant amounts of AMT-061 in such a way that the vector will be able to transduce hepatocytes and mediate detectable hFIX expression is extremely low. It would entail unintended intravenous infusion of 25 mL vector preparation or more. In addition, the probability that such unintended exposure would result in overexpression of hFIX expression levels is extremely low, as the target levels for intended exposure are close to 5% of normal. Finally, the probability that overexpression of hFIX would have any clinical consequence for a third party is low, as already in the normal population there is considerable ‘over’ expression in otherwise healthy individuals, and non-clinical studies suggest that even extreme overexpression holds negligible biological consequence. The overall risk that overexpression of hFIX in third parties due to unintentional exposure will result in observable effects is therefore negligible.

**13. Likelihood of postrelease shifts in biological interactions or in host range**

**Potential post-dosing recombination events – AAV sequences**

AMT-061 itself is replication deficient. Nonetheless, scenarios in which it would replicate *in vivo*, or even revert to a replication-competent AAV, are theoretically conceivable. In any case, such scenarios would require Rep and Cap sequences to be present in the same cell, and hence would require co-infection with wild type AAV. Presence of wild type AAV would however still not circumvent the dependence on a helper virus infection. Hence, replication or revertance of the GMO would require simultaneous infection of one-and-the-same cell with (1) AAV5-hFIX, (2) wild type AAV, and (3) a helper virus. This scenario of triple infection is in itself unlikely. Nonetheless, the conceivable scenarios upon triple infection are outlined below.

Without recombination taking place, triple infection will result in replication of the AAV5-hFIX vector genome by the Rep proteins provided by wild type AAV. Consequently, the triple-infected cell would in this case produce replication-deficient AAV particles of the wild type serotype containing the AAV5-hFIX vector genome, as well as wild type AAV particles. In any case, no replication-competent AAV5-hFIX particles would be formed regardless of the presence or absence of helper virus. Consequently it can be concluded that the presence of helper virus in patients does not impact the risk profile of the product and its intended use. Therefore, helper virus positive patients can safely be included in the proposed study.

Homologous recombination would only involve the ITRs present in the AAV5-hFIX vector genome and the ITRs present in the wild type AAV, as only these sequences may share homology. Essentially, homologous recombination could yield the AAV5-hFIX expression cassette flanked by wild type AAV-derived ITRs, and the wild type AAV-derived genome flanked by AAV5-hFIX-derived ITRs. The likelihood of homologous recombination would depend on the degree of homology between the AAV5-hFIX-derived ITRs (derived from wild type AAV2) and the wild type AAV-derived ITRs, which logically depends on the serotype of the wild type AAV in question. In the case of triple infection, both recombinants would be subject to intracellular replication by the Rep protein provided by wild type AAV. As a result, the triple-infected cell would in this case produce replication-defective AAV particles containing the AAV5-hFIX vector genome, wild type AAV particles, and AAV particles containing either of the recombined genomes. Also in this scenario, no replication-competent AAV5-hFIX particles would be formed regardless of the presence or absence of helper virus.

Non-homologous recombination could theoretically produce a hybrid sequence containing wild type AAV-derived rep and cap sequences as well as the hFIX expression cassette. The likelihood of such recombination events is intrinsically much smaller than the likelihood of homologous recombination. Depending on its primary structure (i.e. depending on whether it is flanked by ITR sequences or not) such hybrid genomes may or may not be valid substrates for Rep protein. In the case of triple infection, hybrids representing valid Rep substrates would be subject to intracellular replication by the Rep protein provided by wild type AAV. Because of their expected size however, such hybrids cannot be packaged into the wild type AAV capsids. The maximum packaging capacity of AAV capsids is approximately 5kb, and rep- and cap- and ITR sequences already make up for 4.7 kb (Daya et al. Clin Microbiol Rev (2008):583-593). The triple-infected

cell would in this case only produce AAV particles containing the AAV5-hFIX vector genome and particles containing the wild type AAV genome, as outlined in the no-recombination scenario described above. Replication-competent AAV5-hFIX particles could however not be formed or released by the infected cell.

Most importantly, in any of the scenarios described above, immunological responses will be mounted against rAAV- and/or wild type AAV-derived Cap proteins. Therefore, adaptive immunity would silence any extracellular manifestation that could result from triple infection, just as it would in the case of a naturally occurring co-infection with wild type AAV and helper virus. The risk of spread to other cells (which would also in these cells require the event of triple infection) is therefore extremely small. It should be noted that cells that are infected with AAV5-hFIX only will not be affected by these immune responses as they do not express AAV-derived proteins. Hence, none of these responses would compromise therapeutic efficacy.
Taken together, the likelihood of recombination is small as it requires concurrence of a constellation of events at a singular moment in time. Replication-competent AAV5-hFIX would not be formed in any case. Recombinant replication-deficient particles could theoretically be formed but none of these would have different characteristics as compared to the vector or wild type AAV, respectively. In any case these particles would not have any significant systemic consequences, as they would be neutralized by the immune system as soon as they leave the cellular compartment. None of these events would affect cells transduced with the therapeutic vector alone. The risk associated with recombination is therefore considered to be negligible.
Even in the very unlikely event of recombination, the products of the outlined recombination events would not increase the likelihood of shedding and transmission based on the shedding and transmission scenarios outlined above. It is therefore concluded that the environmental impact will not be affected by any of the theoretical and unlikely recombination events.

**Potential post-dosing recombination events – SV40 sequences**
The AAV5-hFIX vector contains an SV40 intron and an SV40 polyA sequence. The possible recombination of these viral sequences with wild-type viruses is discussed below.
Recombination of the SV40-derived sequences present in the AAV5-hFIX vector genome with other virus-derived sequences depends on two likelihoods, being (1) the likelihood that homologous sequences are present, and (2) the likelihood that in such case recombination occurs (the likelihood of non-homologous recombination was taken to be negligible).

The simian SV40 virus can infect humans, as approximately 1 in every 5 individuals may be seropositive for this virus (Taronna et al, PLosOne, 2013(8): e61182), (Corallini et al, Hum Immunol, 2012(73):502-10), (Mazzoni et al, PLoS One, 2014(9): p. e110923). Infection in any case remains subclinical (Garcea et al. J Virol 2003(77):5039-5045). Persistence of SV40 DNA in the normal population is a matter of debate. A review article summarized that half of the available screening studies suggested SV40 is not present in the normal population, while the other half suggested that it is, at frequencies of 5 to 25% (Paracchini et al, Mut Res, 2006(612):77-83). It is not unthinkable that SV40 sequences are present in patients during AAV5-hFIX

administration but the likelihood is low. Using BLAST search engines, no human viruses were found that displayed significant homology with the SV40 sequences present in the AAV5-hFIX vector genome [http://blast.ncbi.nlm.nih.gov], suggesting that, in humans, presence of virus-derived sequences (SV40 or other) with significant homology is not a likely event.

In the hypothetical case that homologous sequences are present, the likelihood of homologous recombination depends on the extent of homology. In a study on this exact relation, the recombination frequency of SV40 DNA in monkey cells sharply dropped when the length of the homologous sequences was less than 200 base pairs (Rubnitz et al, Mol Cell Biol (1984):2253-58). The SV40 intron in the AAV5-hFIX vector genome is 94 base pairs in length, the SV40 polyA signal sequence is 133 bp in length. As such, even in the presence of homologous sequences, recombination is an unlikely event.

**14. Known or predicted interactions with non-target organisms in the environment, including competitors, preys, hosts, symbionts, predators, parasites and pathogens**

Not applicable since the vector is not capable of replication and does not code for sequences which would provide a detrimental environmental advantage to microorganisms or plants.

**15. Known or predicted involvement in biogeochemical processes**

Due to the low numbers of vector DNA copies potentially released into the environment through shedding, horizontal gene transfer is highly unlikely. Even if horizontal gene transfer occurred, the sequences would not confer a selective advantage to other organisms such as bacteria since AMT-061 does not contain any prokaryotic promoters, any antibiotic or other types of resistance genes or any genes, which would enhance or constrain their growth. Therefore, it is unlikely that AMT-061 would interfere with the control of pathogenic microorganisms or that it would have an effect on the natural dynamics of microbial populations or the biogeochemical cycles at any given site in the environment.

**16. Other potential interactions with the environment.**

Please see section 8.B.15.

## 6. INFORMATION ON MONITORING, CONTROL, WASTE TREATMENT AND EMERGENCY RESPONSE PLANS

### A. Monitoring techniques

There is no monitoring plan to identify the effects on human health and the environment. Due to the negligible risk presented by AMT-061 to the environment, as described in the sections above, no further monitoring is considered necessary.

### B. Control of the release

**1. Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of release or the designated area for use**

**GMO storage**
The hospital pharmacy, or equivalent, will receive the AMT-061 product and store in accordance with GMO legislation and/or local hospital procedures. Storage will be in a facility with restricted access using equipment that is labeled in accordance with GMO legislation and/or local hospital procedures.

**Preparation in hospital pharmacy or equivalent**
In the hospital pharmacy, or equivalent, the AMT-061 will be pulled from the supplied glass vials into an infusion bag in a Class II Biological Safety Cabinet. The standard precautions that are applied in the hospital pharmacy, or equivalent setting, to control exposure to hazardous substances such as cytotoxic agents are appropriate and will be applied during the preparation of AMT-061. Therefore, no additional measures have been taken. During the preparation of AMT-061 personnel will wear protective clothing and gloves.

**Transportation within the hospital**
The AMT-061 infusion bag will be transported to the treatment room in accordance with GMO requirements and/or local hospital procedures.

**Administration in the clinic**
The standard precautions that are applied to mitigate spillage and/ or aerosol formation of hazardous substances, such as cytotoxic agents, are appropriate and will be applied during the preparation and administration of AMT-061. Therefore, no additional measures have been taken. Accidental exposure and/or spilling of AMT-061 are mitigated by the standard precautions mentioned in Section 4.A.8. The standard precautions will be described in the Investigational Medicinal Product handling manual which will be provided to all clinical staff involved in the preparation and administration of the product. Furthermore, after administration the treatment room will be decontaminated with a disinfectant. A 250-ppm chlorine solution will be used for regular disinfection on used surfaces. In case of a spill the surface will be treated with 1000 ppm chlorine solution.

**Observation of patients**
Observation of patients will be by means of post-administration surveillance for up to 24 hours. Note that the post administration surveillance is not related to any anticipated environmental risk. Subjects will be followed with respect to safety and efficacy parameters for at least 5 years.

**2. Methods and procedures to protect the site from intrusion by unauthorised individuals**
The pharmacy has limited access with badge control.

**3. Methods and procedures to prevent other organisms from entering the site.**
The hospitals have many procedures in place to prevent and control infections.

### C. Waste treatment

**1. Type of waste generated**
The nature of the waste generated includes (partially) used vials, ancillary components used for preparation and administration (e.g. tubing, needles, syringes, infusion bag, personal protective equipment, etc.) and components used for collecting body fluids samples after administration.

**2. Expected amount of waste**
Following handling and administration of AMT-061, the unused product remaining in the opened vials and all the materials that have been in contact with the GMO (such as gloves, syringes, needles, tubing and infusion bag) will be disposed of in accordance with GMO Regulation.
Unused (non-opened) vials of AMT-061 will be returned to uniQure or disposed per study procedures, in accordance with GMO Regulation. Up to 10 patients will be treated in Belgium.

**3. Description of treatment envisaged.**
Following handling and administration, the unused AMT-061 remaining in the opened vials and all the materials that have been in contact with AMT-061 (such as gloves, syringes, needles, tubing and infusion bag) will be disposed per GMO legislation and/or local hospital procedures. Unused (non-opened) vials of AMT-061 will be returned to the Sponsor or disposed as per study procedures, in accordance with GMO legislation and/ or local hospital procedures. Following handling and administration of AMT-061 the unused product remaining in the vials, and ancillary materials, will be disposed of per GMO legislation and/or local hospital procedures.

### D. Emergency response plans

**1. Methods and procedures for controlling the GMOs in case of unexpected spread**
Accidental exposure of health care professionals to AAV5-hFIX should be treated according to the measures listed below. These are standard measures for which it should be indicated that they are not needed to reduce the already negligible risk.

**Table 4: Exposure of GMO and concomitant measures**

| Type of exposure | Measure |
|---|---|
| Needle stick | Encourage bleeding of the wound. Wash injection area well with soap and water. Obtain medical attention. |
| Eye contact | Immediately flush eyes with water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Obtain medical attention. |
| Inhalation | When inhaled, move person into fresh air. Obtain medical attention. |
| Ingestion | Rinse mouth with water. Obtain medical attention. |
| Skin contact | Wash-off with a gauze soaked in a viricidal disinfectant (not alcohol solution) and subsequently wash with soap and plenty of water. Obtain medical attention. |

**2. Methods for decontamination of the areas affected, for example eradication of the GMOs**

The spill procedures are similar to those already in place for handling hazard substance such as cytotoxics. A spill kit will be provided to the clinical sites and pharmacy. The spill kit should be available during all the steps. The spill kit will contain:

- Disinfectant sachets
- Absorbent paper towels
- Disposal forceps
- Biohazard incineration bags
- Emergency contact number
- Copy of the spillage procedure

The spill procedure will be described in the Investigational Medicinal Product (IMP) handling manual.

**3. Methods for disposal or sanitation of plants, animals, soils, etc., that were exposed during or after the spread**

The likelihood of gene transfer to species other than humans and (some) primates is low, given the host preference of AAV.

As far as (unintentional) gene transfer to humans and primates is concerned, the likelihood is low given the selective disadvantage conferred to the GMO under conditions of proposed release.

**4. Methods for the isolation of the area affected by the spread**

The spill procedures for handling AMT-061 are described in section 5.D.2.

Furthermore, connecting the infusion bag to the main infusion tubing in the clinic, creates an increased likelihood of spillage and/or aerosol formation. For AMT-061 administration, the possibility of spillage and /or aerosol formation is reduced as the hospital pharmacy, or equivalent, will prepare and provide the infusion bag connected to a pre-filled tubing with an appropriate, suitable and sterile diluent (e.g. WFI or sterile saline solution). In the exceptional case spillage and/or aerosol formation should occur, this will only be the diluent.

**5. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect.**

Under conditions of release, persons working with the GMO could potentially come into contact with the GMO prior to or during administration, when the GMO is handled as distributed, at high concentration and fully functional.

Standard procedures to mitigate spillage and/or aerosol formation will be applied. Blood samples taken shortly after administration of the GMO should be regarded as containing active (infectious) GMO.

Should persons working with the GMO come into direct contact with the GMO (through inhalation or accidental injection during administration, or via blood samples taken shortly after administration), no immediate and/or delayed effects different from those expected for the recipients (test subjects) are expected: a (dose-dependent) immune response to the GMO could occur that will not affect subjects' general well-being.

It should be noted that humans are natural hosts for AAV, infections are asymptomatic and AAV is not known to cause any noticeable pathology. Similarly, dose-dependent administration of AAV/based GMO´s to humans has been shown to be safe. As noted above, a dose-dependent immune response does occur in a recipient and is without clinical consequence.

Overall, no immediate and/or delayed effects on human health are expected for persons working with the GMO or coming into contact with or in the vicinity of the GMO as it is released.

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