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RIJKSINSTITUUT VOOR VOLKSGEZONDHEID EN MILIEU
NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

RIVM report 605200 001

# Gene therapeutics and DNA vaccines; quality and regulatory aspects

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June 2001

This investigation has been performed by order and for the account of Board of Directors, within the framework of project 605200, New control and characterisation methods for biologicals.

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# Abstract

Transfer of genes to cells and the subsequent expression of these genes can alleviate the symptoms of a disease (gene therapy), or prevent infectious diseases (DNA vaccination). Gene therapy and DNA vaccination are based on relatively new technologies. The first gene therapeutics are expected to enter the market as registered medicinal products within 2-4 years. The vectors used to transfer the gene of interest to target cells are genetically modified viruses or plasmids. Viral vectors and plasmids are produced biotechnologically and are therefore considered to be biological medicinal products ('biologicals'). Since the production of biologicals is inherently variable and difficult to control, these products require extra safety measures and regulations. The Laboratory for Medicines and Medical Devices of the RIVM, involved in the regulatory assessment and control authority batch-release testing of biologicals, has carried out an inventory study on both the analytical methods used for characterisation of viral and plasmid vectors and the regulatory issues affecting these products. The results of this study indicate that many quality and safety considerations applicable to viral and plasmid vectors are identical to those applied to other biologicals. Novel analytical methods are those for testing the identity of the vector, measuring transduction efficiency, testing for replication-competent viruses, assays for particle count and infectious virus titer, as well as tests for genetic and chemical stability and structure of plasmid vectors. These analytical methods are not yet standardised. However, international initiatives have been started for standardisation. Guidelines for the production and characterisation of gene transfer medicinal products, as provided by regulatory agencies like EMEA and FDA, have been formulated in general terms, and remain subject to frequent revision to keep them up-to-date.

# Preface

As a result of the developments in the field of genomics and biotechnology, biotechnological techniques are becoming more and more important in the production and characterisation of medicines. Consequently the number of biologicals to enter the market is expected to increase. The Laboratory for Medicines and Medical devices is involved in both the regulatory assessment for marketing authorisation and control authority release testing of biologicals in the Netherlands. In order to keep up-to-date with new developments in the field of production and characterisation of biologicals the research project 'New control and characterisation methods for biologicals' has been started that aims at setting up new analytical methods for characterisation of biologicals in order to evaluate the suitability of these assays and to contribute to future standardisation of these biologicals.

Relatively new types of biologicals are gene therapeutics and DNA vaccines. We carried out an inventory study on the analytical methods used for the characterisation of gene therapeutics and DNA vaccines and on the regulatory issues affecting these products. The inventory study was carried out by an extensive literature search and by interviewing scientists and hospital pharmacists in the field of gene therapy and DNA vaccination. Hereby we would like to thank those people who were so kindly to provide us with information about the production and characterisation of gene therapeutics and DNA vaccines and shared with us their enthusiasm for this exciting new field: Dr. W.R. Gerritsen of the Dutch society of gene therapy, Dr. A.G. Vulto, Drs. M.A.L. Pluim, Drs. F.J. Rijnja, Dr. J.G.W. Kosterink and Dr. A.R. Wafelman, hospital pharmacists at the academic hospitals in the Netherlands, who are responsible for the quality of gene therapeutics used in clinical trials, Dr. R. Lardenoije from Crucell, Dr. K.B. Islam from the Karolinska institute and Dr. M.J.H. Kenter from the CCMO.

# Contents

# Samenvatting

Gentherapie en DNA vaccinatie zijn relatief nieuwe technieken die zijn gericht op de behandeling of preventie van ziektes door de toediening van een gen dat codeert voor een therapeutisch of immuniserend eiwit aan een patiënt. Aanvankelijk was gentherapie gericht op de behandeling van monogenetische aandoeningen door de vervanging van een gemuteerd gen door een gezond gen. Echter, tegenwoordig zijn de meeste van de klinische trials die worden uitgevoerd met gentherapie, gericht op de behandeling van kanker en andere verworven aandoeningen. DNA vaccins zijn voornamelijk gericht op de voorkoming van infectieuze ziektes die tot dusver met behulp van conventionele vaccins niet bestreden kunnen worden.

Hoewel de verwachtingen van gentherapie hoog waren, vielen de klinische resultaten aanvankelijk tegen. Het jaar 2000 is evenwel een doorbraak op het gebied van gentherapie omdat in dit jaar verschillende klinische successen werden behaald. De verwachting is dat de eerste gentherapeutica de markt als geregistreerde geneesmiddelen zullen bereiken in 2 tot 4 jaar. Echter, voor veel gentherapie strategieën is nog veel fundamenteel onderzoek nodig om de klinische efficiëntie te kunnen verbeteren. Voor DNA vaccinatie is een eerste proof-of-principle geleverd in mensen, maar ook deze technologie vereist nog veel onderzoek voordat producten kunnen worden aangemeld voor registratie.

Voor de overdracht van genetisch materiaal naar cellen in het menselijk lichaam worden virale vectoren of plasmiden gebruikt. Virale vectoren zijn virussen waarin een therapeutisch of immuniserend gen (ook wel transgen genoemd) is gekloneerd en die dusdanig gemodificeerd zijn dat ze niet meer kunnen repliceren. Plasmides zijn kleine ringen van dubbel-strengs DNA, waarin een transgen is gekloneerd. De virale of plasmide vectoren worden direct toegediend aan het lichaam van de patiënt (*in vivo* gentherapie) of worden gebruikt om het gen toe te dienen aan cellen buiten het lichaam van de patiënt, waarna deze gemodificeerde cellen worden toegediend aan de patiënt (*ex vivo* gentherapie).

Virale en plasmide vectoren worden geproduceerd op biotechnologische wijze en worden daarom beschouwd als geneesmiddelen van biologische oorsprong ('biologicals'). De productie van geneesmiddelen van biologische oorsprong is moeilijk te standaardiseren en te controleren en vereist daarom extra veiligheidsmaatregelen en regulering om de mogelijke risico's van deze producten te verminderen. Met name na de dood van een 18-jarige jongen als gevolg van een klinisch onderzoek met gentherapie in de Verenigde Staten zijn de veiligheidseisen voor gentherapie aangescherpt. Het Laboratorium voor Geneesmiddelen en Medische Hulpmiddelen van het RIVM is betrokken bij zowel de beoordelingen ten behoeve van registratie als de vrijgifte testen van biologische geneesmiddelen in Nederland. Anticiperend op toekomstige adviestaken heeft het laboratorium een inventariserende studie

uitgevoerd naar de kwaliteits- en reguleringsaspecten die een rol spelen bij gentherapeutica en DNA vaccins.

Veel van de kwaliteits- en veiligheidsaspecten die van toepassing zijn voor virale en plasmide vectoren zijn in essentie hetzelfde als voor andere biologische geneesmiddelen, zoals recombinant DNA (rDNA) eiwitgeneesmiddelen. Net als voor rDNA producten is het noodzakelijk dat virale en plasmide vectoren worden geproduceerd onder GMP condities, dat het productie proces gevalideerd is en dat het eindproduct grondig wordt gekarakteriseerd. De cellijnen die nodig zijn voor productie worden gebruikt in een working cell bank/master cell bank systeem. De cellijnen moeten getest worden op identiteit en contaminatie met vreemde agentia. Verder moeten de eindproducten worden getest op de aanwezigheid van vreemde agentia en proces-afhankelijke onzuiverheden zoals gastheer DNA, gastheer eiwitten en pyrogenen.

De kwaliteits- en veiligheidstesten die specifiek zijn voor virale en plasmide vectoren zijn de analytische testen die worden uitgevoerd voor het bepalen van de identiteit en de werkzaamheid van de vector. Verder is voor virale vectoren de mogelijkheid dat replicatie-competente virussen worden gevormd tijdens de productie een belangrijke zorg. Daarom moeten de cellijnen die worden gebruikt voor productie en het eindproduct worden getest op de aanwezigheid van replicatie-competente virussen. De assays die hiervoor worden gebruikt zijn celkweekmethodes en daardoor tijdrovend. Verder is het essentieel om het aantal deeltjes en het aantal infectieuze virussen te bepalen in het eindproduct. Hiervoor bestaan veel benaderingen die leiden tot veel variatie tussen laboratoria. Voor plasmide vectoren is het bepalen van de integriteit van de sequentie en structuur een belangrijke test voor de karakterisatie.

De analytische methodes die worden gebruikt voor karakterisatie van virale en plasmide vectoren zijn nog in ontwikkeling en standaard methodes zijn nog niet ontwikkeld. Echter, om de uitkomst van verschillende klinische onderzoeken te kunnen vergelijken is standaardisatie essentieel. Recentelijk hebben verschillende instanties, zoals de Food and Drug Administration (FDA) in de Verenigde Staten en het National Institute for Biological Standards and Control (NIBSC) in Londen, initiatieven genomen tot de standaardisatie van gentherapie vectoren. Door registratie autoriteiten zijn richtlijnen ontwikkeld voor de productie en karakterisatie van gentherapeutica en DNA vaccins.

# Summary

Gene therapy and DNA vaccination are relatively new techniques that are developed for the treatment of diseases by the transfer of a gene that encodes for a therapeutic or immunising protein to cells inside the human body. Initially gene therapy was aimed at the treatment of monogenic diseases by the replacement of a mutated gene with a correct gene but more recently most of the clinical trials that are being conducted by gene therapy are aimed at cancer and various other acquired diseases. DNA vaccines are mainly aimed at the prevention of infectious diseases for which the development of conventional vaccines was unsuccessful.

Although the expectations for gene therapy and DNA vaccination were high many clinical results have been disappointing. But the year 2000 was a breakthrough in the field of gene therapy since in this year several clinical successes have been reported. The first gene therapeutics are expected to reach the market within 2-4 years. However, for many gene therapy strategies still a lot of basic research has to be performed to improve the clinical efficacy. For DNA vaccination proof-of-principle has been delivered in patients but also for this approach still a lot of research has to be performed.

For the transfer of genetic material to cells, viral vectors or plasmids are used. Viral vectors are viruses in which a therapeutic or immunising gene (called transgene) is cloned and which are genetically modified in such a way that they are replication-deficient. Plasmids are small rings of double-stranded DNA, in which a transgene is cloned. The viral or plasmid vectors are delivered directly inside the patients' body (*in vivo* gene therapy) or are used to transfer the gene of interest to cells outside the patients' body after which the transferred cells are delivered to the patient (*ex vivo* gene therapy).

Viral and plasmid vectors are synthesised in a biotechnological way and are therefore considered as biological medicinal products (biologicals). The production of biologicals is difficult to standardise and control and therefore requires extra safety measures and regulation to minimise the potential risks. The death of an 18-year old boy as a result of a clinical trial with gene therapy in the USA was the direct cause for more strict regulations for gene therapy. The Laboratory for Medicines and Medical devices of the RIVM is involved in both the regulatory assessment and control authority release testing of biologicals in the Netherlands. In order to prepare for future advice functions the laboratory carried out an inventory study on the quality and regulatory issues that are associated with gene therapeutics and DNA vaccines.

Many of the quality and safety considerations that are applicable to viral and plasmid vectors are essentially the same as for other biologicals, like recombinant DNA (rDNA) protein pharmaceuticals. Just like for rDNA products it is necessary that viral vectors and plasmid vectors are manufactured under GMP conditions, that the production process is validated and

that the finished product is thoroughly characterised. The cell lines used for production are handled in a working bank/cell bank system and have to be tested for identity and contamination with adventitious agents. Furthermore, the intermediate products and finished product are tested for adventitious agents and for process-related impurities like host cell DNA, host cell proteins and pyrogens.

The quality and safety tests for viral and plasmid vectors that are unique for these products are identity tests and tests for the establishment of the potency of the vector. Furthermore for viral vectors an important safety concern is the possibility that replication-competent viruses are generated during production. Therefore the finished products and the cellines that are used for production have to be tested for the presence of replication-competent viruses. The assays that are used are cell culture-based and therefore time-consuming assays. Furthermore it is essential to estimate the number of particles and infectious viruses in the finished product. Many approaches exist for particle count and titer determination, which are subject to variation between laboratories. For plasmid vectors the characterisation of the integrity of the sequence and structure is an important quality test.

The analytical methods that are used for characterisation of viral and plasmid vectors are still in development and standard methods have not been developed yet. However, in order to be able to compare the outcome of different clinical trials, standardisation is essential. Recently, several organisations, like the Food and Drug Administration (FDA) in the USA and the National Institute for Biological Standards and Control (NIBSC) in London, have initiated standardisation for gene therapy vectors. Guidelines for the production and characterisation of gene therapeutics and DNA vaccines have been developed by regulatory agencies.

# 1. Introduction

Transfer of genes to cells and the subsequent expression of the genes can alleviate the symptoms of a disease (gene therapy) or prevent infectious diseases (DNA vaccination). Gene therapy has the potential to treat a variety of hereditary monogenic diseases as well as acquired diseases like cancer, vascular diseases and neurological disorders. Initially gene therapy strategies were mainly aimed at the treatment of monogenic disorders but more recently most of the clinical trials that are being conducted are aimed at cancer (see table 1). DNA vaccines are mainly aimed at the prevention of infectious diseases for which no vaccines exist yet, like malaria and AIDS.

*Table 1. Diseases for which clinical trials are being conducted by gene therapy and DNA vaccination*

| Diseases | % of clinical trials | Number of clinical trials |
|---|---|---|
| Cancer | 62.2 % | 331 |
| Monogenic diseases | 13.3 % | 71 |
| Gene-marking | 9.0 % | 48 |
| Infectious diseases | 6.8 % | 36 |
| Vascular diseases | 6.8 % | 36 |
| Other | 1.9 % | 10 |

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In 1990, the first clinical trial for the treatment of a disease by gene therapy was started. The gene therapy was aimed at the treatment of severe combined immuno-deficiency (SCID) in two patients that had a defect in the adenosine deaminase (ADA) gene (1). The results of this clinical trial were promising and the expectations of gene therapy were high. However, the results of many subsequent clinical trials have been disappointing. In September 1999, a fatal incident with an 18-year old boy in Pennsylvania, USA put gene therapy in discredit and was the immediate cause for more strict requirements for gene therapy clinical trials (2). But also progress was made. In the opinion of experts, the year 2000 can be considered as the turning point in gene therapy because in this year important clinical successes were reported, e.g. the cure of SCID patients by gene therapy with retroviral vectors (3), clear clinical progress in the treatment of factor IX deficiency. (4), the treatment of patients with advanced non-small cell lung cancer (5) and the treatment of patients with head and neck cancer by gene therapy (6). It is expected that the first gene therapeutics or DNA vaccines will reach the market as registered medicinal products within 2-4 years. However, it should be realised that only a few clinical trials are yet in phase III and still a lot of basic research, pre-clinical and clinical studies will have to be performed to establish the efficacy and safety of many gene therapy and DNA vaccination strategies.

As is the case with any new medical technology there is concern about the safety of gene therapy and DNA vaccination and therefore these new technologies require specific regulation to minimise the potential risks. Since gene therapy and DNA vaccination are rapidly evolving techniques, regulations should keep in pace accordingly.

The governmental agencies that will in the near future be responsible for the licensing of gene therapeutics and DNA vaccines must be well informed on the potential risks associated with these products and have to be aware of the type of characterisation that these products require. Official medicines control laboratories (OMCLs) that are involved in standardisation and release testing of biologicals have to be up-to-date with the suitable tests for these products. The Laboratory for Medicines and Medical devices of the National Institute of Public Health and The Environment in the Netherlands, which is involved in both the regulatory assessment and control authority batch release testing of biologicals in the Netherlands, has carried out an inventory study on both the quality and regulatory issues affecting gene therapy and DNA vaccines. The results of this study are described in this report.

## 1.1 Gene Therapy – Therapeutic applications

Gene therapy was originally aimed at treating hereditary, monogenic diseases by the addition of a correct gene to cells with an affected gene. However, gene therapy strategies are now also being developed for the treatment of acquired diseases like cancer and vascular diseases by the transfer of a therapeutic gene. Also gene therapy is used for the purpose of gene marking, which allows the tracing of transferred cells. Thus the potential applications of gene therapy are diverse. This paragraph gives an overview of the most important therapeutic applications at this moment.

**In vivo *and* ex vivo *gene therapy***

Gene therapy can be divided in *in vivo* and *ex vivo* therapy. In *in vivo* therapy the vector containing the therapeutic gene is delivered directly to the patient. For *ex vivo* therapy the target cells are removed from the patient's body and grown *in vitro*. Subsequently, the therapeutic gene is transferred to the target cells, which are then transplanted back into the body. This latter form of therapy is also considered as a form of cell therapy by the FDA (7).

***Monogenic diseases***

Monogenic, hereditary diseases that are suitable for treatment by gene therapy include severe combined immuno-deficiency (SCID), haemophilia, cystic fibrosis (CF) and Gaucher's disease. The first clinical successes with gene therapy for monogenic diseases have now been reported. Gene therapy corrected the disease-phenotype in two patients suffering from X-linked SCID (3). This was the first study in which a complete cure of a disease was observed with gene therapy. Three haemophilia B patients that were treated with the gene for factor IX

showed expression of factor IX at sites of injection resulting in a reduced necessity for intravenous infusion of factor IX (4).

***Cancer***

Genetic anti-cancer therapies have various approaches (for review see (8)). Tumour cells are often defective in tumour suppressor genes like p53, p16 or BRCA1. By gene therapy the tumour-suppressor function may be corrected by transfer of the tumour suppressor gene to tumour cells in order to induce growth arrest. The first success of this approach was seen in a study with patients with advanced non-small cell lung cancer that received gene therapy with p53. An antitumoural effect with prolonged tumour stability or even regression was observed (5). Several phase III clinical trials have been started for this type of gene therapy with patients with ovarian cancer and head- and neck-cancer.

Inactivation of oncogene expression (e.g. ras genes) is addressed by antisense technology. For this technology a DNA-adduct is administered to the cells that binds to the oncogene mRNA and inhibits protein synthesis. Good results with this approach were reported in animal experiments (8).

Gene therapy can also be used to transform tumour cells with cytokine genes, or other genes that stimulate the immune system in order to generate an immune response against the tumour. Transfer of the human gene encoding HLA-B7 antigen helps the immune system to recognise and attack cancer cells. This therapy is in phase II and phase III testing in certain patients with metastatic melanoma and in phase II testing in patients with head- and neck-cancer. Partial clinical responses have been reported so far with this type of gene therapy (9).

Another approach of genetic anti-cancer therapy, is suicide gene therapy. Tumour cells are transduced with a gene whose product can convert a non-toxic pro-drug to a toxic metabolite. Subsequent delivery of the pro-drug results in a cytotoxic effect in the tumour. For example the herpes simplex virus thymidine kinase (HSV-*tk*) gene product can convert ganciclovir into the cytotoxic ganciclovir triphosphate. Gene therapy with HSV-*tk* can also be used to prevent the potentially lethal effects of graft versus host disease (GvHD) in patients that receive allogeneic bone marrow transplantation for treatment of hematologic malignancies. The HSV-*tk* gene was transferred in lymphocytes obtained from the graft donor and these were infused in patients. Those patients that developed GvHD were treated with ganciclovir resulting in a disappearance of transduced donor lymphocytes and GvHD clinical and biochemical signs (10).

The efficacy of cancer-chemotherapy is often limited by its toxicity. Gene therapy can be used to make chemotherapy more efficient. Bone marrow is selectively protected from chemotherapy by transfer of drug-resistance genes. This allows the exposure to higher doses of chemotherapy with less toxicity. An example of a drug-resistance gene is MDR1 (multiple drug resistance gene 1). With this kind of therapy the concern is that cancer cells in the

marrow may also be transduced with the drug-resistance gene or that higher doses of chemotherapy do not lead to higher response rates. No results of clinical studies are reported yet with this gene therapy approach (reviewed by Roth and Cristiano (8)).

***Vascular diseases***

Cardiovascular gene therapy is expected to become one of the most promising areas of gene therapy (11). The transfer of the gene encoding for Vascular Endothelial Growth Factor (VEGF) or Fibroblast Growth Factor (FGF-5) can stimulate the development of arteries in coronary and peripheral ischaemia. Some other candidate genes also stimulate angiogenesis. Clinical evidence of angiogenesis was first demonstrated by the transfer of the VEGF gene for treatment of critical limb ischaemia (12). Beneficial effects were reported in clinical trials for the treatment of critical limb ischaemia (13) and myocardial ischaemia (14). A potential risk of this kind of therapy is the production of leaky vessels or stimulation of angiogenesis in tumours (11).

***Gene-marking***

Gene marking studies are not intended to treat patients but to gain information about stem cell biology. Gene transfer can be used to transfer a gene to hematopoietic stem cells (HSC) from patients with leukaemia or neuroblastoma in order to mark these cells. After reintroduction of these transduced cells the transfected cells can be traced and the source of relapse after HSC transplantation can be determined (reviewed by Brenner (15)).

## 1.2 DNA vaccines

Conventional vaccines can be subdivided in three types, namely live attenuated vaccines, killed whole vaccines and purified component vaccines (subunit vaccines). Vaccine development can be hampered because inactivated whole cell vaccines or subunit vaccines do not provide sufficient protection or live-attenuated vaccines pose unacceptable risks. A novel approach to the development of vaccines is the use of DNA for immunisation. A DNA vaccine is a polynucleotide, carrying a sequence that encodes the immunogen(s) of interest and is expressed as a protein when it is administered to the host cell. Hence, a DNA vaccine is a kind of subunit vaccine, which indirectly presents only selected components of a pathogen to the immune system.

DNA vaccines have the advantage over inactivated whole cell vaccines and purified component vaccines that they may better mimic a natural infection by producing the immunising material in the host and stimulate both B- and T-cell responses. They share this property with live attenuated vaccines; however, they cannot replicate and thus are safer as live attenuated vaccines (16).

Animal studies have shown that DNA vaccination can raise protective immunity against a number of infectious diseases (17-19). Most of the pre-clinical studies that have been

performed in animals were aimed at viral infectious diseases, such as influenza and Human Immunodeficiency Virus (HIV) but also at parasitic diseases such as malaria and at diseases of bacterial origin (for review see (20)). Clinical trials that are being conducted with humans are mainly aimed at HIV and malaria. Clinical studies in humans have shown lower efficacy than what had been expected based on animal studies. Immune responses in humans against HIV gene products and a gene product of *Plasmodium falciparum* (the causative agent of malaria) have been reported after administration of a DNA vaccine (21-23). However it was not clear whether these responses generated a sufficient protective immunity. Recently, in a clinical study with a DNA vaccine encoding the surface antigen of Hepatitis B Virus (HBV) it was shown that all 12 healthy volunteers developed protective antibody responses. This was the first study that demonstrated protective antibody titers and both humoral and cell-mediated immune responses, induced by a DNA vaccine in humans (24).

## 1.3 The transfer of genetic material to cells

The genetic transformation of target cells requires the use of a gene transport vehicle referred to as a vector. As vectors viruses, plasmids or oligonucleotides can be used.

Viral vectors make use of the capacity of viruses to infect cells and to deliver their genome inside the cell (transduction). Viral vectors are viruses in which a therapeutic or immunising gene (called transgene) is cloned and which are genetically modified in such a way that they are not replication competent, i.e. they cannot multiply in recipient cells (although replication competent, attenuated vectors and conditionally replicating vectors are also being used). However, viral vectors are still capable to infect target cells and deliver their genome containing the transgene inside the cells. The viral genome can integrate into the genomic DNA of the target cell, resulting in stable expression of the transgene (e.g. retrovirus). Or alternatively, the viral genome can not integrate and is present in the target cell as an episomal element (e.g. adenovirus). In the latter case the viral genome will disappear after several cell divisions and as a result the transgene is only expressed during a relatively short period of time (transient expression).

Plasmids are small rings of double-stranded DNA, which naturally occur in bacteria. A DNA sequence for the immunogenic or therapeutic protein as well as eukaryotic control elements (promoter and poly A sequences) can be cloned into the plasmid by recombinant DNA techniques. The eukaryotic control elements allow expression of the protein after transfer of the plasmid in eukaryotic cells. Plasmid vectors need to be formulated and administered in a way that increases their cytoplasmic uptake.

Viral and plasmid vectors are synthesised in a biotechnological way and are therefore considered to be biological medicinal products (‘biologicals’). The production of biologicals is difficult to standardise and control and therefore requires extra safety measures. Oligonucleotides, as used in antisense therapeutic products, are chemically synthesised and

are not considered to be biologicals. They are outside the scope of this report and will not be discussed. This report focuses on the quality, and regulatory aspects of viral and plasmid vectors.

Plasmid vectors are inherently safer than viral vectors, because they are produced in prokaryotes, are not infectious and are easier to manufacture in a consistent way. Although transfer efficiency strongly depends on the pharmaceutical formulation of the plasmid DNA vector, transfer of plasmid DNA to cells is in general less efficient than transfer of viral vectors. Most clinical trials for cancer and monogenic diseases are conducted with viral vectors. DNA vaccination for preventing infectious diseases is in most cases performed with plasmid vectors.

A safety concern of plasmids is that antibodies against injected DNA could be formed and this may lead to autoimmune reactions. A risk of viral vectors is that strong immune- and inflammatory responses against viral components can be generated. The death of the 18-year old boy in September 1999 as a result of gene therapy was probably caused by an inflammatory response that was triggered by a high dose of the adenoviral vector (25). For both plasmid vectors and viral vectors insertion of the vector in the host genome could result in inactivation or activation of certain genes (e.g. tumour suppressor genes), which is also an important safety concern. Furthermore, long-term expression of a foreign antigen may result in an undesired immuno-pathological reaction or the expressed antigen may have undesired biological activity. Another concern for both types of vectors is the transmission of the vector to the germ-line. These safety issues are outside the scope of this report.

This report mainly focuses on the aspects of quality control testing (e.g. product identity, consistency and potency) and product-related safety issues (e.g. presence of replication competent viruses, adventitious agents or other process contaminants). Being medicinal products of biological origin (biologicals), gene therapy products should be produced, controlled and regulated in line with existing requirements such as Good Manufacturing Practice (GMP). The issues that are associated with the production of conventional biologicals, like the presence of adventitious viruses or pyrogens in the finished product, also apply to viral vectors and plasmid vectors. But viral vectors and plasmids also have their own unique safety and characterisation issues, which will be discussed in chapter 2 and chapter 3 respectively. Regulatory issues are discussed in chapter 4.

# 2. Viral vectors

Different kinds of viral vectors have been developed each with their own specific characteristics. The main difference between the vectors is their ability to infect dividing or non-dividing cells, their cell type specificity, their ability to integrate into the genomic DNA of the target cell and their cloning capacity. The choice of a viral vector depends on the target cell, the level and duration of expression wanted and cloning capacity needed. The number of different kind of vectors that are being developed is rapidly growing. Below an overview of the most frequently used viral vectors is given (for review see (8;26;27)).

***Retroviral vectors***
Retroviral vectors are derived from retroviruses, which are RNA viruses. Retroviral vectors integrate randomly into the genome of the target cell. This results in stable expression of the transgene. Only dividing cells are transducted and therefore this vector is suitable for cancer applications. Retroviral vectors are employed in about 50% of the clinical trials because of their high gene transfer efficiency and high expression of the transgene (28). The technical limitations of retrovirus production are the low viral titres that are obtained and low stability of the vector. Therefore, retroviral vectors are not very suitable for *in vivo* gene therapy. Furthermore retroviruses have a limited cloning capacity (max. 8kb).

***Lentiviral vectors***
Lentiviruses (e.g. HIV) belong to the family of retroviruses but can infect both dividing and non-dividing cells. Lentiviral vectors are suitable for *in vivo* therapy and they integrate into the host genome, resulting in stable expression of the transgene. However, due to the high potential risk extensive studies are needed to test the safety of lentiviral vectors before they can be used in humans.

***Adenoviral vectors***
Adenoviruses, which are double-stranded DNA (dsDNA) viruses, cause respiratory-tract infections in humans. Adenoviral vectors have a high transduction efficiency for many cell types, both dividing and non-dividing, except hematopoietic cells. Furthermore, they have a high cloning capacity for foreign genes. Since the viral genome does not integrate into the host genome the therapeutic gene is only transiently expressed.

Recently also studies were reported with a conditional replicating adenoviral vector, called ONYX-015 (6). This vector only replicates in cancer cells that are defective in their p53 function. As a result the cancer cells die. In normal cells the vector does not replicate and hence normal cells are not affected.

Together with retroviral vectors, adenoviral vectors are the most frequently used vectors in clinical trials. Adenoviral vectors are often used in cancer therapies because they achieve

high transgene expression and efficient gene transfer. The advantage of adenoviral vectors over retroviral vectors is that their high titre ensures higher transfer rate in *in vivo* gene therapy. However, the expression of the transgene lasts only during a short period of time, since the viral genome is not integrated into the host genome. A disadvantage of adenoviral vectors is that an immune response is generated against the vector, which results in inactivation of the vector when it is administered for a second or a third time or that the patient already has antibodies against adenoviruses. Furthermore, adenoviral vectors have the disadvantage that leaky expression of viral genes can cause cellular toxicity.

***Adeno-associated viral vectors***
Adeno-associated viruses (AAV) have a small, linear, single-stranded DNA genome. AAV infects dividing and non-dividing cells and hematopoietic cells. AAV integrates into the genome, resulting in stable expression of the transgene. Since most of the viral genome is being replaced with the transgene there is little risk that the vector will generate an immune response. The vector has only a limited cloning capacity (max. 3.5-4 kb).

***Herpes simplex virus***
Herpes simplex virus (HSV) is a large dsDNA virus that infects cells of the nervous system. HSV has a large cloning capacity (max. 30-50 kb). Infection with HSV results in transient expression of the transgene.

***Other viruses***
Numerous other viruses are used for pre-clinical and/or clinical gene therapy studies like Poxvirus (e.g. vaccinia virus), Papilloma virus, Polyoma virus, SV40, Baculovirus, Epstein-Barr virus, Sindbis virus and Semliki Forest virus.

Many viral vectors pose the problem that transduction efficiency and expression of the transgene is too low to have a therapeutic effect. New developments in vector construction focus on vectors with higher transduction efficiency. Furthermore, vectors are being developed that target specific cells and that have tissue-specific enhancer-promoter elements that allow a better control of transcription of the transgene. Also chimaeric vectors are being developed that combine the advantage of different types of vectors.

## 2.1 Construction of viral vectors

The basic principles for the construction and production of viral vectors are the same for all types of viral vectors. First, recombinant viral vector DNA is constructed by replacing the viral genes required for replication and packaging (fig. 1A) with the transgene (fig. 1B). The resulting recombinant viral genomes cannot replicate autonomously and cannot package themselves into virus particles. For replication and packaging of the recombinant viral genome, the viral genes have to be provided by a special cell, called packaging cell. A packaging cell contains the viral genes, either integrated into its genome or present in the cell

on a plasmid. Upon transfer of the recombinant viral genome inside the packaging cell (the cell is now also called a producer cell) (fig. 1C) the viral genome is replicated inside the nucleus of the cell (fig. 1D) and the viral genomes are then packaged into viral proteins. The resulting viral particles are excreted from the packaging cell (fig. 1E).

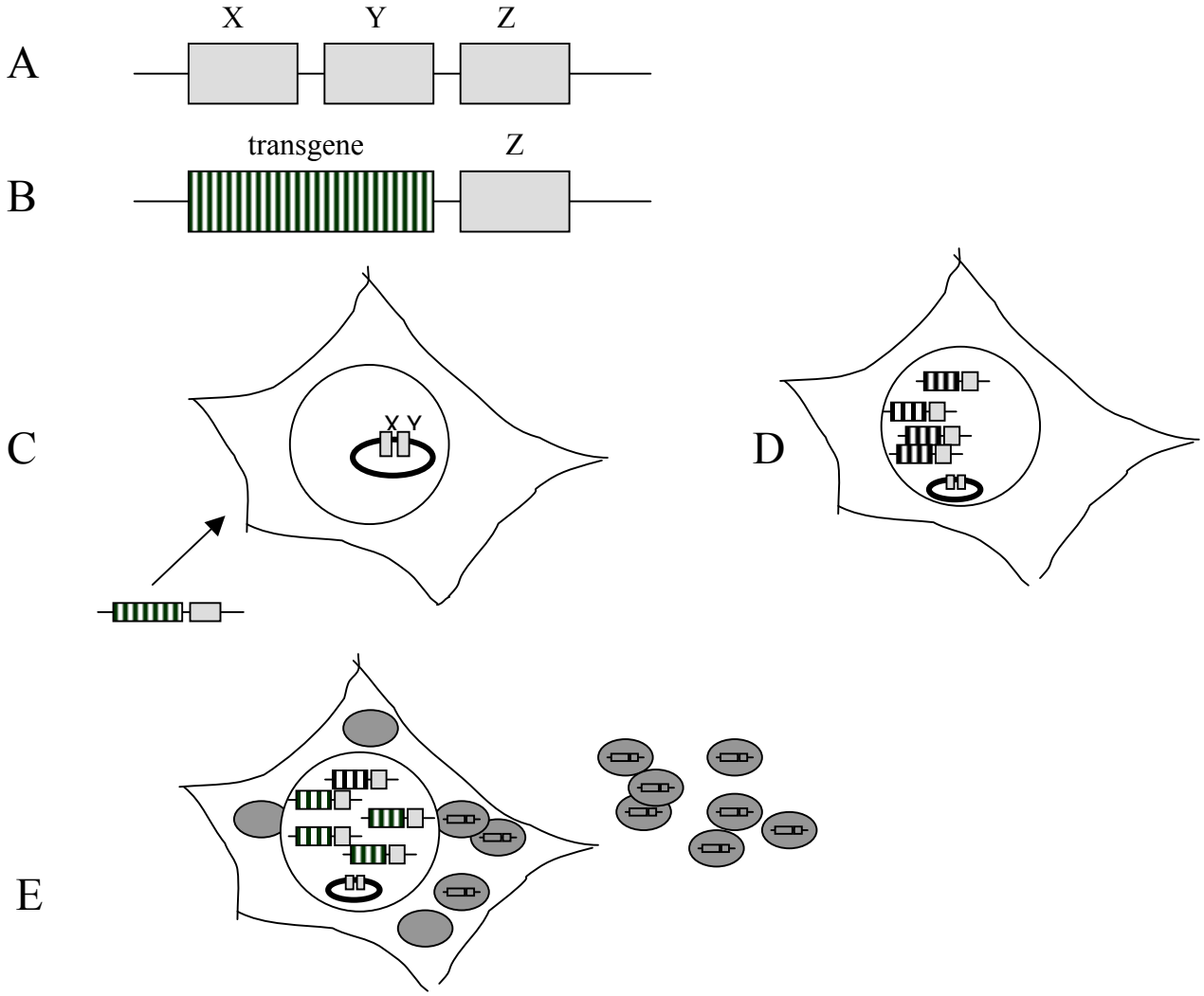


*Figure 1. Scheme for the production of a viral vector. In a viral genome with viral genes X, Y and Z (**A**) the genes X and Y are replaced with the transgene (**B**). The recombinant viral genome is transferred to a packaging cell that contains the viral genes X and Y (**C**). The recombinant viral genome is replicated within the nucleus of the packaging cell (**D**). The recombinant viral genomes are packaged within viral proteins and the resulting viral particles are excreted (**E**).*

The viral genes within the packaging cell help the vector to replicate by providing their functions in *trans.* In *trans* means that these genes are present outside the viral vector genome, but deliver their function to the viral vector. Viral sequences that are present on the viral vector genome are called *cis* acting sequences.

The packaging cell is called a stable packaging cell if the viral genes that are necessary for replication and packaging are integrated in the genomic DNA of the cell. If the viral genes

are present on a plasmid, which is present as an episomal element in the cell, the cell is called a transiently transfected packaging cell (as in fig. 1). These packaging cells have to be transfected with the plasmid containing the viral genes prior to each production cycle.

If the recombinant viral vector genome is integrated in the genomic DNA of the packaging cell, the cell is called a stable producer cell. A transient producer cell line has to be transfected with the recombinant viral vector prior to each production cycle. The recombinant viral vector that is used to transfect the cell (also called virus seed or vector seed) can either be a virus particle or a plasmid, containing the recombinant genome. A stable producer cell line has the advantage that production can easily be scaled up, while production with a transiently transfected producer cell line is more difficult to control. However, viral yield is often higher when a transiently transfected producer cell line is used.

***Construction of retroviral vectors***

Retroviral vectors that are developed so far are predominantly based on the genomic sequence of Murine Leukemia Viruses (MLV). The genome of retroviruses consists of three genes; *gag*, *pol* and *env*, which are flanked by long terminal repeats (LTRs) and packaging sequences ($\psi$) (see fig. 2A). The LTRs contain sequences that are required for integration of the viral genome into the host genome, and the viral promoter and enhancers that regulate transcription of the viral genes. The packaging sequences ($\psi$) allow the viral genome to be packaged by the viral proteins. The *gag*, *pol* and *env* genes provide the viral structural proteins, enzymatic proteins and the viral envelope protein respectively. These genes are essential for replication and packaging.

Upon infection of a cell and transfer of the viral genome inside the cell, the viral RNA is reverse-transcribed into pro-virus DNA, which is integrated into the host cell genome. For vector construction, pro-virus DNA instead of viral RNA is used since DNA is easier to manipulate than RNA.

To construct the retroviral vector, all the viral genes (*gag*, *pol* and *env*) are replaced with the transgene (see fig. 2A). The vector retains only the LTRs and packaging sequence, which are the only *cis* acting sequences that are required for packaging of virus particles and for integration of the viral genome into the host genome. The *gag*, *pol* and *env* genes are provided in *trans* by a packaging cell line. Upon transfer of the vector DNA to the packaging cell the vector DNA is transcribed into vector RNA inside the nucleus of the cell. The structural viral proteins, which are provided by the packaging cells, package the RNA molecules into viral particles. A couple of days after transfection recombinant viruses can be harvested from the cells and culture medium (fig. 2B). Since these particles lack all the viral genes they are replication-deficient. However, they still contain the LTR sequences and are thus able to integrate their genome into the host genome and regulate transcription of the transgene after transduction of cells.

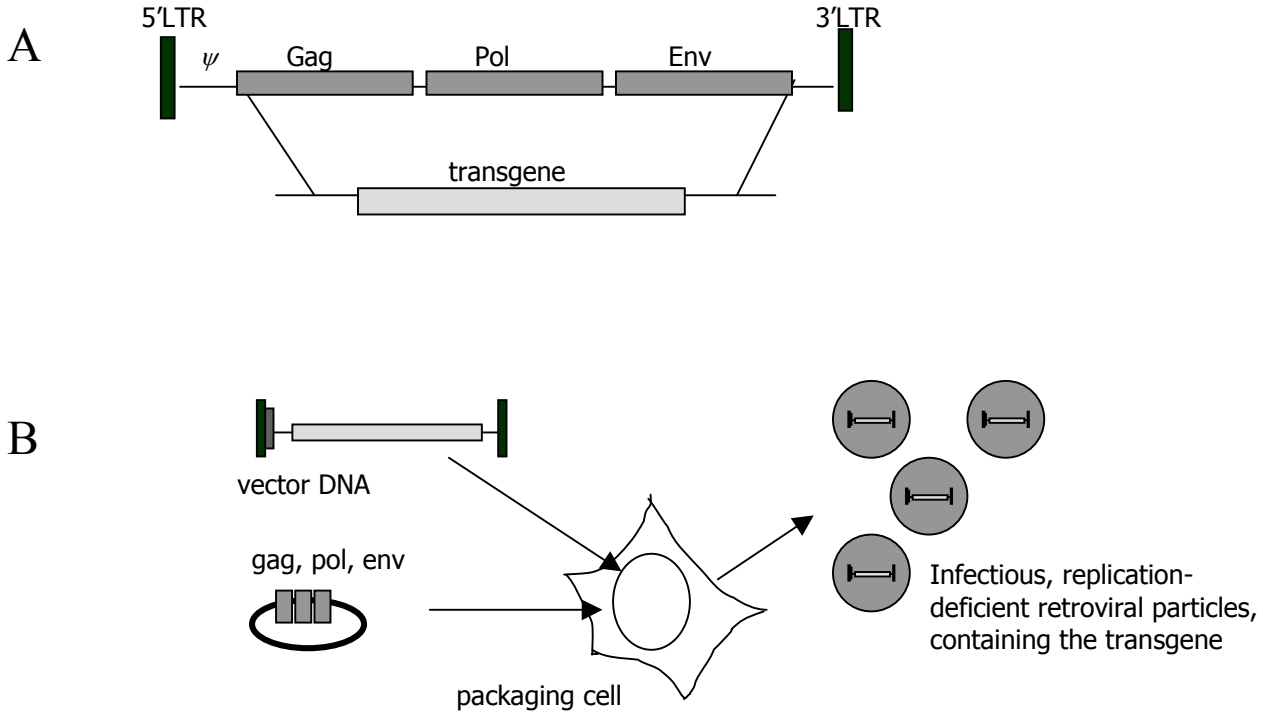


*Figure 2. Construction of a retroviral vector (A) and production of retroviral vector particles (B)*

A risk of the above described production method is that recombinational events between the retroviral vector genome and the viral genes within the packaging cell can lead to the formation of replication-competent retroviruses (RCRs). To minimise this risk, the *gag* and *pol* genes can be provided on a different plasmid than the *env* gene. In this packaging cell three recombinational events will be necessary to lead to RCRs and since this event is very exceptional, this is a rather safe production method (29).

***Pseudotyped vectors***

The cells that are infectable by viruses are determined primarily by the envelope protein (env) of the virus and the presence of appropriate receptors for this protein on the surface of infected cells. In pseudotyped vectors the MLV env protein is replaced by the env protein from another virus, e.g. Gibbon ape Leukemia Virus (GLV). GLV can infect many mammalian species. When a packaging cell line is used that expresses the env protein of GLV the viral particles that are packaged in this cell line contain the GLV env protein and can infect the same cells as GLV can (30).

***Construction of adenoviral vectors***

Adenoviral genomes contain the viral genes, the inverted terminal repeat (ITR) sequences, which regulate transcription of the viral genes, and the packaging sequences (see fig. 3A). The *E1* genes are two of the early genes and are essential for replication of the viral DNA. If *E1* is absent the other viral genes will not be expressed. In first generation adenovirus vectors

the *E1* genes in the adenoviral backbone are replaced with the transgene (see fig. 3A, for review see (31)). For replication and packaging of these adenoviruses a packaging cell is required that provides the *E1* genes in *trans* (fig. 3B). For this purpose human embryo kidney cells (HEK293 cells) were transformed with adenovirus type 5 (32). As a result HEK293 cells contain the *E1* genes integrated into their genome and thus provide the missing *E1* genes in *trans*.

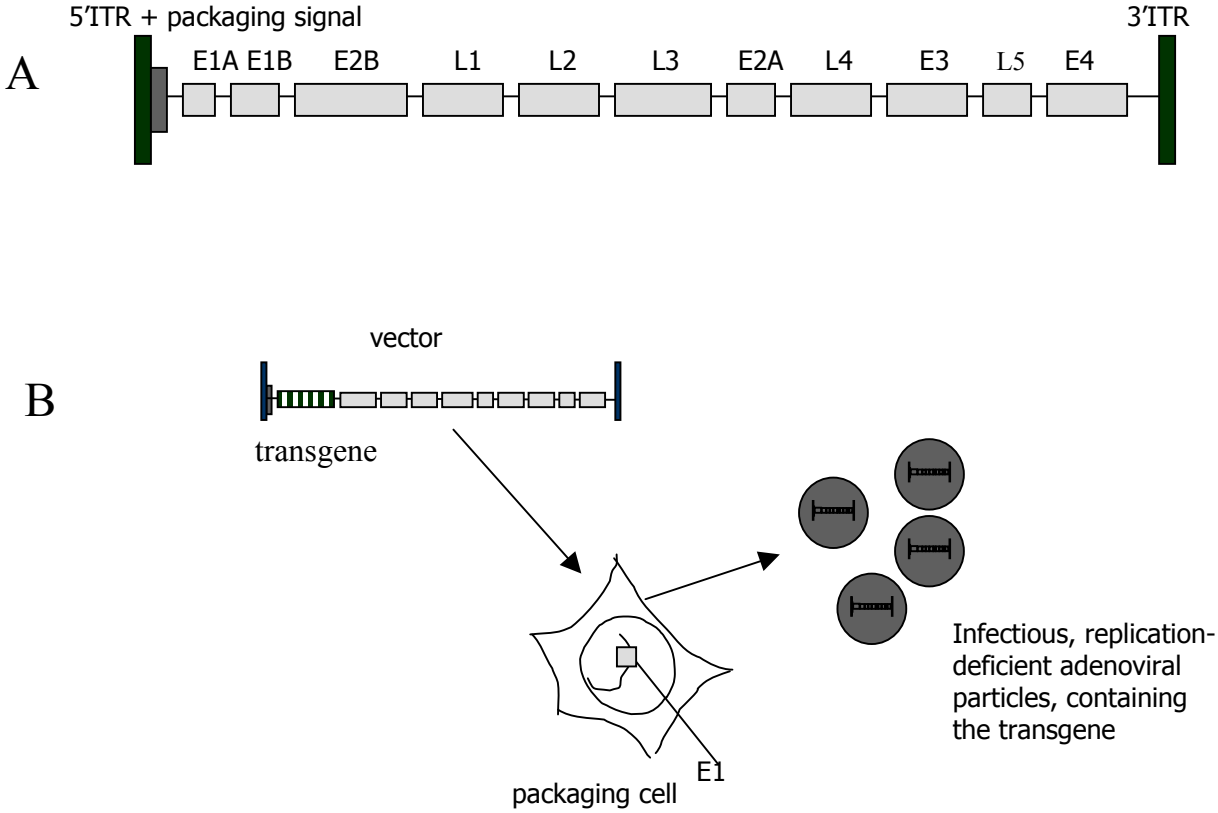


*Figure 3. Schematic representation of adenovirus genome (A), and production of recombinant adenoviruses in which the E1 genes have been replaced with a transgene (B)*

Generation of recombinant adenoviruses in packaging cells can result in vector preparations that are contaminated with replication-competent adenoviruses (RCAs). This is probably a result of homologous recombination events between the adenoviral sequence in the packaging cell and the adenoviral vector genome. The packaging cell line PER.C6 contains only the *E1* genes integrated in its genomic DNA under control of the human phosphoglycerate (PGK) promoter, without flanking adenoviral sequences. Since no sequence overlap exists between the adenoviral vector genome and adenoviral sequences within the PER.C6 cells, homologous recombination cannot occur. As a result RCAs are not generated when using the PER.C6 celline as a packaging cell line (33).

In second-generation adenovirus vectors also other early genes are deleted from the adenoviral backbone so that the vector can accommodate more foreign gene material. Third generation adenoviral vectors lack all the adenoviral genes and are also called gut-less vectors. The advantage of these vectors is that they do not have the problem of an immune response being mounted against adenoviral vectors in the patient's body, because of leaky expression of adenoviral genes. For packaging of these vectors all the necessary adenoviral genes should be provided in *trans*. Furthermore adenoviral vectors are created that are based on adenovirus types to which humans have no or low amount of neutralising antibodies.

***Construction of adeno-associated viral vectors***

Recombinant adeno-associated virus (rAAV) vectors are based on the defective and non-pathogenic parvovirus adeno-associated virus type 2 (AAV-2). This virus has two genes: *cap* and *rep*, which are replaced with a transgene (see fig. 4A). The AAV replication (*rep*) and capsid (*cap*) gene products are provided in *trans* on a helper plasmid in a packaging cell (fig. 4B). Because AAV is a defective parvovirus it is also dependent on a helper virus for replication. As helper virus an adenovirus is usually used that misses the *E1* gene and thus is replication-deficient (fig. 4B). For generation of rAAV particles, packaging cells are usually first infected with (a replication-deficient) adenovirus followed by transfection with two plasmids; one plasmid that contains the transgene between the viral ITRs (AAV vector) and one plasmid that contains the AAV *rep* and *cap* genes (packaging plasmid). As packaging cells HEK 293 cells can be used that provide the missing *E1* gene for the adeno-helper virus in *trans*.

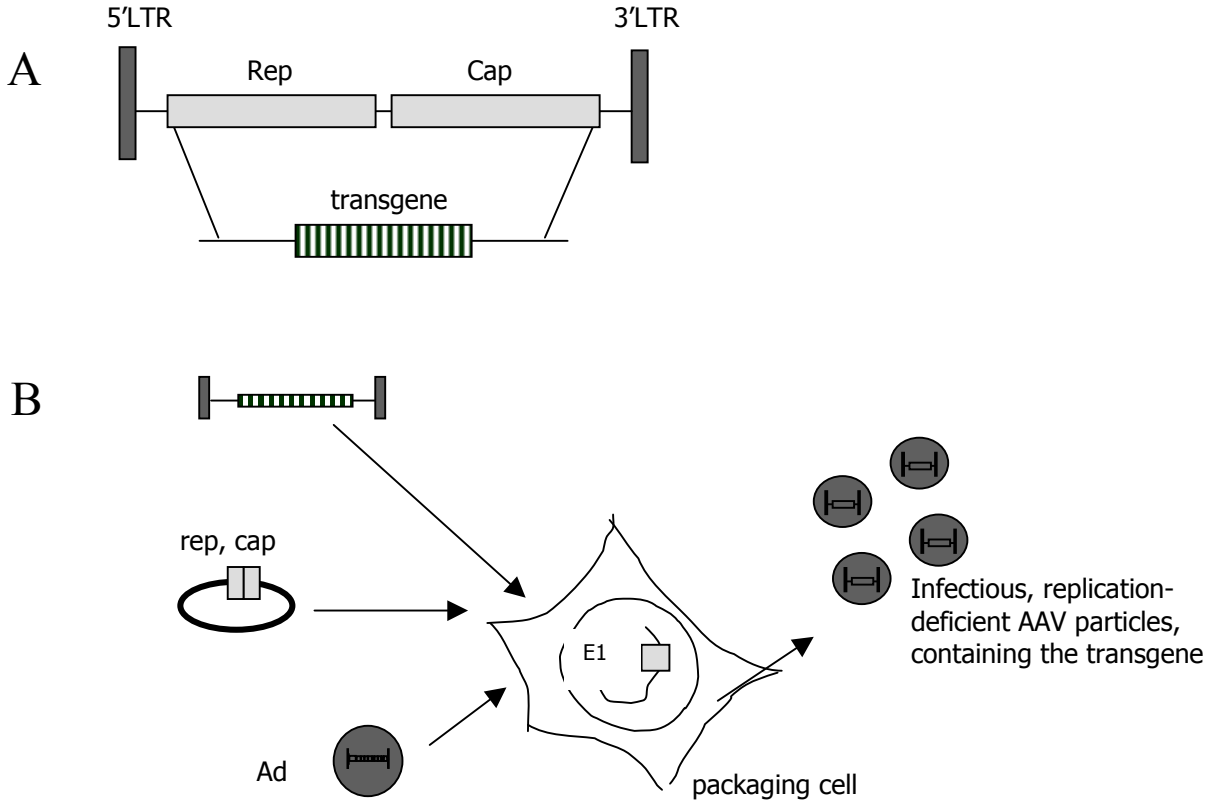


*Figure 4. Construction of an AAV vector (A) and production of viral AAV vector particles (B)*

The presence of adenovirus or adenoviral proteins in the finished product will induce unwanted immune response to rAAV transduced cells. To produce AAV particles that are free of adeno-helper virus, the adenovirus helper functions can be delivered from a plasmid, which contains the essential adenoviral helper genes but lacks the adenoviral structural and replication genes (34). Briefly, packaging cells are transfected with the AAV vector, the AAV packaging plasmid and the adenovirus helper plasmid to produce AAV particles without adenoviral proteins.

## 2.2 Production of viral vectors

***Good manufacturing practice (GMP)***

Production of viral vectors, as all biologicals, should be performed under good manufacturing practice (GMP) standards in order to ensure safe and controlled production and effective characterisation. GMP encompasses production control and quality control.

***Master stocks and working stocks***

To ensure the identity and quality of the packaging or producer cell line a large stock culture is made and characterised which is called the master cell bank (MCB) and which is divided and stored in multiple vials. From one or a few vials of this MCB a large working cell bank (WCB) is created which is used for production of the viral vector. The virus seed or vector seed that is used to transfect the packaging cell line is also divided in a master stock and a working stock. The master and working stocks are generated under GMP conditions.

## 2.3 Purification of viral vectors

Purification of viral vector preparations in order to remove contaminating agents is minimal, as is the case for live viral vaccines. Crude cell supernatants have been administered directly to patients in many clinical trials (35). Larger scale productions of virus preparations with a higher viral titre are required in the future. As a result, downstream purification processes become more important to ensure product purity and consistency (36). Indeed more recently many different purification strategies have been reported (37).

AAV vector preparations always require a purification step since the supernatant of recombinant AAV (rAAV) producing packaging cells is often contaminated with adenovirus particles or adenoviral proteins. Residual adenovirus particles are removed by CsCl gradient, by column chromatography or by a heat-denaturation step, which inactivates any residual particles. The disadvantage of CsCl purification is that it is laborious and that only a small volume of vector supernatant can be purified in each run. An alternative to CsCl purification is affinity chromatography using a heparin column (38-40).

Since retroviral vectors are relatively labile, purification can lead to loss of infectivity (41). Adenoviral vectors however, are relatively stable and can more easily be purified and

concentrated. CsCl gradient or chromatography techniques are useful to remove cellular and viral proteins and nucleic acids. After purification the vector is dialysed and concentrated. After formulation, the vector is sterilised by filtration.

## 2.4 Characterisation and routine control of viral vectors

Viral vectors have to be characterised for identity, purity, potency and safety (for review see (35)). Since purification of viral vector preparations is often still minimal it is extremely important that also cell banks and virus seeds that are used for production are well-characterised and tested thoroughly for contaminations.

The analytical methods for routine control of viral vectors are still under development and therefore standard methods for testing viral vectors have not yet been established. Standardisation is required in order to be able to compare the different clinical studies. Different initiatives for standardisation have recently been initiated; The FDA has started a working group with the objective to develop an adenoviral vector standard in order to calibrate test methods. At the University of Florida, USA a standard for AAV is developed. In Europe, Généthon (Evry, France) provides standardised gene therapy reagents (42). The European Gene Vector Database and Repository brings together 15 European Laboratories to establish reference standard samples and procedures for gene therapy, for example all viral and non-viral vectors, cell lines, production and purification procedures (42). Furthermore, in March 2001, the National Institute for Biological Standards and Control (NIBSC) in London, UK has initiated an informal Working group on Biological Standardisation in Gene Therapy (WBSGT) with as goal biological standardisation in gene therapy. The United States Pharmacopeia Convention (USP) published in the Pharmacopeial Forum (PF) an overview of currently used analytical methods (37).

In this paragraph an overview is given of analytical methods and product specifications that are described in the PF, in literature and in guidelines provided by regulatory agencies (see also chapter 4).

***Characterisation of Master Cell Bank and Working Cell Bank***

The identity tests for cell banks include morphology, doubling time, product expression rates, karyology, isoenzyme analysis and other markers that may be relevant for production (35). MCB and WCB have to be screened for adventitious agents by classical testing for the absence of bacteria, fungi and mycoplasma and by testing for potential viral contaminations. These tests are comparable to the type of tests that are required for cell banks for the production of recombinant DNA products.

***Characterisation of Virus seed***

The virus seed stocks can be identified by sequencing and restriction enzyme mapping and should furthermore be tested for adventitious agents and potential viral contaminations and

mycoplasma. In the case of DNA, the absence of protein contamination can be measured by spectrophotometric analysis; the $A_{260}/A_{280}$ ratio should be 1.75-1.85 for pure DNA. Furthermore, the activity of the virus seed (transgene specific protein expression) and titer should be determined.

***Control of production process***

To ensure the quality and consistency of the viral vectors also the raw materials used in the production process should be characterised. Materials that have a toxic effect or are of biological origin require special attention. Characterisation tests include identity, purity, functionality and freedom from adventitious or microbial contaminants and suitability for intended use (37). Especially fetal bovine serum (FBS) and other biological reagents require special attention due to bacteria, mycoplasma and viruses that can be associated with these reagents and the potential risk of transmissible spongiform encephalopathies (TSE) transmissions (37;43).

The production process is controlled by in-process controls. For viral vectors these may include quantity and viability of cells during bioreactor culture, quantity of virus after culture, specific activity of virus in fractions after column chromatography and quantity of host-cell DNA in fractions after column chromatography (37).

***Characterisation of virus preparation***

Many approaches have been described for characterisation of identity, purity, potency and safety of viral vectors. In general an extensive characterisation will be performed on a limited number of lots (43). The characterisation will be the basis for the selection of test methods needed for routine control.

*Identity.* Identity of clinical lots of viral particles may be confirmed by sequence analysis, restriction enzyme mapping or PCR. The expression product of the transgene can be identified by transducting appropriate cells with the vector and showing the presence of the expression product by an immunological assay. Viral particles can furthermore be identified by analysis of capsid proteins, immunological markers and phenotypic characteristics such as host range.

*Purity.* Impurities can either be process-related impurities or product-related impurities. Product-related impurities in viral vector preparations include aggregates and defective particles. Aggregates can be detected by laser light-scattering, sedimentation rate analysis or native PAGE followed by staining of the gel or western blot analysis (37). Defective particles, e.g. empty particles, can be discriminated by chromatographic techniques. Process-related impurities include host-cell proteins, host-cell DNA and endotoxins. Presence of host cell proteins or proteins from cell culture can be analysed by SDS-PAGE and/or western blotting (38) or ELISA. Presence of contaminating DNA in the vector preparation can be demonstrated by Southern blotting or quantitative PCR. Limits for the presence of protein

and DNA impurities should be set. A limulus amoebocyte lysate (LAL) assay can be used to test for the absence of endotoxins.

*Potency.* The dose of a vector can be expressed as the concentration of the viral particles or the concentration of infectious viruses. The number of infectious viruses is usually a small number of the total number of particles. For example for rAAV the DNA containing particle to infectious unit ratio (P: I ratio) is normally around 60-120:1 (38). Given the potential toxicity of the adenoviral particles themselves, the measurement of P: I ratio of adenoviral vectors is important. The P: I ratio should be determined and the upper limit should be set (43). CBER recommends that for adenoviruses patient dosing is based on particle number and that a ratio in the product of viral particles to biologically active virus of less than 100:1 is employed in phase I studies (7). Transduction efficiency of the viral vector and protein expression is measured by *in vitro* assays. Whenever possible, a potency assay should measure the biological activity of the expressed gene product (7).

At present, no methods for the determination of the virus particle concentration and for infectious titer exist that are standardised and widely accepted . Different particle quantification methods and methods for determining the number of infectious units exist and inter-assay and inter-laboratory variations are high. (27;44). This makes comparing data from different preclinical and clinical studies difficult. Therefore standardisation of these methods is essential. (27;44). Especially after the death of an 18-year old boy in Pennsylvania, which was linked with an extremely high dose of adenoviral vector, the need for standardisation of adenoviral dose was recognised (45). Initiatives have now been started to develop standards for vectors for gene therapy with the aim to diminish the inter-assay and inter-laboratory variation (42).

The FDA started a working group, which has the objective to develop an adenovirus type 5 wild type (Ad 5 WT)-based adenovirus standard. (46). A subsequent goal is to develop a replication-defective adenovirus standard. Production of this second standard is started once the first is underway. It is not the aim of the working group to standardise analytical methods, but the vector standard as a calibrator will make comparison of different analytical methods possible.

The particle concentration in viral preparations can be measured by different methods; E.g. the adenoviral particle concentration can be measured at 260 nm in a 0.1% (w/v) SDS solution, because the relationship between absorption and particle concentration has been established for adenovirus (37). Other particle counting methods are electron microscopy, anion exchange chromatography, quantification of structural proteins of the virion with known molecular mass and copy number by reverse-phase chromatography and quantification of the amount of vector nucleic acid by dot blot assays or quantitative PCR techniques (37).

The estimation of the number of infectious units can be performed by cell plaque assays or tissue culture infective dose (TCID50) assays. The type of assay that can be used (e.g. type of celline used) depends on the type of vector. A number of assays are described below.

Infectious particle quantitation of adenovirus vectors is performed by HEK293 cell plaque assay (47). Also TCID50 assays can be used that measure cytopathic effect or detect the transferred DNA or the expressed vector protein. Zhu *et al.* (48) developed an infectivity assay for adenovirus in which HEK 293 cells are infected with an adenovirus preparation and after harvesting of the cells, the cells are stained with an fluorescently labelled anti-adenovirus monoclonal antibody followed by FACS analysis.

Titer determination of rAAV preparations is performed by transfecting Hela cells that contain a cassette with the wild type AAV *rep*/*cap* genes with serial dilutions of rAAV and adenovirus. Cells are harvested after 24 hours and detection of rAAV is performed by dot-blot hybridisation with a transgene-specific probe (49) or by quantitative PCR (38;50). PCR appeared to be 100× more sensitive as dot-blot hybridisation (50).

For titer determination of retroviral vectors a PCR-method is developed. Cells are first infected with retroviral preparations. The viral region that is transcribed during first strand cDNA synthesis by viral reverse transcriptase upon infection of cells is then amplified by PCR and quantitated (51;52).

If a selection marker is present on the vector, titer determination can be performed by infecting cells with the vector and growing them under selection pressure followed by colony counting.

Transduction efficiency can be measured by immunological methods, such as ELISA or FACS.

*Safety.* The risk that is associated with viral vectors is their pathogenic nature. Although viral vectors are manipulated in such a way that they are replication-deficient and loose their pathogenicity, there is always concern about the possibility that replication-competent viruses (RCVs) are generated. Replication-competent viruses can be formed during production through recombination with viral sequences within the packaging cell or within the patient if he/she was infected with certain viruses prior to the therapy. Replication competent retroviruses (RCRs) can spread in the patient's body, which can result in multiple integrations of the viruses into the patient's genomic DNA, which could lead to the formation of tumours. Immunosuppressed monkeys developed lymphomas after administration of replication competent retroviruses (53). Therefore especially RCRs are considered as a serious safety problem. Measures can be taken to minimise the risk of the formation of RCRs, such as the use of packaging cell lines in which the formation of RCRs is less likely to occur, limiting the number of harvests per culture and developing a consistent production process. At this

moment still each batch has to be tested for RCVs. The formation of RCVs is not yet well understood. It is a random process and therefore testing of each batch probably remains necessary. A batch that contains RCRs should be rejected (43). For replication competent adenoviruses (RCA), which pose less risk to a patient, a limit of one RCA per dose is considered acceptable (7;37).

Screening for replication competent retroviruses (RCRs) is performed by cell culture assays. The two assays that are described in literature are $S^+L^-$ focus-forming assay (54) and marker rescue assay (55). The $S^+L^-$ assay utilises cells that harbour a defective sarcoma virus genome. After infection of these cells by RCR the defective genome is mobilised by the infecting virus leading to discrete foci in the cell layer. In the marker rescue assay, vector preparations are inoculated onto *Mus dunni* cells that contain a replication-deficient viral vector with the *lacZ* gene. Presence of RCR will lead to rescue of the *lacZ*-encoding vector. When after several days the supernatant is harvested and inoculated onto indicator cells, the rescued vector will transduce these cells. This will lead to blue colonies after X-gal staining. Detection of RCR by either assay is equally sensitive (56). In order to increase the sensitivity of the assays, RCRs are amplified by seeding the vector preparations on non-indicator cells and passaging the cells several times (43). The type of non-indicator cells that can be used depends on the env protein of the virus. Commonly *mus dunni* cells are used for amplification. Retroviral vectors pseudotyped with the GLV envelope do not infect *mus dunni* cells. Therefore an assay using HEK-293-cells is developed for these vectors (57). A RCR standard has been developed by CBER and is available through the American Type Culture Collection (ATCC) and can be used as a reference in RCR assays (58;59).

Presence of RCR should be tested in the MCB, WCB, vector products and *ex vivo* transduced cells (43;59). In situations where *ex vivo* transduced cells cannot be cryopreserved during testing, and must be administered to patients prior to the availability of testing results, culture assays should be initiated at the time of patient administration. In these situations, alternative methods such as PCR may be appropriate to provide an initial analysis (59).

Monitoring of patients for RCR after treatment during clinical trials is performed by detection of MLV sequences in peripheral blood mononuclear cells by PCR or by the detection of anti-MLV specific immuno-globulines by ELISA (43;59;60).

RCAs can be measured by a cell culture/cytopathic effect method by which RCAs are first amplified by growing the vector preparation on non-indicator cells (e.g. HeLa-S3 cells) and then passed on to indicator cells (e.g. A549). A cytopathic effect on monolayers of indicator cells is indicative for the presence of RCA. Since high titres of adenovirus are used in gene therapy protocols the RCA assay should be able to detect 1 or 2 RCAs in $10^{12}$ virus vectors.

Because of the association of AAV with adenovirus, testing for AAV is currently recommended in the master cell bank, the master virus seed stock and the final adenoviral product (7).

To assess the presence of wild-type adenovirus in rAAV preparations, HEK293 cells are exposed to recombinant AAV and assessed for cytopathic effects (61).

The virus preparation has furthermore to be screened for adventitious agents by classical bacteria, fungi and mycoplasma testing and by testing for potential viral contaminations.

*Table 2. The recommended assays for control of viral vector production*

| **Parameter** | **Assay** |
|---|---|
| Characterisation of master cell bank and working cell bank | |
| Identity | Morphology, doubling time, product expression rates, karyology, isoenzyme analysis |
| Safety | Sterility tests |
| | Test for mycoplasma |
| | Tests for viral contaminations |
| Characterisation of virus seed | |
| Identity | Sequencing and restriction enzyme mapping |
| | Transgene specific protein expression |
| Purity | $A_{260}/A_{280}$ ratio |
| Potency | Particle count |
| | Infectious titer |
| Safety | Sterility tests |
| | Test for mycoplasma |
| | Tests for viral contaminations |
| Control of production process | |
| | Characterisation of raw materials |
| | In-process controls |
| Characterisation of virus preparation | |
| Identity | Sequencing and restriction enzyme mapping or PCR |
| | Immunoassay for expressed gene |
| Purity | LAL test (endotoxins) |
| | Southern blotting/PCR (host cell DNA) |
| | SDS-PAGE/Western blotting/ ELISA (host cell proteins) |
| | Test for residual RNA |
| | Test for aggregates and defective particles |
| | Test for process contaminants |
| Potency | Particle count |
| | Infectious titer |
| | *In vitro* assay to test function of expressed gene |
| Safety | Test for replication competent viruses |
| | Sterility tests |
| | Test for mycoplasma |
| | Tests for viral contaminations |

# 3. Plasmid vectors

An immunogenic or therapeutic gene can be cloned into a plasmid by recombinant DNA techniques. Plasmids have a limiting size of about 15-16 kb. Therefore, smaller plasmids have a higher cloning capacity for the transgene. Plasmids have a bacterial origin of replication (*ori*), which allows them to replicate in bacteria. For production, the plasmid is transformed in a bacterial host (usually *Escherichia coli*), which grows and produces many plasmid copies. A selectable marker (e.g. antibiotic resistance gene) is often cloned into the plasmid by which bacteria containing the plasmid can be selected. Guidelines recommend to avoid the use of selection markers if feasible (43) or at least to avoid the use of those that confer resistance to antibiotics in significant clinical use (7). After growth of the bacteria, the plasmid DNA is released, isolated and purified.

There are different delivery methods for administration of the plasmid to the patient. The plasmid DNA can be delivered by needle injection into the muscle and different layers of the skin. By this method the DNA is delivered in the extracellular space after which it still has to enter the cell. An alternative to needle injection is gene-gun delivery by which the skin is bombarded with gold-beads coated with DNA. Because this method delivers the DNA directly into the cell, gene-gun delivery is more efficient as injection. Only 0.4 $\mu$g of DNA was needed to protect animals from influenza virus when using the gene-gun delivery method, while at least 100 $\mu$g of DNA was required when DNA was injected (18). However, only skin tissue can be inoculated by the gene-gun method while both muscle and skin can be inoculated by injection. Alternative delivery methods are intranasal or oral delivery using liquid droplets or a spray or the application of electroporation after needle injection of DNA.

DNA can be administered as naked DNA in a solution of PBS or saline or complexed with materials, which facilitate cellular uptake and protect the DNA. For example plasmid DNA can be complexed with cationic lipids. The cationic lipids interact with the negatively charged DNA to form condensed DNA with a protective lipid coating. Other formulations for plasmid DNA can be cationic polymers or cationic peptides.

Unmethylated cytosine-guanine dinucleotide (CpG) motifs within a nucleotide sequence are more frequently found in bacterial than in mammalian DNA. These motifs stimulate the immune response in humans. Therefore, oligonucleotides that contain these CpG motifs are sometimes delivered in combination with the plasmid DNA. Other systems under investigation to induce the immune response are cytokines, monophosphoryl lipid (MPL) A, Ubeninex (an anti-cancer modulator), QS-21 (a highly purified tryterpene glycoside saponin) and co-injection of CD154 (for review see (20)).

To prevent insertional mutagenesis, homology of plasmid DNA sequences to known sequences in the human genome should be avoided if possible (43;62;63). Also retroviral-like

long terminal repeats and oncogenes should be avoided and the plasmid should not include sequences of biological significance (63).

## 3.1 Production and purification of plasmid vectors

Since relatively large amounts of supercoiled plasmid DNA are required for vaccination and gene therapy, large-scale production and purification methods are required (for review see (64-66). In laboratory-scale production and purification methods, toxic chemicals (EtBr, phenol, CsCl) or animal-derived enzymes (RNase) are used, which might raise concerns about residues in the final product. Therefore, various new production and purification processes have been developed. These processes include fermentation, harvesting and purification steps (see fig. 5).

*Fermentation.* After plasmid construction the plasmid is transferred to a bacterial host. After selection of the bacteria containing the plasmid, the bacteria are grown and master cell banks (MCB) and working cell banks (WCB) are constructed. For production, bacteria from the WCB are grown. For optimal plasmid yield it is important that a high-copy-number plasmid, such as pUC19, is chosen. The production of plasmids can be hampered by instability problems caused by defective partitioning of plasmids between daughter cells during division or by recombination. Therefore the right host should be chosen to overcome these problems (for review see (66)). For optimal growth the right media and growth conditions should be chosen. Plasmid yield as high as 220 mg/L can be achieved (67).

*Harvest.* Cells are recovered by centrifugation or microfiltration. The cells are then resuspended in a buffer and lysed by an alkaline-lysis procedure. The lysis reagent (NaOH/SDS) should be added and mixed in such a way that local pH extremes are avoided, because values higher than pH 12.5 cause irreversible plasmid denaturation. The release of genomic DNA (gDNA) during lysis increases the viscosity of the solution, making mixing more difficult. Mixing should be very gentle to avoid shearing of the plasmid DNA and the gDNA.

*Purification.* Addition of high-salt neutralisation solution promotes the formation of aggregates of gDNA and SDS-protein complexes. By the neutralisation step, plasmids renature and recover their original supercoiled form. The aggregates are removed by centrifugation or filtration. Subsequently, plasmids are concentrated by alcohol- or polyethylene glycol (PEG) precipitation. The clarification and concentration steps remove host proteins and some host nucleic acids and reduce further the volume prior to chromatography.

Plasmid DNA should be further purified from RNA, gDNA fragments, endotoxins and plasmid variants. The similarities of these molecules to plasmid DNA and their molecular weight range make purification difficult. Several chromatographic methods can be used to

further purify the plasmid DNA like size exclusion chromatography, ion-exchange chromatography, reverse-phase hydrophobic interaction chromatography, reverse-phase ion-pair chromatography and affinity chromatography (for review see (64)). To purify large amounts of plasmid DNA, two chromatographic steps should be performed. Anion-exchange and hydrophobic-interaction chromatography are the most suitable methods for the initial purification of supercoiled plasmid DNA. These methods enable the removal of gDNA fragments, RNA, proteins and endotoxins. However, a final size-exclusion chromatography step is needed to separate supercoiled plasmid DNA from other plasmid topoisomers and to exchange the plasmid's medium for the appropriate formulation or storage buffer. Finally, the resulting plasmid preparation has to be filter-sterilised (68).

The need to use organic solvents to elute the plasmid DNA from reverse-phase columns is a disadvantage of this purification technique since most of the organic solvents are toxic. However, these techniques are useful for the characterisation of the final plasmid DNA preparations.

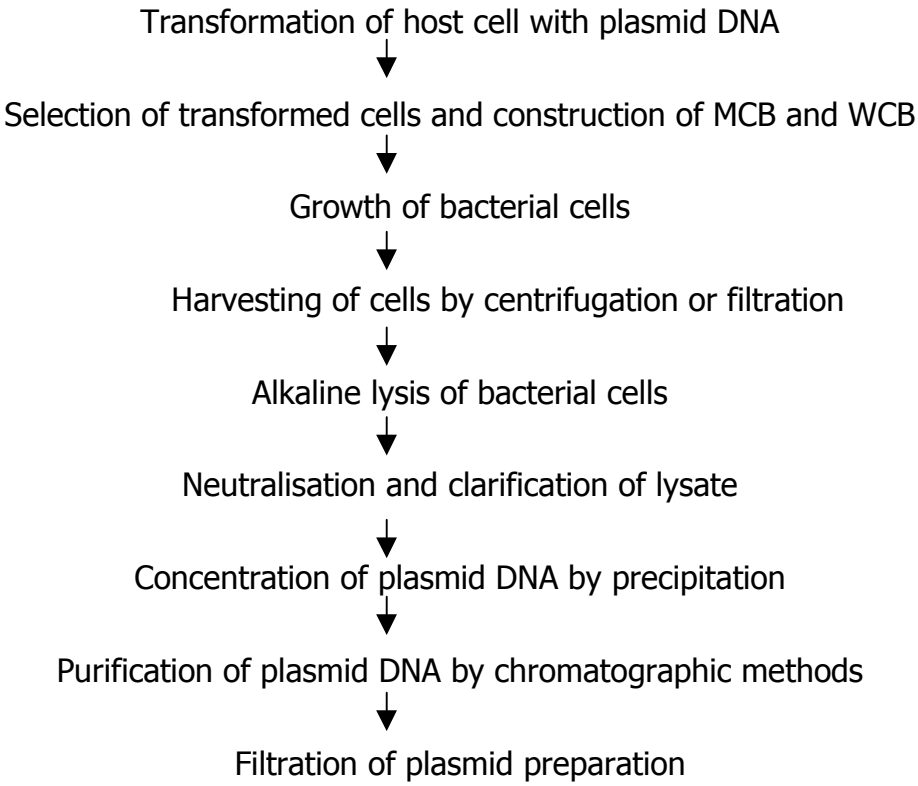


*Figure 5. Flow diagram for production of plasmid DNA*

## 3.2 Characterisation and routine control of plasmid vectors

As for viral vectors, plasmids have to be produced under GMP conditions. The final product has to meet certain specifications for identity, purity, stability, potency and safety (see table 3). Middaugh *et al*. consider the possibility of treating plasmid DNA-based pharmaceuticals as chemically well-defined drugs rather than biological agents which must be tested for their biological activity lot-by-lot (69). The FDA has added therapeutic DNA plasmid products to the list of well-characterised biotechnology products (70).

The bacterial host that is transfected with the plasmid and is used for production is handled in a master cell bank/working cell bank system similar as the packaging cell lines used for viral vectors. These MCBs and WCBs also have to be characterised.

For product characterisation and in-process controls, guidelines are provided by regulatory agencies (see chapter 4). Furthermore, analytical methods and product specifications for plasmid DNA are described exhaustively in literature (65;68;69;71;72) and at company websites (73). The United States Pharmacopeia (USP) published in the Pharmacopeial Forum (PF) an overview of currently used analytical methods (37).

This paragraph gives an overview of product specifications and analytical methods used for plasmid vectors.

***Characterisation of Master Cell Bank and Working Cell Bank***

The MCB and WCB have to be characterised for identity and safety. Especially control for adventitious agents and viral contaminations are important safety tests for cell banks.

***Control of production process***

Just like for viral vectors the raw materials used in the production process should be characterised. Furthermore the production process should be controlled by in-process controls. For plasmid vectors these include the control of the amount of plasmid prior to culture harvesting, the amount and form of plasmid after extraction steps and the absence of endotoxins in the plasmid pool after extraction steps (37).

***Characterisation of plasmid vector***

*Identity.*

- *Size.* Identification of a plasmid can be performed by restriction enzyme mapping. Restriction fragments can be separated by agarose gel electrophoresis, capillary electrophoresis or HPLC. Capillary electrophoretic separation of restriction fragments is superior to gel separation in resolving power, analysis time and quantification (74;75).

- *Sequence.* Sequencing of the whole plasmid or its insert can be performed by an automated sequencing method and can be used to check the identity of the plasmid. Chip

technology is a promising new technology that allows confirmation of a sequence of several kb. The currently available sequencing methods cannot identify small quantities of a mutated site (< 10%) present in a plasmid preparation.

- *Expressed gene.* The expression product of the transgene can be identified by transfecting appropriate cells with the plasmid and showing the presence of the expression product by an immunological assay.

*Purity.* Impurities and contaminants in the DNA vaccine can induce unwanted immunological and biological responses. Therefore, downstream plasmid purification is essentially aimed at eliminating cellular components of the host organism such as bacterial proteins, lipids, lipopolysaccharides, bacterial host DNA and RNA.

Process-related impurities:
- *Bacterial host DNA.* Linear *E. coli* chromosomal DNA can integrate into the genome of transfected cells (76). Regulatory standards will require that the level of host cell genomic DNA contamination (gDNA) is kept below 10 $\mu$g/ml plasmid DNA (63). Host cell DNA contamination can be quantified by Southern blot techniques with a probe specific for the host cell gDNA (77) or by PCR techniques (78;79). Real-time PCR with 23S rDNA as target is 10-100$\times$ more sensitive than the Southern blot method (79). However, sensitive PCR-based detection methods for bacterial DNA can be problematic due to bacterial contaminations in *Taq* DNA polymerase and other PCR reagents (80).

- *Proteins.* Final plasmid preparations should be free of proteins (<10 ng / dose; (81)). Proteins should be undetectable by bicinchoninic acid (BCA) assay or silver-stained gel (68). Protein contaminations can furthermore be detected by measuring the $A_{260}/A_{280}$ ratio, which should be 1.75-1.85 for pure DNA.

- *RNA.* RNA should not be visible on a 0.8% agarose gel or by analytical anion-exchange chromatography (68).

- *Endotoxins.* Endotoxins (lipopolysaccharides) are major components of the outer cell wall of gram-negative bacteria and can copurify with the plasmid DNA (82). Endotoxins can cause side effects if administered to the recipient (65;82;83). They should be less than 1 endotoxin unit/kg body weight (81) or less than 0.1 endotoxin unit/$\mu$g plasmid DNA (68). Endotoxin contamination can be measured using a limulus amoebocyte lysate (LAL) test.

Product-related impurities:
- *Plasmid isoforms.* Plasmids are isolated as covalently closed circular (ccc) DNA molecules in negatively supercoiled (sc) forms. If one of the strands of a supercoiled plasmid is nicked, supercoiling is lost and an open circular (oc) form is created. If a nick occurs in both strands at or near the same point, a linear DNA molecule is generated. A

plasmid can also have intact strands and not being supercoiled but have the oc configuration instead (also called relaxed). Furthermore, a plasmid can occur in a multimeric form or in a (partially) denatured form. Plasmid vectors should be mostly in the supercoiled form, which is thought to be more effective at transferring gene expression than open-circular, linear, multimeric or partially denatured DNA (for review see (65)). A specification should therefore exist for the minimum amount of plasmid in the supercoiled form. Regulations may require > 90% of the plasmid to be in the supercoiled form. However, there are also arguments against this specification (65).

The proportion of supercoiled and open circular plasmid DNA can be quantified by densitometric scanning of an agarose gel, capillary electrophoresis or chromatographic techniques. Denatured supercoiled plasmid runs at the same speed as supercoiled plasmid in agarose gels. This form can be discriminated by HPLC using anion exchange chromatography. The denatured supercoiled plasmid elutes shortly after the main plasmid peak (84). A simple and rapid technique to quantify the proportion of supercoiled circular DNA is SCFluo (85), which is a fluorimetric method that is based on the reversible denaturation of sc DNA and the high specificity of the PicoGreen fluorochrome for double-stranded DNA.

- *DNA modifications*. Consideration should be given to any modification of the DNA, which may have taken place during its production such as bacterial specific methylation (43;63).

*Stability.* Possible pathways of degradation of plasmid DNA and methods of analysis are reviewed by Middaugh *et al*. (69). For the stability of plasmid DNA it is important that it is free of contaminating nucleases. Other possible ways of degradation are deamination of cytosine, precipitation, aggregation, adsorption onto container surfaces and introduction of a single break in the DNA backbone. The latter will convert supercoiled plasmid DNA to the open circular form. Loss of supercoiled structure can be analysed as described above. One of the major degradative pathways for DNA is the two-step process of depurination and $\beta$-elimination, which leads to cleavage of the phosphodiester backbone (69). This process will occur in almost any aqueous solution near neutral pH. The reaction is acidic catalysed and therefore the stability is extended by storing the DNA at a higher pH (pH 8.5). However, raising the pH of the formulation will lead to pain and necrosis of tissue on intramuscular injection (86;87). At pH 7.4, DNA is theoretically stable for 2 years at 5 °C if no other degradative processes are occurring (69). Product stability can be increased by lyophilising the DNA vaccine (88).

Another important source of DNA damage is free radical oxidation. The final result of oxidation is usually strand breakage. Therefore, analysis of the DNA structure as described above also seems to be the most convenient way to monitor DNA damage by oxidation. The rate of cytosine deamination is far too slow to be a major factor in pharmaceutical

formulations. However, deamination can occur during purification. Therefore, assays should be performed to estimate the amount of cytosine deamination that has occurred in the DNA prior to formulation. A sensitive GC/MS method that can detect one uracil molecule per $3 \times 10^7$ bases has been described by Blount and Ames (89).

*Potency.* DNA-concentration can be measured by spectrophotometric analysis. An $A_{260}$ of 1 equals a concentration of 50 $\mu$g/ml. For low-concentration plasmid DNA preparations concentration can be measured by adding fluorescent DNA-binding dyes to the DNA (e.g. PicoGreen, Hoechst 33258) and measuring fluorescence.

Transfection efficiency and subsequent expression of the gene can be determined by transfection of appropriate cells with the plasmid DNA followed by demonstration of antigen expression by an appropriate assay. Horn *et al*. (68) describe an assay for gene expression analysis whereby human cells are transfected with the plasmid DNA and 48 hours post-transfection stained with a fluorescent-labelled antibody for the transgene and quantitated by FACS analysis.

*Safety.* Sterility tests should be performed on the final bulk plasmid to rule out the presence of adventitious agents such as bacteria and fungi (65).

***Characterisation of pharmaceutical formulation***

If lipids are used to formulate the DNA, their identity must be tested by procedures used for traditional pharmaceuticals such as gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (37). If proteins are used their identity should be established (37). For delivery systems using cationic lipids, liposomes or cationic polymer, the surface properties should be adequately characterised including the surface charge. The mean particle size as well as the size distribution should be characterised. The ligand of ligand-mediated gene delivery constructs should be chemically characterised (43).

*Table 3. The release specifications and recommended assays for assessing the identity, purity, potency and safety of plasmid DNA preparations*

| **Parameter** | **Recommended assay** | **Approval specification** |
|---|---|---|
| Characterisation of MCB and WCB | | |
| Identity | Characterisation tests | |
| Safety | Tests for adventitious agents and viral contaminations | |
| Control of production process | | |
| | Characterisation of raw materials | |
| | In-process controls | |
| Characterisation of plasmid DNA | | |
| Identity | | |
| Size | Restriction enzyme digestion followed by agarose gel-electrophoresis or capillary electrophoresis | Coherent fragments with the plasmid restriction map |
| DNA Sequence | Sequencing | |
| Expressed gene | Immunoassay for expressed gene | |
| Purity | Analytical anion exchange chromatography | One single peak |
| Genomic DNA | Agarose-gel electrophoresis | Undetectable |
| | Southern blot | < 10 $\mu$g/mg plasmid |
| | Real-time PCR | < 10 $\mu$g/mg plasmid |
| Host proteins | Spectrophotometric analysis | $A_{260}/A_{280}$ = 1.75-1.85 |
| | BCA protein assay, Silver-stained SDS-PAGE | Undetectable |
| RNA | Agarose-gel electrophoresis | Undetectable |
| Endotoxins | LAL test | < 0.1 EU/$\mu$g plasmid |
| Isoforms | Agarose-gel electrophoresis, HPLC, capillary electrophoresis | < 10% plasmid isoforms (linear, relaxed, denatured) |
| Process contaminants | | |
| Potency | | |
| Dose | Spectrophotometric analysis ($A_{260}$) | |
| Efficacy | *In vivo* assay for gene expression and function of expressed gene | Comparable with plasmid standards |
| Safety | | |
| Sterility | Tryptose broth culture | No colonies after 21-day culture |
| | Culture | No growth after 14 days in rich media |
| Viral safety | Test for viral contaminations | |
| Characterisation of pharmaceutical formulation | | |
| | Identity testing | |
| | Particle size | |

# 4. Regulatory issues

Specific regulations have been established to provide guidance for production and quality control of viral and plasmid vectors for use in clinical trials and for application for marketing authorisation. Regulatory guidance is given on the areas environmental protection and patient safety. This overview reflects to the regulations related to protection of the patient and not to environmental protection in Europe and in the USA.

***Regulation in Europe***
The European Medicines Evaluation Agency (EMEA) is responsible for the market authorisation of gene therapy and DNA vaccine products in Europe and for these products a centralised EEC procedure for evaluation of medicinal products has to be followed, which is valid for all Member States at once. For clinical trials, the regulation for the production and use of gene therapeutics and DNA vaccines differs between the member states (reviewed by Cohen-Haguenauer (90)). This could have as consequence that some countries in which regulation is more strict are disfavoured for the performance of clinical trials.

A lot of similarities exist between gene therapeutics and other biological medicinal products (biologicals), like rDNA products and live viral vaccines. Several OMCLs experienced in the quality control and standardisation of biologicals have taken the first steps towards standardisation of assay-methods for gene therapeutics.

***Regulation in the Netherlands***
In the Netherlands, protocols for gene therapy clinical trials and DNA vaccination are reviewed by the COGEM (Commissie Genetische Modificatie) and by the CCMO (Centrale Commissie Mensgebonden Onderzoek) (91). The COGEM mainly reviews the biosafety issues of gene therapeutics. The CCMO controls the medical relevance of the protocol, the safety for the patient and ethical issues since 1999. The CCMO follows the FDA guidelines and can also demand additional requirements.

In the Netherlands, clinical trials with gene therapy are in general performed on a small scale in academic hospitals. The viral vectors are produced by specialised manufacturers outside the hospital. The hospital pharmacists, who are responsible for the product as delivered to the patient, have at their disposal special units for handling the viral vectors prior to application to the patient. The expertise with respect to the quality control and safety aspects of viral vector and preparations is new to hospital pharmacists, but experience is growing.

***Regulation in the USA***
Within the USA, the Center for Biologics Evaluation and Research (CBER) of the FDA is responsible for the market authorisation of gene therapy and DNA vaccine products and for reviewing gene therapy and DNA vaccination protocols for clinical trials. CBER focuses on safety and efficacy of gene therapy protocols. The recombinant DNA Advisory Committee

(RAC), established to formulate standards for recombinant DNA research and to oversee its progress, considers ethical issues concerning gene therapy. However, the RAC cannot block any protocols (2). Recently the policy of the FDA towards reviewing gene therapy protocols changed drastically as a result of the death of the 18-year old boy in Pennsylvania during a gene therapy clinical trial. The safety information about a gene therapy clinical trial will no longer be kept confidential but will have to be made public by the scientists conducting the clinical trial.

CBER has abbreviated testing and reduced documentation for gene therapy investigational new drugs (INDs) in order to facilitate progress towards effective therapy whenever this can be done while preserving patient safety (7). Certain changes, for example minor modifications in the genetic insert or changes in the antibiotic resistance gene do not necessarily require a new IND or full product retesting and can be dealt with by an information amendment. Exploratory phase I trials for somatic cell and gene therapy products should be based on data that assure reasonable safety and rationale. Less data may be submitted to support beginning exploratory trials than may be submitted at later stages of product development, especially in the case of severe or life-threatening diseases (7).

***Good Manufacturing Practice facilities***

Both viral and plasmid vectors for clinical use have to be manufactured according to GMP standards to ensure purity and consistency of the product. Academic institutions have difficulties in producing gene therapy products at GMP level. Therefore, The National Gene Vector Laboratories (NGVL) in the USA provide manufacturing facility for the production of clinical grade viral and non-viral vectors, following guidelines of the FDA, for use by US investigators in phase I or phase II clinical trials. This way investigators have easier access to GMP-grade vectors. Quality and safety testing is performed in house, or could as an alternative, be performed at official control laboratories as suggested by Cohen-Haguenauer (92).

In Europe, such a non-commercial production facility is only established in Sweden at the Karolinska Institute in Stockholm. In the Netherlands a study has been performed to investigate the feasibility of a central GMP facility for vector production (93). The Minister of Health has decided not to establish such a central facility in the Netherlands but instead to stimulate research to gene therapy (94).

***Guidelines***

In the EU and the USA, guidelines have been established by respectively the Committee for Proprietary Medicinal Products (CPMP) of the EMEA and CBER of the FDA for the production and control of gene therapetics and DNA vaccines.

The CPMP has established the following guideline for gene therapeutics and DNA vaccines;

- Note for guidance (NfG) on the quality, pre-clinical and clinical aspects of gene transfer medicinal products (43).

In the USA, CBER has produced the following guidelines that address somatic cell and gene therapy and DNA vaccines;
- Guidance for Industry, Guidance for human somatic cell and gene therapy (7).
- Draft guidance for industry, Supplemental guidance on testing for replication competent retrovirus in retroviral vector based gene therapy products and during follow-up of patients in clinical trials using retroviral vectors (59).
- Points to consider on plasmid DNA vaccines for preventive infectious disease indications (62).

Furthermore, the WHO has established the following guideline:
- Guidelines for assuring the quality of DNA vaccines (63).

All guidelines require that vectors are produced under GMP standards. The guidelines also require the full description of each element of the vector, including complete sequence, if possible. The methods and reagents involved in the production and purification process should be defined and the cells used for production should be described. Quality control of the manufacturing process as well as of the final product is necessary. The guidelines provide recommendations about what kind of controls should be performed on the bulk products and on the final products. Some guidelines give global recommendations while other guidelines describe specific tests with specifications. Where applicable the guidelines refer to more general guidelines for production of biological products.

Specific issues mentioned in the guidelines are described below;

*CPMP, Note for guidance (NfG) on the quality, pre-clinical and clinical aspects of gene transfer medicinal products.* This NfG replaces the NfG 'Gene therapy products – quality aspects in the production of vectors and genetically modified somatic cells' and the annex to this guideline 'Safety studies for gene therapy products'. The NfG defines under gene transfer the deliberate introduction of genetic material into somatic cells for therapeutic, prophylactic or diagnostic purposes. Gene transfer products are defined as medicinal products of biological origin, which include naked DNA, complexed nucleic acids, viral vectors and genetically modified cells (allogenic or xenogenic).

The NfG provides recommendations with respect to the quality, pre-clinical and clinical aspects of gene transfer in medicinal products and assistance in preparing data for submissions supporting marketing authorisation applications within the European Community. Also the NfG covers biosafety issues of medicinal products that contain or consist of genetically modified organisms (GMOs). The NfG also discusses cell-based products. The NfG is not intended for chemically synthesised polynucleotides.

The NfG mentions that many of the quality considerations for rDNA products and other biologicals will apply to the manufacture of gene transfer products. E.g. it is essential to purify and characterise the vector DNA as thoroughly as possible, as is the case with expression constructs for rDNA-derived protein production. The NfG gives also some recommendations about the testing of pharmaceutical formulations of plasmid DNA products, which is hardly mentioned in other guidelines.

The draft NfG describes which parameters are important for product characterisation but hardly recommends tests or product specifications. Within this aspect the NfG is not very practical.

*CBER, Guidance for human somatic cell and gene therapy.* In the guidance of CBER, gene therapy is defined as a medical intervention based on modification of the genetic material of living cells. Cells may be modified *ex vivo* for subsequent administration to humans, or may be altered *in vivo* by gene therapy given directly to the subject. The genetic manipulation may be intended to have a therapeutic or prophylactic effect, or may provide a way of marking cells for later identification. When the genetic manipulation is performed *ex vivo* on cells, which are then administered to the patient, this is also a form of somatic cell therapy. Somatic cell therapy is defined as the administration to humans of autologous, allogenic, or xenogenic living cells, which have been manipulated or processed *ex vivo*.

Guidance for human somatic cell and gene therapy does not cover virus or DNA preparations used as preventive vaccines. The guidance does cover cell therapy issues and pre-clinical evaluation of gene therapy and cell therapy products. The guidance describes the important characterisation and safety issues and recommends specific tests for identity, purity, safety and potency testing of the vector. This is in contrast to the guideline of the CPMP, which provides more global guidance. This difference is probably due to the longer experience that exists in the USA and the FDA with gene therapy. Probably for this reason, even in Europe the CBER guideline is used preferably by manufacturers of gene therapeutics and scientists that conduct clinical trials.

RCR testing is described in this guideline but recently a supplement on this issue has been provided (59) (see below).

*CBER, Supplemental guidance on testing for replication competent retrovirus in retroviral vector based gene therapy products and during follow-up of patients in clinical trials using retroviral vectors.* This supplemental guidance differs from the guidance for human somatic cell therapy and gene therapy at the following points: 1. It effectively reduces the volume of supernatant required for testing, especially in the case of large volume of supernatant production lots, 2. It revises the time points tested and types of assays which should be used to monitor patients who are treated in gene therapy clinical trials which involve the use of

retroviral vectors, 3. It changes the recommendations for life-long monitoring from active monitoring on an annual basis to the collection and archiving of patient samples and tracking of relevant clinical history on an annual basis.

*CBER, Points to consider on plasmid DNA vaccines for preventive infectious disease indications.* Plasmid DNA vaccines are defined as purified preparations of plasmid DNA designed to contain a gene or genes for the intended vaccine antigen as well as genes incorporated into the construct to allow for production in a suitable host system. The guideline recommends certain tests for identity, safety, purity and potency testing.

*WHO guidelines for DNA vaccines.* The purposes of the WHO guideline are to indicate appropriate methods for the production and control of plasmid DNA vaccines and to specify what information should be included in submissions by manufacturers to national control authorities in support of applications for the authorisation of clinical trials and marketing. The guideline focuses on plasmid vectors meant for vaccination. The guideline tells what characterisation and safety aspects are important and recommends certain tests.

# 5. Conclusions

Gene therapy and DNA vaccination are relatively new and rapidly evolving technologies. But although the expectations are high for gene therapeutics and DNA vaccines, only a few clinical trials have yet shown promising results. The viral and plasmid vectors that are used for gene therapy and DNA vaccination are still in development. It is still too early to say what kind of vectors will be the vectors of choice in the future. Consequently, the analytical methods that are used for quality and safety control of viral and plasmid vectors are also still in development and therefore standard methods for testing these vectors have not been established yet. Also the guidelines that are being developed for these products are still subject to revisions.

Many of the quality and safety considerations that are applicable to viral and plasmid vectors are essentially the same as for other biologicals, like recombinant DNA protein therapeutics. As is the case with rDNA products it is necessary that gene therapeutics and DNA vaccines are manufactured under GMP conditions. The production process has to be validated and the finished products thoroughly characterised. The cell lines used for production should be handled in a working bank/cell bank system and have to be tested for identity and contamination with adventitious agents. Furthermore, the intermediate products and finished product are tested for adventitious agents and for process-related impurities like host cell DNA, host cell proteins and pyrogens. These assays are familiar to official control laboratories and regulatory agencies.

The quality and safety issues that are specific to viral and plasmid vectors are the product-related issues like identity of the vector, presence of replication-competent vector-related viruses, establishment of the potency of the vector, and genetic and chemical stability of the vector. Analytical methods for these issues are mainly molecular biological assays, cell-culture-based assays and chromatographic assays. In order to be able to compare the outcome of different clinical trials, these assays require (international) standardisation.

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