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5.14. GENE TRANSFER MEDICINAL PRODUCTS FOR HUMAN USE

This general chapter is published for information.

This general chapter contains a series of texts on gene transfer medicinal products for human use. The texts provide a framework of requirements applicable to the production and control of these products. For a specific medicinal product, application of these requirements and the need for any further texts is decided by the competent authority. The texts are designed to be applicable to approved products; the need for application of part or all of the texts to products used during the different phases of clinical trials is decided by the competent authority. The provisions of the chapter do not exclude the use of alternative production and control methods that are acceptable to the competent authority.

Further detailed recommendations on gene transfer medicinal products for human use are provided by the Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99) and the Guideline on Development and Manufacture of Lentiviral Vectors (CHMP/BWP/2458/03) of the Committee for Medicinal Products for Human Use (including any subsequent revisions of these documents).

DEFINITION

For the purposes of this general chapter, gene transfer medicinal product (GTMP) shall mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either *in vivo* or *ex vivo*, of a prophylactic, diagnostic or therapeutic gene (i.e. a piece of nucleic acid) to human/animal cells, and its subsequent expression *in vivo*. The gene transfer involves an expression system known as a vector, which can be of viral as well as non-viral origin. The vector can also be included in a human or animal cell.

Recombinant vectors, such as viral vectors and plasmids. Recombinant vectors are either injected directly into the patient's body (*in vivo* gene transfer) or transferred into host cells before administration of these genetically modified cells to the patient (*ex vivo* gene transfer). Viral vectors are derived from various viruses (for example, adenoviruses, poxviruses, retroviruses, lentiviruses, adeno-associated-viruses, herpesviruses). These vectors can be replicative, non-replicative or conditionally replicative. Plasmid vectors include nucleic acids in a simple formulation (for example, naked DNA) or complexed to various molecules (synthetic vectors such as lipids or polymers). Genetic material transferred by GTMPs consists of nucleotide sequences, which may encode gene products, antisense transcripts or ribozymes. Chemically synthesised oligonucleotides are not within the scope of this general chapter. After transfer, the genetic material may remain either cytoplasmic or episomal, or may be integrated into the host cell genome, depending on the integrating or non-integrating status of the vector.

Genetically modified cells. Genetically modified eukaryotic or bacterial cells are modified by vectors to express a product of interest.

PRODUCTION

Substances used in production. The raw materials used during the manufacturing process, including viral seed lot and cell bank establishment, where applicable, are qualified. Unless otherwise justified, all substances used are produced within a recognised quality assurance system using suitable production facilities. Suitable specifications are established to control notably their identity, potency (where applicable), purity and safety in terms of microbiological quality and bacterial endotoxin contamination. The quality of water used complies with the relevant corresponding monographs (*Purified water (0008), Highly purified water (1927), Water for injections (0169)*). The use of antibiotics is avoided wherever possible during production.

Viral safety. The requirements of chapter *5.1.7* apply.

Transmissible spongiform encephalopathies (*5.2.8*). A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out and suitable measures are taken to minimise such risk.

Recombinant vectors

PRODUCTION

GENERAL PROVISIONS

For viral vectors, production is based on a cell bank system and a virus seed-lot system, wherever possible.

For plasmid vectors, production is based on a bacterial cell bank system.

The production method shall have been shown to yield a vector of consistent quality in order to guarantee efficacy and safety in man. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages or subcultures from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

SUBSTRATE FOR VECTOR PROPAGATION

The substrates used comply with relevant requirements of the European Pharmacopoeia (*5.2.2, 5.2.3*, and the section at the end of this general chapter: Bacterial cells used for the manufacture of plasmid vectors for human use).

CHARACTERISATION OF THE VECTOR

Historical records of vector construction are documented, including the origin of the vector and its subsequent manipulation, notably deleted or modified regions.

The vector is characterised using suitable and validated methods.

The genetic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

PROPAGATION AND HARVEST

All processing of the cell banks and subsequent cell cultures is done in an area where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The purity of the harvest is verified by suitable tests as defined in the corresponding specific sections.

PURIFIED HARVEST

The bulk of active substance is defined as a lot of purified recombinant vectors (viral vectors, or naked or complexed plasmids).

FINAL LOT

Unless otherwise justified and authorised, formulation and distribution of the final bulk is carried out under aseptic conditions using sterile containers (*3.2*).

The stability of the final lot is assessed using stability protocols including the duration, storage conditions, number of lots to be tested, test schedule and assays to be performed.

ASSAYS AND TESTS

The GTMPs comply with assays and tests described in the corresponding specific sections.

Genetically modified cells

For cells to be modified with a recombinant vector, the data related to the recombinant vector are documented above, under Recombinant vectors.

PRODUCTION

CELL SUBSTRATE

For xenogeneic cell lines, including bacterial cells, a cell bank system comprising a master cell bank and working cell banks is established.

For autologous and allogeneic cells, a cell banking system comprising a master cell bank and working cell banks is established wherever possible.

TRANSFECTION / TRANSDUCTION

Cells are transduced or transfected using a recombinant vector (plasmid or viral vector) qualified as described under Recombinant vectors; the process is validated. They are handled under aseptic conditions in an area where no other cells or vectors are handled at the same time. All reagents used during cell manipulation steps are fully qualified. Antibiotics are avoided unless otherwise justified and authorised. Transfection or transduction is carried out under aseptic conditions.

FINAL LOT

In the case of frozen storage, the viability of genetically modified cells is assessed before freezing and after thawing.

If the cells are not used within a short period, stability is determined by verifying cell viability and expression of the genetic insert.

In the case of genetically modified cells encapsulated before implantation in man, any encapsulating component used is considered as part of the final product, and is therefore quality-controlled and fully characterised (for example, physical integrity, selective permeability, sterility).

ASSAYS AND TESTS

Controls of xenogeneic, allogeneic or autologous cells include the following:

– identity, counting and viability of cells;

– overall integrity, functionality, copies per cell, transfer and expression efficiency of the genetic insert;

– microbiological controls (*2.6.1* or *2.6.27*), endotoxin content, mycoplasma contamination (*2.6.7*), adventitious virus contamination and, where applicable, replicative vector generation.

The competent authority may approve a reduced testing programme where necessary because of limited availability of cells. Where necessary because of time constraints, the product may be released for use before the completion of certain tests.

ADENOVIRUS VECTORS FOR HUMAN USE

DEFINITION

Adenovirus vectors for human use are freeze-dried or liquid preparations of recombinant adenoviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

There are different approaches for the design and construction of an adenovirus vector. The purpose of clinical use determines which approach is optimal. A method is chosen that minimises the risk of generating replication-competent adenovirus vectors or that effectively eliminates potential helper viruses during production.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality in order to guarantee efficacy and safety in man. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector construct at or beyond the maximum passage level used for production is assessed by suitable methods.

SUBSTRATE FOR VECTOR PROPAGATION

The vector is propagated in continuous cell lines (*5.2.3*) based on a cell bank system. The occurrence of replication-competent adenoviruses (RCAs) may be significant when large regions of homology exist between the viral genome and the genome of the complementation cells. This occurrence may be minimised by minimising the homology between both genomes. The use of cells with no sequence homology with the vector is recommended for production.

VECTOR SEED LOT

Production of the vector is based on a seed-lot system.

The strain of adenovirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

Identification. The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*) or nucleic acid amplification techniques (NATs) (*2.6.21*).

Genetic and phenotypic characterisation. The following tests are carried out.

– The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the

theoretical sequence based on vector construction and available databases.

– Restriction analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.
– A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.

Vector concentration. The titre of infectious vector or the concentration of vector particles in the master seed lot and each working seed lot are determined.

Extraneous agents (*2.6.16*). The master seed lot and each working seed lot comply with the tests for extraneous agents.

Replication-competent adenoviruses (RCAs). RCAs are generated by homologous recombination between the recombinant viral DNA and the adenovirus sequences integrated into the genome of the complementation cells.

Detection of RCAs is performed by a suitable method approved by the competent authority. It is generally performed by an infectivity assay on sensitive detector cell lines, which are not able to complement for the genes deleted from the vector. Other indicators of viral replication may be used as appropriate.

When RCAs are not supposed to be present in the test sample, considering vector construction and cell lines used, at least 2, but preferably 3 or 4 successive passages are performed on the detector cell line, where applicable. Detection of a cytopathic effect at the end of the passages reveals the presence of RCAs in the preparation. Positive controls are included in each assay to monitor its sensitivity.

When RCAs are expected to be present in the test sample, plaque-assays or limit dilution assays on a detector cell line may be performed.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification. The vector is identified by immunochemical methods (*2.7.1*) or NATs (*2.6.21*).

Vector concentration. The titre of infectious vector and the concentration of vector particles in single harvests are determined.

Extraneous agents (*2.6.16*). The single harvest complies with the tests for extraneous agents.

Control cells. Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

PURIFIED HARVEST

Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate the satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Vector concentration. The titre of infectious vector and the concentration of vector particles in purified harvests are determined.

Identification. The vector is identified by immunochemical methods (*2.7.1*) or NATs (*2.6.21*).

Genomic integrity. Genomic integrity of the vector is verified by suitable methods such as restriction analysis.

Residual host-cell protein. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative polymerase chain reaction (PCR) is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents. Where reagents are used during the purification process, tests (for example, liquid chromatography or atomic absorption spectrometry) for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and RCAs have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*) or NATs (*2.6.21*) or restriction analysis.

TESTS

Osmolality (*2.2.35*): within the limits approved for the particular preparation.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin: not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

Replication-competent adenovirus (RCA) concentration: within the limits approved for the particular preparation.

Vector aggregates. Vector aggregates are determined by suitable methods (for example, light scattering).

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

Thermal stability. Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

Vector particle concentration. Physical titration is performed by a suitable technique (for example, liquid chromatography, absorbance measurement or NATs (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

The vector particle concentration of the preparation to be examined is not less than the concentration stated on the label.

Infectious vector titre. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The assay is invalid if:

- the confidence interval (P = 0.95) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector particle concentration to infectious vector titre: within the limits approved for the particular preparation.

Expression of the genetic insert product. The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the content of active substance;
- the recommended human dose, expressed in vector particle concentration;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

POXVIRUS VECTORS FOR HUMAN USE

DEFINITION

Poxvirus vectors for human use are freeze-dried or liquid preparations of recombinant poxviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

The general design of a poxvirus vector is currently as follows: the genetic insert is inserted downstream of a poxvirus promoter. This expression cassette is inserted into the poxvirus genome in such a manner that it interrupts a viral gene non-essential for replication or is positioned between 2 virus open reading frames.

In most strategies used so far for the construction of the vector, the expression cassette is first inserted within the target site of a virus DNA fragment cloned into a bacterial plasmid. The plasmid is then introduced into host cells, cultured *in vitro*, which are simultaneously infected with the parental poxvirus. DNA recombination occurs within the infected cells, between homologous sequences in the viral genome and viral sequences in the plasmid so as to transfer the genetic insert into the targeted site of the viral genome. The correct targeting of the inserted DNA is checked by restriction-enzyme mapping, NATs (*2.6.21*) and sequencing. Successive plaque-cloning steps are performed to purify the recombinant poxvirus from the mixture of parental and recombinant poxviruses. A variety of methods (for example, foreign marker genes, DNA hybridisation, immunological detection, phenotypic changes in the virus) are employed to facilitate recognition and/or selection of the recombinant poxvirus from the background of parental virus. Where foreign marker genes have been transiently employed, they are removed by appropriate methods from the final recombinant poxvirus.

An alternative strategy for creating poxvirus vectors begins with the *in vitro* construction of a full-length virus genome harbouring the expression cassette within a chosen target site. This recombinant genome is then introduced into host cells simultaneously infected with a helper poxvirus that is unable to multiply. The helper virus may be a poxvirus of the same species whose ability to multiply has been inactivated, or another poxvirus species that does not multiply in the host cells.

The construction of non-replicative poxvirus vectors relies on specific host cell lines or primary cells that are naturally permissive, or on host cell lines that have been modified to express an essential poxvirus gene. These cells fulfill the general requirements for the production of medicinal products (*5.2.3*) and do not allow the generation of replicative vectors.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality in order to guarantee efficacy and safety in man. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector construct at or beyond the maximum passage level used for production is assessed by suitable methods.

SUBSTRATE FOR VECTOR PROPAGATION

The vector is propagated under aseptic conditions in human diploid cells (*5.2.3*), in continuous cell lines (*5.2.3*) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (*5.2.2*). When the vector is propagated in a continuous cell line or in human diploid cells, a cell-bank system is established.

VECTOR SEED LOT

Production of the vector is based on a seed-lot system.

The strain of poxvirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

Identification. The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*) or NATs (*2.6.21*).

Genetic and phenotypic characterisation. The following tests are carried out.

- The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Restriction analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.
- A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.
- The host range is verified by determining the replication properties of the vector and comparing them with that of the parental virus, at a passage level comparable to a production batch.

Infectious vector titre. The titre of infectious vector in the master seed lot and each working seed lot is determined.

Extraneous agents (*2.6.16*). The master seed lot and each working seed lot comply with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell culture is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification. The vector is identified by immunochemical methods (*2.7.1*) or NATs (*2.6.21*).

Infectious vector titre. The titre of infectious vector in single harvests is determined.

Extraneous agents (*2.6.16*). The single harvest complies with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

Control cells. If human diploid cells or a continuous cell line are used for production, the control cells comply with a test for identification (*5.2.3*). They comply with the tests for extraneous agents (*2.6.16*).

PURIFIED HARVEST

Processing is carried out under aseptic conditions. Several single harvests may be pooled before the purification process. The harvest is first clarified to remove cells and then, where applicable, purified by validated methods. Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Infectious vector titre. The titre of infectious vector in purified harvests is determined.

Identification. The vector is identified by immunochemical methods (*2.7.1*) or NATs (*2.6.21*).

Genomic integrity. Genomic integrity of the vector is verified by suitable methods such as restriction analysis.

Ratio of infectious vector titre to total protein concentration. The total protein concentration is determined by a suitable method (*2.5.33*). The ratio between infectious vector titre and total protein concentration is calculated.

Residual host-cell protein. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents. Where reagents are used during the purification process, tests (for example, liquid chromatography or atomic absorption spectrometry) for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for bovine serum albumin (when bovine serum is used to manufacture the vector) has been carried out with satisfactory results on the final bulk, it may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*) or NATs (*2.6.21*).

TESTS

Osmolality (*2.2.35*): within the limits approved for the particular preparation.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin: not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

Thermal stability. Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

Infectious vector titre. Titrate at least 3 vials of the preparation to be examined by inoculation into cell cultures. Titrate a vial of an appropriate vector reference standard to validate each assay.

The vector titre of the preparation to be examined is not less than the minimum amount stated on the label.

The assay is invalid if:

- the confidence interval (P = 0.95) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Expression of the genetic insert product. The expression of the genetic insert product(s) is determined, wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity. Unless otherwise justifed and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

PLASMID VECTORS FOR HUMAN USE

DEFINITION

Plasmid vectors for human use are double-stranded circular forms of bacterial DNA that carry a gene of interest or a nucleotide sequence encoding antisense sequences or ribozymes and its expression cassette; they are amplified in bacteria at an extrachromosomal location. They are used to transfer genetic material into human somatic cells *in vivo* or to genetically modify autologous, allogeneic, xenogeneic or bacterial cells before administration to humans. Plasmid vectors may be presented as naked DNA or may be formulated with synthetic delivery systems such as lipids (lipoplexes), polymers (polyplexes) and/or peptide ligands that facilitate transfer across the cell membrane and delivery to the cell, or that target delivery via specific receptors.

Plasmids formulated with synthetic delivery systems are not within the scope of this section.

PRODUCTION

PLASMID CONSTRUCTION

A typical plasmid vector is composed of:

- the plasmid vector backbone that contains multiple restriction endonuclease recognition sites for insertion of the genetic insert and the bacterial elements necessary for plasmid production, such as selectable genetic markers for the identification of cells that carry the recombinant vector;
- the required regulatory genetic elements to facilitate expression of the genetic insert;
- the genetic insert;
- a polyadenylation signal.

A complete description of the plasmid DNA, including its nucleotide sequence, is established with the identification, source, means of isolation and nucleotide sequence of the genetic insert. The source and function of component parts of the plasmid, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

GENERAL PROVISIONS

Cell banks. Production of plasmid vectors is based on a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs), which comply with the section at the end of this general chapter: Bacterial cells used for the manufacture of plasmid vectors for human use. The raw materials used during the manufacturing process, including cell bank establishment, are qualified.

Selection techniques. Unless otherwise justified and authorised, antibiotic-resistance genes used as selectable genetic markers, particularly for clinically useful antibiotics, are not included in the vector construct. Other selection techniques for the recombinant plasmid are preferred.

Reference standards. A suitable batch of the formulated plasmid, preferably one that has been clinically evaluated, is fully characterised and retained for use as a reference standard as necessary in routine control tests.

PROPAGATION AND HARVEST

Plasmid DNA is transferred to host strain bacterial cells and a single clone of transformed bacteria is expanded to create the MCB. The WCB is then derived from the MCB. The EOPCs are obtained from the WCB by fermentation in production conditions.

Plasmid DNA is isolated from harvested cells using an extraction step and is purified to obtain the bulk product.

Unless otherwise justified and authorised, caesium chloride-ethidium bromide density gradients are not used for production.

PURIFIED PLASMID

The production process is optimised to remove impurities consistently while retaining product activity. The requirement to test for a particular impurity depends on the following:

- the demonstrated capability of the manufacture and purification processes to remove or inactivate the impurity through process validation, using specific quantification methods;
- the potential toxicity associated with the impurity;
- the potential decrease of the efficacy of the genetic insert product associated with the impurity.

If selective resistance to specific antibiotics has been used for selection, data from validation studies of purification procedures are required to demonstrate the clearance capability for residual antibiotics.

Relevant in-process controls are performed to ensure that the process is continuously under control, for example, amount and form of plasmid after the extraction steps and amount of endotoxins after the extraction steps.

Only a batch of purified plasmid that complies with the following requirements may be used.

Identity and integrity of the purified plasmid. Identity and integrity of the purified plasmid are established by suitable methods such as sequencing or nucleic acid amplification techniques (*2.6.21*); restriction-digest analysis may be used where it is sufficient to detect potential critical modifications in the plasmid and confirm the plasmid identity.

Plasmid DNA. The following indications are given as examples.

DNA concentrations greater than 500 ng/ml may be determined using absorbance measurement at 260 nm. A 50 µg/ml double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/ml are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

DNA forms. Plasmid DNA is characterised in terms of the proportions of supercoiled, multimeric, relaxed monomer and linear forms, using suitable analytical methods, examples of which are given below. For quantification of supercoiled forms, anion-exchange high performance liquid chromatography (HPLC) or capillary electrophoresis may be used. Capillary electrophoresis is also suitable for the quantification of other forms.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual RNA. The content of residual RNA is determined, unless the process has been validated to demonstrate suitable clearance. Reverse-phase HPLC (RP-HPLC) may be used, or quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (*2.6.21*) when a lower limit of detection is required.

Residual host-cell protein. The concentration of residual host-cell protein is determined using standard protein assays (*2.5.33*), SDS-PAGE followed by silver staining, or specific immuno-assays such as western blot or ELISA, unless the process has been validated to demonstrate suitable clearance.

Microbiological control. Depending on the preparation concerned, it complies with the test for sterility (*2.6.1*) or the bioburden is determined (*2.6.12*).

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

The plasmid vector is identified by restriction-digest analysis or by sequencing. The test for biological activity also serves to identify the product.

TESTS

Tests carried out on the final lot include the following.

Appearance.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

DNA forms. The percentage of the specific monomeric supercoiled form is determined as described for the purified plasmid.

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

Plasmid DNA: not less than the quantity stated on the label, determined, for example, by one of the following methods.

DNA concentrations greater than 500 ng/ml may be determined using absorbance measurement at 260 nm. A 50 µg/ml double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/ml are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Biological activity. Wherever possible, biological activity is assessed through *in vitro* or *in vivo* bioassays. A well-defined, representative reference standard is required as a positive control for the assay. Bioassays employed to assay plasmid vectors generally involve transfection of a relevant cell line *in vitro*, followed by some functional measure of the expressed genetic insert. Such functional assays provide information about the activity of the product encoded by the genetic insert instead of the expression level of the genetic insert itself.

It may be necessary to supplement the bioassay with western-blot and ELISA assays to assess the integrity and quantity of the expressed product.

LABELLING

The label states:

- the plasmid DNA concentration;
- the recommended human dose;
- for freeze-dried preparations:
  - the name and volume of the liquid to be added;
  - the time within which the product is to be used after reconstitution.

BACTERIAL CELLS USED FOR THE MANUFACTURE OF PLASMID VECTORS FOR HUMAN USE

Production of plasmid vectors for human use is based on the use of a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs). A bacterial cell bank for the manufacture of plasmid vectors is a collection of vials containing bacterial cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single clone of a transformed host strain. The MCB has a known, documented history; it is preferably derived from a qualified repository source. The WCB is produced by expanding one or more vials of the MCB. Methods and reagents used to produce the bank and storage conditions are documented.

MCBs and WCBs are qualified by testing an aliquot of the banked material or testing a subculture of the cell bank.

The following table indicates the tests required at each stage of production.

Assay	Host strain	MCB	WCB	EOPCs*
Identity and purity				
Viability	+	+	+	+
Bacterial strain characterisation	+	+	–	+
Genotyping / phenotyping	+	+	–	+
Presence of the plasmid				
– Sequence of the DNA plasmid	–	+	–	+
– Copy number	–	+	+	+
– Restriction map	–	+	+	+
– Percentage of cells retaining the plasmid	–	+	+	+
Adventitious agents				
Purity by plating	+	+	+	+
Presence of bacteriophage	+	+	–	+

* EOPCs are cells with a passage number at least equivalent to that used for production. The analysis has to be done once to validate each new WCB, except for purity, which has to be tested for each fermentation.

IDENTITY AND PURITY TESTING

Viability. The number of viable cells is determined by plating a diluted aliquot of bacterial cells on an appropriate medium and counting individual colonies.

Biochemical and physiological bacterial strain characterisation. Depending on the bacterial strain used for production, relevant biochemical and physiological characterisation is performed to confirm cell identity at the species level.

Genotyping / phenotyping. The genotype of bacterial cells is verified by determination of the suitable specific phenotypic markers or by appropriate genetic analysis.

Presence of the plasmid

Sequencing. The whole nucleotide sequence of the plasmid is verified.

Copy number. The plasmid DNA is isolated and purified from a known number of bacteria and the copy number determined by a validated method such as quantitative PCR (*2.6.21*).

Restriction map. Restriction endonuclease digestion is performed with sufficient resolution to verify that the structure of the plasmid is unaltered in bacterial cells.

Percentage of cells retaining the plasmid. Bacterial elements present in the plasmid, such as selectable genetic markers, are used to define the percentage of bacteria retaining the plasmid.

ADVENTITIOUS AGENTS AND ENDOGENOUS VIRUSES

Purity by plating. Bacterial cells are streaked out onto suitable media and incubated in the required conditions in order to detect potential bacterial contaminants. In order to test for inhibition of the growth of contaminating organisms, additional tests in the presence of a definite amount of relevant positive control bacteria are carried out. A suitable number of colonies is examined; no contamination is detected.

Presence of bacteriophage. Bacterial cells are plated and incubated in a medium allowing proliferation of bacteriophages, to test for bacteriophage presence. The test is validated by the use of a reference bacteriophage strain and permissive cells as positive controls. A suitable number of colonies is examined; no contamination is detected.