Review

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Manufacturing of viral vectors for gene therapy: part I. Upstream processing

Manufacturing technologies for the production of clinical grade viral vectors have been significantly improved in recent years. This is of utmost importance for gene therapy approaches used in the treatment of inherited or acquired diseases. This article briefly describes the general principles for the production of viral vectors. The specific sections are dedicated to more detailed descriptions of the production of adenoviral, AAV, γ-retroviral and lentiviral vectors. A subsequent article (the second part) will then deal with downstream processing (purification) of viral vectors.

Otto-Wilhelm Merten*[,1], Matthias Schweizer[2], Parminder Chahal[3] & Amine A Kamen[4]

[1]Généthon, 1, Rue de l'Internationale, BP60, 91002 Evry Cedex 2, France

[2]Paul-Ehrlich-Institut, Paul-Ehrlich-Str. 51–59, 63225 Langen, Germany

[3]Research Officer, Human Health Therapeutics Portfolio, National Research Council Canada, 6100 Avenue Royalmount, Montréal, QC, H4P 2R2, Canada

[4]Bioengineering Department, Macdonald Engineering Building 270, McGill University, 817 Sherbrooke Street West, Montreal QC, H3A 0C3, Canada

*Author for correspondence: omerten@genethon.fr

Over the last decade, manufacturing technologies for the production of clinical grade viral vectors have been significantly improved. This is of utmost importance for gene therapy approaches used in the treatment of inherited or acquired diseases. Up to January 2014, more than half of all gene therapy clinical trials have made use of adenoviral, adeno-associated viral (AAV), lentiviral or retroviral vectors [1]. Currently, there is a clear trend in an increased use of AAV and lentiviral vectors for gene delivery, respectively, *in vivo* and *ex vivo*. In October 2012, the first European marketing authorization for a human gene therapy product was granted by the European Commission for Glybera®, which contains an AAV1 vector for treatment of patients with lipoprotein lipase deficiency [2].

Viral vector production

Viral vectors are derived from viruses that naturally infect human cells or other mammalian cells, thus their production is essentially using animal cell cultures and for some systems, insect cell cultures. Therefore, animal cell culture processes, involving cell culture bioreactors are needed for the industrial production of viral vectors. In the following, cell culture technologies and processes are described, focusing on their use for the specific production of viral vectors. Depending on the amount of the viral vector to be produced as well as of the cell system used, different culture systems are available for the production of viral vectors. With respect to cell culture processes the following characteristics are of importance and are briefly described: constitutive versus induced vector production, culture of adherently growing cells versus suspension cultures, multiple process (parallel processing units) versus unit process culture systems, and the use of serum-free versus serum-containing media.

Mode of vector production

In general, there are two modes of vector productions: stable producer cell lines and transient (inducible) production system. The induction of the vector production is performed either by transfection where the cells are transfected with one or several plasmids providing the helper functions as well as the vector construct or by infection of the cells with one or several virus(es) providing the functions required for the production of the viral vector. In principle, the transient (or inducible) production mode can be used for the manufacture of all the viral vectors, including retroviral vectors when stable producer cell lines are not available.

Adherent cell versus suspension cell

In general, all cell lines except those derived from the blood system as well as tumor cells

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and some insect derived cell lines grow adherently. Since tumor cells have an increased tendency to grow in suspension they can be adapted to suspension growth [3]. The key difference between anchorage dependent and suspension cells from a process standpoint is the way of subculturing or passaging the cells. This can be as simple as dilution of the suspension cells in fresh medium or as complicated as detaching the anchorage dependent cells from a surface and plating them on a new surface in fresh medium. Different ways to detach surface adherent cells for subcultivation have been reviewed by Merten [4]. With regard to process scale up these differences become very important. For suspension cultures the size of the cell culture vessel can easily be scaled up from a spinner or shaker flask to a laboratory-scale stirred tank reactor (STR) then to an industrial scale STR that might have a total volume of up to 30,000 l [5], whereas, for adherently growing cells, the scale-up is limited by the surface available for the cell growth. In some cases, anchorage dependent cells have been adapted to suspension culture addressing this limitation. Another option largely adopted by the vaccine industry to culture anchorage dependent cells is the use of microcarriers [6], which provides large surface areas in large volume bioreactors. Currently, virus production has been scaled up to 6000 l using microcarriers [7]. However, since the required vector quantities might be low for some applications, viral vector manufacturers have evaluated either fixed bed reactors or parallel processing units, such as roller bottles or CellFactories (CFs).

Unit process versus multiple parallel processes

Finally, cell culture systems can be distinguished by their scalability. Unit process systems [8,9] are characterized by scalability based on the increase in the size/volume of the culture system ('bioreactor') whereas multiple culture systems [10] are characterized by scalability via the increase in the number of identical culture systems (e.g., increase in the number of roller bottles or CFs for producing larger quantities of a given product).

In addition to the excellent scalability, further advantages of the unit process systems over the multiple parallel processes are:

- Practicable to monitor and control a range of process parameters with the use of detectors and control loops;
- Easy to generate and keep records of process conditions, enabling a better understanding of the process, which often results in improved cell growth and higher yields and provides extensive documentation for regulatory agencies;
- Low operation costs as compared with the multiple parallel processes;
- Better monitored and controlled operations to insure optimal culture conditions.

In addition, multiple process systems (T-flasks, roller bottles, CFs) are labor intensive, characterized by an elevated contamination risk due to the need to open and close flasks for seeding, medium change, and harvesting and can be used only in a batch, fed batch or repeated batch mode, whereas bioreactor systems (stirred tank reactor for suspension and microcarrier cultures, fixed bed, fluidized bed) can also be used in a perfusion mode – an option attractive for the production of γ-retroviral and lentiviral vectors. Whereas fixed bed reactor systems show mass transfer limitations, hydrodynamic shear effects are the drawbacks of suspension and microcarrier cultures.

Currently used cell culture systems for the production of different viral vectors

Small scale/laboratory scale cell culture systems

Adherent cells are cultured in static cell culture systems including Petri dishes and T-flasks. T-flasks provide culture surface ranging from 25 to 225 cm^2. The general applications are subculture, generation of seed material for starting larger scale productions as well as small-scale productions of viral vectors, using batch, fed batch or repeated batch culture modes.

When larger viral vector quantities are needed for research or development purposes, anchorage dependent cells can be cultured in roller bottles (Figure 1A) or multilayer tissue culture flasks such as CFs or CellSTACKS® (Figure 1B). Roller bottles provide culture surfaces in the range of 490 cm^2, 850 cm^2 and 1750 cm^2, whereas CFs can provide up to 25,100 cm^2 for 40 stacks.

Roller bottles are simpler and easier to use than CFs but require a mechanical device for rotation of the roller bottles at a maximum speed of 1.5–2 rotations/min. The volume of the culture can be varied between 25 and 100%, which is equivalent to 125 and 500 ml for a roller bottle with a surface area of 850 cm^2, thus allowing higher product concentrations at lower culture volumes. When large numbers of roller bottles have to be used, then two solutions can be envisaged:

- The use of automatic Cellmate processing systems as proposed by The Automation Partnership [102], a system which allows the automatic handling of all cell culture steps; such systems have been used by the biopharmaceutical industry for the production of viral vaccines for human and veterinary use as well as recombinant proteins;

- The use of the RollerCell system from Cellon [103], which is a self-contained roller bottle processing system. This system is designed to automate all steps of roller bottle based tissue culture – from cell inoculation, incubation, medium change and trypsinisation to final harvest. A single unit can process the equivalent of 200 standard roller bottles simultaneously. A standard pack (20 bottles) provides a culture surface of 36,000 or 85,000 cm^2 when using flat wall or expanded wall surface (pleated roller bottles), respectively. The same system in configuration of the 'RollerCell' 40 or 'RC 40' provides a culture surface of 2 × 36,000 cm^2.

On the other hand, the CF system is a static culture system, mainly dedicated to the culture of adherently growing cells. This system shows its limitation with respect to the possible variations in the culture volume (600–1000 ml for the ten stack system). An additional limitation of this culture system is a sufficient gas exchange, and it has been shown that active gassing of ten stack CF systems led to an increase in viral vector production (adenovirus, AAV) by improving CO_2 and air exchange and maintaining culture pH [11]. The use of HYPERFlasks which provide a culture surface of ten-times 175 cm^2 by maintaining the volumetric needs of a 175 cm^2 culture flask, make use of a gas permeable film to provide gas exchange between the cells and the medium and the atmospheric environment surrounding it. Such an improved gas exchange was also shown to lead to an improved (LV) vector production [12]. The advantage of the HYPERFlasks is the much easier handling than CFs or CellSTACKS.

Generally the ten-stack version and the largest 40-stack version are the most used. The CF-40 stack system is a semi-closed system and requires specific equipment for handling [13]. In comparison to the production done with a standard roller bottle process, the use of CF allows a production under semi-closed conditions, when all CF units, bottles, medium and harvest bags are connected using silicon tubing.

However, as for the roller bottle system only a linear scale-up is feasible because any increase in the production capacity requires the addition of supplementary culture units.

Both culture systems have been used for the production of preclinical and clinical vector batches of γ-retroviral (Table 1), lentiviral [14,15] or AAV vectors [16] with either constitutive or transient transfection based expression systems.

For suspension cells, small-scale cultures are typically performed in shake flasks or spinner flasks allowing adjustment of the culture volumes within the limits of the oxygen transfer capacity of the respective systems and the number of flasks to the production needs. In particular, since spinner systems have no active gassing (sparging) at high cell densities they are oxygen transfer limited by the available headspace. These culture systems may be used for the amplification of cell seed for initiation of large-scale suspension cultures.

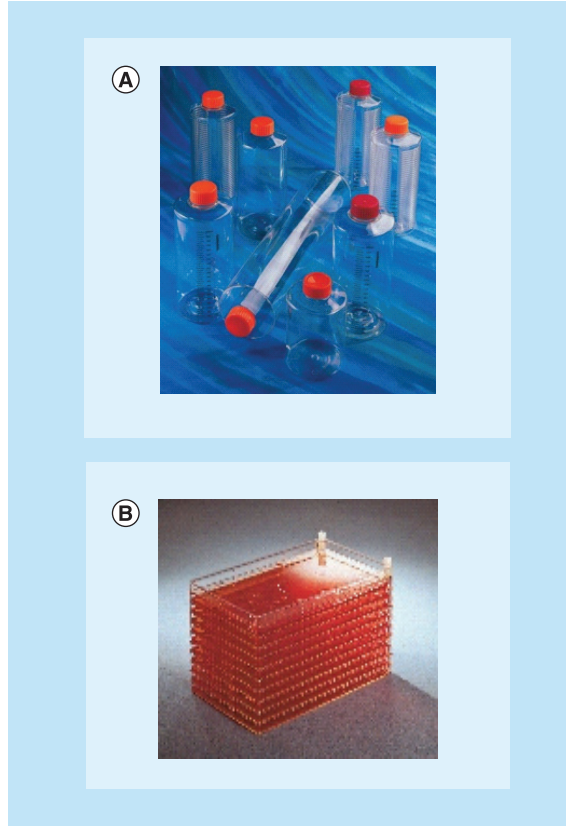

Figure 1. Small scale/laboratory scale cell culture systems routinely used for viral vector production.
(A) Roller bottles: three different sizes are shown: 490, 850 and 1750 cm^2. **(B)** CellFactory (10 stack version).

Large scale cell culture systems

In this section only the bioreactors that are routinely used for the production of viral vectors for research and development as well as manufacturing of clinical-grade material are described. These are STRs, fixed bed bioreactors and WAVE-type bioreactors.

STR system

STR systems can be employed for the cultivation of suspension and adherent cells grown on microcarriers suspended in the medium. Broadly speaking, these reactors are vessels that are equipped with an agitator [35], with or without a draft tube [36] or baffles [37]. The largest industrial-scale cultivation of animal cells is a volume of 20,000–30,000 l [5]. The cell density is limited to approximately 1–5 × 10^6 c/ml for batch or continuous cultures without cell retention. This depends

Table 1. Large scale production of MLV vectors for clinical applications.

Production system (available culture surface)	Cell line	Volume produced (l)	Daily perfusion rate (l/day)	Daily perfusion rate (ml/(day.cm²))	Total vector quantity produced per day	Duration of production (days)	Ref.
CellCube							
1 CellCube module 10 (8500 cm²)	Phoenix Frape 1 and Phoenix Frape 3	10–10.5	12.5–13.12	0.588–0.618	12–18 × 10^9 TU	2	[28]
1 CellCube module 25 (21,250 cm²)	Phoenix Frape 1 and Phoenix Frape 3	19.4–21.5	6.46–7.16	0.304–0.337	6.45 × 10^{10}TU	3	[28]
1 CellCube module 25 (21,250 cm²)	PA317 – G1NaSvAd.24	30	1.3	0.061	2.4 × 10^{11} CFU	23	[29]
4 CellCubes module 25 (84,900 cm²)	PA317 – G1TK1SvNa.7	210	2.625	0.124	4.2–6.3 × 10^{12} CFU	>20	[Zhang S, Pers. Comm.]
1 CellCube module 400 (340,000 cm²)	HT1080 (A) –HAII/pCF8 (hFVIII)	200–400	0.96–1.91	0.045–0.09	5–10 × 10^{12} CFU	13	[30]
Roller bottle							
100 roller bottles (850 cm²)	PA317 – G1TK1SvNa.7	80	266.6	0.314	1.6 × 10^{12} CFU	3	[Zhang S, Pers. Comm.]
90 roller bottles (850 cm²)	PUZIkat2 – CC49ζ	54	200	0.235	1–3.8 × 10^{11} TU	3	[31]
40 roller bottles (2 x 36,000 cm²)	Phoenix Frape 1	30	118	0.139	3.68 × 10^{11}	3	[28]
Cell Factory							
1 Cell Factory (CF-40)	PG13/SF1m (CS78)	9.6	600	0.095	1.85–2.69 × 10^{10} ip	4	[32,51]
4 Cell Factories (CF-10)	PG13NIT clone 2	7.8	650	0.103	3.3 × 10^9 TU	3	[34]

on both the maximal possible growth rates of the cultivated cells, cell size, and on the culture conditions (for instance medium composition). High cell density culture systems that have achieved cell densities ranging from 5 × 10^6 to 50 × 10^6 cells per ml [38] are of interest for increasing the reactor productivity. Such cultures have been performed using a perfusion mode in order to maintain the cells at an optimal metabolic state. In order to maintain the active biomass within the bioreactor, the bioreactor has to be equipped with one of the existing retention devices in order to run the cultures in perfusion mode [39]. Various biologics, including, viruses, and viral vectors, have been produced in low and high-density processes with single cell suspension or microcarrier culture systems. These systems are of high interest because they allow the installation of highly flexible production units with reduced set-up costs.

The advantage of STR systems is the expertise on their scale-up and use which has been generated over many decades of use for different applications. From a hydrodynamic point of view these systems have been extensively studied and a wealth of literature is available (e.g., [40]). The main disadvantages are certainly the fact that surface adherent cells can only be cultured in the STR system either after adaptation to growth in suspension or after establishment of a microcarrier culture system which is less straightforward to use than real single suspension cultures. Adverse hydrodynamic effects on cells grown on microcarriers in stirred tank bioreactor can lead to cell removal from the carriers, reduced cell growth, and cell death and may impact the cell metabolism [41]. In such a case, other systems, in which the cells are protected from hydrodynamic stress, such as fixed bed reactor systems (see below) or hollow fiber systems should be used.

In addition, cell biomass generation for starting a production reactor is less simple than for suspension cultures because adherent cells have to be detached using a protease (often trypsin), eventually washed or treated with trypsin inhibitor and then put into large culture vessels for further amplification

For many years now, disposable STR systems with volume reaching 2000 l [42] have been developed.

Fixed bed reactor/packed bed reactor

In order to increase the reactor cell density, the use of fixed or packed bed reactors is of interest because very high cell densities (0.5–2 × 10^8 c/ml carrier) can be obtained. The attached and/or entrapped cells grow on and/or in the carrier matrix and the culture medium, conditioned for optimal pH and pO_2, is circulated through the fixed bed. Two configurations are possible: the medium is circulated from a conditioning vessel to the fixed bed and back to the conditioning vessel (Figure 2 – the CellCube system); or the fixed bed is integrated in bioreactor and the medium conditioned within this bioreactor is circulated through this bed. New Brunswick Scientific (NBS), for instance, has developed such a fixed bed reactor system (basket reactor), which provides in the smallest reactor version a bed volume of 700 ml (Figure 3). The CellCube system provides a surface range of 8500 cm^2 (module 10), 21,250 cm^2 (module 25), 85,000 cm^2 (module 100), and 340,000 cm^2 (module 400), allowing vector preparations for early stage clinical studies. There are two main drawbacks: the system is limited in scale because the largest scale is the module 400 scale, and the system is only partially disposable (the cell culture stack) which is an advantage, however, the conditioning vessel, the oxygen probe support as well as the pump head for medium circulation are re-usable and have to be prepared (washed, cleaned, autoclaved) after each production run. In addition, the disposable and the re-usable part have to be assembled in a laminar airflow bench, which is associated with a slightly elevated contamination risk.

On the other side, the basket reactor from NBS is a real laboratory system although it has been used for the production of the first licensed gene therapy vector (AdV) in China [43]. In this context, ATMI has commercialized a disposable fixed bed reactor system (the iCELLis™ system) which is comparable to the reactor system developed by NBS [44]. ATMI proposes a range of different production scales ranging from 0.2 to 25 l bed volume, by keeping the maximal height of the fixed bed constant signifying that eventual gradients over the fixed bed will not change during scale-up.

The main drawbacks are the following: the scalability of the packed bed is limited due to the generation of concentration gradients over the fixed bed, meaning that a maximal height of the fixed bed should not

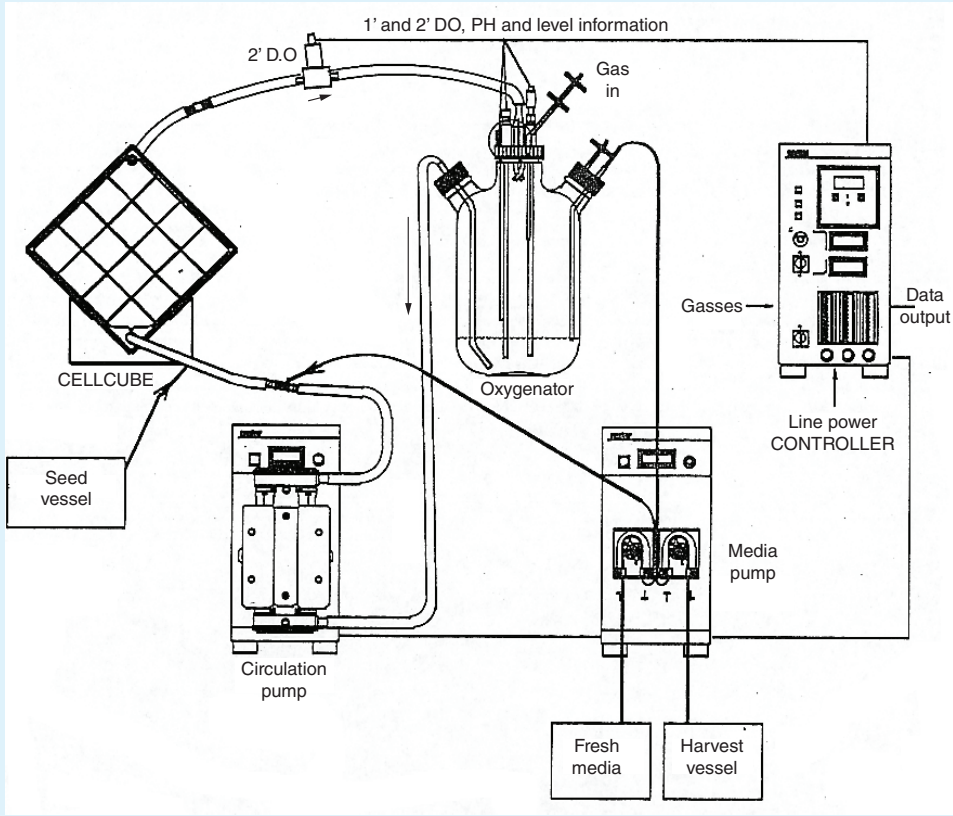

Figure 2. CellCube, laboratory version (module 25) (Corning).

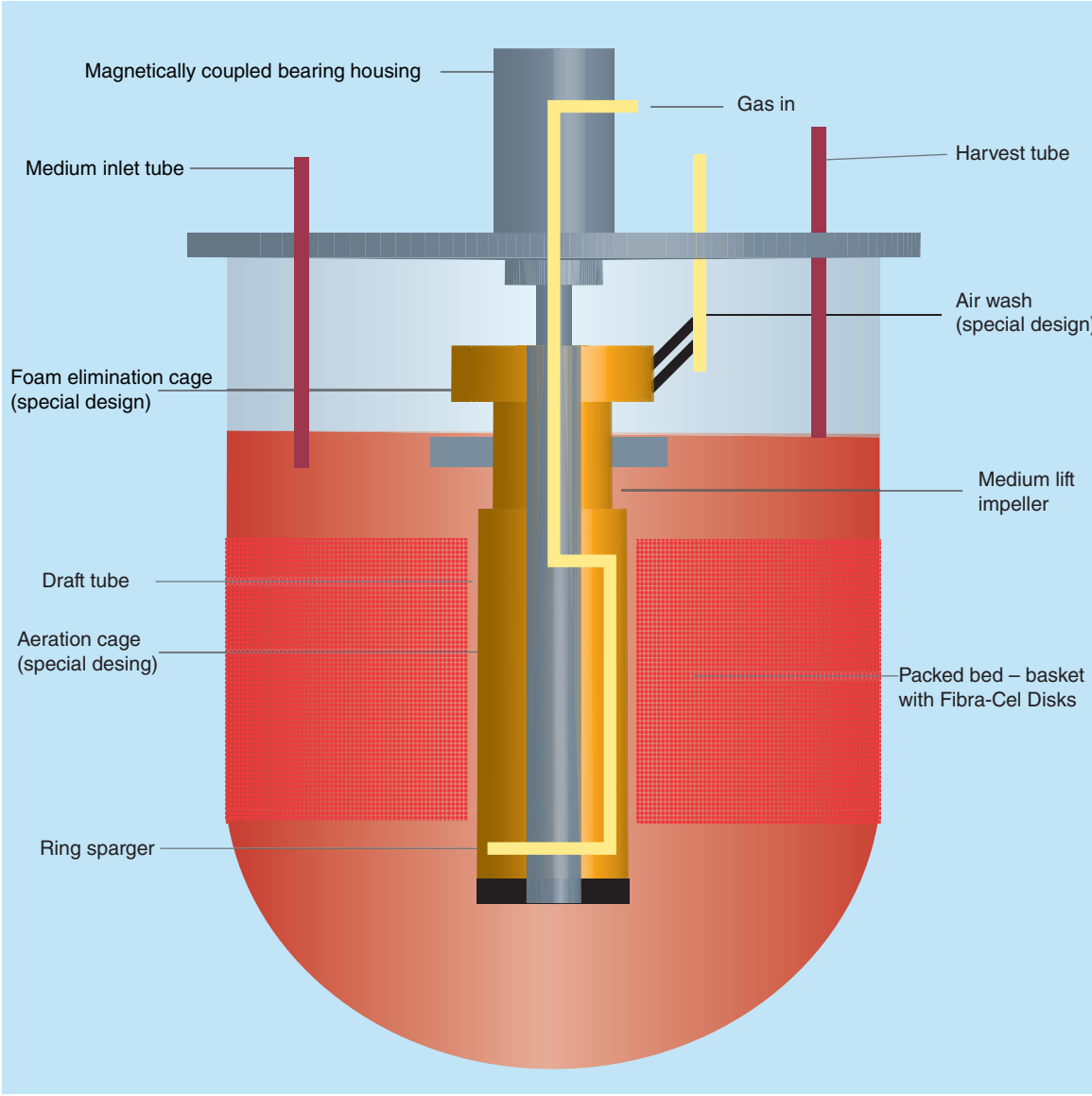

Figure 3. Principle of a packed bed reactor.

be exceeded; the system is non-homogeneous because of the generation of a nutrient/oxygen/CO_2 gradient over the height of the fixed bed; sampling of the viable biomass within the fixed bed is difficult or impossible; and the outgrowth of cells may lead to channel blockage [45].

WAVE bioreactor system

The WAVE bioreactor system (Figure 4) has been used for the cultivation of animal, insect, and plant cells exploiting wave agitation induced by a rocking motion [46]. This agitation system provides good nutrient distribution, off-bottom suspension, and efficient oxygen transfer with reduced generation of fluid shear and absence of gas bubbles, both of importance for microcarrier-cultures. In addition, it is easier to operate than a STR. The largest developed scale is 500 l [42]. The bioreactor is disposable, and therefore requires no cleaning or sterilization. The main disadvantage is the limited mass (O_2) transfer capacity thus *a priori* limiting the scalability to a maximum of 500 l at low cell densities.

The WAVE bioreactor system is well adapted for the production of biomass for starting production reactors or virus seed stocks for production purposes. Various cell types have been cultured in the WAVE Bioreactor including: recombinant mouse myeloma cells (NSO) in suspension; adenovirus production using human embryonic kidney (HEK293) cells in suspension; insect cell (Sf9), for instance used for the production of AAV after infection with recombinant baculovirus [47]; BHK21 cells grown in suspension in view of the pro-

duction of AAV after infection with two different herpes simplex virus [24]; adherent HEK293 cells grown on microcarriers [46]; and stable inducible producer cells for production of lentiviral vectors [48].

Serum-containing versus serum-free media

Traditionally, serum-containing media are used because without much development or adaptation most of the cells can easily be cultured. Despite these advantages, the general trend is towards the use of serum-free media in view of the augmentation of the overall biosafety of the manufacturing system and for getting chemically defined media which allow a much better understanding of the metabolic needs of the producer cell line and a knowledge based optimization – for a general review, see Merten [49]. Another advantage of the use of serum-free medium is the fact that most of continuous cell lines detach from the culture surface and start to grow in suspension. Although often these cells tend to form clumps, clumping can be reduced or avoided by either reducing the Ca++ concentration of the medium or by adding chelators (for chelating Ca++) or substances, such dextran-sulphate or heparin, for reducing cell clumping. With respect to viral vector production, in the past most vector productions were performed in the presence of serum, either because the cells had not been adapted to serum-free media, or the process was developed based on adherent cells requiring the presence of serum for maintaining firm attachment (this is valid for all transfection-based vector productions of AAV [16] or LV vectors [15], for instance, but in the past this was also the case for NIH 3T3 and TeFly based γ-retroviral vector producer cells which require surface adherence [50]. In order to reduce the contamination by residual serum proteins, several processes have been developed in which the biomass generation phase was performed in the presence of serum, whereas for the vector production phase only serum-free media were used. Although such processes do not have an advantage with respect to biosafety of final vector preparation, the advantage is that the crude vector preparation is less contaminated by serum proteins, which is advantageous for the succeeding downstream processing. Such processes have been developed, for instance, for the production of γ-retroviral vector using stable producer cell lines [32,51] as well for the production of AAV [33] and lentiviral vectors [95] using transfection protocols.

It is evident that the most optimal process (at least most optimal from a biosafety point of view) is a manufacturing process which is performed in the absence of serum and ideally performed as an 'animal-free' process, a process in which no animal derived components are utilized. Such manufacturing processes have essentially been developed using bioreactors because the cells used for the production of the vectors do not adhere to a culture surface in the absence of serum. Using transfection methods in suspension culture, complete serum-free processes have been developed for the production of AAV [52] and LV [53] vectors, although

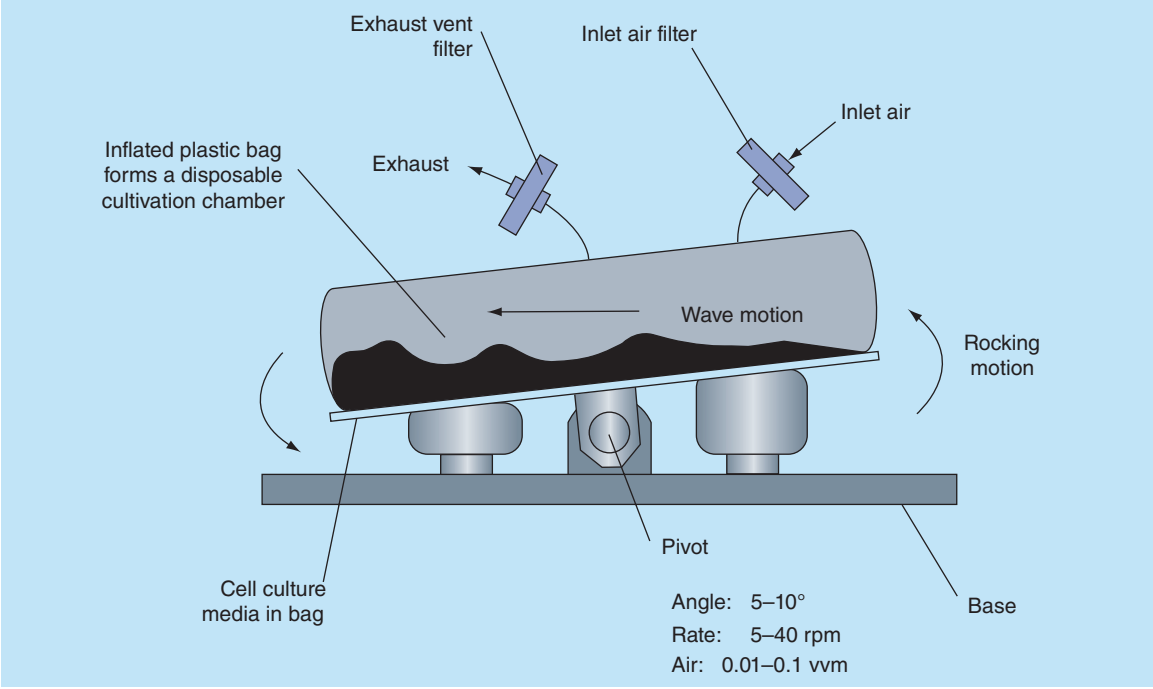

Figure 4. WAVE reactor design.
Adapted from with permission from Springer Science and Business Media [46].

only at laboratory scale for research purposes. Based on viral infection for inducing vector production, several serum-free production processes for different viral vectors have been developed, including the generation of adenoviral vectors using HEK293 and Per.C6 cells infected with adenovirus (references in [54]) and the production of AAV vectors via the infection of insect cells using 1–3 recombinant baculovirus(es) [26,55], BHK-21 cells using two recombinant herpes simplex viruses [24] or stable HeLa based producer cell lines using wild-type adenovirus [22,25]. All these processes are based on the use of STRs [22,25–26,55] or WAVE Bioreactors [24] and have been developed at a large scale for the production of viral vector lots for clinical use.

Bioprocessing of viral vectors

Here we review the most current methods used for the production of viral gene therapy products focusing on the four viral vectors that have been the most extensively used in clinical trials: adenoviral vectors (AdV), adeno-associated viral vectors (AAV), and γ-retroviral and lentiviral vectors.

AdV vector production

The first and second generation AdV vectors are propagated by infection of a trans-complementing cell line such as the E1 complementing HEK 293 human embryonic kidney cell line. Whereas, the third generation or helper-dependent adenovirus (HDV) vector propagation rely on co-infection of complementing cell lines with HDV and a helper virus, involving a primary rescue of the HDV after transfection of HDV-DNA. Most of the production steps are common to both generations of AdV vectors; however, some steps are very specific to the last AdV vector generation. This section covers standard AdV production protocols and provides indications on the HDV production.

Any process development for an efficient, robust and scalable AdV production aims at maximizing the cell specific vector yield. As a theoretical target, one might consider a specific yield as high as 30,000 infectious viral particles (IVP) per cell as achieved by replicating a wild-type adenovirus on a permissive cell line [56]. The scale of production is mainly determined by the number of virus particles required per batch. For adenovirus production, a scale associated with cultivation of up to 10^{15} virus particles (VP)/batch or less may be normally produced in up to 100 l bioreactor for gene therapy applications.

The production of AdV involves the following steps: growing the cells to a desired cell density for infection; infecting with AdV stock at predetermined optimal multiplicity of infection (MOI); and finally, harvesting the virus at a predetermined optimal time post infection. There are numbers of serum-free formulations available commercially that have been optimized for Adenovirus production using HEK293 (human embryonic kidney-derived cells) or PER.C6 cells (human embryonic retina-derived cell). In general, for potential clinical applications, it is recommended to select media that are free of animal-derived components and are preferentially chemically defined. The important criteria used for infection is MOI, defined as the number of viral particles per cell. In order to minimize the amount of virus stock and to get batch-to-batch reproducibility, it is recommended to determine the lowest possible MOI for a given system to obtain the optimal virus yields. The AdV infection process is rapid and lytic; therefore, normally an MOI greater than 1 (typically between 5 and 10) is used to have all the cells infected in the first round of infection.

Cultivation parameters such as temperature, pH, dissolved oxygen (DO) and osmolarity must be evaluated for optimal production of Adenovirus. Temperature is usually maintained at 37°C. The optimal pH for virus production has been reported to be 7.2 and 7.3 for HEK293 or PER.C6 cells, respectively. DO is normally maintained in the bioreactor at higher than 30% of air saturation. On-line monitoring of production parameters of the culture allows real-time supervision and control of critical phases of the process and could be used to support process characterization and validation. Osmolarity has been shown to play an important role in the productivity and should be carefully assessed during the cell growth and infection phase [57].

When the cells are infected with virus, the growth rate decreases but the specific oxygen and glucose consumption rates are elevated during the first 24 h post infection (hpi), probably directly related to the increase in energy demand for DNA replication and viral protein production. Virus assembly and DNA packaging occurs between 20 and 48 hpi. Since AdV is typically purified from the cell mass, it is critical that cells are collected before they are lysed. Viability (typically 80–40%) is usually used as criterion to harvest the culture. Normally, the harvest is done approximately 48 hpi, but is highly dependent on the MOI used (e.g., higher MOI triggers early drop in viable cells), AdV serotype and the transgene expressed.

Upon complementing cell infection, the replication of viral DNA, encapsidation, maturation and expression of viral particles translates in a batch culture process in cell infection, cell growth cessation, virus particles accumulation in the cells as early as 24 hpi with a maximum titer reaching approximately 40–48 hpi and cell death thereafter. Maximal viable cell densities as high as 8×10^6 cells/ml are routinely achieved

in suspension bioreactor cultures [58]. However, with current commercial serum-free media formulations, productions of AdV vectors that maintain a high specific yield in batch operation is limited at cell densities of approximately 1–2 × 10^6 cells/ml [59]. These results strongly suggest a limitation either due to key nutrient depletion or inhibitor byproduct accumulation. High specific productivities might be maintained in high cell density cultures if the cells are infected in a fresh medium. Large-scale cell separation can be realized using a continuous centrifuge. Infection is normally done at 2 × 10^6 cells/ml in fresh medium. Subsequently, medium is further exchanged (~90% volume) if necessary at approximately 24 hpi. AdV is harvested when maximum yield is observed between 40–48 hpi [59].

Easy to operate and readily scalable, the fed-batch mode is employed to extend culture lifetime by supplementing limiting nutrients. Fed-batch additions of glucose, glutamine and amino acids allow infections at cell densities up to 3 × 10^6 cells/ml. The development of an efficient feeding strategy to successfully operate at higher cell densities require prior identification of the factors limiting or inhibiting the virus replication beyond a cell density of 1–2 × 10^6 cells/ml, depending on the type of media used. To date, despite considerable efforts, fed-batch cultures during adenovirus production have not translated into significant increase in cell densities at infection or high yields of AdV.

To overcome metabolic limitations at high cell densities using batch, sequential-batch, or fed-batch modes, the use of perfusion mode with low-shear cell retention devices is now a common trend in adenovirus manufacturing. However, the perfusion parameters should be optimized that includes feed rate, infection and harvest times, and shear reduction (reduced agitation and recirculation). Typically feeding is supplied at the rate of 2–3 reactor volumes per day. The perfusion has been shown to be a feasible mode for producing adenovirus to circumvent metabolic limitations and to allow infections at cell densities up to 3 × 10^6 cells/ml [60] by removing toxic metabolites generated during virus production.

Large-scale production cultures using HEK293 and PER.C6 cells have been performed at scales of several hundred liters under serum-free conditions. A HEK293 based manufacturing process at a 500 l scale represents a commercial process for the production of an Adenovirus vectored veterinary rabies vaccine for use in wildlife. Up to the date of publication more than 17,000 l of culture supernatant have been produced [61]. For the production of adenoviral vectors for cancer gene therapy treatment, a commercial HEK293 based manufacturing process has been established in China [43] however, no details on the actual process or process scale are available. Using PER.C6 cells, Xie *et al.* [62] published a study performed at a 250 l scale as model in view of the optimization of the hydrodynamic conditions with respect to cell growth and adenoviral vector production at a 10,000 l manufacturing scale.

Helper-dependent adenovirus production

The helper-dependent adenovirus (HDV) vector propagation cannot rely only on complementing cell lines, but needs helper virus. The challenge is to remove the AdV helper from the HDV final preparation. The helper to HDV ratio should be better than 1:10^4. It is beneficial for the downstream processing if during HDV production the AdV accumulation can be minimized. The standard HDV production is a two-step process: rescue and amplification.

The replication of HDV at rescue step is limited by the transfection efficiency and therefore, gives low yields relative to the infection processes. Therefore, multiple rounds of HDV amplifications are required to meet the demand of HDV quantity required for large-scale operation. Normally, crude lysate from the preceding harvest is used to infect the cells for the next stage and this process is repeated until sufficient HDV is obtained.

The purpose of the rescue step is to recover HDV from HDV DNA plasmid. The complementing cells, which also express recombinase (such as HEK293 FLPe cells) are transfected with the linearized HDV genome, followed by an infection with the first generation AdV vector as the helper virus between 8 to 18 h post-transfection (hpt) at MOI of 30–100 VP/cell. The site specific recombination catalyzed by FLP recombinase results in rescue of HDV [63]. When cytopathic effect is visible around 40 to 48 hpt, the viral lysate containing the HDV is recovered. As an example, two successful rescue procedures have been described for HEK 293 cells expressing the FLPe recombinase. The first method relies on transfecting HEK293 FLPe cells at 0.5 × 10^6 cells/ml with 1 μg/ml HDV linearized DNA plasmid with 3 μg/ml linear 25 kDa polyethylenimine (PEI) one hour prior to infection by Helper AdV at an MOI of 5 IU/cell [64]. Whereas a second protocol potentially amenable to large-scale production uses the adenofection. This protocol combines the transfection and the infection in one single step and takes advantage of the AdV infection process to improve the transfection efficiency resulting in a net synergistic effect. Complexes of HDV DNA, PEI and Helper AdV are formed by simple mixing and added to the producer cells to generate HDV. This can be achieved not only with adherent cells but also with cells in suspension

cultures. Practically, 1 µg/ml HDV linearized DNA plasmid is mixed with 3 µg linear PEI /ml to which helper AdV is added at an MOI of 50 VP/cell. The mixture is then added to HEK293 FLPe cells in suspension at 0.5 × 10^6 cells/ml. The HDV is collected 48 h post-adenofection [64].

Amplification steps are carried on thereafter to build a viral HDV stock. Because the HDV titre is normally low at the end of the rescue step (10^2–10^5 infectious units [IU] of HDV/ml), further amplification of HDV is required. Typical amplification protocols consist of exhaustive passages of viral lysate on an increasing number of cells. The drawbacks of such amplification protocols are process time length, fluctuation in titer, and possible viral recombination. However, by operating with a controlled MOI protocol for the HDV and the Helper AdV it is possible to minimize the passage number while favoring HDV amplification and limiting Helper AdV contamination. A practical example described uses HEK293 FLPe cells at 0.5–1.0 × 10^6 cells/ml. The cells are co-infected with Helper AdV and HDV from the rescue step or from preceding amplification stages at a total MOI of 100 VP/cell with HDV to Helper AdV ratio of 1:1. Harvesting is completed at 40–48 hpi [64].

AAV Vector production

In the general design of AAV vectors the inverted terminal repeats (ITR) are retained and the exogenous sequences to be transferred are cloned in-between. The Rep and Cap functions have therefore to be supplied in *trans*. The helper functions have to be provided either from auxiliary virus such as Adenovirus or by plasmid coding for the helper virus sequences.

The traditional laboratory-scale production method is based on the transfection of HEK293 cells co-transfected with 3 plasmids at an equimolar ratio that contain the rAAV vector (ITR-transgene-ITR), the *rep* and *cap* genes, and the Adenovirus helper genes, respectively (Figure 5). Specific production levels are in the range of 10^3 to 2 × 10^4vg (nuclease resistant genome containing vectors) per cell [65,66]. The main advantage of this system is its high flexibility consisting in an easy switch from one ITR-transgene-ITR cassette to another one and the possibility to switch from one serotype to another one because of the compatibility of different capsid serotypes with the ITRs and rep proteins from the AAV2 serotype which has been extensively developed [67]. In the vast majority of the studies, a pseudotyping strategy has been adopted for simplicity reasons. Basically, all recombinant genomes

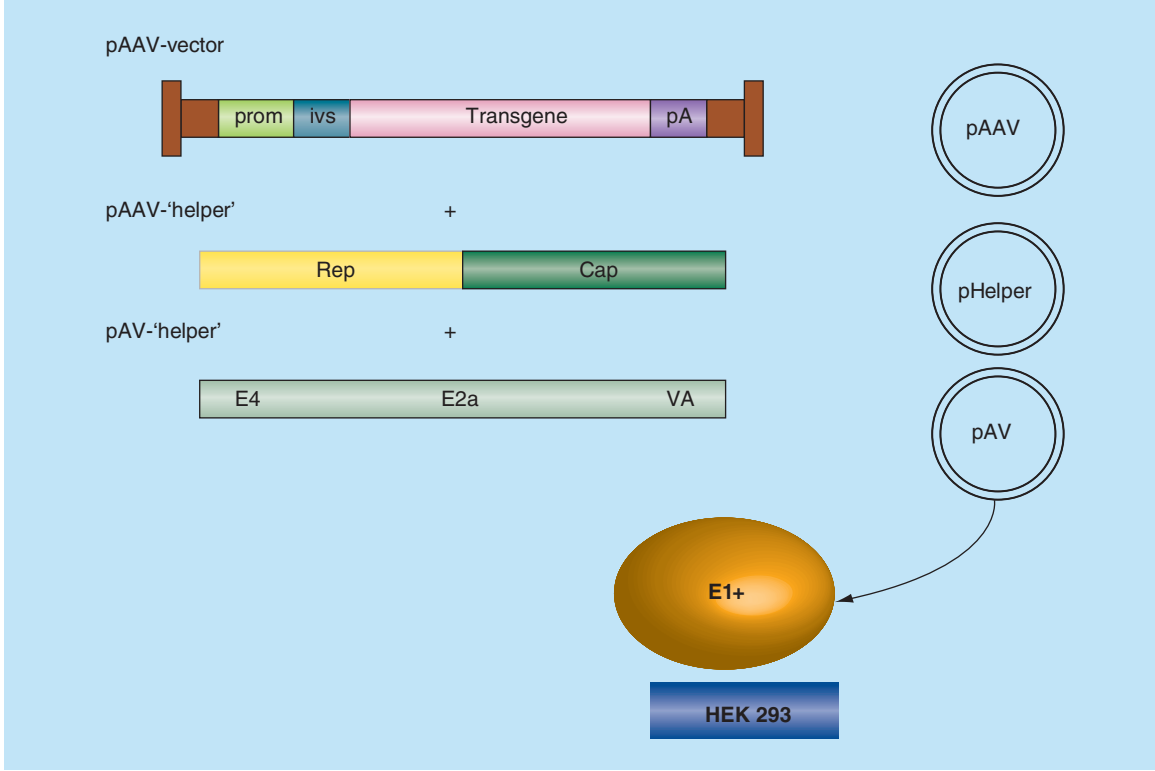

Figure 5. Classical transfection production principle using HEK293 cells. In a transient production system, the pAAV-'helper' (carrying *rep* and *cap* functions), the pAV-'helper', a mini-AV plasmid (such as pXX6), carrying the AdV helper functions E4, E2a and VA, and the pAAV-vector plasmid (carrying the sequence of the gene of interest and the regulatory sequences such as promoter (prom), intervening sequence (ivs) and the polyadenylation site (pA)) will be brought to the producer cells (HEK293) by transfection.

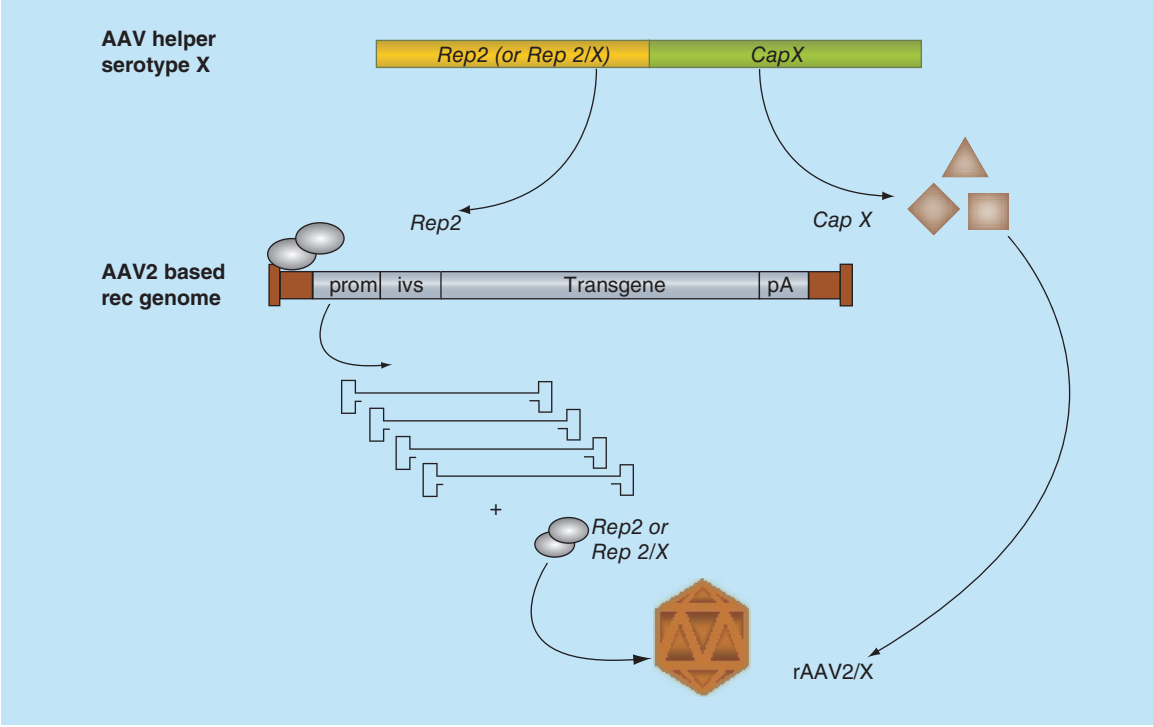

Figure 6. Pseudotyped rAAV design and production principle. Pseudotyped vectors have been extensively and preferentially derived since the early 2000s. The general principle for the genetic trans-complementing system is depicted here. The recombinant AAV genome bearing the expression cassette is framed by type 2 inverted terminal repeats. Although the Rep protein from all serotypes but AAV5 can bind and ensure the replication process, it has been found more convenient and efficient to maintain the *rep orf* from type 2 or to create a chimeric *rep* gene with its 5′ part of type 2 origin and the 3′ of the serotype of interest [69]. The auxiliary virus functions are provided as for rAAV2 production.

are based on AAV2 ITRs, the *rep* functions are derived from AAV2, and only the capsid is of the serotype of interest (Figure 6). Thus, the production strategies for pseudotyped particles are exactly the same as for the AAV2 vectors. More details can be found in Merten *et al.* [17] and Ayuso *et al.* [68].

To overcome the limited scalability due to the use of anchorage dependent cell cultures, suspension based transfection processes as well as three different scalable AAV vector production systems have been developed. These are based on the use of: suspension based transfection process; stable HeLa (an immortal cell line derived from cervical cancer cells) or A549 cell clones (a cell line derived from cancerous lung tissue); the recombinant herpes simplex expression system; and the SF9/baculovirus system:

- Suspension based transfection process: The most advanced approaches take advantage of the transfection of suspension culture of HEK293 cells using serum-free media. Several studies have been performed reporting production levels of 1.4×10^4–3×10^4 vector genome/cell(vg/c) at 'production' scales ranging from 60 ml to 300 ml, 3 l, and to 3.5 l [52,70–72]. Recently, Grieger and Samulski [21] reported preliminary data on a selected HEK293 clone, producing up to 10^5vg/c when grown in an optimized serum-free (animal-free) medium in WAVE bioreactors with a perspective of scale-up to 50 to 100 l for the production of clinical grade material. Although production scales of 50–200 l are possible, transfection of cells in suspension cultures remains limited because of the considerable costs related to the preparation of GMP or high quality grade plasmids when transferring the process to GMP manufacturing. Another caveat is the inherent variability associated with the use of a transfection method. In addition, transfection based productions are characterized by potential recombination events between the plasmids leading potentially to the generation of replication competent viruses, even at very low frequencies [72], which should be avoided. Thus other processes using culture of cells in suspension have been developed and are used for the production of AAV vectors for clinical and/or commercial use;
- Stable HeLa or A549 cell clones: This system is based on the use of stable HeLa- or A549-cell clones. Only HeLa-derived producer cells have

been used for large scale AAV production. As packaging cell lines they contain the AAV *rep-cap* genes for packaging AAV DNA [73] whereas as producer cell clones they contain both *rep-cap* genes and the rAAV-vector sequence [74–77]. They are infected with a wild-type Adenovirus and a hybrid Adenovirus-AAV vector, or only with a wild-type Adenovirus, respectively, for inducing AAV production. The use of producer cell lines is more straightforward than the use of packaging cell lines requiring a sequential double infection. Specific vector production rates beyond 10^4 vg/c have been reported.

- HeLa based producer cell lines have been developed and rigorously characterized by Targeted Genetics Corp. [78] and AAV production had been scaled up to 250 l using cultures performed in STRs [25], generating consistently in the order of 10^{16} DRP (DNase resistant particles or vg) after infection with wildtype Adenovirus 5. Such vector quantities are required for late stage clinical trials. A 2000 l scale is planned [22,25] and will be the largest production scale ever been considered for the production of AAV vectors;

- Recombinant herpes simplex expression system: This system is based on the use of Herpes simplex virus 1 (HSV-1). It has been demonstrated that replication defective HSV vectors, deficient in ICP-27 gene are useful vectors for the generation of AAV vectors using mammalian cells [79,80]. A highly efficient rHSV-based rAAV complementation system has been reported [81]. It uses two rHSV vectors, one harbouring the ITR-flanked gene of interest (rAAV-vector) and the second one bearing the *rep* and *cap* genes. In principle, HEK293 (adherent, but can be adapted to suspension growth) and BHK-21 cells (suspension) can be used for AAV production, however, BHK-21 are advantageous because of a higher specific AAV vector productivity. The infection of BHK-21 cells at 1.4–2.1 × 10^6 c/ml in suspension culture using a 10 l-WAVE bioreactor with both rHSV-1 viruses (4:2 MOI ratio for rHSV-rep/cap:rHSV-AAV vector) led to the production of 85,400 DRP/cell. Thomas *et al.* [23] reported average volumetric productivities of 2.4 × 10^{14} DRP/l when using disposable 10 l-WAVE bioreactor, which is the largest scale published so far for this specific production;

- SF9/baculovirus system: This system is based on the use of Sf9 insect cells grown is suspension and infected at a cell density of approximately 10^6 c/ml with two recombinant baculoviruses providing the AAV *rep* and *cap* genes on one baculovirus and the rAAV vector on a second baculovirus (MOI = 0.05 per baculovirus) [82]. The volumetric production yields are in the range of 1.1 × 10^{14} vg/l (AAV1) when using 10 l STR thus achieving productivities comparable with the HSV-1 based production system and larger production scales of up to 200 l have been established and are routinely used [27].

Table 2 compares the different production systems as well as the established production scales. Concerning the specific vector yield per cell, all expression systems except the transfection system based on adherently grown HEK293 cells are comparable and the specific yield ranges between 10^4 and 10^5 vg/c. However, as already underlined, a scalable expression system should be based on the use of a suspension culture process.

All suspension based production systems, including the suspension based transfection process, generated vector titers ranging from 5 × 10^{13} to 2 × 10^{14} vg/l or DNAse resistant particles/l (DRP/l). These differences are essentially due to different production cell densities and differences in the vector specific production rates. In addition, it has to be kept in mind that the titration methods are not harmonized making a real comparison challenging.

The full to empty particle ratio is also an important issue for clinical use of rAAV vectors, although some purification protocols are capable to reduce or remove the fraction of empty particles, either by classical gradient centrifugation [83] or ion-exchange chromatography [84]. However, it is evident that if the ratio between full and empty particles is high at the harvest stage then the overall purification protocol is more efficient because of the reduction or absence of competition between full and empty particles for ligands of any chromatography step. Although none of the large scale AAV vector preparation protocols have been compared side by side to get a clear view on the ratios of full to empty particles, literature indicates that this ratio is beyond 70% of full particles for the use of HeLa cell based stable producer cell lines [85], essentially free of empty particles for the Herpes simplex based production system [24], and beyond 50% for the Sf9/baculovirus system [55]. However, it is important to underline that the size and the nature of the transgene as well as the production of single-stranded AAV or self-complementary AAV can have an important impact on the full to empty particle ratio.

With respect to genetic stability, cell substrates used in cell suspension cultures have shown stability during productions at scales of several thousand liters. Genetic stability of HeLa-derived stable producer cell lines has been demonstrated for more than 60 popula-

Table 2. Production yields of rAAV using different production systems.

Production method	Cell number at induction of AAV production	Final vector titre	Yield (vg/cell)	Scale-up, production system	Ref.
Adherently grown HEK293, triple transfection†	10^9c /CF10 = 151 × 10^3 c/cm^2	1–2 × 10^{12} vg/CF-10, 1 CF-10 ≈ 1 l	10^3 – 2 × 10^4	Limited scale-up: CellFactory (CF-10), roller, CellCube	[17–20]
HEK293 cells grown in suspension, tripletransfection†	5–8 × 10^5 c/ml	>10^{14} vg/l	≈10^5	WAVE bioreactor (probably several tens of l)	[21]
HeLa based producer cell, rAAV production induced by infection with wt Ad5‡	Information not available (probably: 10^6 c/ml)	>5 × 10^{13} DRP/l	>5 × 10^4	250 l stirred tank reactor, perspective of 2000 l scale production	[22]
rHSV-1 expression system: sBHK cells are infected with 2 different rHSV-1 vectors	1.4 × 10^6–2.1 × 10^6 c/ml	≈2.4 × 10^{14} DRP/l (AAV1)	7.4 × 10^4 – 1.1 × 10^5	10 l WAVE reactor	[23,24]
Baculovirus system: Sf9 infected with 3 recombinant baculoviruses	Approximately 10^6 c/ml	9.4 × 10^{13} vg/l (AAV1)	10^4–10^5	50 l stirred tank reactor	[25]
Sf9 infected with two different reccombinant baculoviruses	1.6 × 10^6 – 1.8 × 10^6 c/ml	8.3 × 10^{13} vg/l (AAV6)		200 l stirred tank reactor	[26]
Sf9 infected with two different reccombinant baculoviruses	Approximately 10^6 c/ml	4.2 × 10^{13} vg/l (AAV8)		200 l stirred tank reactor	[27]

†Triple transfection means the transfection is done using three different plasmids.
‡Production levels not shown for AAV packaging cell lines.

tion doublings [25] and the recombinant viruses for the herpes simplex and the baculovirus based production systems have been shown to be stable for 13 [81] and 7 [82] successive passages, respectively. In addition, no generation of rcAAV has been reported for any of the suspension processes except for the transfection based production process [72].

From a practical point of view all suspension based production systems can be used in WAVE and stirred tank bioreactors, with a certain advantage in favour of the STR due to the easiness in scaling-up as well as the possibility to infect cultures at higher cell densities than 10^6 c/ml due to the better mass transfer characteristics of STR. Today bioreactor scales of 200–250 l are routinely used for the production of clinical grade AAV vectors and the use of a 2000 l bioreactor scale is under consideration [22,25]. Such a scale is also foreseeable for the Herpes simplex and the baculovirus-based production systems.

All AAV production systems using suspension cell culture processes described in this section were used for the production of preclinical or clinical vector preparations either for performing bridging studies for the switch from the transfection system to the Herpes simplex/BHK production system [86] or for various clinical trials in the case of the use of stable producer cell lines (HeLa cell-based) [25]. The Sf9/baculovirus system is already used for commercial production after having received the marketing authorization by the EMA for Glybera® [87].

γ-retroviral vector production

In contrast to most of the other viral vectors, γ-retroviral vectors can be produced using stable producer cell lines which have been developed first using mouse fibroblast cell lines and later human cell lines mainly due to safety considerations (for more details, see Stacey and Merten [88]). Today the generally recommended and accepted method is to use stable packaging cell lines in which a plasmid expressing *gag* and *pol* along with a plasmid expressing an *env* gene have been incorporated into the cell line (Figure 7). A vector plasmid can then be introduced, and stable transfectants are obtained with the aid of a drug selection marker. The population or individual high titer clones can then be used to generate a stable Master Cell Bank (MCB). Only in the case that no stable producer cell line is available transient production based on transfection can be envisaged.

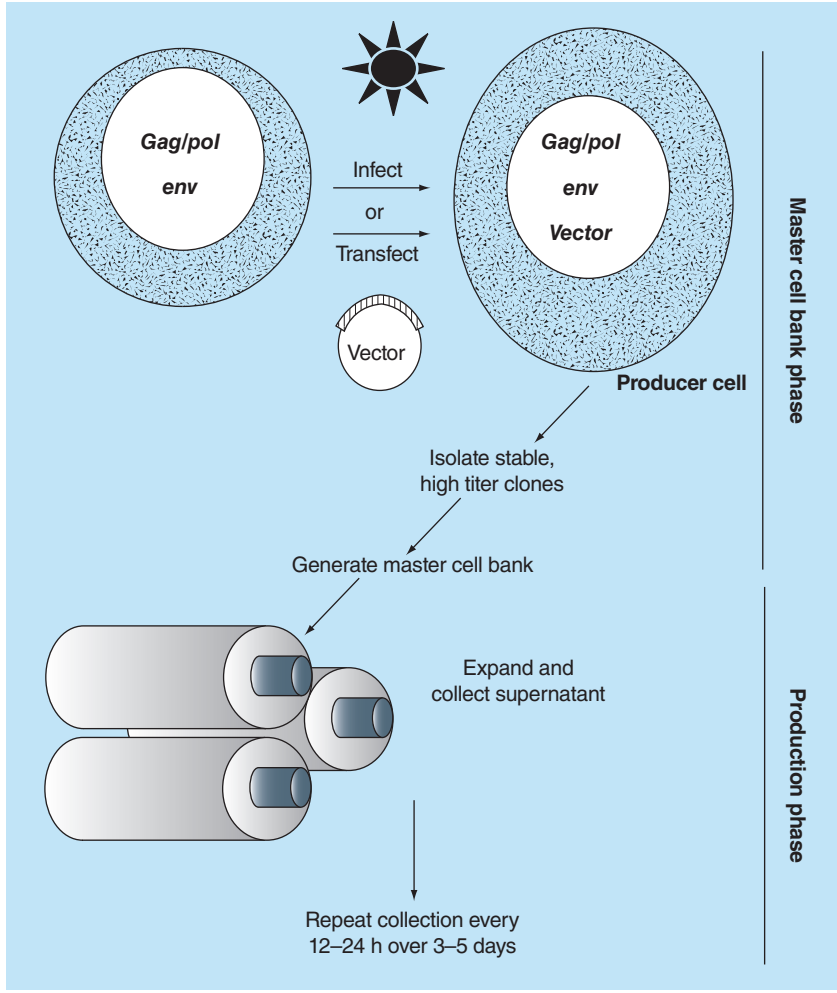

Figure 7. Retroviral vector production using producer cell lines. Producer cell lines are generated from packaging cell lines that are stable cell lines expressing the viral *gag*, *pol*, and *env*genes. A vector plasmid is introduced by transfection or the vector is introduced by infection with vector particles. Stable, high-titer clones are then isolated. The optimal clone is expanded to a MCB that is then certified. A vial of the MCB is expanded to generate vector. The cells generally maintain high titer throughout multiple harvests for 3–5 days. In the case of transient vector production cells are transfected with three plasmids and the supernatant is harvested during several days after transfection without selection of a stable producer cell line.
Reproduced with permission from Springer Science and Business Media [89].

Stable cell clones can then be used for small scale and large-scale productions according to the quantities required. In the case of large-scale vector productions, optimization of critical culture and production parameters is of high importance. Data on the impact of the variation of the following parameters on vector production have been reviewed by Merten [31]: use of butyrate, glycocorticoids, serum (10% FCS – serum-free), and different sugar sources (glucose, fructose, galactose, mannose, or others), pH, pO_2, production temperature (32°C, 34°C, 37°C), and medium perfusion rate or medium change rate per day.

Production systems for the large scale manufacturing of MLV vectors for preclinical & clinical applications

For clinical purposes large-scale production systems are needed in order to produce the quantity of γ-retroviral vector required for the treatment of the patient and for lot certification (by quality control – QC). Although γ-retroviral vectors are secreted into the supernatant, as for most of the recombinant proteins produced by the biotech industry, there are some very important differences between secreted recombinant proteins and secreted γ-retroviral vectors. Amongst these differences, the overall instability

and limited storage stability of the produced vector should be mentioned.

It has been shown that retroviral vectors are not stable when stored at 37°C [31] therefore after release of the vector into the supernatant the vector has to be harvested as rapidly as possible and stored at low temperature for further treatment and finally stored at -80°C. This implies for the manufacturing process that the supernatant has to be rapidly harvested between once and up to several times a day if the production is performed with roller bottles and CFs (repeated batch mode) or continuously if a bioreactor based perfusion process is used. Although many different culture systems have been tested and described (for details, the reader is referred to the review by Merten [31]) only very few systems have really been used for the production of clinical grade MLV vectors. Smaller preclinical and clinical grade quantities were generally produced with roller bottles [28,30] or CFs [29,32,34,51], whereas large vector preparations have been produced using fixed bed bioreactors (CellCube) [30,34,90–91] as well as Wave bioreactor [92]

Although roller bottles and CFs could be used for more than one week (see above) all large- scale productions were limited to the harvests on 3–4 consecutive days (Table 1). For both production systems, depending on the number of culture units the overall harvest volume could be adapted to the needs. Whereas, for the CellFactory systems 600 to 650 ml of supernatant/medium (medium volume required for culture) were changed per day (reference: 1 CF-10 unit = 6320cm^2) representing a change of about 0.1 ml/(day.cm^2), the medium change rates per day were higher for the roller bottle system and varied between 0.14 and 0.31 ml/(day.cm^2) (or 118–267 ml per roller bottle and harvest (using 850 cm^2 in roller bottle as a reference)) indicating that the CellFactory system would produce high titers at a more reduced volume than the roller bottle system (Table 1). Data from literature indicate volumes ranging from 30 l [30], to 54 l [28] and 80 l [Zhang, Pers. Comm.]. Using the CellFactory systems (4x CF-10 or 1x CF-40), only 7.8–9.6 l were produced within 3–4 days.

For both systems, the overall vector production cannot really be compared because different cell lines, different vector constructs and different titration methods were used. However, the overall vector quantities are indicated, for information, in Table 1.

With respect to the CellCube system, literature data indicate overall production volumes ranging from 10 l [30] to 400 l [34] resulting from the scale used and/or number of harvests. The perfusion rate can be chosen according to the requirements of the cell metabolism and/or the production needs. Literature data indicate perfusion rates ranging between 0.045 and 0.618 ml/(day.cm^2) (Table 1). This is an important difference in comparison to roller bottle or CellFactory system. In principle it is possible to produce higher vector titers using reduced perfusion rates, however, this depends on the medium used and the rate limiting components present in the medium for cellular functions. In contrast to the roller bottle and CellFactory system, the duration of the production phase can be easily prolonged beyond 3 weeks, which can be easily achieved because perfusion culture can be automated, allowing thus production of large quantities of vector supernatant. All the supernatant collections are kept at 4°C to minimize virus degradation, which are later pooled for further processing. It is important to make sure that the cells do not loose partially their vector production yield when confluent.

Table 1 presents the scales of the systems used for clinical grade vector production, the type of the producer cells, the volume produced, the daily perfusion rates the total daily vector quantity produced as well as the duration of production.

Since only few comparative studies on the different production systems have been done, it is very difficult to indicate which system is the most optimal for MLV vector production. By comparing the system performance based on equivalent cell culture surface, the CellCube system is preferable because after optimization it delivers up to five-times higher vector quantities in comparison to the roller bottle system [90] although Wikström *et al.* [90] have reported the opposite. This might be explained by insufficient optimization or an insufficient cell number in relation to the very high perfusion rate used (0.30–0.62 ml/(day.cm^2)) whereas Kotani *et al.* [90] used a perfusion rate of 0.061 ml/(day.cm^2) (Table 1). No comparative study between the CellFactory and the CellCube production systems has been published in literature; however, the general advantage of a bioreactor system is that the pH and pO_2 values can be set to values optimal for cell culture and production of the vector. For the roller bottle or CellFactory production systems, these parameters are not controlled and vary during the course of the culture. Together with other fixed bed systems such as the basket reactor system from NBS (Celligen), the CellCube system is the most feasible production system of MLV vectors when adherent producer cells are used [31,91].

The main disadvantages of the CellCube system is that some parts, such as the oxygenator and the probes have to be cleaned and autoclaved between subsequent production runs because it is a semi-disposable system. This feature is specific for the CellCube system and does not concern other fixed bed reactor systems (e.g., basket reactor system from NBS or the novel iCEL-

Lis system from ATMI). In general, classical reactor systems were conceived as reusable systems, needing cleaning, sterilization as well as extensive validations which are not necessary for disposable systems, such as the CellFactory, the roller bottle and the iCELLis systems.

Recently, the iCELLisNano system (ATMI) was evaluated for the production of γ-retroviral vectors in view of replacing a CellFactory based production protocol allowing the production of approximately 24 l over a duration of 10 days producing thus 9.23×10^{11} TU (in a pool of ten harvests) whereas the parallel production performed with a CellFactory based process described by Przybylowski *et al.* [29] delivered only 1.9×10^{11} TU (pool of three harvests using six CF-10 units) [93]. These recent data indicate very clearly the evolution of the large-scale production processes for MLV and in the future for LV vectors whenever efficient stable cell lines will be available from multiple parallel process systems like roller bottles or CFs to scalable bioreactor systems allowing the continuous semi-automatic or automatic production of these viral vectors.

Transient production of MLV vectors

Whenever the development of a stable producer cell line is impossible because of the toxicity of either the envelope protein (e.g., VSV-g) or the expressed transgene or in the case of speeding up the time to production at a larger scale, the same plasmid constructs utilized for the establishment of the packaging cell lines, can also be used for the production of preclinical and clinical grade material via transient transfection. Generally, three plasmids are used to encode the vector, the viral *gag* and *pol* gene, and the envelope gene sequences (Figure 7). These can be introduced using various techniques, such as calcium phosphate transfection, lipofection, PEI or electroporation. Cell lines such as HEK293/HEK293T and HT1080 cells tend to be the cells of choice due to their high transfection efficiency and ability to produce vectors at high titers. Recently, Schambach *et al.* [94] reported on a large scale transfection process using a WAVE bioreactor in which after transfection 293T cells are cultivated on Fibra-Cel carriers (100 g carriers for a WAVE bioreactor of 10 l) for several days generating three vector harvests. This production system was adapted for the production of clinical vector batches.

Currently, there is rather limited process development in the field of γ-retroviral vector (MLV) production because MLV vectors are being gradually replaced by lentiviral vectors for most of the applications and development efforts are focusing on LV vectors. However, all the lessons learned in developing MLV production processes are very valuable for the development of advanced LV production processes.

Lentiviral vector production

Due to the difficulties to establish stable LV producer cell lines because of the cytotoxicity of several vector components, such as the VSV-g env protein, large-scale LV manufacturing is essentially exploiting the transient transfection method with 2–5 plasmids to provide all the required functions. However, recently the situation started to change because new stable producer cell lines that have the characteristics required for the production of clinical grade material are becoming available.

Production of lentiviral vectors based on the use of transient transfection protocols

The optimization of the process considers essentially few parameters such as the selection of the transfection agent, the ratio between transfection agent and DNA as well as the ratio between the plasmids required for inducing vector production. Culture and production parameters can be optimized as already mentioned for the production of γ-retroviral vectors. Further details can be found in a review by Ansorge *et al.* [95].

Traditionally, large-scale productions for pre-clinical and clinical applications are essentially based on the use of multiple units of 2 or 10 stack CFs, which can be harvested once or several times after transfection. The transfection is performed either by the calcium-phosphate precipitation method or by using PEI [95]. Rather recently, suspension based transfection processes have been evaluated using either PEI based transfection [96] or flow electroporation which has the advantage of performing transfection without the concomitant addition of transfection agents [97]. Large-scale production runs are often harvested one- to three-times while maintaining the overall vector productivity, whereas for research grade productions up to five harvests may be performed.

Depending on the production volume (between 600 and 1000 ml per CF-10 CellFactory – as above mentioned) and the number of harvests, the maximum volume of the non-purified bulk product batches ranges between 20 l and 52 l when using 12 to 24 CF-10 CFs [14,98]. In order to produce larger volumes of a drug product (purified and vialed product) than possible with a given set of CFs, several harvests of successive campaigns can be pooled [99]. The harvests of single runs are treated separately leading to the purified bulk product per run which is separately certified for quality. For generating a lot of drug product the necessary number of aliquots of purified bulk is thawed, pooled and assessed for sterility [99] and vialed. As an

example, the Beckman Research Institute performs up to six successive production cycles of 20 CF-10 that are weekly started from subcultured producer cells. This production scheme can be maintained for up to 6 weeks allowing thus the production of 120 l in total of vector containing supernatant (1 l/CF-10) [99].

Generally vector titers reached in the unprocessed supernatants range from 0.1 to 5 × 10^7TU/ml, and are thus in the range published in previous reports using similar transient production processes with 293T cells.

For more information the reader is referred to Schweizer and Merten [15].

Production of lentiviral vectors using stable producer cell lines

The recent development of more efficient producer cell lines of LV vectors has opened the way to the production of clinical grade LV vectors and today two different cell lines have been or are under development for GMP production. It has to be kept in mind that inducible cell lines are required in the case where cytotoxic vector components are used for vector production, including the traditionally used VSV-g env protein derived from the Vesicular Stomatitis Virus (VSV), and the non-modified lentiviral protease. Greene *et al.* [48] have evaluated an HEK293T/17-based inducible producer cell line (tet-off induction) producing a VSV-g pseudotyped LV vectors for vector production in a WAVE bioreactor. Since the medium has to be changed for removing doxycycline in order to induce vector production the cells are grown on a support (Fibra-Cel disks). Such a production system (bag scale: 10 l [for developmental purpose] or 50 l [for production purposes]) can generate 5 l and 25 l of vector-containing supernatant per batch harvest, respectively. The harvests between day 3 and day 6–8 post-induction are pooled and purified. Large-scale production runs generated between 130 and 140 l per run with a vector titer ranging from 1 to 2 × 10^7 TU/ml.

Recently, Stornaiuolo *et al.* [100] generated a novel HEK293T based producer cell line producing RD114-TR pseudotyped lentiviral vectors. Due to the lack of toxicity of the RD114-TR envelope protein, this cell line is non-inducible and vector production is constitutive, as for classical MLV producer cell lines, at a level of approximately 10^6 TU/ml. Although for the moment no large scale data are available, this cell line is of high interest for the large scale production of LV vectors for the transduction of hematopoietic stem cells for which RD114 pseudotyped vectors have shown a 2 log higher efficiency than VSV-g pseudotypedlentiviral vectors.

Conclusion & future perspective

Cell culture technologies have been successfully adapted for the production of viral vectors to meet

Executive Summary

Introduction
- Bioproduction of clinical grade viral vectors has implications for gene therapy approaches used in the treatment of various inherited or acquired diseases.

Viral vector production
- Different technologies have been adapted for commercial-scale cultivation of animal cells to produce viral vectors. For a larger amount of product, the most effective method is suspension cell culture, as compared with processes using adherent cells. Suspension cell processes in serum-free media have been operated in batch, fed-batch or perfusion mode enabling high yielding productions

Bioprocessing of viral vectors
- The four viral vectors that have been the most extensively used in clinical trials include adenoviral vectors, adeno-associated viral vectors, γ-retroviral and lentiviral vectors.
- Production of adenoviral vectors includes the process 'Helper-Dependent Adenovirus Production'.
- Suspension-based transfection processes, as well as three different scalable AAV vector production systems, have been developed for the production of adeno-associated viral vectors.
- Large-scale lentiviral vector production essentially exploits the transient transfection method.

Conclusions and future perspective
- Cell culture technologies have been successfully adapted for the production of viral vectors, however subject to various product critical attributes and process critical parameters scale-up might be still limited requiring more research and development
- Currently the development of inducible or constitutively expressed lentiviral vector producer cell lines is making significant progress.
- With the recent marketing authorization of the first gene therapy medicine, it is envisaged that production technologies will be further improved and scaled-up in the near future.

Downstream processing
- Part II of this article will address the downstream processing of adenoviral, AAV, γ-retroviral and lentiviral vectors.

the needs of early and advanced clinical trial phases. However, depending on the type of vector and the cell culture production platforms selected scale-up might be still limited. In general, for all suspension culture production systems using either constitutive or infection induced expression further scale-up does not present significant challenges. This is the case for the manufacturing of adenoviral and AAV viral vectors and eventually γ-retroviral (MLV) vectors, but in the case of lentiviral vectors, a scalable production method still remains to be developed. Currently the development of inducible [101] or constitutively expressed [100] lentiviral vector producer cell lines growing in suspension is making significant progress. The first lentival vector lot produced by a stable producer cell lines was recently used within a clinical trial context [48]. Clearly gene therapy is showing remarkable progress with extensive safety and efficacy data accumulated in numerous clinical trials. Furthermore, with the recent marketing authorization of the first gene therapy medicine in Europe, production technologies will be further improved and scaled-up for providing viral vectors with well-defined quality attributes to respond to increasing needs in the broad field of gene therapy.

Downstream processing

All viral vectors have to be rigorously purified when they are destined for a clinical application. Part II of this article will address the downstream processing of adenoviral, AAV, retroviral and lentiviral vectors.

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