

State-of-the-art of the production of retroviral vectors

O.-W. Merten*

Généthon, 1bis Rue de l'Internationale, BP 60, F-91002 Evry-Cedex, France

*Correspondence to: O.-W. Merten, Généthon, 1bis Rue de l'Internationale, BP 60, F-91002 Evry-Cedex, France. E-mail: omerten@genethon.fr

Summary

Retroviral vectors are still the vectors that are used in the majority of gene therapy trials for treatment of acquired or inherited diseases. In this review, the present state-of-the-art of the production of retroviral vectors and the most important parameters, such as the choice of the producer cell line, stability issues, medium additives, serum, type of bioreactor, that influence production issues is presented and discussed in light of an optimal vector production. The available literature data clearly indicate that, on one hand, the choice of the producer cell line is of utmost importance for obtaining a high level producer cell line, and that, on the other hand, the optimization of the medium, e.g. the replacement of glucose by fructose, has a potential for improving vector production rates and titers. Finally, the use of high-density perfusion culture systems for adherent as well as for suspension cells presents the best choice for a production system, because high cell densities imply high reactor specific production rates, which must be associated with a rapid harvest of the produced vector, thus avoiding vector inactivation due to an extended residence time. The overall optimization of the cultivation and production parameters will have a significant impact on the use of retroviral vectors for gene therapy purposes. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords retroviral vectors; bioreactors; culture conditions; stability; producer cell lines

Introduction

The successful implementation of gene therapy approaches in the clinic still awaits the development of optimized processes for the manufacture of gene transfer vectors. To date, a majority of clinical trials have used vectors derived from murine leukemia viruses (MLV) ([1], <http://www.wiley.co.uk/genmed>). Despite their limitations (i.e. relatively low titer and activity, limited application to dividing cells), MLV vectors lead to permanent gene transfer and expression and the first clinical success of gene therapy was obtained using this technology [2].

From the technological point of view, the main problem associated with the use of retroviral vectors is the relatively low vector titer generally obtained in non-perfused low scale and/or, in general, in non-optimized production systems. The titers seldom rise above 10^6 infectious particles (ip) per mL. At the production temperature of 37°C, which is used in most of the production protocols, a vector half-life of only 2 to 9 h was observed [3–8]. In order to harvest the retroviral vector before inactivation, and also to improve the whole culture and production conditions, perfusion processes, or in general processes allowing a rapid and regular exchange of the production medium, are required. The optimization of such processes with respect to both process conditions and also medium composition leads to a much improved vector production and productivity ([9], and unpublished results).

This overview describes the present state of the production of retroviral vectors in various bioreactor systems, compares these systems, and tries to give an answer to the question "what is the best system for cell growth and retroviral vector production".

Choice of the production cell line

Vector construction and problems with RCRs

The first packaging/complementation cells for producing retroviral vectors were based on the use of mouse NIH 3T3 cells. First- and second-generation cell lines, such as psi-2 and the PA317 cells, respectively, were constructed by using only one plasmid containing all necessary functions for the synthesis of retroviral vectors. In order to avoid the production of replication competent retroviruses (RCRs), some retroviral functions were deleted or inactivated in the plasmid, e.g. the ψ packaging signal (in the case of the first-generation cell lines) or the ψ packaging signal as well as the 3'-LTR sequence (in the case of the second-generation cell lines). Such constructions are rather simple and only one or two homologous recombination events are necessary to achieve the generation of RCRs [10]. This problem led to the development of third-generation packaging cell lines with a split genome, indicating that the necessary functions were separately transfected into the cells. To construct such cells, three plasmids are used, containing the gag-pol, the env, and the vector with the transgene, respectively (Figures 1 and 2). The plasmids are constructed in such a way as to reduce sequence overlaps as much as possible, because already an overlap of eight consecutive nucleotides is sufficient to increase the probability of homologous recombination and, thus, RCR production [11]. In addition, the gag-pol and env plasmids no longer contain the retroviral LTRs (Figure 2). In these third-generation complementation cells such possibilities of homologous recombination events between plasmid DNAs used for transfection are generally avoided, although RCR-generation caused by recombination events between the vector, one of the packaging constructs, and endogenous retroviral sequences in a third-generation murine packaging cell line has been described [12,13]. More details can be found in the reviews by [14] and [15].

Historically, packaging cell lines were based on the use of mouse cell lines (NIH 3T3). However, certain limitations of these packaging cell lines have initiated the search to improve them. Principally, mouse cell lines are associated with the following drawbacks: They produce relatively low titers (see later). Furthermore, murine retroviral sequences that are present in murine packaging cells can be selectively packaged into retroviral particles [16] increasing the possibility of generating RCRs. Therefore, the use of human cell lines for the establishment of packaging cells is a step towards

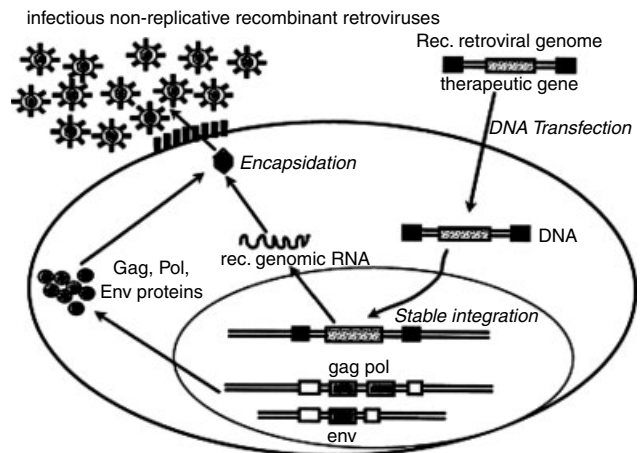


Figure 1. Functioning of a third-generation MLV-packaging cell line (from [14])

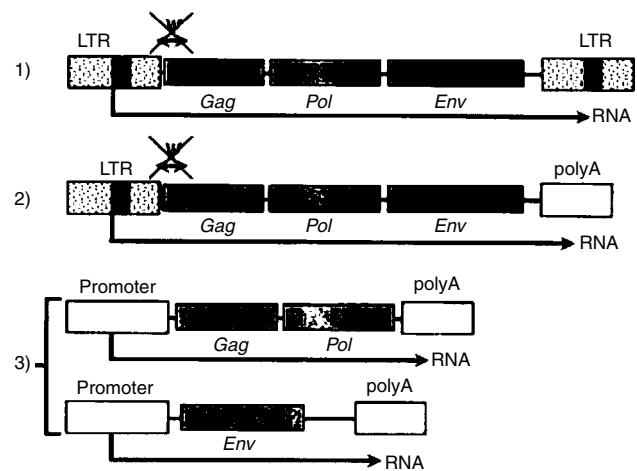


Figure 2. Structures of some expression plasmids used to generate packaging cells: early generation of packaging cells containing complete viral genomes deleted only from the ψ packaging signal (1) or from both ψ and the 3'-LTR (2). Such cells (e.g. psi-2 and PA 317 cell lines) can produce potentially pathogenic replication-competent retrovirus (RCR) particles by recombination of the vector DNA with viral sequences stably integrated in the packaging cells. Improved complementation cell lines were more recently produced by transfection of expression plasmids with the MLV gag, pol, and env genes under the control of heterologous promoter and polyadenylation signals (3) (from [14])

increased biological safety, because they lack endogenous murine retroviruses [10,17,18]. In fact, viral supernatants or producer cells derived from human cells have never given a positive test result for RCRs in small- or large-scale assays [19].

Patience *et al.* [20] showed that retroviral vectors interact with human packaging cells (FLY cells which are based on HT1080 cells) to produce retroviral particles that are far less contaminated by endogenous viral sequences or other types of extraneous particles than murine packaging cells (the murine AM12 packaging system which is based on the use of NIH 3T3 cells). Farson *et al.* [21] showed, that, in contrast to the mouse

system, where the ratio of the transmission of recombinant retrovirus and of the murine VL30 was about 1 : 1, the 293-based production system transmitted HERV-H elements at a ratio of at least as low as 1 : 5×10^5 . In addition, because packaging cell lines derived from human cells lack endogenous murine retroviral sequences, the likelihood of producing RCRs is minimized [22].

Glycosylation and complement inactivation

It seems that the glycosylation of the retroviral particles (glycosylation of the env protein and of cellular proteins incorporated into the particles, lipid associated carbohydrates) has an impact on the stability/retention of retroviral particles in human serum. It is known that retroviral particles when produced with mouse packaging cell lines are inactivated by the human complement within 20 min after injection. This is generally considered to be due to the presence of the galactosyl(α 1-3)galactosyl carbohydrate moiety on the vectors produced by murine packaging cells, whereas such vectors produced in human or primate cells do not have this glycostructure and therefore are resistant to complement inactivation [17,23]. However, it has been shown that even retroviral vectors produced from a galactosyl (α 1-3)galactosyl carbohydrate positive ferret brain cell line (Mpf) are resistant to complement inactivation, signifying that it is not only the structure of the glycosylation of the env protein, but also other epitopes, such as lipid-associated carbohydrates (galactosyl(α 1-3)galactosyl carbohydrate moiety being only one of them), present on the surface

of the viral membrane where antibody binds and/or complement acts [24].

Adherent versus suspension growth

Finally, the question of the use of a cell line growing in an attached mode versus a suspension cell line is also of importance because production processes based on the use of suspension cell lines can be scaled up much easier than processes making use of adherently growing cells.

These reasons led to the development of human-based packaging cell lines, in general, and of human cell lines growing in suspension, in particular (Table 1).

All murine packaging cell lines are based on the use of NIH 3T3 cells which are strictly adherently growing cells, signifying that only surface culture systems (T-flasks, cell-factories, fixed bed reactors, such as CellCube or the NewBrunswick Scientific basket reactor), microcarriers, macrocarriers, or hollow fiber reactors) can be used for cell growth and vector production. The human packaging cell lines FLY [23] and Te FLY [25] also grow adherently and lead to the formation of large clumps when grown in suspension ([124]: FLYRD18, [9]: Te FLY GA18). Such clump cultures generally show a strongly reduced growth rate (only the cells at the periphery are growing), and the titers obtained are 10 to 100 times lower than those obtained in adherent cultures [9]. Packaging cells based on the human embryonic kidney cell line, 293, can grow as attached cells when in serum-containing medium at normal Ca^{2+} concentration (DMEM: 264 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per L); however, these cells tend to enter into suspension already in serum-containing low Ca^{2+} media [27], and

Table 1. Some characteristics of packaging cell lines used for the production of retroviral vectors for gene therapy

Cell line	Species of origin	Type of growth: Suspension – adherent	Maximal titers at standard conditions (37 °C, T-flask culture)	Reference
PA317-GINa.40	NIH 3T3 (mouse)	adherent	3×10^6 CFU/mL	[3]
ψ CRIP-LLZA	NIH 3T3 (mouse)	adherent	6×10^6 FFU/mL	[64]
pMFG/ ψ CRIP			9×10^5 CFU/mL	[4]
PG13-MND-GFP	NIH 3T3 (mouse)	adherent	5×10^5 ip/mL	[47]
PG13-MFG-GFP			0.7×10^5 ip/mL	
Mpf	Ferret brain	adherent	8×10^2 to 6×10^4 CFU/mL	[24]
FLY A4 lacZ	HT1080 (human)	adherent	2×10^7 iu/mL	[23,120]
FLY RD18/LNC-hB7			1.2×10^5 CFU/mL	
HA-LB	HT1080 (human)	adherent	10^7 CFU/mL	[19]
Te FLY GA18	TE671 (human)	adherent	$1-5 \times 10^6$ ip/mL	[9]
ProPak-clones	293 (human)	adherent/suspension	2–3 times better transduction efficiency than for PA317 cells	[18]
Phoenix-clones	293 (human)	adherent/suspension	10^6 ip/mL	Our unpublished results
293-SPA-clones	293 (human)	adherent/suspension	Same titer as for ψ CRIP cells; however, 5–10 times better transduction efficiency	[22]
PUZikat2	293 (human)	adherent/suspension	$2-7 \times 10^6$ TU/mL (roller process)	[21]
DA	293 (human)	adherent/suspension	2×10^7 CFU/mL	[19]
CEM FLY-clones	CEM (human)	suspension	1.8×10^4 to 2.2×10^6 IU/mL	[7]

practically do not attach any more, when serum-free low Ca^{2+} media are used [28,29]. This indicates that they can be cultivated in T-flasks, cell-factories, fixed bed reactors (NewBrunswick Scientific basket reactor; however, not in the CellCube due to shear forces) and on microcarriers (e.g. Cytodex III [30]) or macrocarriers, and in hollow fiber reactors when they grow in an attached mode. In the case that these 293-based producer cell lines grow under suspension conditions (low serum and/or low Ca^{2+} levels), they can be cultivated in T-flasks and cell-factories (which is not very efficient); however, all reactor systems specially developed for suspension cultures can be used: spinner flasks, stirred tank reactors with and without cell retention, fixed bed reactor systems (NewBrunswick Scientific basket reactor), fluidized bed reactors. This is also valid for CEM FLY based suspension cells which are absolute suspension cells [7].

Influences on vector production and vector titers

The maximum titers obtained in small-scale cultures of murine packaging cells (Table 1) range from 0.7×10^5 (PG13-MND-GFP) to 6×10^6 units per mL (ψ CRIP-LLZ), which are only slightly inferior to those obtained in human packaging cells ranging from 1.2×10^5 (FLYRD18/LNC-hB7) to 2×10^7 (FLYA4 LacZ, DA). Several issues have to be considered in this context:

1. Construction of the vector backbone and the transgene to transfer

The construction of both, the vector backbone [31] and the transgene to transfer, has an impact on vector production rates and titers. By using the same type of complementation cell line, Byun *et al.* [31] showed that titers and transgene expression levels were higher with MFG- than with LNCX-based vectors. In agreement with this observation, we observed that the use of the MND backbone (transgene: GFP) led generally to lower vector titers (0.7×10^5 ip/mL) than the MFG backbone (2×10^5 ip/mL) (Table 1). In addition, the expression of the transgene can be toxic for the producer cells (e.g. GFP) or can significantly reduce vector production (e.g. overexpression of the Wiskott-Aldrich syndrome protein (WASP) in cells producing retroviral vectors with the WASP-transgene led to a considerable reduction in vector production rates (L. Dupré, personal communication), probably due to the interaction of the WASP with lipid rafts which are the privileged sites for the synthesis and budding of viral particles [32–34]). Thus, in order to obtain an optimized producer cell line, the vector backbone sequence and the promoter have to be optimized for the ultimate application.

2. Stoichiometric amounts of viral proteins and viral RNA, vector copy number, etc.

The design of the genetic elements and the optimal ratio of viral proteins and retroviral genomic RNA are important

factors in order to achieve high-level productions of retroviral vectors. It is known that there is a threshold number of provirus copies required for efficient virus production [35], and also that the copy number of the provector in the producer cells has a direct influence on vector yield. However, this is often associated with an increased probability to produce RCRs (e.g. in the case that 'ping-pong' strategies are employed [36,37]). In this context, Sheridan *et al.* [19] demonstrated that the incorporation of multiple provector copies into the producer cells (following a high multiplicity of infection of vectors with reduced sequence homology) led to higher vector titers without the occurrence of RCRs. They obtained producer clones able to produce titers up to 2×10^8 CFU/mL. In the case that high levels of gag, pol, and env proteins are expressed with a balanced stoichiometry, it seems that the titer of the recombinant retrovirus is determined primarily by the levels of packagable RNA, suggesting that viral RNA synthesis may be the rate-limiting step in the production of active retroviruses [38]. An additional important issue is the sufficient production of the env protein, in particular in producer cell lines synthesizing the corresponding receptor in parallel to the env protein. In transient production studies, Yap *et al.* [39] showed that vector titers from 293 cells transiently producing amphotropic viruses were influenced by the availability of the amphotropic envelope protein. However, this was not the case with the ecotropic vector because the 293 cells do not express the ecotropic receptor. This suggests that the premature interaction between envelope and receptor in producer cells could limit the amount of envelope available for virion incorporation.

Thus the optimal choice of the elements to construct producer cell lines is a very important prerequisite for achieving high titer vector preparations.

3. Extracellular factors

All adherent cells produce variable quantities of extracellular matrix proteins partially consisting of proteoglycans. These macromolecules are negatively charged, are of variable size, and act as inhibitors during cell transduction, thus eventually reducing significantly the transduction efficiency despite high vector titers [40–43].

Indeed, the choice of the packaging cell line is of utmost importance. Le Doux *et al.* [40] observed that NIH 3T3 based producer cell lines (ψ CRIP) produced important quantities of proteoglycans, which, at $0.2 \mu\text{g/mL}$, were strongly inhibitory for cell transduction. In similar studies, we observed a relatively important production of proteoglycans in reactor cultures of Te FLY cells. At the end of CellCube cultures (11 days after inoculation, total cell number: 28×10^9 c/module 25), $12\text{--}19 \mu\text{g/mL}$ of proteoglycans were detected. Titration assays clearly showed that higher titers were obtained in serial dilutions (= non linear dose-response curve) indicating thus the presence of transduction inhibitors (unpublished results comparable to those from [40] for NIH 3T3

derived retroviral vectors). As all adherently growing cells produce extracellular matrix, variable inhibitory effects could be observed in function of the quantity of proteoglycans produced. However, as suspension cells or those adherently growing cells which have a tendency to grow in suspension produce much less or no extracellular matrix, they are expected to produce retroviral vector preparations with a much higher transduction efficiency, as has been shown by [10,18,44] and [21]. Davis *et al.* [22] observed similar titers with ψ CRIP and 293-SPA cells. However, the transduction efficiency of the vectors prepared from 293-SPA cells was 5 to 10 times higher than that of the vectors prepared from ψ CRIP cells. Forestell *et al.* [18] also reported that the transduction efficiency of ProPak (293 based) vector supernatants was 2–3 times higher than that of PA317 vector supernatants (Table 1).

However, for the moment, no comparative data on transduction efficiencies or on the eventual production of inhibitory substances by FLY and CEM FLY cells are available.

Culture conditions – medium composition – metabolic effects

Not only the choice of the producer cell line, nor the construction of the plasmids alone, is responsible for the production of high titers and high specific production rates. The medium composition and the culture conditions in general also have an important impact on the production rates and final titers. Factors described to improve vector production are briefly presented and discussed.

Butyrate

The addition of sodium butyrate (mM range) in the culture medium increased retroviral vector production in mouse producer cell lines (PA317, PG13) by 20- to more than 1000-fold, probably due to an increase in steady-state levels of full-length vector RNA [45]. Pagès *et al.* [46] observed a five-fold increase in vector production with ψ CRIP cells in the presence of 5 mM sodium butyrate. This increase was associated with increased levels of genomic vector RNA. GP+E-86 based producer cells can equally be induced to higher vector production after sodium butyrate treatment. Although this approach is viable for certain producer cells, producer clones based on the use of Te FLY packaging cells did not show any positive effect on production rates and titers in the presence of butyrate (our unpublished results).

Olsen and Sechelski [45] also showed that the addition of sodium butyrate in association with a reduced cultivation temperature led to a synergistic increase in vector titers. The addition of sodium butyrate (15 mM) led to an 55-fold increase with respect to the reference condition (in sodium butyrate-free medium at 37 °C), whereas the increase was 330-fold when 15 mM of sodium butyrate

was added to cultures incubated at 32 °C (The reduction of the culture temperature from 37 to 32 °C in the absence of butyrate led only to a 8-fold increase in vector production.) Furthermore, positive effects of the addition of sodium butyrate were observed for the transient production of retroviral vectors using PA317 cells as well as for the production of HIV (as discussed by [45]).

Glucocorticoid

As MLV-LTRs contain different responsive elements, such as glucocorticoid responsive elements, Pagès *et al.* [46] showed that the joint addition of butyrate and dexamethason to retroviral vector producing cells could increase the vector production by a factor of 10. Again, like for the single addition of butyrate, this might be due to increased levels of genomic vector RNA. However, the single use of dexamethason was not sufficient. Similar results were observed when cholesterol, a precursor of the glucocorticoid synthesis, was added to low serum cultures of Te FLY GA18 cells. The addition of 5 to 10 μ g/mL led to an increase in the vector titer as well as in the vector production rate by a factor of 5 to 6 (our unpublished results). As no Northern blot analysis was performed to measure the viral genome levels within the cells, the reason for the increase is still unclear. Because cholesterol is an important component of cell membranes and of viral envelopes, the increase might also be due to an improved membrane synthesis.

Serum

Serum was also found to increase retroviral vector production by ψ CRIP cells, as a result of better cell growth [4]. However, with FLY RD18 cells, Gerin *et al.* [26] observed a 4-fold increase in virus titers under serum-free conditions in comparison with medium supplemented with 10% fetal calf serum (FCS), with a dose-dependent negative effect. The increase was probably due to the absence of protease inhibitors present in serum, which seem to inhibit virus production. However, for other cell lines, such as Te FLY GA18, the elimination of serum led to a significant reduction in vector production (our unpublished results).

Different sugar sources

Most of the cell culture media are supplemented with glucose, which presents together with glutamine the source for carbon and energy. The drawbacks of the use of glucose are its rapid consumption and inefficient metabolization associated with a rapid production of lactate and a reduction of the culture pH (due to the joint production of lactate and CO₂). At higher concentrations, lactate becomes toxic, and, beyond 5 mM, it inhibits growth of Te FLY GA18 cells and vector production [9], whereas PG13 cells were more resistant to high lactate

concentrations [47]. In principle, there are two ways to reduce the production of lactate and CO_2 : reduction of the concentration of glucose in the medium to very low levels or the use of a sugar which is differently and more slowly metabolized than glucose leading to reduced lactate production [47]. We could show that replacing glucose by mannose, fructose at a concentration of 5.5 mM did not alter the growth rate of Te FLY GA18, whereas the growth was reduced by a factor of 2–3 in the galactose-supplemented medium. Lactate production was 5–6 times reduced in fructose- and galactose-supplemented media, whereas it was identical in glucose- and mannose-supplemented media. The vector titers were 3–8 times higher than in glucose-supplemented medium, indicating an augmentation of the specific vector production rate by a factor of 2–3 in the glucose-free media (Figure 3, Table 2). Finally, fructose was kept for replacing glucose, because, at 24.75 mM, cell growth was higher in fructose than in galactose-supplemented medium at identical lactate excretion rates.

The positive effect of replacing glucose by fructose was not observed with only the Te FLY GA18 cell line, but it seems to be of general validity. In fact, we could show that different retroviral vector producing cell lines behaved in a similar way: Te FLY GA18, two clones of PG13 (Table 2), and two Tel CEB clones [8].

This enhancing effect of fructose was significantly increased in bioreactor cultures [8]. Te FLY GA18 cells gained a 1.4 times higher biomass after the passage of 20 L of medium when cultured in a NBS basket reactor. The fructose culture produced about five times more total infectious vectors than the glucose cultures, indicating that the specific vector production rate increased by a factor of 2.8 (Table 3). Although PG13-MFG-GFP cells grew in fructose-supplemented medium in a CellCube reactor (module 25) only at a rate of about 30% of that observed in the glucose supplemented medium, the cumulative vector production was about 8 times higher in the fructose medium. This was due to a 26-fold increased vector production in comparison with the glucose cultures (Table 3).

The reason for the enhanced vector production in the presence of fructose is not clear for the moment. As fructose is metabolized differently from glucose, it is possible that the glycosylation of the proteins and lipids (including retroviral proteins and glycolipids), as well as lipid metabolism and the synthesis of lipid precursors, are modified. In addition, it is known that fructose is utilized to a large extent via the pentose-phosphate cycle [48] necessary to produce precursors for the nucleic acid synthesis. Since the viral genomic RNA is often the bottleneck for the production of high titer vector

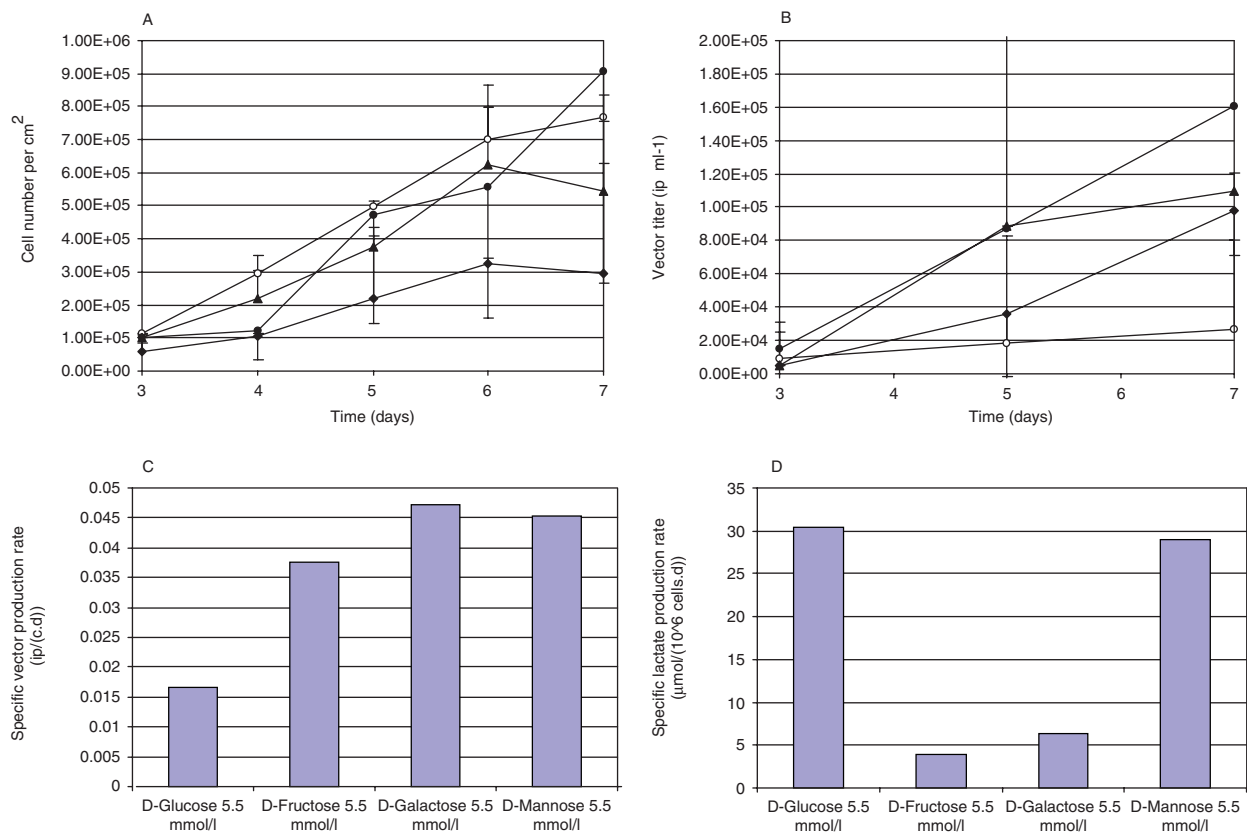


Figure 3. Effect of the addition of different sugars (at 5.5 mmol L⁻¹) on cell growth (A), vector titer (B), average specific vector production rate (C) and specific lactate production rate (D) of Te FLY GA18 cells. In (A and B), the standard deviations are added, and the following symbols were used: glucose (○) fructose (▲), galactose supplemented with 1 mmol L⁻¹ of pyruvate (◆), and mannose (●). Note: the cell-specific vector production and cell-specific lactate production rates are means of the specific rates calculated for each day (our unpublished results)

Table 2. Cell growth and vector production of three vector-producing cells in glucose- and fructose-supplemented media [8]

Cell line	Sugar source	Te FLY GA18		PG13-MND-GFP		PG13-MFG-GFP		
		Glucose 4.5 g/L	Fructose 15 g/L	Glucose 4.5 g/L	Fructose 20 g/L	Glucose 4.5 g/L	Fructose 20 g/L	Fructose 25 g/L
CELL GROWTH	Max. cell density per cm ²	6.31 × 10 ⁵	6.88 × 10 ⁵	3.56 × 10 ⁵	3.00 × 10 ⁵	8.61 × 10 ⁵	5.57 × 10 ⁵	5.65 × 10 ⁵
	Specific growth rate (1/h)	2.02 × 10 ⁻²	2.06 × 10 ⁻²	2.09 × 10 ⁻²	2.07 × 10 ⁻²	2.74 × 10 ⁻²	2.46 × 10 ⁻²	2.47 × 10 ⁻²
VECTOR PRODUCTION	Max. vector titer (ip/mL)	2.48 × 10 ⁶	1.13 × 10 ⁷	7.42 × 10 ⁴	1.73 × 10 ⁵	1.96 × 10 ⁵	3.07 × 10 ⁵	3.65 × 10 ⁵
	Average specific vector production rate (ip/c.d)	1	4.2	0.06	0.122	0.389	0.768	1

Note: The cultures were inoculated at a cell density of 12 000 cells per cm². The cells had been adapted to the fructose concentrations during three passages. The fructose concentrations chosen had been previously established as optimal. In all cases, the basal medium was DMEM supplemented with 5% FCS and glucose or fructose. After 3 days, the medium was changed every 24 h. The total culture duration was 8 days.

Table 3. Bioreactor cultures of Te FLY GA18 and PG13-MFG-GFP cells in glucose- and fructose-supplemented media [47]

	Glucose based-medium	Fructose based-medium	Fructose/glucose ratio
Te FLY GA18, NewBrunswick Scientific basket reactor	4.5 g/l glucose	15 g/l fructose	
Innoculum cell density	6.0 × 10 ⁷	6.0 × 10 ⁷	
Culture duration (h)	213.5	217	
Cumulative biomasse (cell)	4.34 × 10 ⁹	7.10 × 10 ⁹	1.64
Cumulative titer (ip)	1.49 × 10 ¹⁰	6.96 × 10 ¹⁰	4.67
Average of specific production (ip/10 ⁶ cell.h)	16 080	45 174	2.8
PG13-MFG-GFP, CellCube module 25	4.5 g/L glucose	25 g/L fructose	
Innoculum cell density	2.049 × 10 ⁸	2.26 × 10 ⁸	1.1
Culture duration (h)	157.5	182.25	1.16
Cumulative biomasse (cell)	4.62 × 10 ¹⁰	1.25 × 10 ¹⁰	0.27
Cumulative titer (ip)	8.95 × 10 ⁹	73.6 × 10 ⁹	8.22
Average of specific production (ip/10 ⁶ cell.h)	1230	32 307	26

Note: The cultures were started with the indicated cell number and the cultures were perfused up to the production of 20 L of culture supernatant. Then the cultures were stopped. In all cases DMEM with 5% fetal calf serum was used, in which the sugar source was either glucose or fructose.

preparations [38], an increased nucleotide synthesis resulting from the advantageous fructose metabolism might lead to increased amounts of viral genomic RNA available. However, these explanations are still speculative and have to be verified in further studies.

Cell growth

The improvement of vector production might be also activated via the modification of cell growth. Since retroviral LTRs are most active in cycling cells [49], enhanced cell growth rates should lead to an increased vector production rate, as shown by several authors. By performing fixed bed reactor cultures of Te FLY GA18 cells, we could show that those cultures with higher specific growth rates showed also increased specific vector production rates [122]. McTaggart and Al-Rubeai [50] came to the same conclusion, after cell cycle studies with FLY RD18/LNC-hB7 producer cells: the vector production was not cell cycle associated but depended directly on cell cycle progression.

Culture conditions

In addition to the cell-associated parameters, the general culture conditions can have an important impact on cell growth and vector production. Little work has been done in this direction. CO₂, an important metabolite of the cellular metabolism, seems to have no effect on the productivity of the packaging cells [3]. Results obtained with FLYRD18/LNC-hB7 and Te FLY GA18 cells by [51] and by us, respectively, indicated that the optimal pH for the specific vector production was 7.2 and that at both higher and lower pH values considerably lower specific production rates were obtained. We could show that for Te FLY A7 cells, the pH should be between 6.9 and 7.2 in order to maintain an optimal vector production rate (unpublished results). Similar conclusions were drawn by [52] who reported that pH 7.0 was optimal for storage of MLV- and HIV-1 based VSV-G pseudotypes vectors (produced by transfecting Te671 cells). The dissolved oxygen level ranging from 20 to 80% of air saturation is optimal for cell growth and vector production for FLYRD18/LNC-hB7 ([51] and Te FLY GA18 (our unpublished results)), whereas oxygen

limitations immediately led to reduced vector production or transduction efficiency ([18], our unpublished results).

Depending on the cell line used, the cultivation temperature can have an important influence on total vector yield and on the specific vector production rate. Whereas no or no significantly positive effect was observed for Te FLY GA18 (our unpublished results), ψ CRIP LLZA (Heard, personal communication), ψ CRIP/MFG-LacZ ([53] increase by a factor of two), or PA317-SVNLZ [54] when the cultivation temperature was shifted from 37 to 32 °C, other authors have reported a positive effect: Kotani *et al.* [3] and Kaptein *et al.* [55] observed 5–15-fold increase in retroviral titers with different PA317 clones at 32 °C in comparison with 37 °C. For hollow-fiber cultures of PA317 producer cells, Pan and Whitley [56] observed also a positive effect at 34 °C. FLYRD18/LNC-hB7 cells showed a 20-fold increased vector productivity and a two-fold reduced specific growth rate at 32 °C in T-flasks as well as in packed bed reactor cultures [51]. However, at a lower cultivation temperature (27 °C), practically no vector production was observed any more [50]. On the other hand, producer cell lines based on GP+envAM12 and PG13 showed a significantly lower vector production at 32 °C in comparison with 37 °C [57].

While the temperature effect may vary according to the cell line, it probably depends also on the vector construction and the overall culture conditions (medium, serum, etc.).

The weak point of all retroviral vectors is their rather low stability. Half-lives of 2–9 h have been reported for vectors produced with clones derived from PA317, ψ CRIP [3–6,54], Te FLY [8], and CEM FLY cells [7] when kept at 37 °C. At lower storage temperature, i.e. at 4 °C, the vector half-life of TeFLY GA derived vectors could be increased up to about 250 h [7]. More data are provided in Table 4. A possible explanation for these large differences in the vector stability might be due to the different membrane characteristics of mother packaging cell lines. Beer *et al.* [58] observed that amphotropic mouse retroviruses (MLV-A) derived from human and hamster producer cells exhibited 2–3-fold longer half-lives compared with those derived from mouse cells.

However, in contrast to other cell lines that exhibit higher virus titers when cultivated at 32 °C (see above), Beer *et al.* [58] observed that the cultivation temperature affected, up to now not noticed, the half-life of MLV-A vectors. The 37 °C to 32 °C shift resulted in a three-fold decrease in viral half-lives compared with MLV-A released from mouse cells at 37 °C. Thus, MLV-A released at 37 °C is phenotypically different from MLV-A synthesized at 32 °C. The authors observed also an inversed correlation between increased virus stability and the level of cholesterol in the viral membrane. In this context, depletion of viral cholesterol *in vitro* resulted in intact virus with increased thermal stability. Thus, retrovirus lability depends on the host cell and parallels the amount of cholesterol in the viral lipid shell. Cruz *et al.* [59] studied the effect of culture conditions and choice of the producer cell on vector stability. They

observed that vectors with the amphotropic envelope were generally more stable at 37 °C than GALV-enveloped vectors produced under the same conditions (producer cell line: Te FLY cells), which corroborates literature data on the impact of envelope proteins on vector stability [60]. They showed also that vectors produced in serum-containing medium were more stable than those produced in serum-free medium, particularly in the case of GALV-enveloped vectors. The production temperature affected significantly the degradation rates during storage although the effects were not the same for vectors with different envelopes. In fact, amphotropic vectors produced in serum-containing medium at 37 °C were more stable than those produced at 32 °C when stored at or below 22 °C. In the case of GALV-enveloped vectors, the temperature of 37 °C also seemed to be more favorable for vector production as it led to vectors with lower degradation rates.

In sugar replacement studies, we showed that the replacement of glucose by fructose led to a 25% increase in the vector half-life, indicating that the culture conditions and thus the cellular metabolism have an important impact on vector stability [8,61] (Table 4) which corroborates well with the data published by [58].

A general observation is that reduced storage temperatures lead to an improvement in the retroviral vector stability. However, for CEM FLY cells, that produce vectors with a low half-life at 37 °C (3.9 h), the increase in the vector half-life is less pronounced at lower storage temperatures than for vectors from cells with a higher half-life at 37 °C (7.5 h) (produced by ψ CRIP cells). These vectors showed a much higher stability at 25 °C (53.3 h) and at 10 °C (268.8 h) than those produced by CEM FLY cells with half-lives of 23.4 and 106 h at 25 and 4 °C, respectively [6,7] (Table 4).

Harvest frequency/perfusion rate

As already mentioned, the storage stability of retroviral vectors is rather short at 37 °C, which is in most cases the production temperature. Thus to obtain intact and functional vectors with low contamination of inactive particles, the vector particles should be harvested shortly after their biosynthesis in a discontinuous or continuous mode.

Using multiple process systems (T-flasks, rollers, multitrays) harvesting is performed discontinuously and can be done every 8 h to every second day, as reported by [57] and [3], respectively. As the half-life at 37 °C ranges between 4 and 9 h, the optimal harvest frequency should be about every 8 h. Reeves *et al.* [57] have observed that optimal harvest times were 8 h for GP+envAM12 producer clones and 24 h for PA317- and PG13-derived producer clones. Reference [3] reported 2 days as optimal harvest frequency.

However, due to the short half-life of retroviral vectors, the harvest frequency should be at least every 24 h (one discontinuous harvest per day), which is used in most of

Table 4. Literature data on half-lives of retroviral vectors produced with various producing cell lines

Packaging cell line	Clone	Half life (h)						Remark	Reference
		37 °C	32 °C	25 °C	20 °C	4 °C	4 °C		
PA317	GINa.40 SVLNZ	Loss of 92% in 24 h 4.1	No loss in 24–48 h 12			123.4		[3] [54]	
ψ/CRIP	POPA/neo4	9			34	92		[55]	
	MFG-LacZ	5			36	132		[53]	
PG13	pMFG-LacZ	5.5						[4]	
	α-SGC-LacZ SF1/m	7.51 ± 0.82 7.5		53.28 ± 3.60	17.7	268.8 ± 6.72 (10 °C) 54.2 (4 °C – 8 °C)	Production done in X-Vivo 10 at 32 °C for 16 h, 8 h in DMEM-10% FCS for cell recovery (4 consecutive cycles)	[42] [63]	
Fly	MND-GFP	4.38					DMEM (Glucose: 4.5 g/l) + 5% FCS	[8]	
	MND-GFP	5.47					DMEM (Fructose: 20 g/l) + 5% FCS	[8]	
Te671	RD18/LNC-hB7	6.9	11			64.3	DMEM/F12 + 5% FCS	[51]	
TeFLY	Transfection (transient) GA18	4.5 ± 1.0 9	24 – 28		55	247.6 [7]	DMEM (Glucose: 4.5 g/l) + 5% FCS	[8]	
CEM FLY	GA18	11.15			67		DMEM (Fructose: 15 g/l) + 5% FCS	[8]	
	AL19	3.9		23		106		[7]	

Table 5. Production of retroviral vectors with perfusion processes

Packaging cell line	Clone	Reactor system	Volume produced (L)	Average vector titer	Maximal vector titer	Duration (d)	Cultivation temperature (°C)	Final reactor cell density	Perfusion rate	Reference
PA317	G1NaSVAd.24	CellCube module 25, 3 L vol.	30	8 × 10 ⁶ CFU/mL	1.2 × 10 ⁷ CFU/mL	23	32	12 × 10 ⁹	About 0.66 reactor vol every 2nd day	[3]
ψ/CRIP	SVLNZ	NBS-basket reactor, 10 g Fibracell, 0.5 L vol.	10	1.45 × 10 ⁶ CFU/mL	ND**	7	32	2.955 × 10 ⁹	4.2 reactor vol per day	[54]
	G1TK15/Na.7	Packed bed reactor: 0.8 L packed bed, 2 L vol.	280	1–10 × 10 ⁷ CFU/mL	ND	30–40	?	40 × 10 ⁹	4–6 reactor vol per day	[121]
TeFLY	ID15	CellCube module 25, 2.7 L vol.	35	3880 U/mL	ND	13	37	6.88 × 10 ⁹	1 reactor vol per day	[64]
	MFG-lacZ	Biolaftite STR*, 5 g/L Cytodex 1, 1.5 L vol. Mini packed bed reactor, 0.7 g Fibracell, 0.01 L	31.2 0.96	840 U/mL	ND	13	37	3.44 × 10 ⁹	1.6 reactor vol per day	[83]
PG13	GA18	Spinner flask, 3 g/L Cytodex 3, 0.1 L CellCube module 25, 2.7 L vol.	0.25 50	≈0.49 × 10 ⁵ CFU/mL	1.56 × 10 ⁵ CFU/mL	7	37	6.9 × 10 ⁷	0.5 reactor vol per day	[122]
	MFG-GFP	NBS-basket reactor, 70 g Fibracell, 1.4 L vol. NBS Celllift, 5 g/L Cytodex 1, 1.4 L vol. NBS Celllift, clump culture, 1.4 L vol. CellCube module 25, 2.7 L vol.	20 18.5 18 20	2.099 × 10 ⁷ ip/mL 2.965 × 10 ⁷ ip/mL 0.954 × 10 ⁷ ip/mL 2.447 × 10 ⁵ ip/mL	3.5 × 10 ⁷ ip/mL 4.31 × 10 ⁷ ip/mL 1.99 × 10 ⁷ ip/mL 0.05 × 10 ⁷ ip/mL	15 12 16.2 10	37 37 37 37	48.9 × 10 ⁹ 19.7 × 10 ⁹ 8.58 × 10 ⁹ 6.11 × 10 ⁹	3.3 reactor vol per day 2.4 reactor vol per day 2 reactor vol per day 1.6 reactor vol per day	[Own unpublished results]
		NBS-basket reactor, 70 g Fibracell, 1.4 L vol.	20	9.2 × 10 ⁵ ip/mL	23.3 × 10 ⁵ ip/mL	9	37	22.1 × 10 ⁹	3.4 reactor vol per day	

Note: *STR = stirred tank reactor. **ND = not determined/not available.

the cases for small- and large-scale productions using roller bottles (e.g. [21,54] or an ImmobaSil-G based packed bed process [51]).

A rather optimal frequency of medium change is twice a day (once in the morning and once in the evening, leaving the incubation times at 8 and 16 h [7,62,63]). The latter authors reduced the serum level in the vector preparations by cultivating confluent PG13 producer clones after three washing steps in serum-free medium (X-VIVO 10) for 16 h (overnight) and keeping the cells for 8 h in DMEM + 10% FCS (Table 4). This procedure was repeated for four consecutive days. Such a protocol was used for a production volume of 9.6 L in a 40 tray Cell Factory yielding a titer ranging from 1.9×10^6 to 2.8×10^6 ip/mL [62]. Using CEM-FLY AL19 cells, Pizzato *et al.* [7] showed that the final titer was equivalent for harvesting frequencies of once in 8, 16, or 24 h, indicating that the optimal harvest frequency for maximal production would be at least every 8 h.

However, this also means that perfusion cultures are the best choice for the production of retroviral vectors when reactor systems are used. The choice of the perfusion rate generally depends in the first instance on the cell number per reactor volume and then on the production rate and the decay rate of the retroviral vectors. This means that cultures at a relatively low cell density of 2.3×10^6 to 6.1×10^6 cells per mL (3.44×10^9 to 8.58×10^9 cells per reactor) (ψ CRIP ID15: CellCube and microcarrier culture; TeFLY GA18: clump and microcarrier culture) [64,122] were conducted with perfusion rates ranging from 1.6 to 2 reactor volumes per day (Table 5). The only exception concerns the data published by [3] who used a very low perfusion rate for their CellCube cultures although the final cell density of the PA317 producer clone was 13×10^9 cells per reactor.

At higher reactor cell densities, the perfusion rates reported in the literature ranged from 2.4 to about 6 reactor volumes per day (Table 5). With respect to the vector half-lives ranging from 4 to 9 h, and the fact that harvest frequencies of about 3 volumes per day for batch processes seemed to be rather optimal, the optimal perfusion rate for high density reactor cultures ($>6 \times 10^6$ cells per mL reactor volume) should approach at least 3 volume changes per day, and must be adapted at a higher rate when higher cell densities have to be alimanted. However, the results obtained by us indicated that a perfusion rate of about 3 to 4 volume changes per day was optimal for cell growth and vector production [8,122].

Reactor cultures – a general overview

Bioreactor systems have two advantages. The first one is the possibility to scale-up the production capacity and size to industrial scales when necessary. This implies that the scale-up is achieved by increasing the size of the vessel and not the number of vessels, as is the

case for multiple process systems. A second advantage is that reactor systems are generally equipped with control and regulation units allowing the use/maintenance of optimized and controlled culture conditions which generally leads to improved cell growth and production yields [18,122]. In addition, multiple process systems (T-flasks, roller bottles, cell factories) are rather labor-intensive and can be used in a batch, fed batch or repeated batch mode only, whereas bioreactor systems (stirred tank reactor for suspension and microcarrier cultures, fixed bed, fluidized bed, and hollow fiber reactor) can also be used in a perfusion mode, which is in any case the culture mode of choice for the production of retroviral vectors. Whereas fixed bed and hollow fiber reactor systems show mass transfer limitations, hydrodynamic shear effects are the drawbacks of suspension and microcarrier cultures. The only system which does not seem to be negatively affected by system inherent drawbacks is the fluidized bed reactor system. Serious scale-up limitations, however, exist for hollow fiber reactors. Fixed and fluidized bed reactor systems are also limited in scaling or at least show a reduced scaling potential. The reactor systems which can be used for the production of retroviral vectors are discussed below:

Stirred tank reactor system

Stirred tank reactor systems can be used for the cultivation of suspension and adherent cells when grown on microcarriers suspended in the medium. In general, these reactors are vessels which are equipped with an agitator [125] and eventually with a draft tube [66] or baffles [30]. The largest available scale for industrial cultivation of animal cells is 12 000 L [67]. An overview of engineering and scaling issues was published by [68]. In the case that batch or normal continuous cultures without cell retention are performed the cell density is limited to about $1-5 \times 10^6$ c/mL, which depends not only on the maximal possible growth rates of the cultivated cells, but also on the cell size and on the culture conditions (e.g. medium composition). As high cell density culture systems are of interest for increasing the reactor productivity, the reactor has to be equipped with one of the existing retention devices. While for cells growing as single cells or in clumps the ultrasound retention device (Applisens) [69] or the continuous centrifuge (Kendro-Sorval, formerly Centritech) [70] seems to be the optimal choice, spinfilter systems [66,71,72,125] and sedimentation devices (New Brunswick Scientific) are well adapted for perfusion cultures of cells growing on microcarriers and also for clump cultures. Using retention devices, the cell densities can be increased to values ranging from 5×10^6 to 50×10^6 cells per mL [73]. The largest scale of perfusion cultures is 2000 L [30].

In many cases adherent cells cannot be adapted to suspension growth and thus cannot grow in suspension. However, as suspension culture systems show the best scalability, suspension growth of adherent cells is

desirable and was achieved by the use of microcarriers. The concept of microcarrier culture is rather simple. It comprises the cultivation of adherent cells on small solid particles (the microcarriers) suspended in the growth medium by stirring. Cells attach and spread on these carriers and gradually grow out on a confluent monolayer, in the case of contact inhibited cells, or eventually to multilayers in the case of absence of any contact inhibition. Classical microcarriers, such as Cytodex I and III (Amersham Biosciences), have a size of about 150–200 μm and are used at a concentration of 10^4 to 2.5×10^4 beads per mL of culture. Thus a quasi-homogeneous system is achieved which resembles the traditional suspension culture with all its advantages, and as used for suspension cells. In microcarrier culture the features of both suspension and monolayer cultures are brought together in one system. More details can be found in a review by [72].

In general the same reactor systems as for classical suspension cultures can be used; however, with special reference to microcarrier cultures, the aeration system should be bubble-free in order to avoid cell damage and loss of carriers due to foaming. For this particular application, the reactors are equipped with bubble-free aeration systems, such as hydrophobic membranes [74,125], metal nets fixed on the agitator shaft [72,125], or a vibrating gassing cylinder (ChemCell System) [75].

As an example, Figure 4B shows confluent microcarriers of a MDCK reactor culture destined for the production

of influenza virus [76], and Figure 4A a laboratory-scale stirred tank reactor, which is equipped with a Cellift impeller (New Brunswick Scientific) allowing a very soft/low shear stress agitation as well as a bubble-free aeration via the metal net fixed on the shaft of the agitator. However, other reactor configurations are equally functional (for review, [125]).

Various biologicals have been produced in low- and high-density suspension processes using single cell or microcarrier systems: monoclonal antibodies, recombinant proteins, viruses, and viral vectors.

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 ($0.5\text{--}2 \times 10^8$ c/mL carrier in the case of the use of a basket reactor) can be obtained. In principle, cells attach to the carriers when solid glass spheres [77], porous carriers (e.g. Siran (glass) spheres) [78], or stainless steel gauze and coupons [79] are used, or attach and are entrapped when porous sponge-like materials, such as Fibracell [80], are used. In both of the latter cases, also cells that do not readily attach (such as hybridoma cells) can be cultivated in packed bed reactors. The attached and/or entrapped cells grow on and/or in the carrier matrix and the culture medium, conditioned for optimal pH and $p\text{O}_2$, is circulated from a conditioning vessel to the fixed bed and back to the conditioning

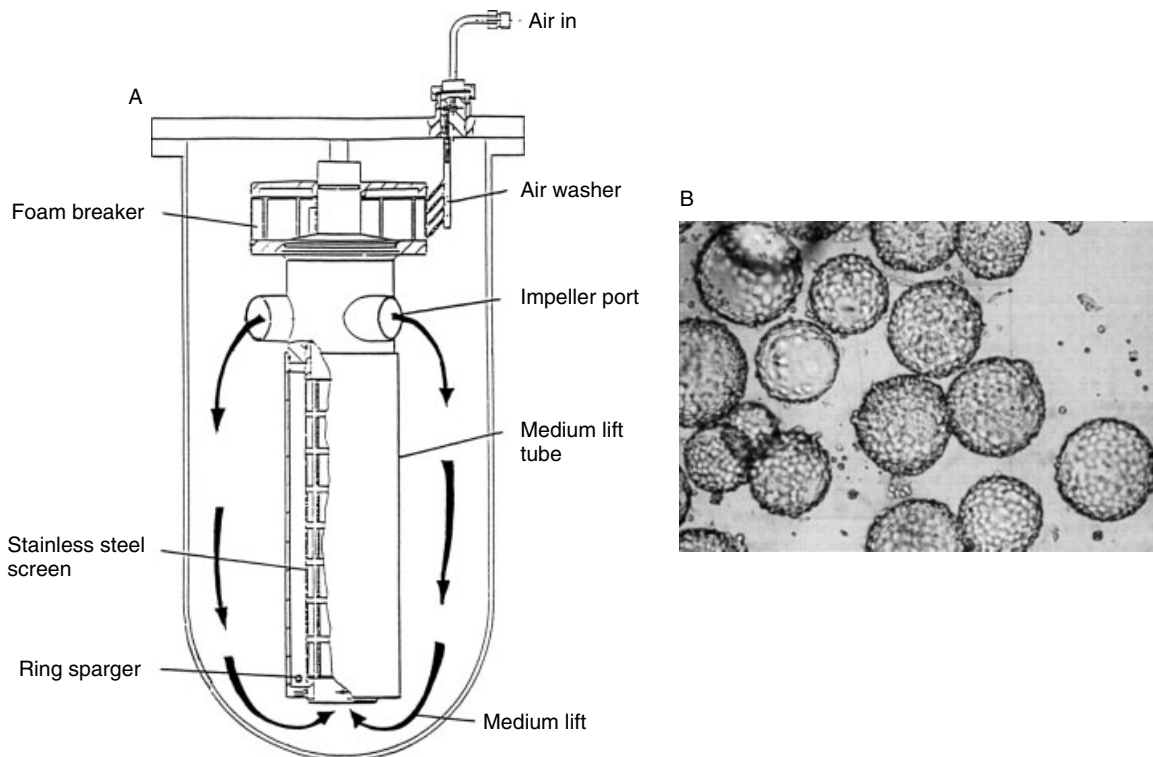


Figure 4. (A) Cell Lift impeller with air wash defoamer (© NewBrunswick Scientific, reproduced with permission). (B) Confluent MDCK – microcarrier cultures: MDCK cells were grown on Cytodex I microcarriers (5 g/L) to a cell density of 5.8×10^6 cells/mL in serum-free medium and examined under a light microscope. The diameter of the microcarrier beads is about 180 μm (range 131–220 μm)

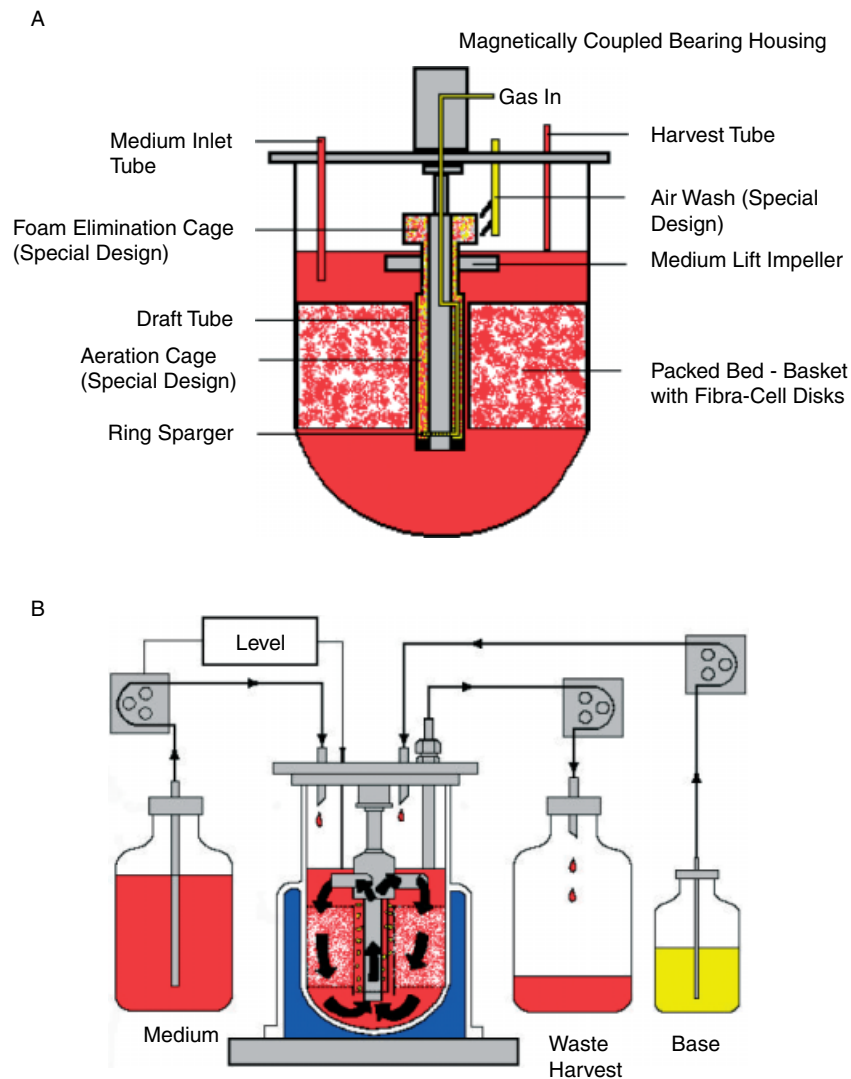


Figure 5. Packed bed perfusion system with harvest and level tube (© New Brunswick Scientific, reproduced with permission). (A) Principle of a packed bed reactor. (B) Complete set-up of a packed bed reactor

vessel (Figure 6) or both systems (cell compartment and conditioning vessel) are integrated, as for the basket reactor provided by New Brunswick Scientific (Figure 5). Figure 5 presents the principle of a packed bed reactor (Figure 5A) as well as a complete set-up of packed bed reactor showing in particular the way in which such a perfusion system is used (the addition of fresh medium is controlled by a level sensor, whereas the waste harvest is eliminated continuously. The base addition is the means for controlling the pH) (Figure 5B).

Basket type packed bed reactors were developed very early, largely pioneered by Spier and co-workers [81], and have been scaled by [77] to an approximate bed volume of about 21 L allowing the production of 200–300 L of supernatant per day. The largest established scale of a packed bed reactor is 100 L of bed volume (Bliem, personal communication). However, to date, no such system is really used under industrial conditions. The main drawbacks are: (i) that the linear scale-up is limited due to concentration gradients, (ii) the system is non-homogeneous, (iii) no sampling of the reactor content is

possible, and (iv) the outgrowth of cells leads to channel blockage [82].

Such reactors have been used for the production of viral vaccines [81], retroviral vectors [83,122], adenoviral vectors [84], recombinant proteins, and monoclonal antibodies [65,77,85,87].

A special version of a fixed bed reactor is the CellCube (Corning, Figure 6). This system was developed for the cultivation of adherently growing cells and has extensively been used for the production of viral vectors for gene therapy purposes for production of retroviral vectors [3,122], of adeno-associated viral vectors [86], as well as for the production of a hepatitis A vaccine [79]. However, the main drawbacks are the limited scalability (largest system: module 400, providing a surface of 340 000 cm²), the relative complexity of the system, the assembly of autoclaved (tubings, pump module, oxygenation reservoir) and non-autoclavable radiation sterilized (the CellCube) parts of the system in a laminar air flow bench, and difficulties in validating the cleaning of the oxygenation reservoir.

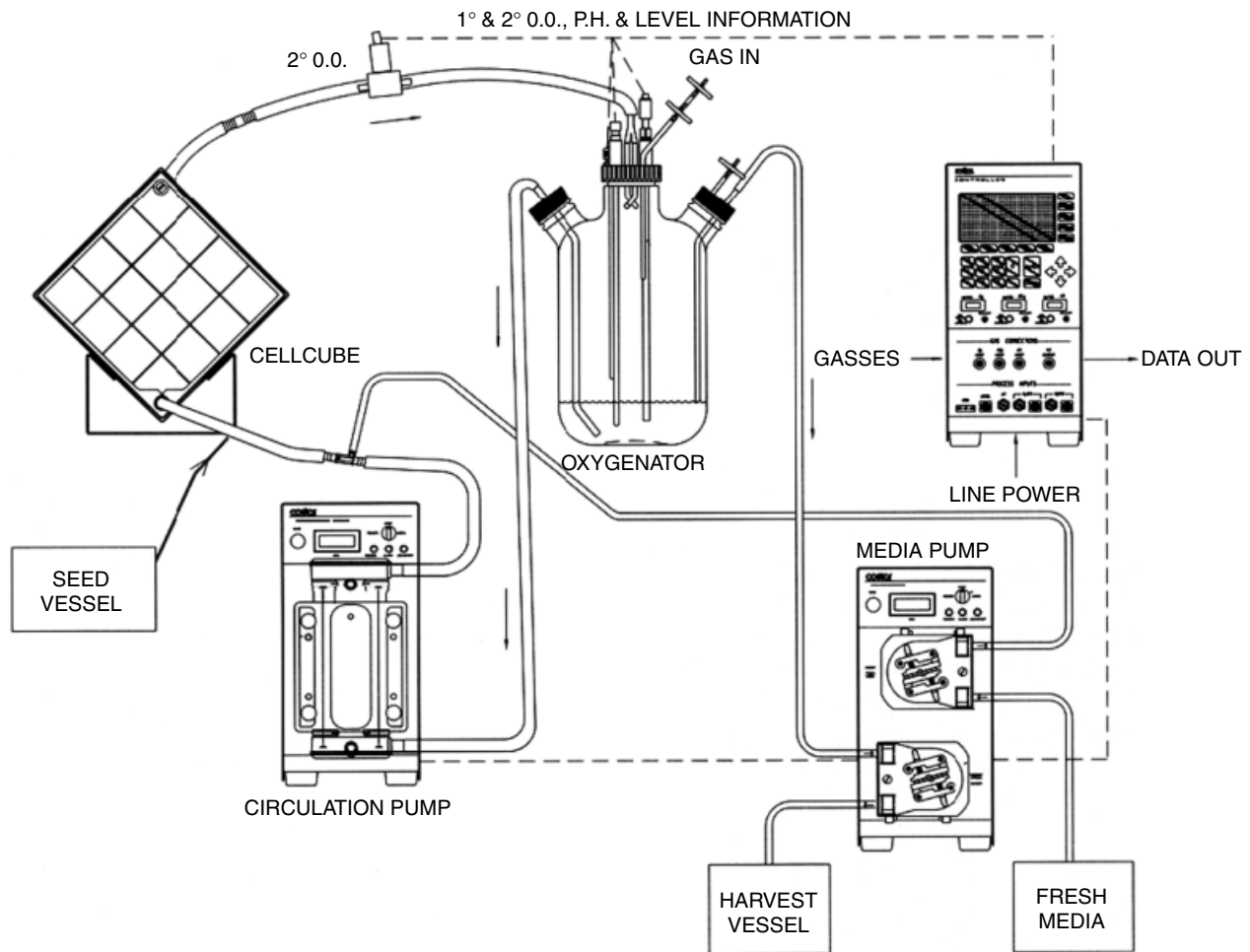


Figure 6. CellCube, lab version (module 25) (Corning). Reproduced with permission

Fluidized bed reactor

This system was originally designed for waste-water treatment, because it has the potential for improving performance with less operational problems (i.e. channel blockage) than fixed bed reactors. The use of fluidized bed reactors for animal cell culture has been reported by several authors [88,89]; however, only Verax [90,91] and, in particular, Amersham Biosciences [92] (Figure 7) have developed an industrial-scale operation: 24 L [93] and 120 L [92], respectively. This system generally makes use of porous carriers, such as Cultisphere (Percell Biolytica), Microsphere (Verax Corp.), or Cytoline 1 (Amersham Biosciences). Their use allows high cell densities of about 5×10^7 CHO cells/mL Cytoline 1 carriers in a protein-free medium, as reported by [94] and [95], or up to 5×10^8 cells/mL Microsphere, as reported by [91]. As for the fixed bed reactor systems both adherent and suspension cells can be cultivated in fluidized bed reactor system because the cells are either attached or entrapped within the carriers. Only the porous carriers (Microsphere and Cytoline 1) can be used for suspension cells because these cells have to be entrapped to be cultivated in a fluidized bed reactor. The great advantage is that outgrowing cells are eliminated by shear forces and perfusion leading to a

quasi steady-state situation. Further advantages are: good mass transfer and mixing characteristics, homogeneous cultivation system, very good potential for volumetric scale-up, sampling of the reactor content possible, no bed clogging by cell growth or gas bubble entrapment.

Fluidized bed reactor systems have been evaluated for the production of monoclonal antibodies [93,96–98], recombinant proteins [90,92,94,98–100], and retroviral vectors [101,123].

Hollow fiber reactor

The principle of the hollow fiber reactor system is that cells are separated from the circulating nutrient medium. They are generally cultivated in the extracapillary space of the cartridge. The medium is cycled from a conditioning vessel (control of pH and pO_2) through the intracapillary space (lumen) of the hollow fiber and back to the conditioning vessel (Figure 8). Depending on the membranes used, the product is retained in the cellular compartment or not. This small-scale system was used for the first time by Knazek *et al.* [102] and allows the production of tissue-like cell masses. In addition, much higher product titers (3–13-fold) can be obtained compared with low cell density cell culture

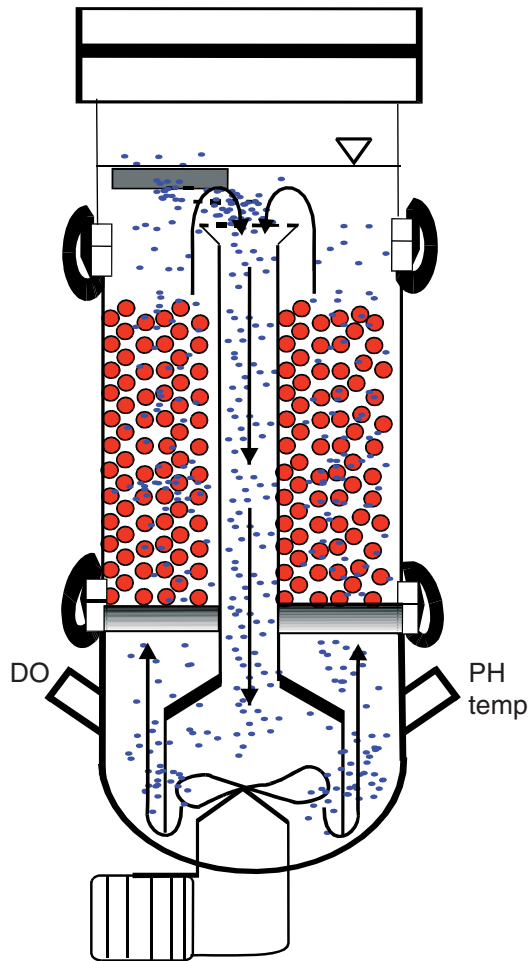


Figure 7. Cytopilot Mini fluidized bed reactor (Amersham Biosciences, reproduced with permission). At the bottom of the vessel, sensors are inserted for controlling the dissolved oxygen (DO), pH, and temperature (temp)

systems, such as roller bottles, multitrays, or batch processes in stirred tank reactor systems, as Knazek *et al.* [102] and Knazek [103] reported for hormone production. A similar increase in the product titers was reported for the production of β -interferon [104] and of monoclonal antibodies [105,106]. This increase is due to tissue-like cell densities that allow a cellular microenvironment obtained by the local accumulation of autocrine substances being favorable for cell growth and production. This generally leads to reduced serum needs on one hand, and often to increased production rates of the biological to be produced (for hollow fiber reactor cultures: [103,105,107]; for cultures of entrapped cells: [98]) on the other. Another advantage is the accumulation of the product in the cell compartment allowing thus 10- to 100-fold concentration, since it is not diluted in the medium.

However, there are several disadvantages associated with the use of hollow fiber reactors: due to axial gradients, this reactor system is rather limited in the scalability, and the formation of axial and radial gradients of nutrients and metabolic waste products implies that the physiological state of the cells is

heterogeneous. In addition, the collection of the cells from the extracapillary space is difficult and can be only incompletely performed. If the product is not regularly collected, then the product residence times can be very long, eventually leading to the inactivation of the product, which, in particular, is an important issue for the production of retroviral vectors.

The following biologicals have been produced by using hollow fiber reactor systems: monoclonal antibodies [106,108–112], natural and recombinant proteins [102–104,107,109,111,112], and retroviral vectors ([56], our unpublished results).

Other immobilization systems

In principle, animal cells can be cultivated in immobilization systems, where the cells are encapsulated [113–117] or entrapped in a polymer matrix, such as agarose [118] or alginate [119]. These capsules or beads are then cultivated in the form of suspensions in stirred tank reactors. The advantage is that high cell densities ($1-2 \times 10^8$ cells/mL capsule or beads) can be obtained. The main disadvantage is that viral vectors will not or, at least, not easily be released from capsules or beads, wherefore these systems are not used for the production of viruses or viral vectors for gene therapy. They are therefore not treated in further detail here.

Comparison of different reactor systems for the production of retroviral vectors

All reactor comparisons were done with adherent packaging cell lines (which are the most frequently used producer cells). No literature references can be found comparing different types of bioreactors for producer cells grown in suspension. To produce retroviral vectors using surface-adherent cells all types of bioreactors have been tested: stirred tank reactors for cells growing in clumps after adaptation to growth in suspension or attached on microcarriers, fluidized bed reactors for cells growing attached on and in macroporous carriers, fixed bed reactors, and hollow fiber reactors.

Figure 9A presents a comparison of the daily vector production, normalized for production per 1 L of reactor volume, with various producer cells cultivated in some of the above-mentioned production systems. The comparison performed by [64] indicated clearly that the use of the CellCube system, multitrays, or roller bottles (Figure 9A, bars 1, 3, and 4) led to an equivalent daily vector production for ψ CRIP ID15 cells, whereas the microcarrier system was about three times less efficient (Figure 9A, bar 2). A similar observation was reported by [83], when they compared the packed bed reactor (use of Fibracell chips) with the microcarrier system for cultivating of ψ CRIP MFG-lacZ cells (Figure 9A, bars 5

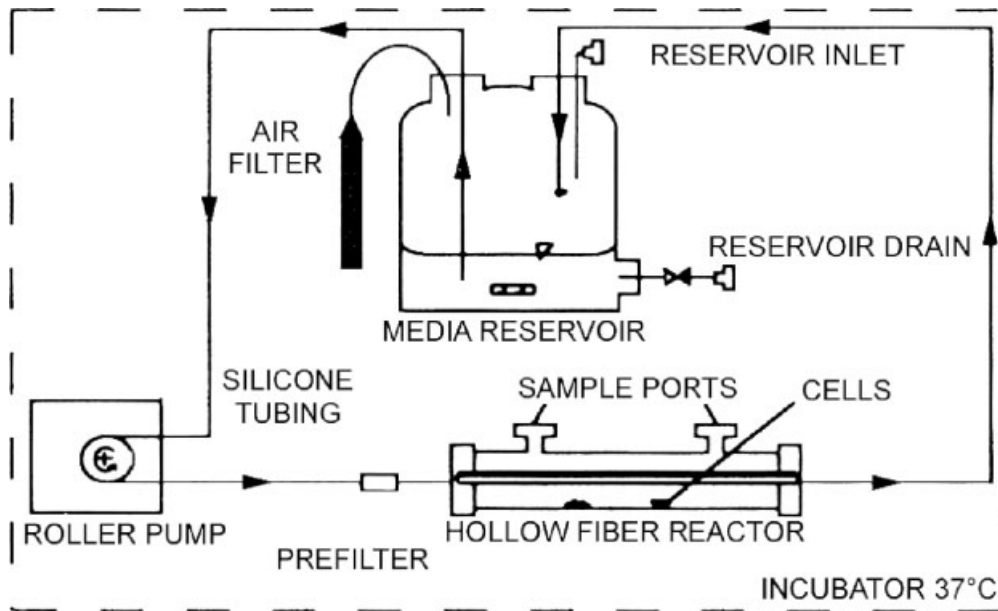


Figure 8. Flow diagram of the hollow fiber reactor system placed inside an incubator at 37°C [108]

and 6). The packed bed reactor system was about 30 times more efficient with respect to the daily vector production than the microcarrier system.

With respect to the cultivation of TeFLY cells, [122] showed that the long-term cultivation and production of retroviral vectors was, with respect to produced vector titers, as efficient in microcarrier systems as in fixed bed reactor systems; however, also that the vector titer had to increase from very low titers (about 10^4 infectious particles per mL) for about 290 h before reaching the same titer as in the packed bed reactor systems (0.5×10^7 to 1×10^7 infectious particles per mL, Figure 9B). However, due to this slow increase in the titer, the average daily vector production was about three times lower than for the fixed bed reactor systems (Figure 9A, bars 7, 8, and 9). Whereas for the use of microcarriers for the cultivation of TeFLY cells this slow but constant increase of the vector titer seemed to be due to the slow saturation of vector absorbing sites on the carriers (Cytodex 1 is an anion-exchanger and can absorb retroviral vectors at physiological conditions), the situation for the cultivation of ψ CRIP cells is apparently different.

Microcarrier cultures performed with ψ CRIP cells never reached the same vector titers and daily reactor production rates as those observed for fixed bed reactor systems. This might be explained by the sensitivity of retroviral particles to shear forces [64,83] and/or due to differences in the lipid composition of the viral envelope which is determined by the physiology of the producer cell line. However, many additional factors, such as production clone, the medium, the cultivation conditions, etc., may influence cell growth and vector production rates (see above) and contribute thus to the differences observed.

Several authors reported that fixed bed reactor culture systems, used in the form of a CellCube [3,8,64,122]

or a basket type reactor [8,54,83,122], were the better systems when compared with microcarrier culture systems [64,83,122] or classical small-scale systems, such as T-flasks, roller bottles, or multitrays [3,64]. With respect to the normalized daily vector production per L reactor volume, ψ CRIP and TeFLY clones produced daily 3–30 times more infectious viral vectors in packed bed reactors than in microcarrier systems (Figure 9A, bars 1, 2, 5, 6, 7–9, 11, and 12) and were more efficient than when small-scale systems (T-flasks) were used (not shown). No significant differences could be observed between the CellCube fixed bed reactor system and the basket reactor system with both TeFLY and PG13 clones although their respective production performances greatly differed (Figure 9A, bars 7, 8, 11, and 12).

The reason for the increased daily production yields in the packed bed reactor systems seems to be mainly due to the high surface-to-volume ratio owing to the parallel plates (CellCube) or the packed beads (basket reactor) leading to much higher reactor cell densities than those generally obtained in microcarrier systems. In addition, the general culture conditions seem to generate fewer shear stresses leading to reduced vector inactivation [83]. Finally, continuous medium exchange (perfusion) renders the fixed bed reactor systems more efficient due to the continuous harvest of a product with a short half-life (see above).

All tested cell lines (ψ CRIP, PG13, TeFLY) are surface-adherent cell lines. In contrast to TeFLY cells, the adaptation of ψ CRIP and PG13 to growth in suspension in the form of clump cultures is not possible. TeFLY cells have a very extended adherence capacity and single cell suspensions are not possible in serum-containing media, wherefore we tried to cultivate them in the form of clumps in spinner and stirred tank reactors [122]. Although this was possible, very low cell growth as well

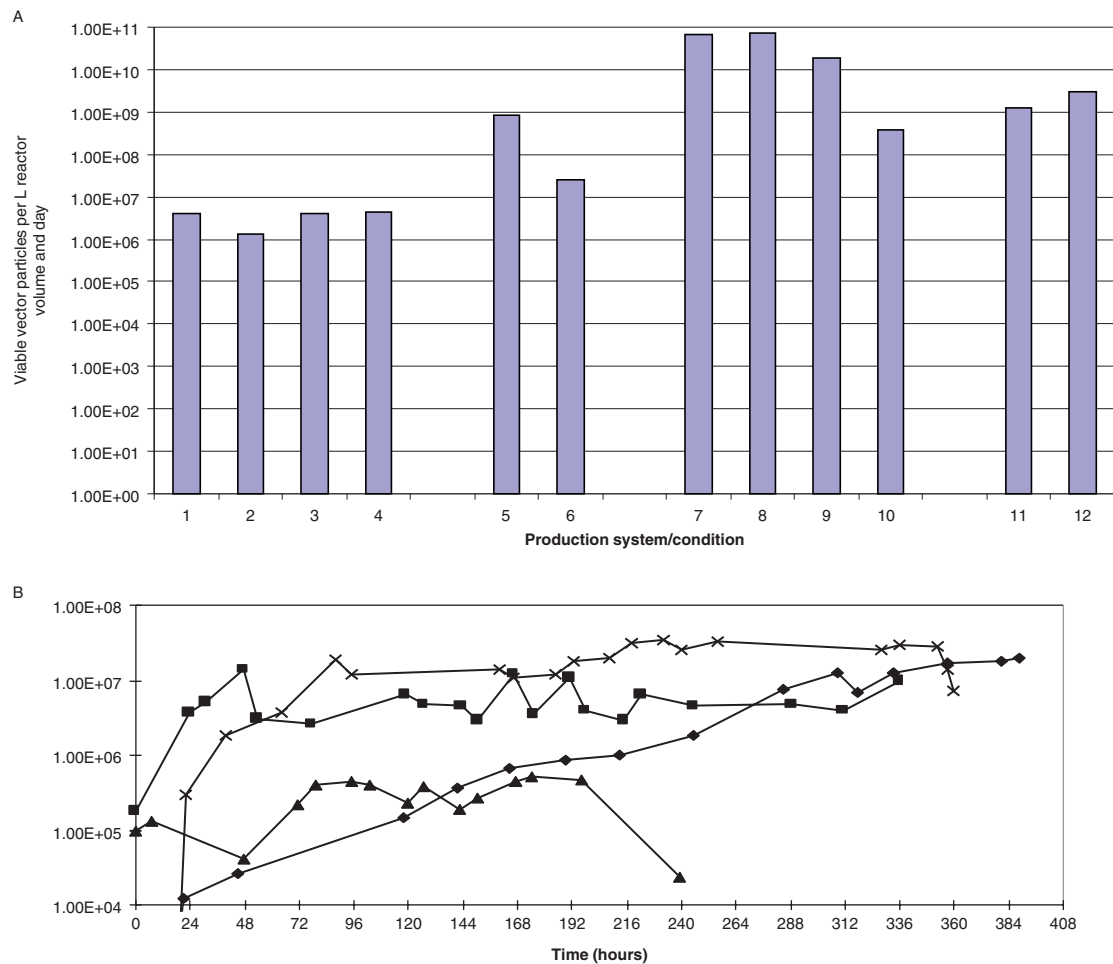


Figure 9. Comparison of bioreactor systems for retroviral vector production. (A) Daily vector production expressed as daily production per 1 L of reactor volume. The following producer cells and reactor systems were tested: 1–4. ψ CRIP-ID15 [64]: 1. CellCube, module 25, 2. Stirred tank reactor (Biolafitte), use of Cytodex 1-microcarriers (5 g/L), 3. Multitray CF10, 4. Roller 850 cm². 5 and 6. ψ CRIP/MFG lacZ [83]: 5. Packed bed-FibraCell, 6. Spinner flask, use of Cytodex 3-microcarriers (3 g/L). 7–10. TeFly-GA18 [122]: 7. CellCube module 25, 8. Packed bed (New Brunswick Scientific)-FibraCell, 9. Stirred tank reactor (New Brunswick Scientific), use of Cytodex 1-microcarriers (5 g/L), 10. Stirred tank reactor (New Brunswick Scientific), clump culture. 11 and 12. PG13-GFP ([own unpublished results]): 11. CellCube module 25, 12. Packed bed (New Brunswick Scientific)-FibraCell. The presented data correspond to the culture data presented in Table 5. (B) Comparison of four different culture systems with respect to retroviral vector production using TeFLY GA18 cells: Cytodex I-microcarrier culture system performed in a New Brunswick Scientific CellLift (MC, ◆), New Brunswick Scientific packed bed reactor system (FB1, ■), clump culture performed in a New Brunswick Scientific CellLift (CL, ▲) and a CellCube packed bed reactor system (CC, x) [122]

as very low titers were observed, leading to a very low reactor productivity, i.e. about 50–200 times less than that observed for the microcarrier or the packed bed reactor systems, as shown in Figures 9A (bars 7–10) and 9B. The reason is that the cells had a tendency to form very dense clumps leading to correct cell growth and vector production only at the periphery of the clumps. Cells in the center of the clumps were poorly alimanted and had to support nutrient limitations, leading to cell death and reduced or no vector production. In addition, virus produced in the clumps is unable to leave them actively and is therefore retained and inactivated.

In addition, it was observed that rapidly growing cells often produced vectors at elevated rates [4,64] because promoter activity of the LTRs are more active in cycling than in resting cells [49]. This would imply that producer

cells in a non-cycling, stationary state, like the cells within the clumps, would be in a non-optimal physiological state for vector production. This signifies that clump cultures are certainly not the optimal way for vector production.

These comparisons showed very clearly that high cell density production systems are superior with respect to vector titers (Table 5) and daily reactor based vector production than microcarrier or clump cultures. This could be confirmed by performance evaluation studies [122].

Two other systems, the fluidized bed reactor system and the hollow fiber system, which are rather promising for retroviral vector production, have not been studied as exhaustively as the culture systems mentioned above.

The fluidized bed reactor (Cytopilot Mini, Figure 7) system has only been tested once for the production of retroviral vectors using PG13 cells by [123]. In this

preliminary study consistent cell growth was obtained when 3×10^8 cells were inoculated on 150 mL of Cytoline 1 carriers. A culture of a duration of 336 h needed about 30 L of culture medium and produced a total vector quantity of 1.33×10^{10} infectious particles (average titer: 4.3×10^5 ip/mL) at an estimated total viable cell number of 0.43×10^{10} cells. The comparison with a standard process based on the use of a CellCube module 25 system led to the conclusion that both systems provided a rather similar vector production. A culture of a duration of 157.5 h produced in total about 1.14×10^{10} infectious particles with an average titer of 6.1×10^5 ip/mL. The total viable cell number was estimated to be 2.2×10^{10} cells. The comparison of the average daily vector production per L bioreactor showed that 6.42×10^9 , 4.58×10^9 , and 3.78×10^9 infectious particles were produced by the cultures performed in CellCube module 25, in Cytopilot Mini with 150 mL, or with 300 mL of Cytoline 1 carriers, respectively. Similar results were obtained when TeFLY A7 cells were cultured on 300 mL of Immobasil HD carriers in a Cytopilot Mini reactor.

This comparison indicated also, that with respect to cell numbers, the use of 600 to 750 mL of Cytoline 1 carriers would lead to equivalent cell numbers and cumulative vector titers as obtained in a CellCube module 25 system. In addition it should be mentioned here that the conditions of the fluidized bed culture were not optimized, whereas those of the CellCube were, indicating that cell growth and production rates can be much improved after some optimization studies. This, as well as the fact that the fluidized bed reactor was already scaled up to 120 L [92], indicates the great potential of this culture system for the large-scale production of retroviral vectors.

Using PA317-based packaging cells, Pan and Whitley [56] showed that hollow fiber systems were also a valuable tool for the production of retroviral vectors. Depending on the clones cultivated they observed an increase in the vector titers by a factor of 14.4 (up to 2.2×10^7 cfu/mL) in comparison with normal T-flask cultures. Up to 3 L of vector supernatant could be generated during a 2-month production run. We could not observe such titer increases for a TeFLY clone when cultivated in hollow fiber systems (unpublished results). Depending on the cut-off of the membrane (70 000 Da, 30 000 Da), the cells produced higher (12.8×10^6) or lower vector titers (1.57×10^6), respectively. Although these titers were equal or about 8 times higher than in T-flask cultures, the highest vector titers were obtained in fixed bed reactor systems [122].

These results, as well as those published by Pan and Whitley [56], indicate that the use of hollow fiber reactors is possible for the production of relatively small quantities of retroviral vectors; however, they indicate also that a careful optimization has to be performed and that the change of the producer cell type eventually would need a completely new process development. As hollow fiber systems are limited in their scalability, they are only of a

certain interest for small- and medium-scale productions of retroviral vectors.

Conclusions

This overview indicates very clearly that many different factors influence the production of retroviral vectors, ranging from the construction and establishment of the producer cell line, over the choice of the culture conditions up to the choice of the production system. Although the optimization of the expression construct has the highest impact on vector titers (up to 10^8 particles per mL have been observed for the best producer cell lines [19]), the optimization of the culture medium and the medium additives as well as the optimization of the reactor system can improve production rates and final vector titers by 10- to 100-fold.

Although only few comparisons on reactor performances have been published up to now, their conclusions are, however, relatively homogeneous and clear. The production of large quantities of retroviral vectors is feasible and the best-adapted culture systems for this purpose are those which can assure high cell densities using a perfusion mode. As retroviral vectors are rather sensitive to shear forces and other environmental influences, these reactor systems should be as smooth as possible in order to keep the vector 'alive'. All comparative studies come to the same conclusion that fixed bed reactor systems are the most efficient ones for cell growth and vector production, and the use of fluidized bed reactor systems might be more appropriate due to their scalability. However, to date, not enough data are available to clearly prove the superiority vis-à-vis fixed bed reactor systems.

As a final and general remark, it was observed that the use of reactor systems led in many cases to an increased vector production and transduction efficiency because of increased production rates, probably caused by an improvement of general culture and production conditions [18,122]. This means that, as soon as clinical studies are planned, the establishment of an optimized reactor production will be a prerequisite in order to assure the quantity of retroviral vectors of appropriate, constant and high quality needed.

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