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# Use of a Fluidized-Bed Bioreactor for the Production of Retroviral Vectors for Gene Therapy Applications

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Gene therapy is a promising medical technology that has the ability to treat inherited and acquired diseases. However, efficient and economical large-scale production of vectors is necessary to meet the potential patient demand. Several approaches have been evaluated for the mass production of retroviral vectors, including fixed-bed bioreactors, suspension cultures, and microcarrier cultures.[1,2] In this article, we report on the use of a Cytopilot fluidized-bed bioreactor for the production of retroviral vectors from the human packaging cell line TEFLYRD.

Scale-up of suspension cell lines is relatively simple and the maximum cell densities achieved in fed-batch cultures can reach 2 x $10^7$ cells/ml. However, high-volume bioreactors are necessary to produce the required amount of product. Consequently, the use of high-density perfusion culture systems presents the best choice for production. The 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.[3]

The Cytopilot is able to support high cell densities by the continuous culture of either adherent or suspension cell lines immobilized on Cytoline microcarriers, which have a large surface area and are of higher density than the culture medium. It has been successfully used in the production of recombinant human proteins, such as human interferon-γ, and erythropoietin.[4,5]

Carriers are fluidized by the upward flow of medium, which is circulated through the bed by an axial flow impeller. Microcarriers are separated from direct aeration and agitation, thus protecting cells from high shear forces that develop as a result of stirring or gassing regimens. The bioreactor configuration allows for the continuous supply of nutrients and removal of virus particles, as well as control of the temperature, pH, and dissolved oxygen. In addition, fluidized-bed bioreactors provide a high mass transfer interface because both cells and fluid are moved, making it a suitable production system for larger scales.[6]

## Materials and Methods

### *Attachment to Microcarriers*

The attachment of TEFLYRD/83 cells to Cytoline 1 and Cytoline 2 microcarriers (Amersham Pharmacia Biotech, Uppsala, Sweden) was assessed. Two milliliters of microcarriers (dry volume) were prepared according to the manufacturer's instructions and placed into 100-ml spinner flasks (Bellco Glass, Vineland, NJ). Microcarriers were inoculated with 4 x $10^6$ cells suspended in 10 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), non-essential amino acid (NEAA) solution, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The flasks were incubated at 37° C and 5% $CO_2$ and stirred every 30 minutes at 40 rpm to obtain uniform distribution of cells on the carriers. A sample of supernatant was taken every hour and the number of cells in suspension was counted under a microscope using a hemocytometer. After four hours, a sample of microcarriers was taken and the cell density was determined using the MTT colorimetric assay.[7]

### *Bioreactor Set-up*

The fluidized-bed bioreactor (Cytopilot Mini; Amersham Pharmacia Biotech) was loaded with 300 ml of Cytoline 1 microcarriers and prepared according to the manufacturer's instructions. The bioreactor control unit was used to maintain the pH at 7.1, temperature at 37° C, and the $DO_2$ at 50% saturation. Medium circulation through the fluidized bed was controlled by a magnetically driven axial flow impeller (Fig. 1).

The Cytopilot was inoculated with 2 x $10^6$ cells/ml microcarrier. Cells were prepared in T-flask cultures. The bioreactor was filled to the work-

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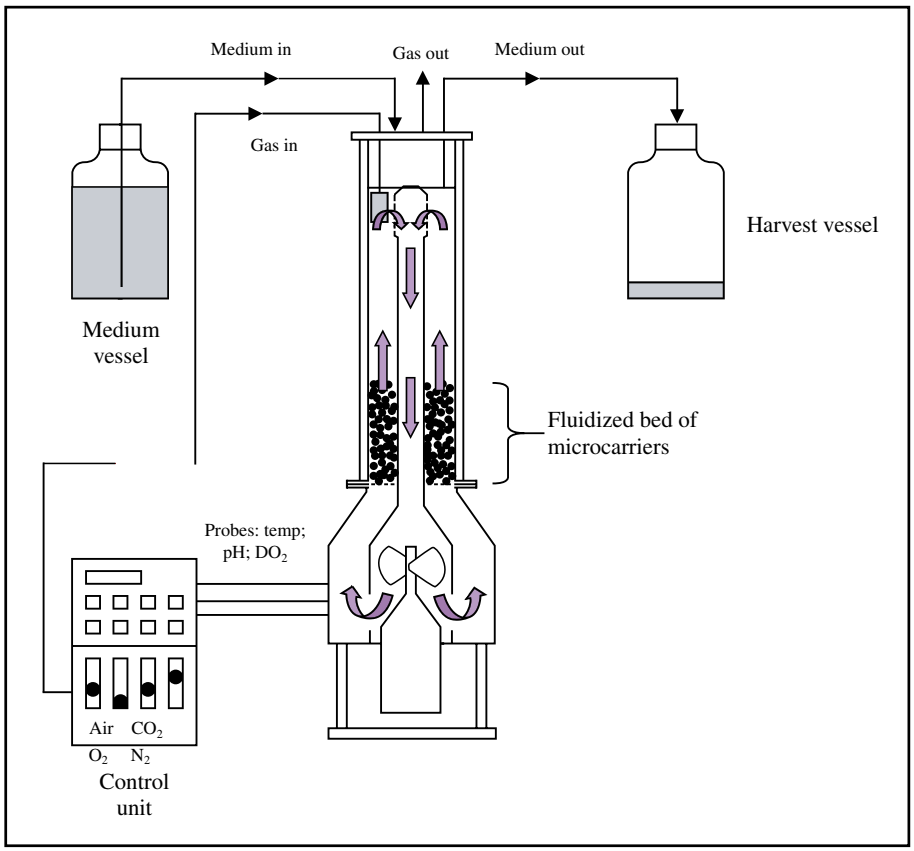


Figure 1. The schematic diagram of the experimental set up of the fluidized-bed bioreactor. Medium was circulated through the bed by a magnetically driven axial flow impeller. The culture set points were: temperature 37° C, pH 7.1, $DO_2$ 50%, and impeller speed 350 rpm.

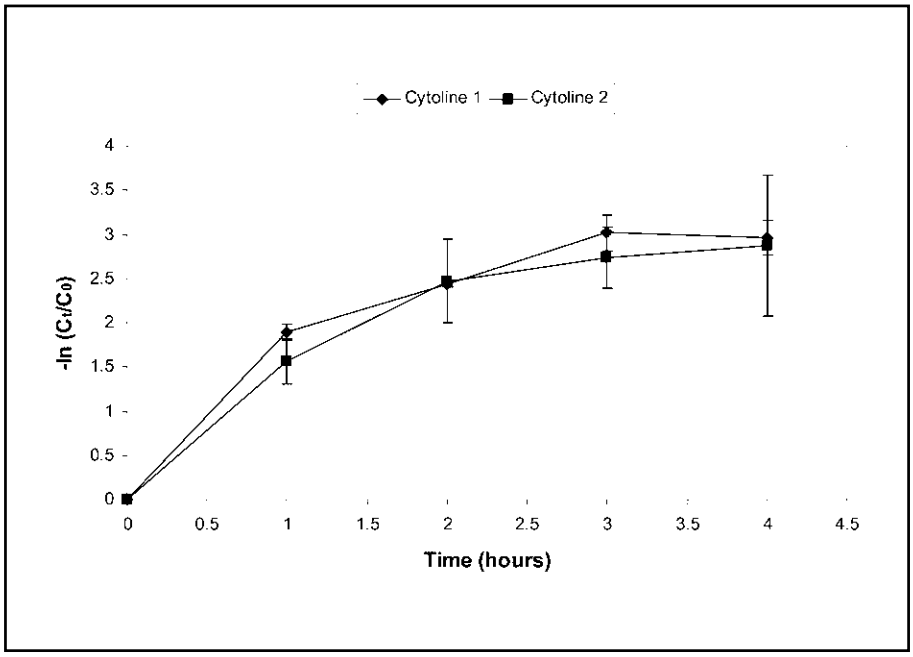


Figure 2. The rate of attachment of TEFLYRD/83 cells to Cytoline 1 and 2 microcarriers. Cells were inoculated at a density of 2 x $10^6$ cells/ml carrier in 10 ml of culture medium. Culture vessels were 100-ml spinner flasks containing 2 ml of carriers. Cultures were stirred every 30 minutes at 40 rpm while incubated at 37° C and 5% $CO_2$. The number of attached cells was determined by counting the number of cells remaining in suspension and deducting this from the initial cell density. Error bars represent standard deviation.

ing volume (two liters) with DMEM supplemented with 10% (v/v) FBS, NEAA, 100 IU/ml penicillin, and 100 µg/ml streptomycin. After cell seeding, the fluidized-bed bioreactor was run in packed-bed mode at 100 rpm with fluidization of the bed for 10 seconds every 30 minutes at 150 rpm. This was repeated for three hours to allow cell attachment. After three hours, the impeller speed was set to 350 rpm to give 100% bed fluidization.

Samples were taken at regular intervals and the glucose concentration was measured. The perfusion rate was adjusted to maintain the glucose concentration above 1.5 $g/L^{-1}$. Cell density was estimated from the glucose uptake rate (GUR). Samples of the supernatant were stored at -80° C for later analysis by the LacZ virus titration assay.

### *LacZ Virus Titration Assay*

HT-1080 cells were seeded into 12-well plates at a density of $10^5$ cells per well and incubated overnight at 37° C in 5% $CO_2$. A tenfold serial dilution was made of each virus sample in culture medium supplemented with polybrene (8 µg/ml). Target cells were transduced with 0.5 ml of virus solution from the most suitable dilution range. After two days, cells were fixed and stained with X-Gal solution.[8] Clusters of LacZ positive (blue) cells were counted at the appropriate dilution of the virus stock after 24 hours of incubation at 37° C under an inverted microscope. Each cluster of blue cells was assumed to have arisen from a single transduction event. The infectious virus titer was presented as the number of LacZ transforming units (LTU) per milliliter.

## Results

### *Analysis of Attachment Kinetics*

The rate of disappearance of cells from the supernatant follows an exponential decay curve, which can be expressed logarithmically as:

$$-1n\ (x_t / x_0) = kt$$

where $x_t$ is the cell concentration at time $t$, $x_0$ is the initial cell concentration,

and $k$ is the attachment rate constant. Consequently, a first-order rate would be represented by a straight line from a plot of -ln $(x_t / x_0)$ vs. time with a gradient $k$. The value of $k$ was interpreted as the specific attachment rate (hour$^{-1}$).

### *Evaluation of Microcarriers for Cell-Bead Attachment*

The rate of bead attachment was rapid, with more than 80% of cells attaching after one hour and more than 90% attaching after two hours. There was no significant difference in the rate of attachment between Cytoline 1 and Cytoline 2. The $k$ values were calculated by linear regression analysis from the data presented in Figure 2 and are given in Table 1.

To confirm that cell disappearance from the medium was caused by attachment to carriers and not to attachment to the vessel walls or cell lysis, a control experiment was performed under identical conditions in the absence of microcarriers. The number of cells in suspension did not change significantly. Furthermore, the results from the MTT assay performed four hours post-inoculation confirmed that cells had attached to the microcarriers (Table 1).

**Table 1. The attachment rate constant, $k$, of TEFLYRD/83 cells to different microcarriers under intermittent stirring modes in 100 ml spinner flasks and the final number of attached cells after four hours.** ***(Data represent mean values ± standard deviation, n = 4.)***

| Microcarrier | Attachment rate constant, $k$ (hour$^{-1}$) | $r^2$ | Final cell number (x $10^6$ cells)* |
|---|---|---|---|
| Cytoline 1 | 1.3556 | 0.8898 | 3.29 ± 0.91 |
| Cytoline 2 | 1.3004 | 0.9730 | 4.53 ± 0.66 |

*The number of attached cells was determined by an MTT assay after 4 hours.

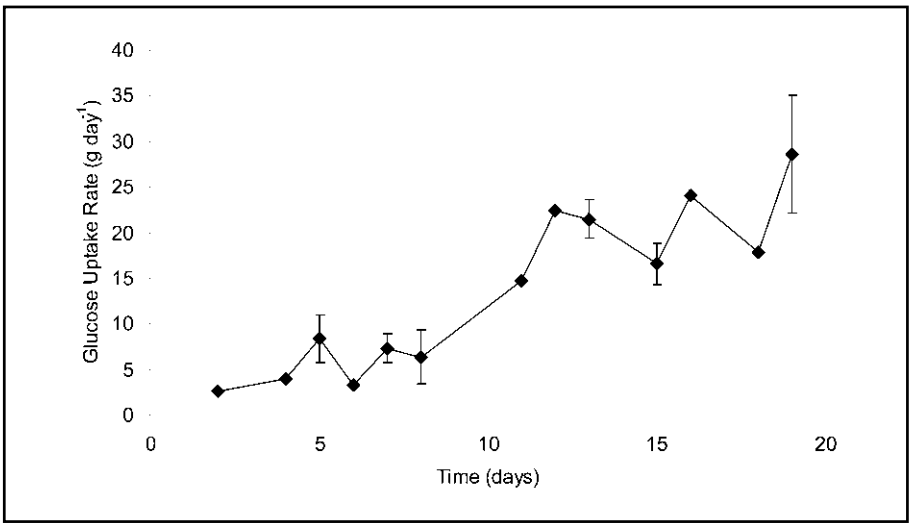


Figure 3. The mean glucose uptake rate (GUR) of a perfusion culture of TEFLYRD/83 cells in a fluidized bed bioreactor. The GUR was calculated from the change in glucose concentration and the perfusion rate. Error bars represent standard deviation.

### *Cell Growth and Virus Production*

The culture was run for 19 days. During the course of the experimental runs, the glucose concentration was maintained above 1.5 g/L$^{-1}$ by adjusting the perfusion rate to prevent substrate limitation. The GUR increased for the duration of the experiments, indicating an increase in cell numbers (Fig. 3). The final cell density was estimated as 5.27 ± 0.83 x $10^6$ cells/ml of microcarrier. This value is comparable with CHO cell cultures under the same conditions at the same time point.[9] The specific cellular growth rate was calculated as 1.09/day$^{-1}$.

Figure 4 shows the virus production in the Cytopilot bioreactor. The virus titer increased throughout the runs, in accordance with the increase in cell density. The highest virus titer was achieved at the end of the run and was $10^7$ LTU/ml. The cumulative virus titer was calculated as 5.03 x $10^{10}$ LTU in 11.4 liters of medium. These data also were used to calculate the specific virus production rate, which was estimated as 4.5 x $10^{-3}$ LTU/cell$^{-1}$/day$^{-1}$.

## Discussion

Cytoline microcarriers were selected because they are the carriers recommended for use with the Cytopilot fluidized-bed bioreactor. Cellular attachment occurs through electrostatic interactions.[10] Charged surfaces support better cell attachment than carriers coated with an extracellular adhesion component such as collagen or fibronectin.[11,12] The rapid attachment of cells to macroporous microcarriers is consistent with previous reports. It has been shown that the FLYRD18 cell line efficiently attaches to a variety of microcarriers within a few hours or less. For example, more than 80 percent of cells were able to attach to Immobasil FS carriers within one hour.[13,14] In contrast, it has been reported that TEFLY producer cells did not easily attach to solid microcarriers and had a tendency to form aggregates.[15] This discrepancy may be due to the surface properties of the carriers and the charge of the substrate; Cytodex solid microcarriers carry a high positive charge due to the N, N-diethylaminoethyl groups, whereas the silica content of Cytoline 1, Cytoline 2, and Immobasil carriers produces a slightly negative charge. Cytoline 1 microcarriers were chosen for the experimental runs due to the greater attachment rate, larger surface area (0.3 m$^2$/g$^{-1}$ vs. 0.1 m$^2$/g$^{-1}$), and higher carrier density. The higher carrier density requires greater circulation through the bed to achieve fluidization, which is

beneficial for nutrient transport to, and product removal from, the cells.

The fluidized-bed bioreactor has a number of features that make it attractive for the production of retroviral vectors. The cell number increased over the course of the experiment and had the potential to grow further based on the available surface area. According to Kong, 300 ml of Cytoline 1 microcarriers have an available surface area of 40.5 $m^2$.[9] However, surface area is not the only consideration when assessing growth potential. Limitations in nutrient transport to cells, removal of toxic metabolites from cells, and changes in local pH lead to the development of necrotic zones in macroporous microcarriers. It was possible to maintain the glucose above 1.5 $g/L^{-1}$ in the bulk medium by increasing the perfusion rate; continuous removal of medium also prevented the buildup of toxic waste products. However, cellular activity is determined by the chemical concentrations in the particle, not in the bulk medium. Concentration gradients can cause cellular viability and functionality to vary greatly, depending on their internal position. The glucose consumption rate for the system is the sum of all the individual uptake rates for each particle. Fluidized-bed bioreactors provide a very high mass transfer interface since both cells and fluid are moved.[6] Consequently, intraparticle mass transport is superior to other production systems such as stirred-tank bioreactors.

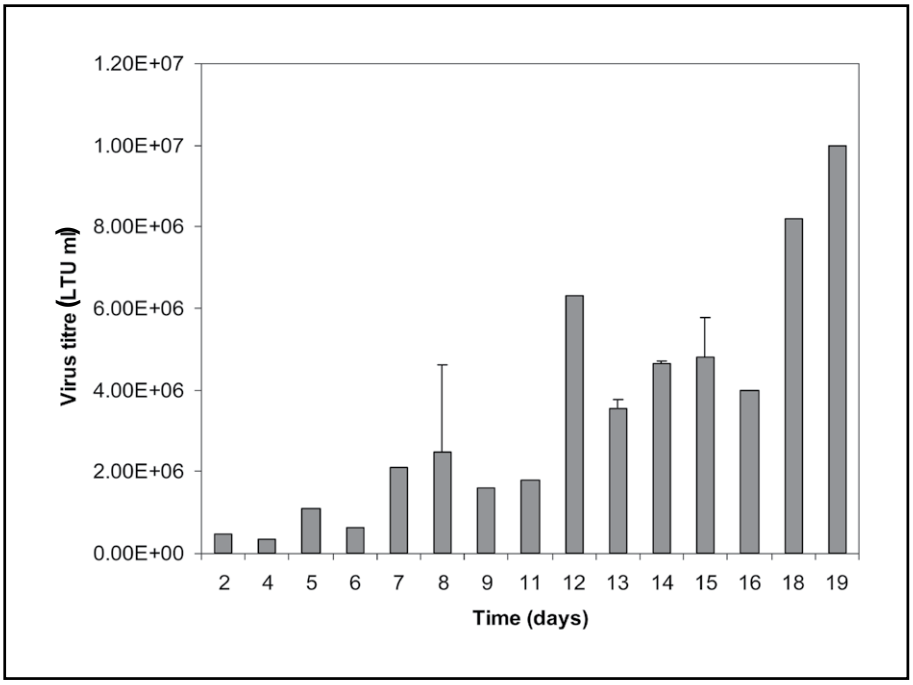


Figure 4. Mean daily virus titers for a perfusion culture of TEFLYRD/83 cells cultured in a fluidized bed bioreactor. The infectious virus titer was measured with the LacZ titration assay. Error bars represent standard deviation.

The fluidized-bed bioreactor demonstrated good vector production, with a maximum virus titer of $10^7$ LTU/ml, and a total viral yield of 5.03 x $10^{10}$ LTU. In roller-bottle cultures, daily titers of 5 x $10^6$ LTU/ml are achieved from 50 ml per bottle (unpublished results). Therefore, the total yield obtained from the Cytopilot over 19 days is equivalent to the yield from 10 daily harvests from 20 roller bottles. It was observed that virus titer increased with an increasing cell number. Therefore, because the Cytopilot has the potential for higher cell numbers in longer runs it also has the potential for greater viral titers. In contrast, the cell number in roller bottles reaches its maximum within 3 to 5 days. Thus, an increase in total viral yield using roller bottles can only be accomplished by an increase in unit number or longer culture periods.

Preliminary data recently published by Merten have also shown the potential of the Cytopilot; the PA317 packaging cell line was cultured on 150 ml of Cytoline 1 for 336 hours and produced a total vector harvest of 1.33 x $10^{10}$ infectious particles.[3] This was comparable with their standard CellCube process. Culture conditions were not optimized in this study, however, indicating that product yield could be greatly improved. Merten also compared microcarrier cultures, clump, and fixed-bed cultures using the TEFLY packaging cell line (Table 2).[1] The maximum virus titers obtained from clump and microcarrier cultures are similar to those

**Table 2. Production of retroviral vectors with alternative perfusion culture systems using the TEFLY packaging cell line.***

| | Fixed-bed bioreactor (70g Fibra-Cel™) | CellCube | Microcarrier culture (5 g $L^{-1}$ Cytodex 1) | Clump culture |
|---|---|---|---|---|
| Duration (days) | 12 | 15 | 16.2 | 10 |
| Average virus titer (x $10^7$ ml) | 2.97 | 2.10 | 0.954 | 2.45 |
| Maximum virus titer (x $10^7$ ml) | 4.31 | 3.50 | 1.99 | 0.05 |
| Perfusion rate (reactor volumes per day) | 2.4 | 3.3 | 2 | 1.6 |

*Merten OW, Cruz PE, Rochette C, Geny-Fiamma C, Bouquet C, Goncalves D, Danos O, and Carrondo MJ. Comparison of different bioreactor systems for the production of high titer retroviral vectors. *Biotechnol Prog* 2001;17:326–335.

obtained in the Cytopilot. However, the maximum virus titers obtained in the fixed-bed cultures, both from the CellCube and the Celligen basket reactor, are approximately fourfold higher than the Cytopilot. There are two possible reasons for this difference in performance. First, the culture medium used by Merten *et al.* contained five percent fetal calf serum compared with 10 percent in this current study. Serum has been shown to have a negative dose-dependent effect on virus production because it contains proteases that inhibit intracellular virus assembly.[13,16] Hence, cells cultured in 5% serum would have a higher specific production rate then cells cultured in 10% serum. Second, the perfusion rate used in the fixed-bed reactor systems was significantly higher than the rate used in the fluidized-bed bioreactor. Characterization of the virus produced by the RD83 clone has shown that its half-life is six hours at 37º C.[17] Therefore, at higher perfusion rates the residence time in the reactor is less and virus particles can be collected before they become noninfectious.

The final consideration when assessing a system for the production of any biopharmaceutical product is the scale-up potential. Current trends in retroviral vector production favor the use of basket-type packed-bed reactors and the CellCube.[3] However, these systems may not be suitable for large-scale production. Basket-type reactors are not used by industry because scale-up is limited by the formation of concentration gradients, and outgrowth of cells leads to channel blockage, dead zone formation, and a non-homogenous system.[18] The largest CellCube system provides a surface area of 34 $m^2$, limiting its potential. Additionally, the complexity of the system, sterilization, and the assembly within a laminar-flow cabinet pose problems for safety and quality-control validation.

The Cytopilot possesses many advantages over other immobilized cell systems. The constant flow of medium through the bed provides good mass transport and mixing within the system, resulting in a homogenous culture. The Cytopilot also has good potential for volumetric scale-up; fully validated reactors are available with a working volume up to 400 L. Furthermore, the unique features of the fluidized bed allow the microcarrier concentration to be 50% of the reactor volume.

In conclusion, the Cytopilot showed efficient cell growth and vector production. The scalability of the bioreactor makes it an attractive option for the commercial production of retroviral vectors for use in gene therapy applications.

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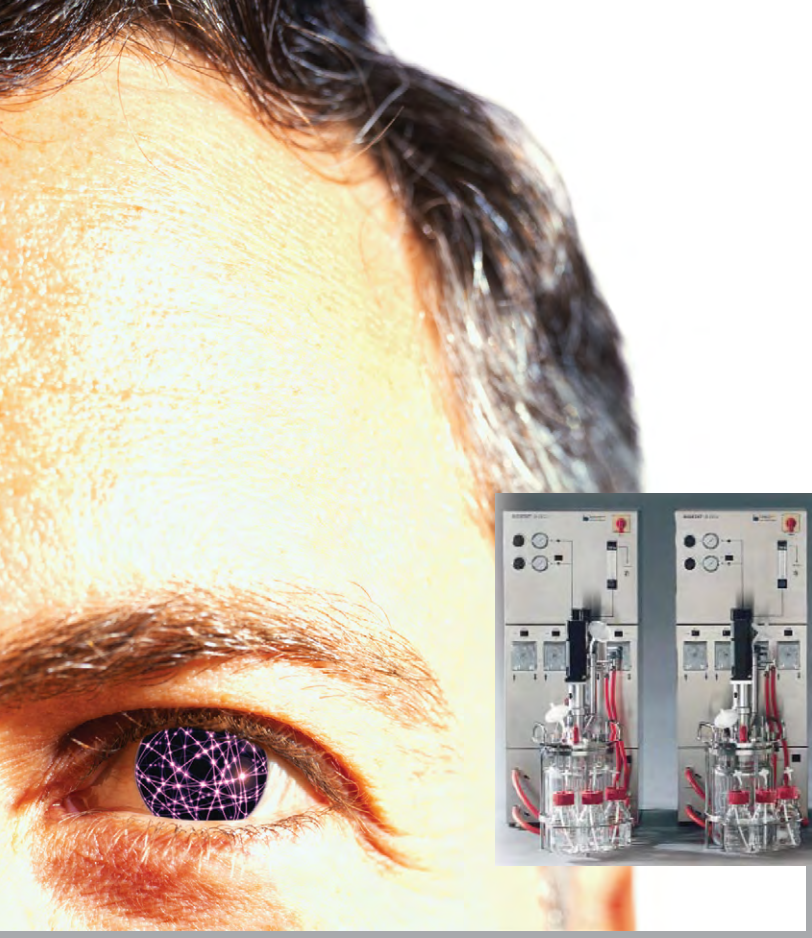

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- double wall vessels
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- mag drive
- pH-dual pH
- DO-dual DO
- vessel temperature
- jacket temperature
- foam
- level
- weight
- redox
- gravimetric feed
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