Volume 10 / Issue 2 • ISSN 1538-8786

BioProcessing JOURNAL

Trends & Developments in BioProcess Technology

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BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Optimising the *Drosophila* S2 Expression System for Production of Therapeutic Vaccines

By CHARLOTTE DYRING

Continuous cell lines of *Drosophila* have now been in use for more than two decades as hosts for the expression of heterologous gene products. The most popular are the Schneider lines 2 and 3 (S2, S3)[1, 2] and a few derivatives of the Kc line.[3] These have been widely employed for both stable and transient gene expression. The S2, in particular, is an exceptionally versatile system that has proven to be useful for high-level protein production. S2 cells have been used for the expression and analysis of intracellular, secreted, and membrane-associated proteins. This includes cytokines, antibodies, receptors, and viral antigens. These have all been shown to be authentically-processed, biologically active, and produced to high levels.

The cell line is ideally suited for the development and industrial-scale manufacturing of a wide range of biopharmaceuticals including vaccines and therapeutic protein products. Compared to conventional technologies, the strengths of the S2 expression technology lie in its quick access to proteins, excellent protein expression capability, scalability, regulatory-friendliness, and applicability to high cell density perfusion cultivation.

Modes of Expression

Insect cell expression can be divided into two different expression modes: 1) lytic expression where cells lyse upon infection with a recombinant baculovirus containing the gene of interest; and 2) stable expression where insect cells are transfected with an expression vector followed by selection and screening of polyclonal or monoclonal high-producers.

Lytic Expression

Insect cell lines used with baculovirus expression vector systems (BEVS) are derived from insects including the fall army worm (*Spodoptera frugiperda*: Sf9 and Sf21[a clonal derivative of Sf9]), the cabbage looper (*Trichioplusia ni*; Hi-5), and derivatives thereof, such as Protein Sciences' expresSF+ cell line (Sf9) and GlaxoSmithKline's Hi-5 cell line (Hi-5 Rixx4446).

Advantages: Quick access to biologically active proteins (weeks rather than months or years compared to mammalian cells); baculoviruses are harmless to humans.

Disadvantages: Proteolysis of the protein (due to its lytic nature); baculovirus infection is an extra step in the process; heterogeneous glycosylation patterns; difficulties in reproducibility and consistency between production runs; production in perfusion mode not possible.

Stable Insect Cell Lines and Non-Lytic Expression

In this mode of expression, an expression vector is stably integrated into the chromosome of the insect cell and the recombinant protein is expressed without

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subsequent cell lysis (comparable to traditional protein expression in mammalian cells). Cell lines used with this expression system include Sf9, Sf21, Hi-5 and *D. melanogaster* cell lines such as the S2. Sf9, Sf21, and Hi-5 are traditionally used with BEVS and S2 cells are the most widely used cell line for non-lytic expression.

Advantages: Homogeneous glycosylation profile; reproducibility between production runs; several options for cultivation modes such as perfusion that allows for long cultivation times with daily harvests; no cell lysis, resulting in lower levels of contaminating host cell proteins.

Disadvantages: Additional stable cell lines need to be established (unless transient transfection is pursued).

The founders of ExpreS²ion Bio have extensive experience with stable transfected S2 expression and have received approval for, and then performed, human phases 1 and 2 clinical trials in patients with metastatic breast cancer (Pharmexa HER-2 AutoVac™) using the S2-derived drug substance. Toxicology and efficacy studies in monkeys with the S2-derived Pharmexa receptor activator of nuclear factor κ-B ligand (RANKL) AutoVac have also been performed. Other groups have also seen the advantages and are actively working with expression systems utilizing stable S2 cell lines: Hawaii Biotech with lead vaccine candidates in clinical development that target the West Nile virus and Dengue virus. Additional recombinant protein subunits vaccine candidates produced from this protein production platform are currently under development. The Dengue fever vaccine unit was recently acquired by Merck Sharp & Dohme.

S2 cells are particularly good at expressing complex proteins and they provide a eukaryotic environment conducive to the post-translational modifications required for biological activity. Glycosylation differs from that of mammalian cells in that it is shorter (Figure 1) which can be beneficial for vaccine production due to enhanced immunogenicity. A consistent glycosylation profile is crucial, and stably transfected S2 cells provide a reliable, reproducible oligosaccharide profile (Figure 2).

The ExpreS² Expression Platform

ExpreS²ion's technology package consists of the following elements:

- a highly efficient expression vector series with different features to be used for expression of recombinant proteins when transfected into *Drosophila* S2 cells;
- a proprietary and optimized expression host derived from *Drosophila* S2 for efficient expression of recombinant protein;
- a cell culture medium developed to sustain increased growth and high-titre production of recombinant proteins in *Drosophila* S2 cells; and
- a very effective transfection reagent to introduce vector DNA into cells at high transfection efficiency.

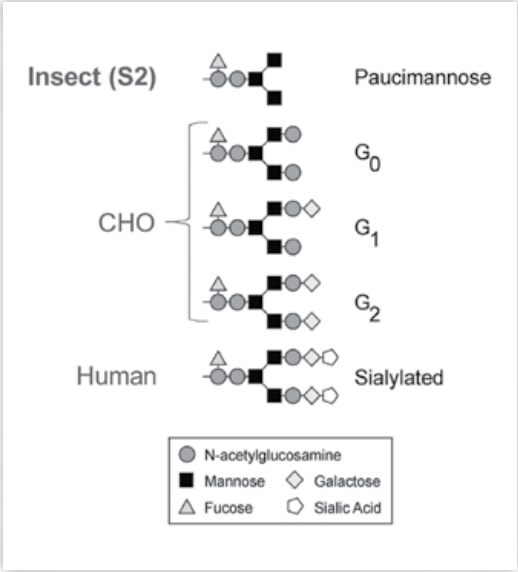

FIGURE 1. Glycosylation in S2 cells differs from that of mammalian cells in that it is shorter and ends on mannose (paucimannose structure) while that of CHO cells is either G0, G1, or G2 depending on the number of terminal galactoses. A non-mammalian glycosylation may be beneficial for vaccines.

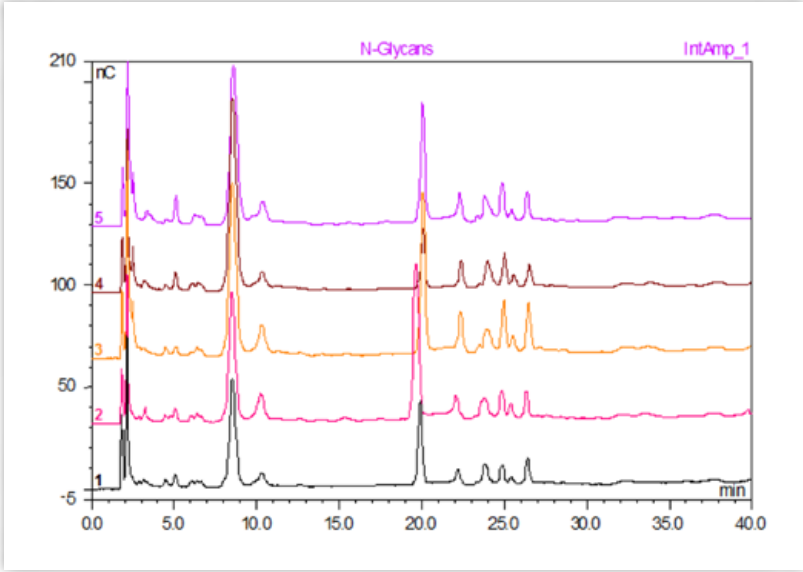

FIGURE 2. Reproducible oligosaccharide profile of five GMP batches produced using an S2 expression system.

The expression package offers ease of use, high success rates, consistently high yields, reproducibility, up-scalability, cost-time savings, and compliance with cGMP and regulatory requirements (Figure 3).

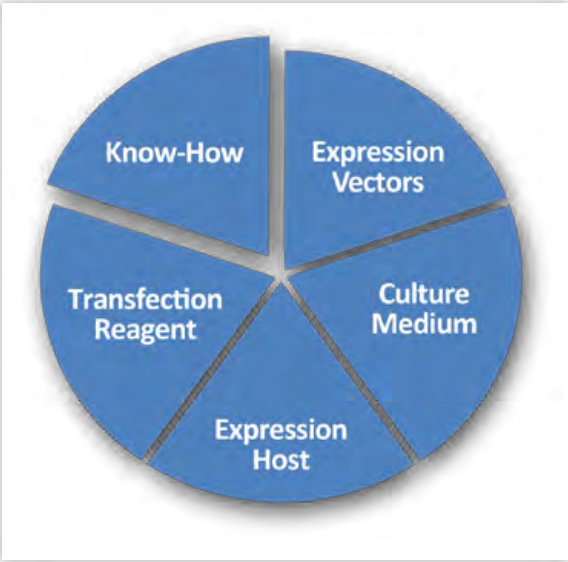

FIGURE 3. The ExpreS^2 protein expression package.

Expression Vectors

Initial vector development focused on screening native *Drosophila* promoters. Two that were of particular interest are associated with heat shock protein 70 (HSP70) and the actin5C protein. These promoters were further investigated by making truncation variants of each and testing the effects on expression levels for transient transfections and stable cell lines. It was found that a truncation variant of the actin5C promoter was constitutively the strongest promoter, but a truncation variant of the HSP70 promoter lead to a similarly strong deregulated promoter. Further truncation studies showed that the core (or shortest) active promoter from the HSP70 promoter was more than twice as "strong" as the core promoter from actin5C. A hybrid promoter was therefore created where the best actin5C truncation variant had its core promoter exchanged with the stronger HSP70 core promoter. This resulted in a 50% increase in expression level over the best actin5C truncation variant (Figure 4).

However, the strength of the promoter is not the only factor influencing the expression level. Chromosomal positioning and export of RNA from the nucleus are also known to play a role. These possible bottlenecks were addressed by the addition of an intron to promote export of the RNA from the nucleus or by flanking the

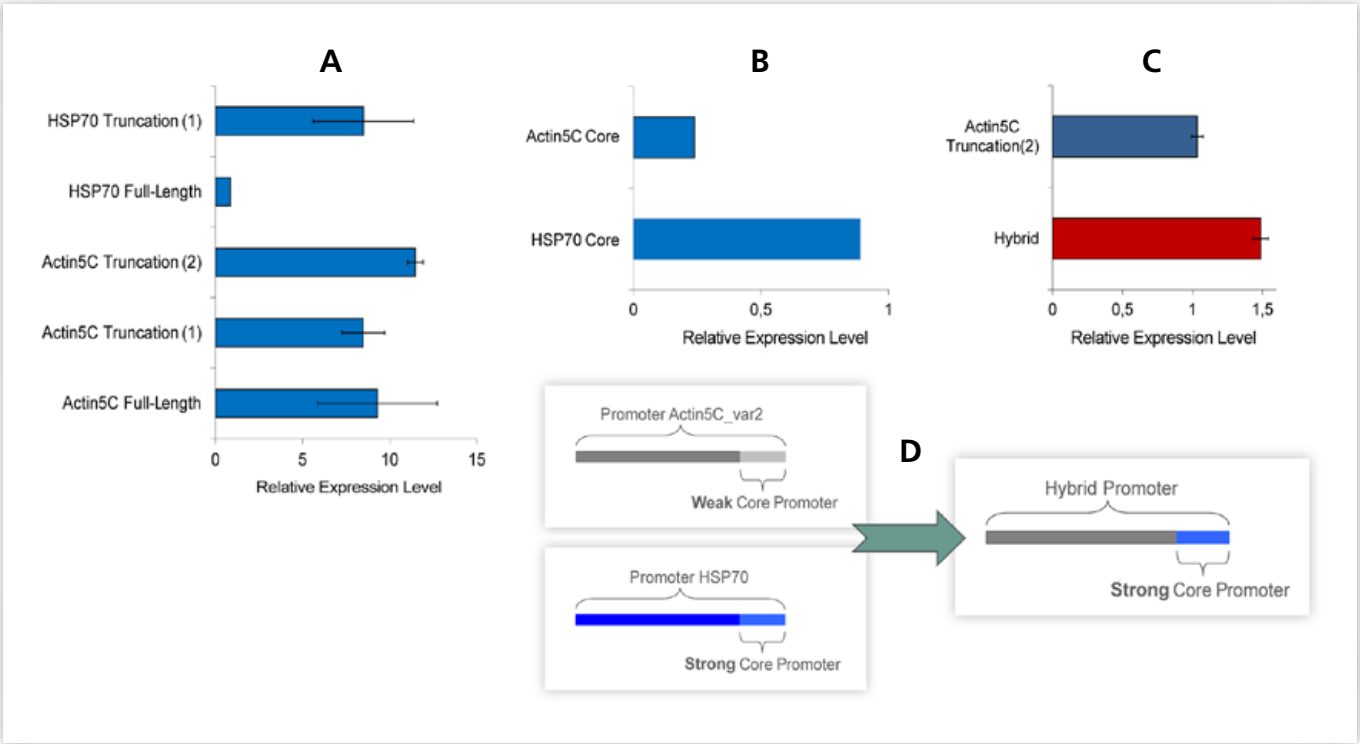

FIGURE 4. The development of the vector is presented here. (A) Initial promoter screen and variant analyses showed that a truncation of HSP70 and actin5c had the highest expression level. (B) Next, the core promoter of HSP70 is demonstrated to be superior to the core actin5C promoter. (C) Finally, the hybrid promoter is shown to be 50% better than the previous best actin5c promoter. (D) Additionally, a graphic representation of the hybrid promoters construction is presented.

expression cassette with a matrix attachment region (MAR) from the *Drosophila* HSP70 locus. MARs insulate the expression cassette from possible negative influences related to chromosomal positioning, thereby ensuring that the gene-of-interest is accessible for expression. Both of these approaches further improved expression by 20% and 50% respectively compared to hybrid promoter in stable cell lines.

The final ExpreS^2 expression vectors were compared to two of the most widely used and commercially available vectors from Invitrogen for the expression of three different proteins: 1) RANKL, a cytokine; 2) hemagglutinin A (HA); and 3) green fluorescent protein (GFP). The ExpreS^2 vectors were up to 20-fold better in terms of both transient and stable transfection (Figures 5 and 6).

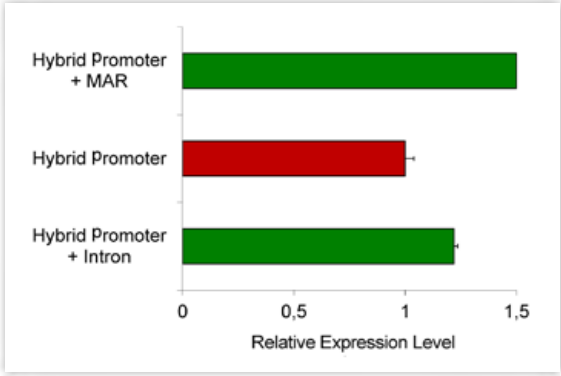

FIGURE 5. The hybrid promoter was compared to two vectors where additional expression enhancing elements were added. The matrix attachment regions (MARs) and intron improved protein expression by 50% and 20%, respectively.

Cell Line

Most S2 cell lines available for purchase from organizations such as ATCC and Invitrogen are limited to research applications and come with a material transfer agreement (MTA) excluding use for commercial purposes.

The ExpreS^2 cell line developed by ExpreS^{2}ion Biotechnologies grows to a higher cell density than standard S2 lines and can be used commercially. The cell line was derived by cloning using limited dilution followed by evaluation of the growth properties of the clones in shake flasks. The best clone was compared to standard S2 cells from ATCC over a period of ten weeks (19 passages — two passages per week) to demonstrate the improved cell growth characteristics. Cells were split by centrifugation every 3 – 4 days to 8E6 cells/mL and counted at day 3 or 4 using an Innovatis CASY TT cell counter (Roche Diagnostics). As can be seen in Figure 7, the clone reached a 40% higher final cell density than standard S2 cells.

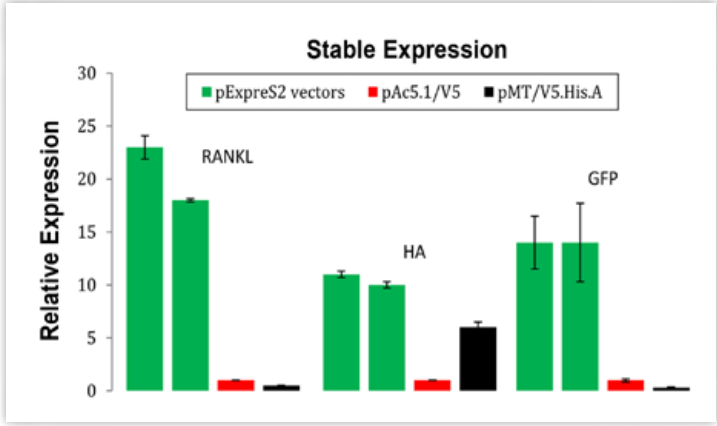

FIGURE 6. The final ExpreS^2 expression vectors were compared to two of the most widely used, commercially available vectors from Invitrogen for expression of three different proteins: RANKL, HA, and GFP. Relative yields from stable polyclonal pools are shown.

Culture Medium

The ExpreS^2 serum-free culture medium (SFM) was developed using a commercially available culture medium for insect cells as a base, and the composition of nutrients (vitamins, lipids,

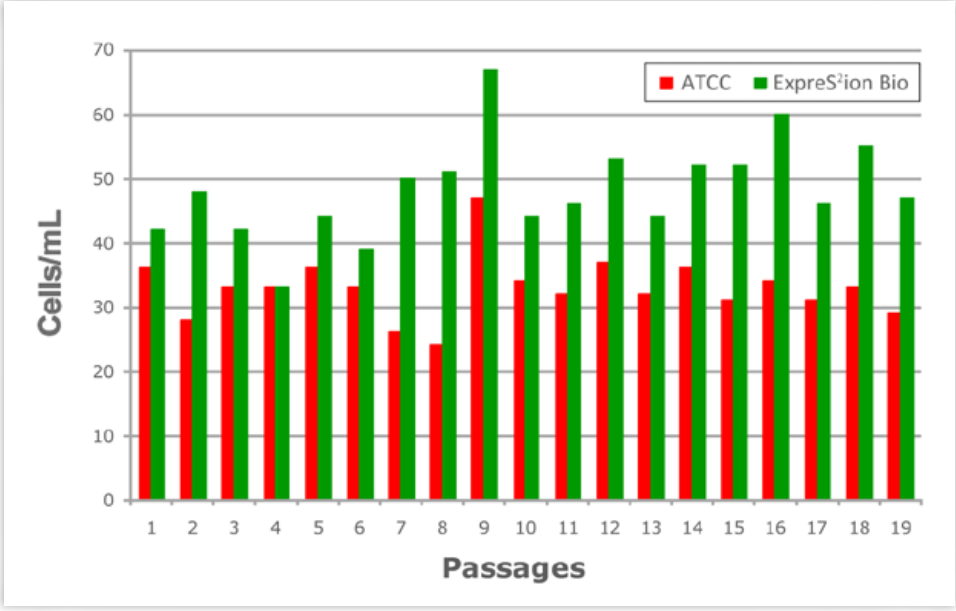

FIGURE 7. Comparison between the ExpreS^2 cell line and S2 cells from ATCC.

amino acids, sugars, trace elements) was adjusted until a three-fold increase in yield had been obtained. The optimisation was performed in shake flasks using a stable monoclonal cell line expressing a cytokine, and the results were confirmed using a polyclonal cell line expressing hemagglutinin A (Figure 8). Increased cell growth was confirmed using non-transfected ATCC S2 cells and ExpreS^2 cells (results not shown).

Transfection Reagent

We also developed and optimised a liposome-based transfection reagent (ExpreS^2 Insect-TR) for S2 cells that is more efficient than other commercially-available transfection reagents that are most often optimised for other cell types but will work in S2 cells. Insect-TR is also very efficient for transfection of Sf9 cells. Using this reagent, we consistently get a good transient transfection — the basis of a good stable cell line.

ExpreS^2 Platform Benefits

The key advantages of the S2 expression system are:

- It is a rapid and stable process.
- Very high cell densities can be achieved without aggregation or toxic metabolite issues.
- The platform is suited for perfusion processes.
- The risk of human viral contaminants is reduced.
- It offers consistency and reproducibility.

Fastest in Achieving a Stable Process

Transient expression can be attained with S2 cells, allowing for fast screening and initial selection of protein candidates for further development. Generation of stable, high yielding cell lines takes only an additional ten days (Figure 9).

Generating the host cell line(s) and the protein production needed for downstream development and animal experiments are usually the time-consuming, production-limiting steps. However, by using the S2 expression system, time from DNA to protein can be dramatically reduced from months to just weeks by using stable polyclonal pools. A hallmark of the S2 system is the one-step gene amplification that occurs during selection generating high-expressing cell lines that don't require the additional amplification often needed in CHO-based systems. In combination with the stability of polyclonal pools—another advantage of the S2 cells—the cumbersome and time-consuming step of clonal isolation to produce recombinant proteins can completely be eliminated in the discovery phase.

This unique feature is particularly useful in expressing many different proteins and/or variants thereof for evaluation and characterisation—both in discovery and R&D—in a short timeframe. Additionally, by applying stable pools of S2 transfectants for protein production, the development of upstream and downstream processes as well as the establishment of analytical tools can be dramatically sped up. They could potentially be in place when cloning, selection, and screening processes are completed. This saves time and money, and exploits a company's manpower and financial resources in the best possible way.

Very High Cell Densities Without Aggregation or Toxic Metabolite Issues

Cultures of S2 cells typically reach cell densities more than ten-fold greater than that of other insect or mammalian cell lines in batch processes (up to 70 million cells/mL). Increased viable cell concentrations usually translate into increased production yields and therefore improved process economics. High volumetric

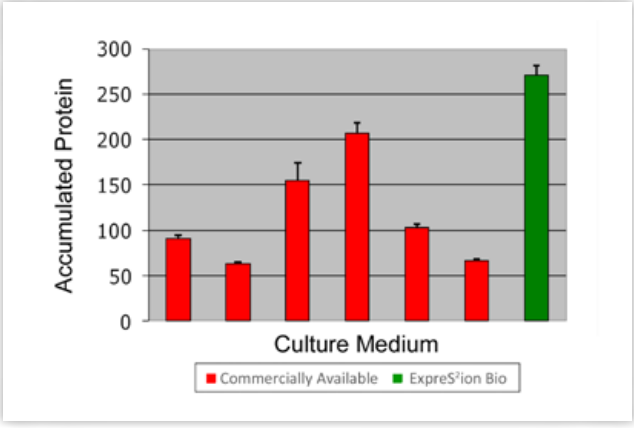

FIGURE 8. The ExpreS^2 SFM was compared to six different commercially available media after weaning. The experiment was performed in triplicate using cell cultures in shake flasks.

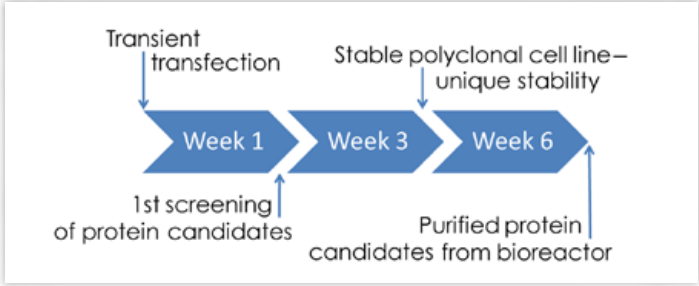

FIGURE 9. From transfection to purified protein from bioreactors in just six weeks: Stable cell lines are established after transfection by culturing the cells in the presence of selection reagent for three weeks. The transient expression level can also be analysed to give a quick answer to expressability of the protein. Once the stable cell line is established, selection reagent is removed from the medium and the cells are expanded into bioreactors.

productivity can be obtained in batch modes facilitating ease of production and up-scaling in the discovery phase.

A macrophage origin of the S2 cell type may explain the preference for cells to grow in suspension even in unagitated and unaerated environments.[4-6] S2 cells are exceptionally well suited for perfusion processes because the cells do not aggregate even at very high cell densities (>150 million cells/mL) (Figures 10 and 11). Other cell lines such as CHO, BHK, and HEK have a tendency to aggregate at high cell densities which complicates both process development and manufacturing. Aggregation may lead to a number of process-related problems such as: 1) a lack of control with the culture due to differentiation in physical culture conditions; 2) decreased cell viability; and 3) the release of proteases and lysed cell components making downstream processing difficult and altering overall product recovery and homogeneity.

Suitability for Perfusion Processes

The perfusion cell culture mode is a cost-effective technology that provides high cell density and volumetric productivity, and allows for continuous protein harvest from the bioreactor. Because S2 cells do not aggregate, perfusion is an ideal method.

Compared to fed-batch fermentation methods, capital and start-up costs are lower for perfusion technologies as smaller upstream and downstream capacities are required and the process uses smaller volumes with fewer seed steps. Batch failure costs due to contamination are reduced in perfusion technologies. Should a contamination occur, product harvested prior to contamination will not be affected and can still be processed. If infection arises early in the perfusion run, then only a small volume of media will have been wasted. If contamination happens further into the run, significant non-contaminated product will have already been harvested. With either scenario, the high cost of failure is reduced when compared to fed-batch where the whole run will usually be lost.

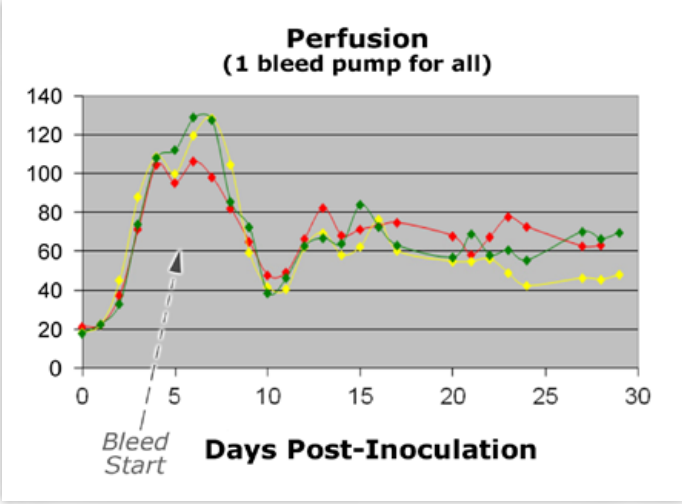

FIGURE 10. High cell densities can be obtained using perfusion and controlled using a bleed pump. In this particular example, three bioreactors were controlled using one and the same bleed pump accounting for the differences in cell density control due to slightly different growth in the three bioreactors.

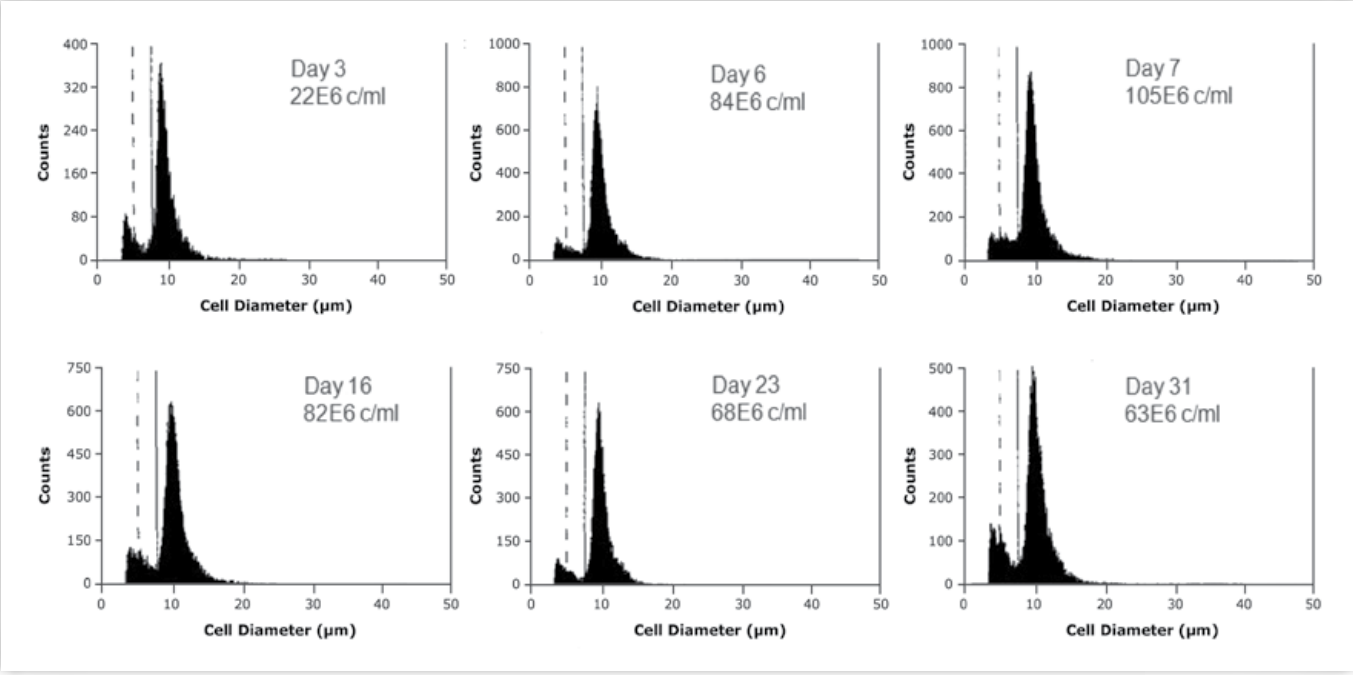

FIGURE 11. Cell size distribution of ExpreS^2 cells in a perfused bioreactor at different days in cultivation and at different cell concentrations (controlled using cell bleed). No cell aggregation was observed.

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Furthermore, S2 cells are resistant to the toxic effects of fermentation waste products such as ammonia and lactate. In contrast to mammalian cell cultures, our studies have shown that S2 cells can tolerate very high concentrations of ammonia, a catabolic by-product of the glutamine in culture medium that does not usually accumulate in S2 cultures. Furthermore, S2 cells do not produce lactate during growth like CHO and other mammalian cells do, except when under conditions of anoxic stress.

Reduced Risk of Viral Contaminants

Producing biological products for human use does come with some inherent risks due to the potential for virus and cell contaminants—and employing the insect cell production system has a relatively short track record. However, the same tried and tested techniques (*e.g.*, electron microscopy, *in vitro* and *in vivo* assays) that have been utilised to ensure the safety of other cell lines used in biologics production can and do provide the same assurance with insect cells. It is also noteworthy that many human pathogens, some of them potentially harmful, are unable to grow and replicate in insect cells. Therefore, unlike human or mammalian cell lines, insect cells can provide a distinct benefit with respect to these safety issues. The risk that a virus can replicate in both human and S2 cells is remote.[7]

Consistency and Reproducibility

It is vitally important to ensure the consistency of the pharmaceutical proteins. Variations in product quality can have a negative impact on product efficacy and safety as well as regulatory acceptance. The consistency of S2-produced material is clearly demonstrated in Figure 12 (rHA protein-specific Western blots) as one single band for each of the HA products (HA1, HA2) as well as trimeric HA2, respectively, in contrast to the multiple bands shown for BEVS-produced material in Sf9 and Sf21 cells.

Because BEVS is a lytic system, and by nature difficult to control, lysing cells can lead to variations in product consistency caused by varying glycosylation and signal peptide cleavage. This is evident in the lanes in each Western blot which correspond to different harvest time points (BEVS: days after infection; S2: days in perfusion) and the consistency of the S2 material which is clearly demonstrated by the day-to-day reproducibility.

FIGURE 12. Western blot showing hemagglutinin A production in different insect cells.

ABOUT THE COMPANY

ExpreS²ion Biotechnologies is a spin-out from Affitech A/S and was established in early 2010. The founders and employees of the company have worked together more than ten years to employ and optimise the S2 expression system for production of therapeutic vaccines. ExpreS²ion's proprietary protein expression platform ExpreS², consists of high yielding expression vectors, an S2 cell line that grows to higher cell densities than standard S2 cells, optimised culture media, as well as a highly efficient transfection reagent optimised for S2 cells (Figure 3 for overview).

Unlike BEVS, ExpreS² does not involve viral infection and the system is amenable to both transient and stable expression which is perhaps the most important feature distinguishing BEVS and ExpreS². The S2 cells are transfected with expression vectors and stable cell pools are selected using standard selection agents (*e.g.*, G418, hygromycin, or zeocin).

ExpreS²ion offers services related to vector development, cell line development, cloning, upstream development, optimization, and production of GLP material using the ExpreS² technology. ExpreS²ion can facilitate GMP production scale-up in collaboration with our own preferred CMO partners, with the customer, or with their preferred CMO. In addition, licenses to the expression platform are offered, and individual components (transfection reagent and culture medium) can be obtained by contacting the company.

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