APPLICATION NOTE No. 479

A Beginner's Guide to Sf9 Culture in Stirred-Tank Bioreactors

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Abstract

This application note presents a protocol for culture of Sf9 insect cells and the production of recombinant proteins via the baculovirus expression vector system in stirred-tank bioreactors. We explain the different workflow steps, including the preparation of viruses for the infection of Sf9 cells, Sf9 cell expansion in shake flasks, and Sf9 culture in bioreactors for recombinant protein production. In this study a bench-scale BioFlo® 120 bioreactor control system equipped with BioBLU® 3c Single-Use Bioreactors was used to produce green fluorescent protein (GFP) in Sf9 cells. The principles in this beginner's guide to Sf9 culture may be applicable to Sf9 cell culture processes at smaller or larger scales.

Introduction

Sf9 cells are derived from pupal ovarian tissue of S*podoptera frugiperda*, the Fall Armyworm. They are commonly used to produce recombinant proteins and to isolate and propagate recombinant baculoviral stocks. They also provide a suitable host for the production of other viral vectors like recombinant Adeno Associated Viruses (rAAV). Sf9 cells have become a popular expression platform due to their ability to be cultured in suspension, relative ease of use, high growth rate, and their capability to perform post-translational modifications.

For protein expression in Sf9 cells the baculovirus expression vector system (BEVS) is frequently used. BEVS is based on the use of insect viruses from the family baculoviridae. They are rod-shaped dsDNA viruses that infect lepidopteran species such as the one Sf9 cells were derived from. The system is widely used on insect cells for the expression of recombinant proteins due to its ability to produce high yields of correctly folded and biologically active proteins. Since its discovery and characterization 40 years ago by Smith et al. [1], BEVS has been used to produce thousands of proteins for various studies [2]. The technology has more recently been used for commercial manufacture of various veterinary and human vaccines, including several flu vaccines and a recently approved COVID-19 vaccine [2]. The flexibility, ease of use, and overall safety make it a valuable tool for researchers.

Baculoviruses have the potential to serve as gene delivery vehicles in the field of gene therapy. They are capable of transducing mammalian cells and therefore can potentially serve as vectors to mediate the expression of a protein or non-coding RNA that has a therapeutic effect. Rather than using baculovirus itself as a gene delivery vector, the BEVS insect cell platform is also being evaluated for the production of recombinant Adeno Associated Viruses (rAAV) for gene therapy applications [2, 5].

Use of the BEVS process starts with the construction of a recombinant baculovirus comprising the gene of interest (GOI). First, the gene of interest is cloned into a transfer

plasmid. This transfer plasmid is then transformed into competent *E. coli* cells resulting in a large recombinant plasmid containing the GOI and baculovirus genome (bacmid). This recombinant bacmid is then extracted and transfected into insect cells for virus production. This initial virus stock designated as passage 1 needs to be amplified to increase the titer and culture volume for use in producing recombinant proteins (Figure 1).

This application note demonstrates a workflow for protein expression in Sf9 cells in stirred-tank bioreactors at bench scale (Figure 2). Sf9 cells were cultivated using a BioFlo 120 bioreactor control system equipped with BioBLU Single-Use Bioreactors. A baculovirus containing green fluorescent protein (GFP) as the GOI was used to analyze protein production.

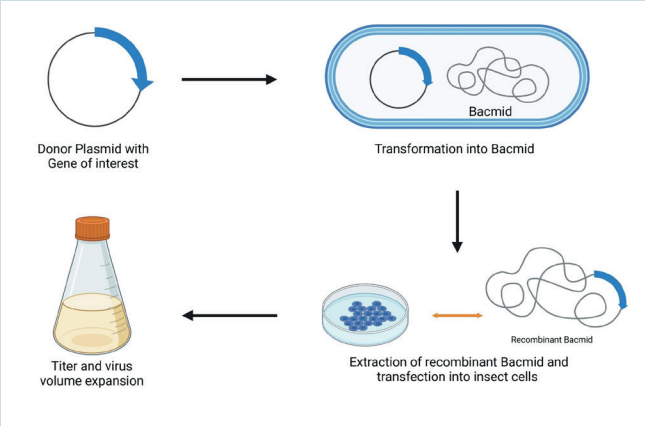

Fig. 1: Baculovirus construct generation. Created with BioRender.com

Material and Methods

Figure 2 shows the workflow steps for recombinant protein production in Sf9 cells described in this study.

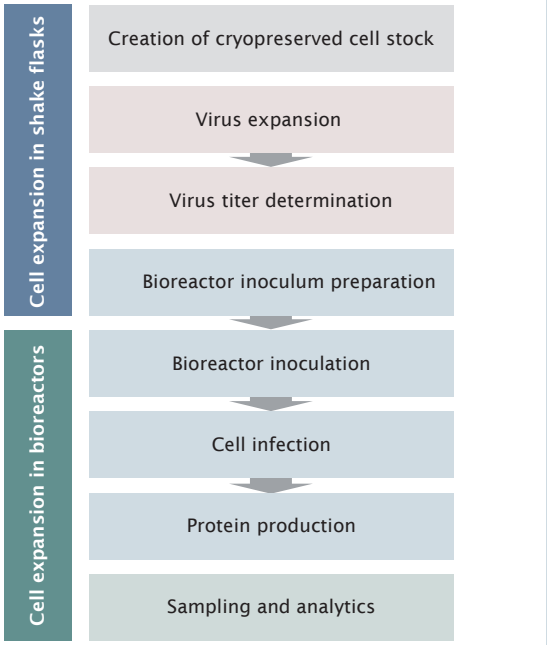

Fig. 2: Workflow steps for recombinant protein production in Sf9 cells described in this study.

Cell line and medium

In all experiments a suspension Sf9 cell line from Thermo Fisher Scientific® (Gibco Sf9 cells, cat. no. 11496015) was used. The cells were cultivated in Sf-900 II SFM (Thermo Fisher Scientific, USA) which is a serum-free, protein-free insect cell culture medium that is optimized for the growth of Sf9 cells. Cells were either cultivated in single-use, flat bottom, non-vented polycarbonate shake flasks (Corning®, USA), which were placed in an Innova® S44i incubator (Eppendorf, Germany), or using a BioFlo 120 bioreactor control system equipped with BioBLU Single-Use Bioreactors (Eppendorf, Germany). In the incubator, the cells were cultivated at 135 rpm and 27 °C. In the bioreactor, the cells were cultivated using the process parameters listed in Table 3.

Preparation of cryopreserved stock

The initial cell vial received was used to create a cell bank. The cells were thawed using a ThawSTAR® CFT2 instrument (MedCision®, USA). The cells were transferred into a 125 mL flask with 25 mL of pre-warmed medium. Cells were cultured in an Innova® S44i incubator set to 135 rpm and 27°C. After 3 days of culture the cells were checked for appropriate cell density and viability according to the product information provided by Thermo Fisher Scientific. Once appropriate

cell growth was confirmed, the cells were cultured for an additional three passages before cryopreservation. The cryopreservation procedure was done as follows with all steps done aseptically: First a 250 mL flask containing 100 mL of medium was seeded at 1.0 × 10^6 cells/mL. The cells were grown to mid-logarithmic phase of between 2.0 – 4.0 x 10^6 cells/mL, and the total cell number was determined for the flask. The cells were pelleted at 130 × g for 3 minutes (Centrifuge 5430 R, Eppendorf, Germany). They were then resuspended in a premade cryopreservation mixture from Table 1 below, at the target cell density of 1.5 × 10^7 cells/mL. The cells were aliquoted at 1 mL volume into 2 mL cryovials (Corning, USA). The vials were placed in a cell freezing container and placed into a –80 °C freezer and allowed to freeze overnight. The cells were then transferred to liquid nitrogen storage for future use in experiments.

Table 1: Cryopreservation medium

Components	Final concentration
Sf-900 II SFM conditioned medium	46.25%
Fresh Sf-900 II SFM	46.25%
Dimethylsulfoxide (DMSO) cryoprotectant	7.5%

Cell expansion in shake flaks and bioreactor inoculum preparation

To expand the virus or to prepare the bioreactor inoculum Sf9 cells were first expanded in shake flasks. A cryopreserved stock vial was thawed as described in the previous section. The cells were transferred into a 125 mL flask with 25 mL of pre-warmed media. After 3 days of culture the cells were checked for appropriate cell density and viability according to product information provided by Thermo Fisher Scientific. Once appropriate cell growth was confirmed the cells were cultured for two weeks before ramping up for virus stock production and bioreactor inoculation. The cells were passaged on Mondays, Wednesdays, and Fridays. The seeding density was 1.0 ×10^6 cells/mL on Monday and Wednesday, and 0.5 × 10^6 cells/mL on Friday. The flask fill volume was maintained between 30 % and 40 % for all flask sizes used. Table 2 below shows the passage and flasks and volumes used for the experiments.

Table 2: Cell culture flask and culture volume used

Cell passage	Flask size	Culture volume
1-2	125 mL	50 mL
3-5	250 mL	100 mL
5+	1000 mL	300 mL

Virus preparation

To demonstrate the production of a recombinant protein, we used a baculovirus containing green fluorescent protein as the gene of interest. Baculovirus producing GFP was obtained from ABM® (Applied Biological Materials Inc., Canada)

Virus expansion

The vial we obtained from the supplier contained 10 mL of virus with a titer of 10^6 infectious units/mL at passage 2. This virus needed to be expanded in order to increase the titer and volume to infect cells in a bioreactor. For expanding and amplifying a virus stock, a multiplicity of infection (MOI) of 0.5 or less is desirable to prevent the buildup of defective virus particles [3].

To expand the virus, a flask was seeded at a density of 1.0 × 10^6 Sf9 cells/mL and incubated overnight to a density of around 1.5 – 2.0 × 10^6 cells/mL. The cells were counted the following day using the Vi-CELL® BLU cell analyzer (Beckman-Coulter®, USA) to determine the total number of cells in the flask. The cells were infected at a multiplicity of infection (MOI) of 0.1 according to the formula below, then incubated as described above.

$$\text{Virus volume for infection [mL]} = \frac{\text{Desired MOI [pfu/cell]} \times \text{total number of cells}}{\text{Titer of viral inoculum [pfu/mL]}}$$

Formula 1: Determination of virus volume for infection
pfu: plaque forming units

The cells were harvested at 48 hours post-infection. To do so, the culture was transferred to centrifuge tubes using a serological pipette in a laminar flow hood, then centrifuged at 250 × g for 5 minutes to remove cells and debris. After centrifuging, the virus was transferred to a storage bottle in the laminar flow hood using a serological pipette and stored at 4 °C protected from light until use. This process was done successively two times for each bioreactor run to increase the virus volume and titer for use in a bioreactor.

Virus titer determination

The virus titer must be obtained to infect a cell culture at the appropriate MOI for protein production. The standard method for titer determination with BEVS is the plaque

assay. This method is labor and time intensive however, requiring many steps and taking over 7-10 days to accomplish. For our experiments, titer was determined for the virus stocks using the method described by Imasaki et al. [4]. The simple protocol allows for quick titer estimation and is simple to conduct. Briefly, a serial dilution of virus is made and used to infect a series of flasks with one uninfected cell growth control flask. The flasks are then counted 24 hours post infection to determine cell density. The assay is based on the assumption that infected cells do not divide. In that case, if all cells are infected, the cell densities are the same at the time of infection and 24 hours after infection. If a subpopulation is infected, it does not divide, while uninfected cells proliferate. Therefore, the initial infectivity can be calculated from the cell density 24 hours after infection and the MOI can be estimated from this. This is used to estimate the virus titer. To sum up, the titer can be calculated given the volume of virus stock added to the cell culture, and the cell density 24 hours after infection. A detailed protocol including the equations used can be found in reference 4.

Sf9 culture in bioreactors

Sf9 cells were cultivated using a BioFlo bioreactor control system (Figure 3). The BioFlo 120 has the ability to control both Eppendorf glass and BioBLU Single-Use Bioreactors. Single-use vessels were chosen for these experiments. Single-use vessels offer several benefits such as eliminating the need for cleaning and autoclaving of bioreactors. This helps in the reduction of preparation time and lowers the contamination risks for experiments.

Inoculation density

All cultures were inoculated at a density between 0.5 -0.6 × 10^6 cells/mL.

Infection

All cultures were infected at an MOI of 2, at different cell densities to see the affects this had on protein production.

Temperature

The temperature setpoint was 27 °C. The temperature was controlled using a heating blanket. The temperature setpoint should be entererd and achieved prior to inoculation.

pH

A gel-filled pH sensor was used to monitor pH. pH was monitored but not controlled for these experiments. We did not control the pH, because for Sf9 cultures in Sf-900 II SFM it is usually stable between roughly 6.1 and 6.5 and this fluctuation generally doesn't affect the run or product made.

Fig. 3: BioFlo 120 bioreactor control system equipped with BioBLU c Single-Use Bioreactor

Technical Features

The BioBLU c Single-Use Bioreactor portfolio covers a working volume range from 100 mL to 40 L. Various BioBLU Single-Use Bioreactor variants are available, which differ in the sparger type, number of impellers, impeller type, and compatible sensor types. Furthermore, single-use septa, tri-ports, and compression fitting adaptors facilitate the flexible use of Pg 13.5 ports.

For more technical information on the BioBLU c Single-Use Bioreactors, please visit www.eppendorf.group/bioblu-c

DO

The DO was measured using a polarographic sensor (Mettler Toledo®, Switzerland) and controlled at 50 % by sparging air and/or O_2 at a flow rate of 0.04 SLPM – 3.0 SLPM using the 3-Gas Auto mode for these experiments. A macrosparger was used.

Usually it is acceptable, if the DO setpoint is not achieved at the time of inoculation. An initial DO value of approximately 100 % is acceptable, it will decrease as the culture metabolizes oxygen.

Agitation

The culture was agitated using one pitched-blade impeller at 115 rpm (0.4 m/s tip speed).

Table 3: Process parameters and setpoints

Parameter	Device/setpoint
Starting volume	2.5 L
Inoculation density	0.5 – 0.6 x 10^6 cell/mL
Agitation	115 rpm (0.4 m/s tip speed)
Temperature	27 °C
DO	50 % (P=2; I=0.09)
Gassing range	Air flow: 0.04 SLPM – 3 SLPM O_2 flow: 0 SLPM – 3 SLPM
Virus volume	MOI of 2

Bioreactor run

After inoculation, bioreactor runs were performed using the process parameters described above.

Two or three days after inoculation the cells were infected with the virus at a MOI of 2. To do so, the virus was aseptically collected and spun down as stated in the virus preparation section and added to a sterile transfer bottle. When the culture reached the desired density, the bottle was connected to the bioreactor by welding and the virus suspension was transferred to the bioreactor using the system's pumps. After infection, cells were further cultured for 4 to 5 days.

Sampling and analytics

Samples were taken from each bioreactor daily. To ensure an aseptic sample was taken from the culture, a sterile 5 mL syringe was connected to the sample port Luer Lock. A 3 mL sample was taken and discarded. A second 3 mL sample in a new syringe was taken to be used for analytics and cell measurements.

Cell growth and viability

Cell number, viability, and cell diameters were measured with a Vi-CELL analyzer. Virus infection results in cell growth arrest and an increasing cell diameter and therefore monitoring these parameters can be used to estimate the success of the infection.

GFP production

Samples were taken daily starting 24 hours after infection for analysis of recombinant GFP production. Samples were taken and frozen at –80 °C. GFP expression was analyzed by fluorescence microscopy and measured using a GFP quantification kit from Abcam® (Abcam, UK).

Results

The purpose of this study was to demonstrate the feasibility of insect cell-based recombinant protein production using the BioFlo 120 controller. To evaluate this, two batch cultures were performed. For the first batch culture a cell density of 2.0 × 10^6 cell/mL was targeted for infection density. For the second batch culture a cell density of 3.0 × 10^6 cells/mL was targeted for infection density.

Analysis of cell growth

In the first run the cells doubled at a rate of roughly 24 hours reaching 2.1 × 10^6 cell/mL on the second day after seeding. The cells were then infected with the virus stock at an MOI of 2. The cell growth was arrested (Figure 4A). The average cell diameter increased from approximately 15.6 µm before infection to 17.5 µm 24 hours after infection. Cell viability began to decrease after infection, as expected for the virus-infected cell culture (Fig. 4A).

For the second batch a higher infection density was chosen, targeting 3.0 × 10^6 cell/mL. The growth of the cells was consistent with the first batch run and on day three reached a viable cell density of 3.1 × 10^6 cells/mL (Figure 4B). The cells were infected with the virus stock at an MOI of 2 as with the first batch run. Cell growth was arrested (Figure 4B). The average cell diameter increased from approximately 16 µm before infection to 17 µm 24 hours after infection.

Analysis of GFP expression

Samples were taken every 24 hours after infection to analyze protein production. GFP expression could be demonstrated by fluorescence microscopy (Figure 5). Furthermore, GFP

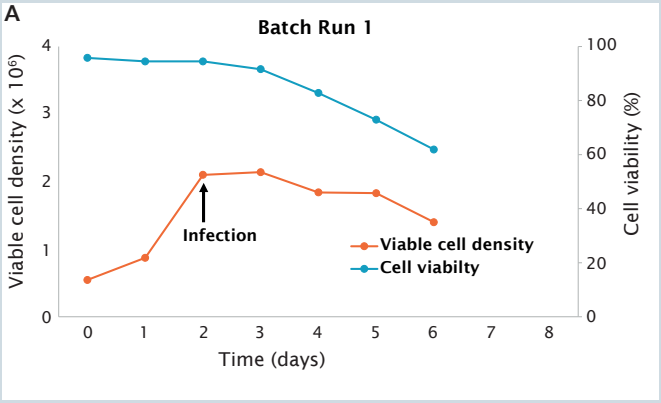

Fig. 4: Viable cell density and cell viability of batch run 1 (A) and 2 (B).

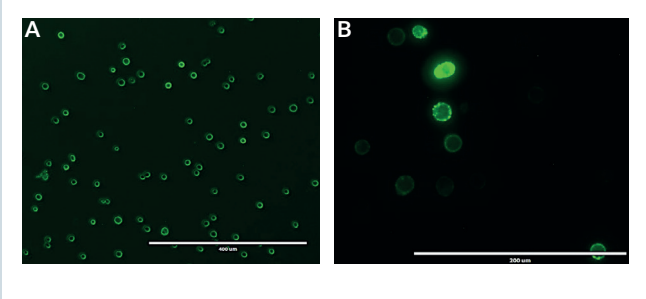

Fig. 5: Fluorescence microscopy of Sf9 cells infected with recombinant baculoviruses for the expression of GFP. Samples were taken 48 hours after infection.
A: Bar: 400 µm. B: Bar: 200 µm.

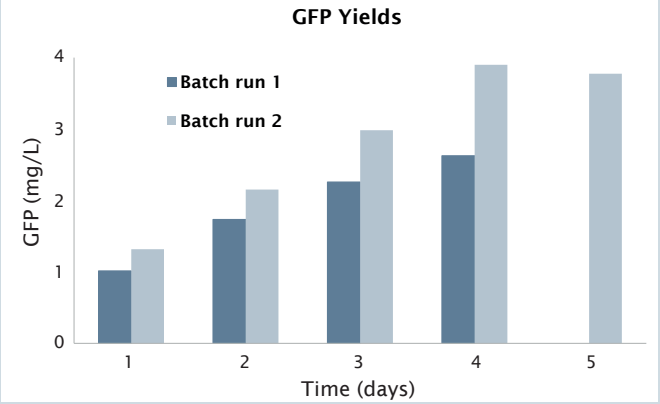

Fig. 6: GFP yield comparison for batch run 1 and batch run 2 at different days post infection.

production was quantified with the results shown in Figure 6. Batch run 2 had higher yields at each sample point with an almost 50 % increase in yield by day 4 as compared to batch run 1. This correlates with the increased density at infection for batch run 2. There was no sample taken on day five for batch run 1. For batch run 2 samples were taken every day until the protein concentration began to decrease, which was observed on day 5.

Conclusions

These experiments showcase a workflow for recombinant protein production in insect cells using BioBLU Single-Use Bioreactors under the control of BioFlo 120 bioreactor control system. Two batch cultures were successfully infected at different cell densities. The GFP analysis of each culture showed increased protein yield when infected at higher densities. The described protocol can serve as a starting point for further optimization.

References

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Ordering information

Description	Order no.
BioFlo® 120, base control station, with water connection	Inquire*
BioBLU® 3c Single-Use Bioreactor, macrosparger, 1 pitched-blade impeller, optical pH, sterile, 1 piece	1386125000
Innova® S44i, stackable incubator-shaker	Inquire*
Centrifuge 5430R, high-speed centrifuge	Inquire*

*Inquire the part number for your country

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