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APPLICATION NOTE No. 471

HEK293 Bioreactor Transfection for Vaccine Applications Using the Eppendorf SciVario® twin Bioprocess Controller: An Example with COVID-19 Spike Protein Production

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Abstract

Bioreactor suspension cell culture platforms are essential tools for vaccine production. They can support very high cell densities, allowing for much higher production yields and reduce the challenges associated with the scalability process of adherent cell culture. Bioreactor transfection is a method of deliberately introducing plasmids into large numbers of cultured cells for protein production. With the current global COVID-19 health crisis caused by the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), it is imperative to produce large quantities of vaccine components such as spike proteins, the predominant antigen of SARS-CoV-2 S vaccines.

To demonstrate feasibility, production of the SARS-CoV-2 S HexaPro spike protein was conducted using the suspension-adapted HEK293 cell platform Expi293F™ transfected with spike-protein-encoding plasmid DNA. To achieve high yield, the transfection was performed using a BioBLU® 1c Single-Use Bioreactor controlled by the SciVario® twin bioreactor control system. During the cell culture run, parameters like cell density and viability, as well as metabolite concentrations were monitored. In the end, the transfection strategy reached a spike protein titer of around 4 mg/L, which is in line with previous reports [1].

Introduction

Vaccines are valuable tools to minimize the risk of infectious diseases, such as the COVID-19 pandemic that started in 2020. Many vaccines subtypes exist, all with the goal to train the immune system to fight certain infectious agents and thus provide protection for future infections.

One such subtype is the protein subunit vaccine which contains only components or antigens rather than the whole pathogen. An example for an antigen used for subunit vaccines is the COVID-19 spike protein (S-protein). After delivering this antigen to the body, it is recognized by the immune system and stimulates immune responses, such as antibody production (Figure 1).

Adjuvanted spike protein vaccines are available on the market, such as the Novavax COVID-19 vaccine NVX-CoV2373 that recently received the emergency use authorization from the FDA [2].

Albeit the expenditure of time to identify the most effective immunostimulant and the requirement of adjuvants in most cases, subunit vaccines are less likely to produce strong adverse reactions [3]. Furthermore, recent market analyses have concluded that the global subunit vaccine market is anticipated to grow at a significant Compound Annual Growth Rate of 10.9 % during the forecast period of 2022-2028 [4].

In order to advance vaccine production and to enable sufficient protection against prevalent and future pathogens, scalable vaccine production strategies are needed. Bioreactors offer a platform to develop such strategies.

In this application note, a bioreactor-based SARS-CoV-2 S HexaPro spike protein production was developed using the suspension cell culture platform Expi293F (Thermo Fisher Scientific). In previous studies, this cell line demonstrated efficient expansion to high densities [5] and was employed for the production of Adeno-associated virus capsids [6], an important component of vaccination but also in the emerging field of gene therapy.

The bioprocess conditions were controlled and monitored by the SciVario twin bioreactor control system in conjunction with the BioBLU 1c Single-Use Bioreactor. Parameters like cell growth, viability, and metabolic activity (glucose, ammonia, and lactate levels within the medium) were analyzed throughout the run. In addition, the SARS-CoV-2 S HexaPro spike protein titer was determined at different time points after transfection by ELISA, and its purity and molecular weight were analyzed by SDS-PAGE.

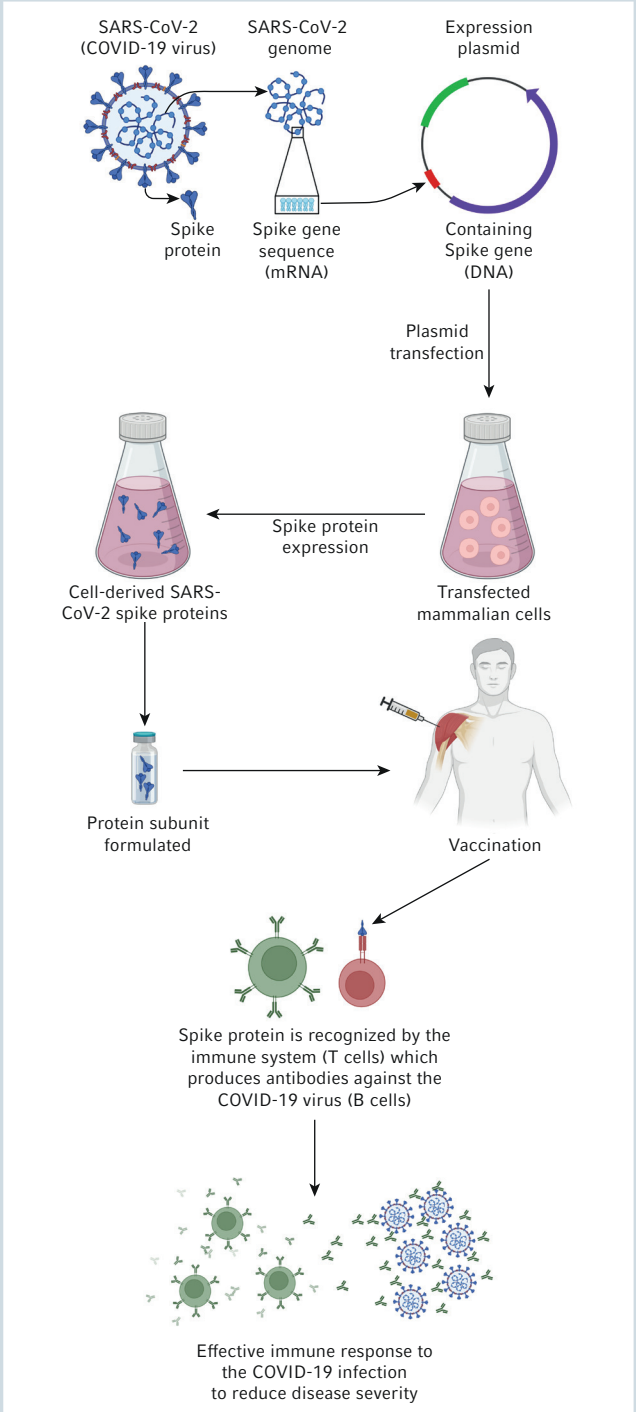

Figure 1: Schematic representation of SARS-CoV-2 S spike protein production and potential immunization procedure. Created with BioRender.com

Material and Methods

SciVario twin bioreactor control system and BioBLU 1c Single-Use Bioreactors

The SciVario twin bioreactor control system was used to perform two batch culture runs simultaneously using BioBLU 1c Single-Use Bioreactors equipped with a single pitched-blade impeller. Each bioreactor control system is equipped with three universal port connectors for pH and dissolved oxygen (DO) sensors, a temperature control block that combines electrical heating and water cooling, agitation control and a gas module that includes a Thermal Mass Flow Controller (TMFC) with standard gas flow rates of 0.1 – 1,200 sL/h (resulting in an ultra-high turndown ratio of 1:12,000), as well as four solenoid valves (see Figure 2).

Figure 2: The SciVario twin bioreactor control system allows the control two glass or single-use bioreactors, either individually or in parallel, at the same time across a wide range of vessel sizes from small- to bench-scale. It was developed for both cell culture and microbial fermentation applications.

To learn more about the possibilities of the SciVario twin bioreactor controller, please visit www.eppendorf.com/scivario

Sensor calibration

Prior to the preparation of the BioBLU 1c Single-Use Bioreactors, ISM® gel-filled pH sensors (Mettler Toledo®) were connected to the SciVario twin bioreactor control system where they were automatically detected by the software. The calibration process was performed according to the operations manual using buffer solutions of pH 7 and pH 4 as "zero" and "span", respectively. Hereafter, the pH sensors were disconnected and sterilized in an autoclavable pouch.

BioBLU 1c Single-Use Biorector preparation and process parameters

Each BioBLU 1c Single-Use Bioreactor was equipped with magnetic drives. The previously sterilized pH sensors were then inserted in a spare PG 13.5 port under aseptic conditions in the biosafety cabinet. In addition, the polarographic DO sensors (Mettler Toledo) were fitted *via* a non-invasive sensor sleeve into both bioreactors. DASGIP® peltier exhaust condensers were connected to each bioreactor and the sparge line (from the controller) was connected to the submerged sparge filter on the bioreactor. Three liquid addition ports were used on each bioreactor: one for inoculation/medium addition, one for base addition and another for the addition of 0.1 % of Sigma Aldrich® Antifoam C Emulsion (Merck). Then, the BioBLU 1c Single-Use Bioreactors were placed in their respective temperature control block to keep the system at a constant temperature. Finally, each bioreactor was filled with HEK ViP NX cell culture medium (Sartorius) supplemented with GlutaMAX™ (Thermo Fisher Scientific) and conditioned for at least 24 hours using the parameters and setpoints listed in Table 1.

Table 1: Process parameters and setpoints of the batch culture experiments.

Working volume	1 L
Agitation	155 rpm (tip speed 0.4 m/s)
Temperature	37 °C
Inoculation density	0.4×10^6 cells/mL
Cell culture medium	HEK ViP NX cell culture medium
DO setpoint	40 % (P = 0.1; I = 3.6/h)
pH setpoint	7.0 (deadband = 0.2), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)
Gassing range	0.1 SLPH – 60 SLPH
Gassing cascade	Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 100 %. Set flow at 0 % controller output to 0.1 SLPH, and at 100 % controller output to 60 SLPH

SARS-CoV-2 S spike protein production workflow

An overview of the entire workflow schematic for SARS-CoV-2 S spike protein production is shown in Figure 3. The individual steps are detailed below.

SARS-CoV-2 HexaPro expression vector

Recombinant SARS-CoV-2 S HexaPro spike protein was produced by transfecting the target cells with SARS-CoV-2 S HexaPro expression vector obtained in-house from a HexaPro variant plasmid in *E. coli* (Addgene, 154754). Briefly, *E. coli* strain DH5 alpha cells were cultured in shake flasks with LB medium (Merck) supplemented with the appropriate selective antibiotic (ampicillin, Merck) for 18 hours at 37 °C and 200 rpm using an Innova® S44i shaker incubator. After reaching the log phase, the cells were pelleted by centrifugation at 11,000 rpm (16,639 × g) for 5 min at 4 °C. Plasmid DNA was purified from culture using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Thermo Fisher Scientific). Finally, purified plasmids were eluted from the HiPure column by gravity flow after adding 15 ml of elution buffer. The absorbance at 260 nm and 280 nm

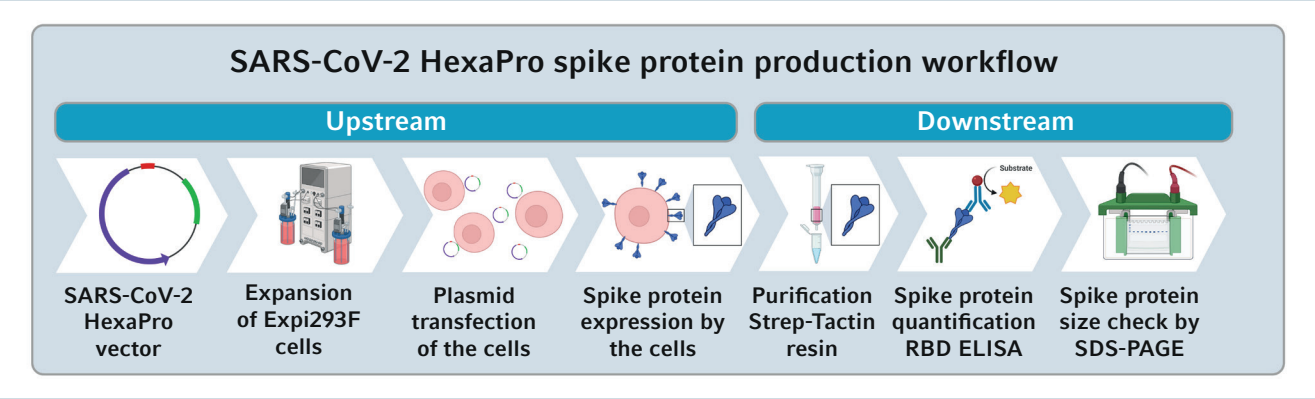

Figure 3: The SARS-CoV-2 S HexaPro spike protein production workflow. Created with BioRender.com

was determined by using a BioSpectrometer® Kinetic D30 spectrophotometer. The ratio of A260/A280 estimates sample purity. Ratios between 1.8 and 2 are commonly accepted as pure DNA. Furthermore, an A260 value of 1 translates to ~50 ng/µL of pure double-stranded DNA. Hence, the concentration and purity of the plasmid were 2.58 mg/mL and 1.85, respectively.

Suspension Cell Line (Expi293F)
In order to produce the SARS-CoV-2 S HexaPro spike protein, the suspension adapted HEK293 cell line Expi293F (Thermo Fisher Scientific) was used for plasmid transfection. In previous transfection experiments for the production of Adeno-associated virus capsids, this suspension cell line demonstrated sufficient protein production and high cell density [5,6].

Expi293F cell inoculum preparation for the BioBLU 1c Single-Use Bioreactor
The cell's expansion process was performed as shown in Figure 4. Cells were cultured in HEK ViP NX Cell culture medium supplemented with GlutaMAX in a New Brunswick S41i CO_2 incubator shaker at 37 °C, 8 % CO_2 and at an agitation speed of 125 rpm. During the expansion process, the inoculation density, percentage fill of the shake flasks and other parameters were kept constant. Finally, 200 mL inoculum containing 400 × 10^6 cells in HEK ViP NX cell culture medium was added to each inoculation bottle.

Expi293F cell culture in BioBLU 1c Single-Use Bioreactors
Both BioBLU 1c Single-Use Bioreactors were inoculated with the inoculum from the inoculation bottles (see section "Expi293F cell inoculum preparation for the BioBLU 1c Single-Use Bioreactor") for a total working volume of 1 L with a cell density of ~0.4 × 10^6 cells/mL and more than 95 % cell viability. In both cases, the temperature was set to 37 °C and the DO setpoint of 40 % was controlled by a cascade (Table 1). To control foam formation, Antifoam C Emulsion was added as needed. The pH setpoint was controlled using a cascade of CO_2 (acid) and 0.45 M sodium bicarbonate (base) (Table 1).

Transfection procedure of Expi293F cells in BioBLU 1c Single-Use Bioreactors
The transfection cell density of approximately 1-1.2 × 10^6 cells/mL was reached at two days after inoculation in both bioreactors. Then, 1 µg of HexaPro vector was diluted in 50 mL of Gibco™ Opti-MEM™ I Reduced Serum Medium supplemented with GlutaMAX (tube 1). The transfection mix was prepared by adding 1 µl of FectoVIR or FectoPRO per µg of plasmid DNA to 50 mL of Opti-MEM medium (tube 2). The content of tube 1 was filtered through a 0.22 µm syringe filter into tube 2 and mixed by inversion. Then, the transfection mix solution was incubated for 20 minutes at room temperature and finally added onto the cells in the bioreactor.

Cell viability and metabolic activity
Samples were collected twice a day from the bioreactors to determine the cell viability, cellular density, and the concentration of metabolites (glucose, ammonia (NH_3) and lactate), by connecting a sterile 5 mL syringe to the Luer Lock sample port. Then, 3 mL of dead volume were discarded and another 3 mL were collected again (using a

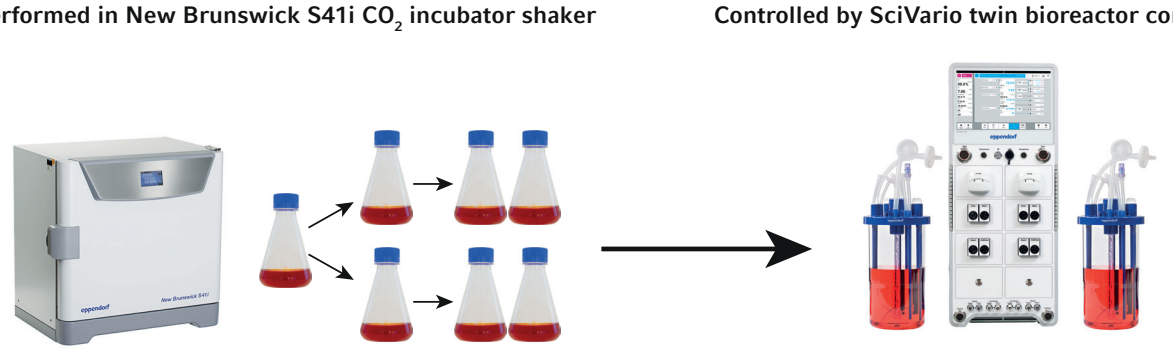

Figure 4: Expi293F cell culture scale-up from shaker to bioreactor for inoculum preparation.

new 5 mL sterile syringe) as a viable sample for analysis. Cell density and viability were measured (via the trypan blue exclusion method) using a Vi-CELL® XR Viability Analyzer (Beckman Coulter®). pH values were monitored offline by using an Orion Star™ 8211 pH-meter (Thermo Fisher Scientific). Using the offline pH value, the pH calibration on the controller was restandardized daily to prevent any discrepancy between online and offline measurements. Glucose, ammonia and lactate were measured using a CEDEX® Bio Analyzer (Roche).

SARS-CoV-2 S HexaPro spike protein purification and titration

Every day (until day 5) after transfection, 60 mL of sample containing Expi293F cells and medium were collected using a Labtainer™ BioProcess Container (Thermo Fisher Scientific) with line sets. The cells were centrifuged at 300 × g in a centrifuge 5430R for 5 minutes to separate the cell pellet from the supernatant. SARS-CoV-2 S spike proteins from the supernatant were purified by affinity chromatography. Briefly, 2 mL of Strep-Tactin® Superflow® resin (as 50 % suspension, IBA LifeSciences) were added to Poly-Prep® Chromatography Columns (Bio-Rad). The resin was equilibrated with 5 mL of Strep-Tactin wash buffer (IBA LifeSciences) (five column volumes). The supernatant was then added to the columns followed by another washing step using Strep-Tactin wash buffer as above. Finally, 4 mL of Strep-Tactin elution buffer (IBA LifeSciences) were added to the column before the eluate was collected in a single tube and concentrated using a 30 kDa cutoff spin concentrator (Amicon® Ultra-15 Centrifugal Filter Units, Merck) at 4000 x g for 5 min (4 °C).

After the purification step, the pure SARS-CoV-2 S HexaPro spike proteins from the supernatant were titrated through the Invitrogen™ Human SARS-CoV-2 RBD ELISA kit (Thermo Fisher Scientific). This ELISA antibody pair detects the SARS-CoV-2 regional binding domain (RBD) of the S1 subunit of the spike protein. Then, the assay was conducted according to the manufacturer's protocol instructions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with the Invitrogen™ NuPAGE™ 4 to 12 %, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels (Thermo Fisher Scientific) under non-reducing conditions. SARS-CoV-2 S HexaPro spike protein samples were diluted in Invitrogen™ NuPAGE™ LDS Sample Buffer (4x) (Thermo Fisher Scientific) and 15 µl per sample were loaded onto the gel. Gels were run in Invitrogen™ NuPAGE™ MES SDS Running Buffer (20×) (Thermo Fisher Scientific) at 200 V

and the electrophoresis was completed in approximately 20 minutes. Then, gels were washed with DI water to remove the SDS and buffer salts, stained with Invitrogen™ SimplyBlue™ SafeStain (Thermo Fisher Scientific) and incubated for 1 hour at room temperature with gentle shaking (30 rpm) using a New Brunswick S41i CO_2 incubator Shaker. Finally, the gels were washed twice with DI water for 1 hour, and photographs were taken with a clear background using an Edvotek™ White Light Box (Thermo Fisher Scientific). A pre-stained protein standard (Invitrogen™ Novex™ Sharp Pre-stained Protein Standard, Thermo Fisher Scientific) was used for accurate molecular weight estimation in a range of 3.5 to 260 kDa.

Results and Discussion

SARS-CoV-2 S HexaPro spike protein production in BioBLU Single-Use Bioreactors

The expansion and transient transfection of Expi293F cells were carried out in a 1 L BioBLU 1c Single-Use Bioreactor culture. For transfection, approximately 1 µg of our in-house-produced HexaPro expression vector was used for 10^6 cells. Total volumetric DNA-to-transfection reagent (FectoVIR or FectoPRO) ratio was 1:1.

Both bioreactors were inoculated with a cell density of approximately 0.4 × 10^6 cell/mL. Transfection was carried out at day 2 post inoculation with a cell density of approximately 1-1.2 × 10^6 cells/mL. As shown in Figure 5, two different growth profiles were obtained. When using FectoVIR, a rapid increase of cell growth was observed up to day 7 of culture, reaching a peak in viable cell density at 9.5 × 10^6 cells/mL. After transfection with FectoPRO, slower cell growth was observed, and the culture reached 6.6 × 10^6 cells/mL at day 8.

It is worth pointing out that both bioreactors showed lower cell growth after transfection compared to the Expi293F cells growth in a 1 L bioreactor under the same conditions but without transfection (data not shown, peak viable cells density was approximately 13 × 10^6 cells/mL).

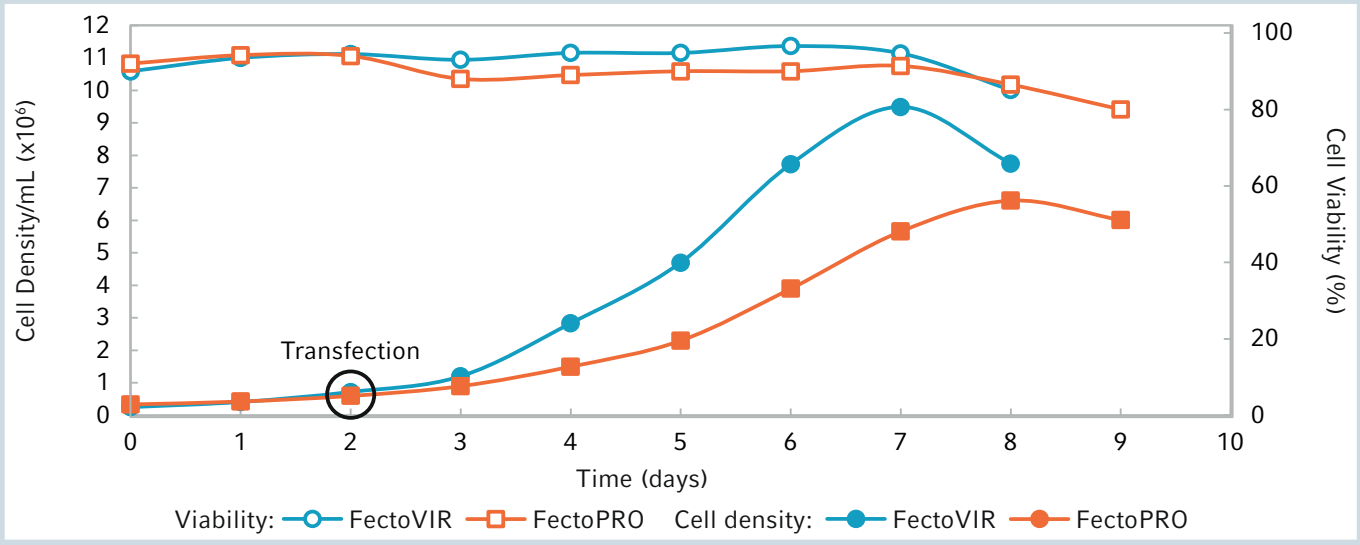

Figure 5:. Expi293F growth profile in BioBLU 1c Single-Use Bioreactors. Expi293F cell density and viability of two different bioreactors containing different transfection mixes were shown.

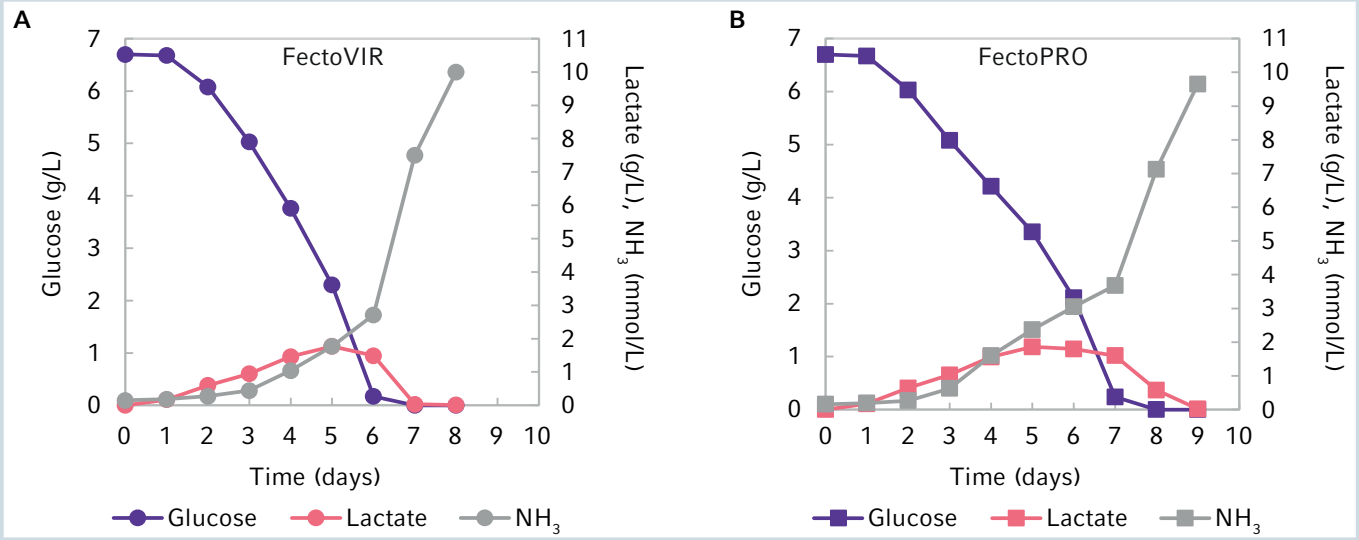

Figure 6: Metabolic profile. A: Expi293F cells transfected using FectoVIR in the transfection mix. B: Expi293F cells transfected using FectoPRO in the transfection mix.

Furthermore, the concentration of glucose, lactate and ammonia (NH_3) was analyzed on a daily basis. The lactate concentration level were below 2 g/L in both bioreactors throughout the run. NH_3 concentrations were between 2 and 3 mmol/L until day 5 in both bioreactors and increased drastically to toxic levels after day 6 towards the end of both runs (Figure 6).

60 mL of sample was harvested daily post-transfection until day 5 to determine the SARS-CoV-2 S HexaPro spike protein titers in the supernatant by ELISA (Figure 7A). Spike protein concentrations were reaching 3.5 mg/L for the FectoVIR-transfected and 4 mg/L for the FectoPRO-transfected cells. Thus, the spike protein concentration in the supernatant from cells transfected with FectoPRO as part of the transfection mixture was noticeably higher (about 4 mg/L) compared to the bioreactor in which FectoVIR was part of the transfection mixture (3.5 mg/L) despite its lower growth post transfection.

Finally, SDS-PAGE was used to analyze the SARS-CoV-2 S HexaPro spike protein produced in both bioreactors. As shown in Figure 7B, both samples showed high purity with apparent molecular weight of ~ 190 kDa as expected [7]. Molecular weight standards in kDa are indicated on the left side of Figure 7B.

Conclusion

This study successfully demonstrated the feasibility of bioreactor-based plasmid transfection in BioBLU 1c Single-Use Bioreactors under control of the SciVario twin bioprocess controller.

COVID-19 spike proteins were produced from the plasmid transfection, representing an important compound of protein subunit vaccines. The suspension cell culture approach allows a more straightforward scale-up into larger bioreactors and offers much desired simplicity, as well as access to a greater variety of production systems over attachment culture-based methods.

The efficient setup of the SciVario twin enabled precise control of the cell culture environment, resulting in robust spike protein titers of 3.5 to 4 mg/L. To conclude, the

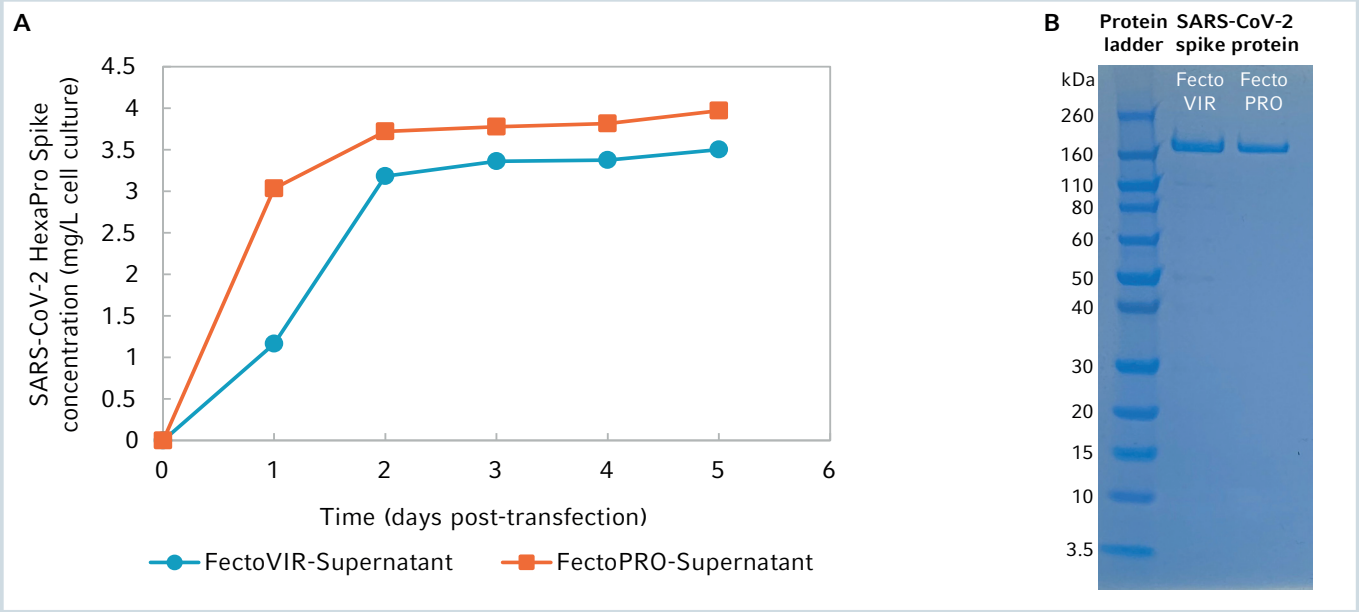

Figure 7: Characterization of purified SARS-CoV-2 S HexaPro spike protein. A: Spike protein concentration obtained every day (until day 5 post-transfection) in the cell culture supernatant of transfected cells. B: SDS-PAGE gel of SARS-CoV-2 S HexaPro spike protein after elution from the Strep-Tactin column.

Expi293F cell line together with the advanced suspension stirred-tank bioreactors and controllers offered by Eppendorf provide an efficient platform for the development of suspension cell-based protein production at various scales.

Literature

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Ordering information

Description	Order no.
SciVario® twin fermenter/bioreactor control system, base unit for 2 bioreactors	7600 100 001
BioBLU® 1c Single-Use Bioreactor, cell culture, open pipe, 1 pitched-blade impeller, optical pH, sterile, 4 pieces	1386 110 400
DASGIP® Peltier Exhaust Condenser Adaptor, for BioBLU® 0.3c/0.3f/1c Single-Use Bioreactors	7820 132 2
New Brunswick S41i, 170 L, CO2 incubator shaker with inner shelf and touch screen control, stackable	S41I 120 010
Innova® S44i, Stackable Incubator Shaker, non-refrigerated, stackable up to 3-fold, orbit 2.5 cm (1 in)	S44I 200 005
Centrifuge MiniSpin® plus, non-refrigerated, with Rotor F-45-12-11	0226 202 07
Centrifuge 5430 R, keypad, refrigerated, with Rotor FA-45-30-11 incl. rotor lid	0226 206 01
Eppendorf Conical Tubes, sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 50 mL	0030 122 178
Eppendorf Conical Tubes, sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 15 mL	0030 122 151

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