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Application Note

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4Cell®BHK-21 CD medium

Adaptation of BHK-21 cells to suspension using chemically defined medium

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Abstract

BHK-21 cell strains have been utilized for several years as a platform for veterinary vaccines and recombinant protein manufacturing. However, these cells are anchorage-dependent, requiring the use of dissociation agents that slow and are traumatic for cells while also creating scale-up limitations. By contrast, suspension culture facilitates large-scale processes, offers options for process intensification and contributes to reduced manufacturing costs due to less process complexity. BHK-21 suspension cells grown in serum-free media that produce high-virus titers are desirable in developing vaccines that are affordable worldwide.

Although some cell lines used in vaccine production still require serum supplementation, BHK-21 cultures have been shown to be propagated in serum-free media. The desire to remove potential uncontrolled contaminants, natural products and by-products from the culture led to the development of more complex media. Such chemically defined media demonstrated that serum could be omitted, without cellular adaptation, if appropriate nutritional and hormonal modifications were made.

In this study, we successfully adapted anchorage serum-dependent BHK-21 cells to suspension in a chemically defined (CD), serum-free, Sartorius medium. This was developed for more consistent performance, easier purification and downstream processing that fulfills regulatory requirements and enables cost-efficient vaccine production.

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Introduction

The purpose of this application study is to assess the performance of the 4Cell® BHK-21 chemically defined medium during stepwise adaptation of a BHK-21 cell line from adherence to growth in suspension.

BHK-21 cell lines have been widely employed for years as a platform for virus and protein manufacturing.

Historically, BHK-21 cells were commonly grown in serum-containing media, however, serum can lead to variability in performance due to lot-to-lot variation, as well as the presence of undefined adventitious agents.

Recently, serum use has also raised regulatory concerns due to an increased potential for contamination in health-care products. These contamination concerns spurred the pharmaceutical industry's recommendation to eliminate serum (and all other undefined raw materials where possible) in the production of biologicals for therapy or vaccines [1, 2].

To address these regulatory concerns, Sartorius developed a chemically defined, non-animal-origin (NAO) medium. In comparison to other serum and animal-origin free media alternatives, this formulation is shown to boost both cellular and viral production in BHK-21 cells adapted to suspension.

Materials and Methods

Materials

Consumables

- Corning®:T-25 flask
- Corning®: 125 mL and 250 mL polycarbonate Erlenmeyer shaker flasks
- Corning®:Falcon™ Conical 50 mL centrifuge tube
- Corning®: Falcon™ Conical 15 mL centrifuge tube
- Cell culture media:
- Sartorius: 4 Cell®BHK-21 CD Medium (Cat: CFV3FA0001, Lot: MSC11001P)
- Sigma: DMEM (Cat: D5523, Lot: SLBS0097V)
- Gibco: FCS (South America Origin, Lot: 10270-098, Ref: 42G9551 K)

Cell line

- BHK-21 cells (Clone 13 - ATCC®CCL-10™)

Equipment

- Vi-CELL™ (Beckman Coulter)
- BIOSTAT®A bioreactors

Fig 1. BIOSTAT®A used for the cultivation of BHK-21 suspension cells and 4Cell® BHK-21 CD Medium.

Method

- Cell enumeration:
  Cell concentration and viability were determined using a Vi-CELL™ (Beckman Coulter) to provide automated Trypan Blue Dye Exclusion.
- Growth promotion test:
  The cells were cultivated in 125 mL shaker flasks with flat cap plain bottom. Inoculation was done at 3×10^5 viable cells/mL with 25 mL working volume. The cultivations were carried out at 36.5 °C with an initial shaking frequency of 125 rpm, 7% CO2 in a humidified' atmosphere.

Cell growth experiments were also performed in Biostat®A bioreactors. Inoculation was done at 3×10^5 viable cells/mL with 1 L working volume. The cultivations were carried out at 36.5 °C with an initial agitation rate of 100 rpm, pH 7.2, Air/N_2/CO_2 on headspace, O_2 on sparger, DO set point 50%.

- Rabies virus production:
  BHK-21 rabies virus strain (adapted to the BHK-21 Clone 13 - ATCC® CCL-10™ grown in monolayer) were provided by Ankara University. The virus was cultivated in suspension in 4Cell® BHK-21 CD Medium to assess virus production. BHK-21 suspension cells (7×10^6 cells/mL) were infected with a Multiplicity of Infection (MOI) of 0.01 in 125 mL shake flasks (25 mL working volume).

Fluorescent Focus Assay (AAF) was performed as described in the Terrestrial Manual [3]. Briefly, BHK-21 Clone 13 - ATCC® CCL-10™ cells produced in 96-well plates for 24 hours were infected with 10-fold virus dilution (50 µl per well) in quadruplets. Infected cells incubated for 72 hours were fixed and stained with FITC anti-rabies conjugate. The test was evaluated after observing total well surface by fluorescent microscopy.

Stepwise adaptation of BHK-21 cells to suspension conditions in 4Cell® BHK-21 CD Medium:
The following steps help for a successful adaptation:

a. Ensure good growing cells in your standard medium (containing e.g. 10% fetal calf serum - FCS) in your standard cultivation procedure.
b. Change growth conditions. Adapt cells to suspension growth conditions in the same medium.
c. Change medium. Transfer cells from your reference medium into 4Cell® BHK-21 CD Medium.
d. Reduce FCS in a stepwise manner.
e. Decrease inoculum cell concentration.
f. Run stock culture long enough under the new standard condition.

Ensure good growing cells:

1. After thawing, BHK-21 adherent cells were passaged a minimum of four times to ensure consistent performance. Subsequently, five T175 cm^2 flasks (35 mL working volume) were inoculated with 1×10^4 to 2×10^4 cells/cm^2 and grown to confluence for three to four days to prepare for suspension adaptation.

Change growth conditions:

2. Following growth to confluence, BHK-21 cells from the five T175 flasks were pooled together and centrifuged at 1000 rpm (revolutions per minute) for four minutes at room temperature. The cell pellet was re-suspended in 20 mL DMEM with 10% FCS to a concentration between 0.8×10^6 to 1.2×10^6 viable cells/mL (working volume 20 mL). The cells were transferred to a 125 mL shake flask and grown for three days under agitation (125 rpm) at 36.5°C ± 0.5°C and 7% CO_2.

Change medium:

3. Following culture, cells were centrifuged for four minutes at 1000 rpm and re-suspended in 5 mL supernatant supplemented with 20 mL of 4Cell® BHK-21 CD Medium + 10% FCS (25mL total working volume) to maintain an inoculation cell concentration between 0.8×10^6 to 1.2×10^6 viable cells/mL. The objective of subsequent passages was to sustain between 0.8×10^6 to 1.2×10^6 viable cells/mL within 250 mL shake flasks of 50 mL working volume. Following centrifugation, the pellet was re-suspended in 10 mL conditioned medium, and augmented with 35 mL 4Cell® BHK-21 CD Medium and 5 mL of FCS.

Reduce FCS in a stepwise manner:

4. To reduce the serum concentration, cells must consistently double, at a minimum, every two to three days for three successive passages. After successful passaging in 4Cell® BHK-21 CD Medium with 10% (v/v) FCS serum, concentration level reduced gradually (i.e. 10%, 5%, 2%, 1%, 0.5% and 0% FCS serum) while maintaining inoculation concentration between 0.8×10^6 to 1.2×10^6 viable cells/mL in 50 mL working volume.

Decrease inoculum cell concentration:

5. After a successful adaptation to suspension and serum-free conditions, the inoculation cell concentration was gradually reduced (i.e. 8, 6, 4, and 3×10^5 cells/mL) when cell concentration was shown to double in two to three days. When cells reached a constant growth rate (viability >90%) for several passages in 4Cell® BHK-21 CD Medium at a 3×10^5 cells/mL inoculation density, the adaptation was considered successful (see flow diagram).

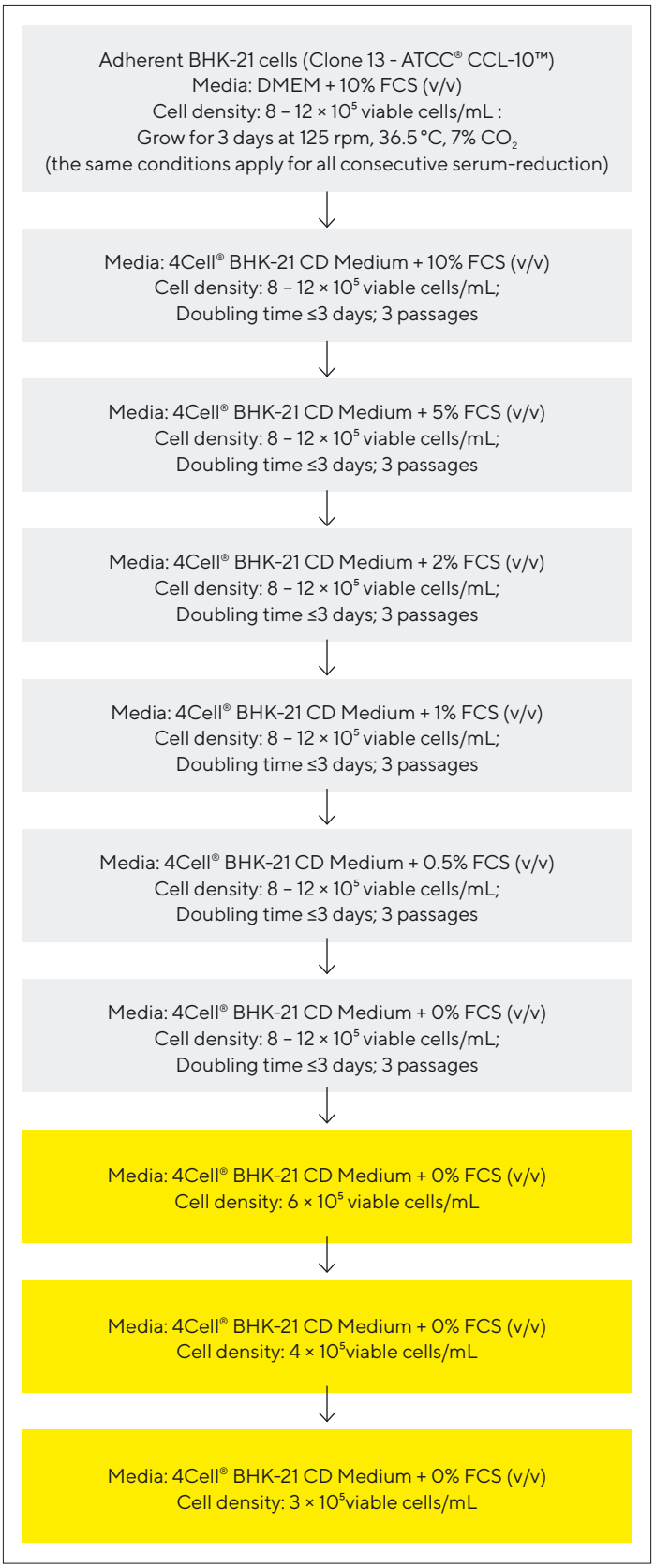

Figure 2. Flow diagram for adaptation of BHK-21 adherent cell to serum-free, chemically defined 4Cell® BHK-21 CD Medium.

Results and Discussion

1. Stability of Suspension

BHK-21 cells following step-by-step adaptation from adherent with serum to suspension serum-free culture conditions in 4Cell® BHK-21 CD Medium (see methods), cells were assessed for their stability. The cells in 4Cell® BHK-21 CD Medium was sub-cultured for more than 20 passages (Figure 3, n=2).

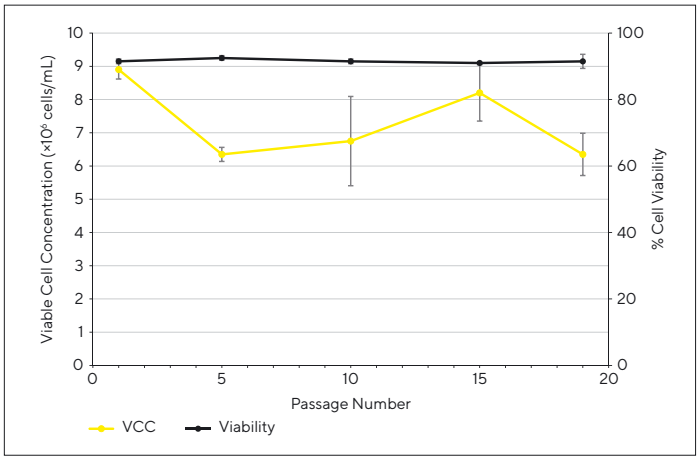

Fig. 3: Stability of BHK-21 cells in Sartorius 4Cell® BHK-21 CD Medium. Cells are stable in the medium up to more than 20 passages (n=2). VCC = Viable Cell Concentration.

Throughout 20 passages of BHK-21, cells consistently achieved an average of 6.5 × 10⁶ viable cells/mL and more than 90% viability in 4Cell® BHK-21 CD Medium media.

2. Growth of Suspension

BHK-21 cells 4Cell® BHK-21 CD Medium supports rapid growth to higher densities (VCC 7 × 10⁶ viable cells/mL), nearly six fold, compared to the serum-free reference NAO (non-animal origin) medium as shown in Figure 4. Throughout four days of batch growth, cells consistently maintained a viability above 80%.

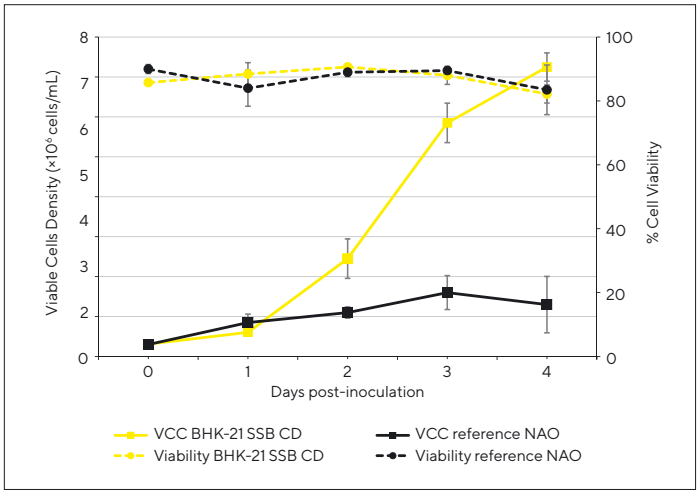

Fig. 4: 4Cell® BHK-21 CD Medium supports rapid growth to high densities compared to a serum-free, non-animal-origin reference medium in Biostat® A bioreactors (n=3).

3. Rabies Virus Productivity

To assess virus productivity, BHK-21 cells adapted to suspension culture in 4Cell® BHK-21 CD Medium was infected with a 0.01 MOI of the reference rabies virus, and the infection was allowed to proceed until viable cell densities dropped below 20 percent. Supernatant was collected and analyzed by fluorescent antibody assay. Initial data obtained resulted in virus equal to 7×10^4 FFU / mL (data not shown). Results need to be reproduced to get statistically relevant data.

Conclusion

Serum used in the biopharmaceutical industry has long provided a multitude of benefits in many processes with its unique composition of growth factors and ancillary components. Although the use of serum brings many benefits, it also has its share of challenges. The greatest is in the quality and consistency of the product (e.g. batch-to-batch variation, quality, source, contamination risk, etc).

Due to unknown discrepancies with serum usage, routine screening for mycoplasma and viruses has also become important. The risk associated with either of these contaminants may detrimentally influence the culture, either falsely implicating the adequacies of the media, or, in worse case scenarios, alter the final product posing safety concerns for clinical use.

As a result, the industry has moved toward non-animal origin, chemically defined materials and media. Sartorius 4Cell® BHK-21 CD Medium provides such an alternative.

This study tested the robustness and efficiency of adapting adherent BHK-21 cells to suspension and subsequent growth in chemically defined, serum-free 4Cell® BHK-21 CD Medium. Adaptation of adherent BHK-21 cells to this medium allowed for an easier process scale-up and was capable of supporting cell growth to high densities and viability, with initial results suggesting that 4Cell® BHK-21 CD Medium can provide a viable option for the production of high-titer rabies virus.

Acknowledgments

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