## Process Guidance

# Cellvento™ BHK-200 cell culture medium

## Introduction

Cellvento™ BHK-200 medium is a serum-free cell culture medium, formulated without any animal-derived component and optimized for the growth and maintenance of suspension BHK21 cells at high density and viability. It is also optimized for the efficient propagation of viruses used in vaccine production, such as the foot-and-mouth disease virus (FMDV). BHK21 cells can be grown as suspension cultures in T-flasks, shaker flasks, spinner bottles or stirred tank bioreactors.

When the cell density reaches a certain level, the virus can be inoculated directly into the reactor without medium exchange or a cell separation/sedimentation step, resulting in high virus replication and thus high antigen content. It is recommended to use only 70% of the maximum bioreactor volume for cell growth. After cells have reached the desired cell density, 30% fresh medium can be added in the reactor before inoculation and propagation of the virus.

Cell growth and metabolism may differ significantly in serum-containing media compared to serum-free media. The serum-free process requires a medium with a well-balanced nutrient composition and well-defined and standardized cell culture conditions. To achieve high performance, it is very important to understand the cell culture method, the cultivation environment and especially to adapt the cells to the new conditions and the serum-free medium. In order to prepare the cells to changes in the environment (i.e. amino acid concentration, removal of growth factors or undefined components present in the serum), the cells need to be adapted through a defined procedure. It is recommended to do this on a small scale (e.g. in shaker bottles) according to one of the following protocols and then to establish a cell bank. When the cell growth in the shaker bottle is stable (equivalent doubling time over several passages), it can be scaled up in a bioreactor. The cell adaptation process only needs to be done once and results in adapted cells which can be used to generate a new master cell bank and a new working cell bank.


EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

## Recommendations and lab equipment

It is recommended to use 'water for injection' (WFI) or ultrapure water (e.g. Milli-Q®) for the preparation of cell culture media and cleaning of the culture containers and pipelines. Alternatively, purified steam can be used to sterilize the culture containers and pipelines. In magnetic stirrer bottles, the mechanical damage and shear force are relatively high, which may affect the cell growth during the adaptation process. As such, it is recommended to use Erlenmeyer flasks (250 mL, plastic with vent filter) and a shaker incubator (37 °C, 80% relative humidity, 5% $CO_2$, agitation at 110 rpm). The use of $CO_2$-controlled conditions is important in serum-free culture since the buffering system is essentially provided by sodium bicarbonate. In bioreactors, the pH should be controlled through sparging with gases.

## Adaptation of suspension BHK21 cells to serum-free conditions

There are several approaches to adapting BHK21 cells to serum-free media. The first approach described in this protocol is very conservative and can be used for every suspension BHK21 clone. Examples of shorter adaptation protocols are also provided and can be used for some very stable, non-clumping clones like the BHK21C13-2P cell line. In principle, the adaptation consists of reducing the serum concentration progressively. It is based on the following basic principles:

- The culture volume should be 100 mL in a 250 mL Erlenmeyer flask
- The shaker bottles/Erlenmeyers should be agitated in a constant-temperature incubator at 36.5–37.0 °C with a rotating speed of 110 rpm, 80% humidity and 5% $CO_2$
- pH should be 7.2–7.4, and never below 6.8 during the adaptation process. This is possible through the use of a $CO_2$ incubator and the bicarbonate buffering system.
- In a given defined step (percentage of serum), passages should only be performed if the viable cell density reaches $1.5\times10^6$ cells/mL
- Before proceeding to the next step (reduced serum concentration), a minimal viable cell density of $1.5\times10^6$ cells/mL and a viability > 95% should be reached.
- If the viability is low (< 90%), transfer cells to a T-flask, sediment for a few minutes, remove the supernatant (containing mainly dead cells), resuspend remaining cells in fresh medium and incubate further in T-flasks for 1 or 2 passages. When viability is recovered (> 95%), transfer back to shaker flasks.

During the adaptation step, the cells should stay in the exponential phase to allow a direct comparison of doubling times. If this is not possible due to weekends (e.g. for the early phases with high serum concentrations), the seeding density can be reduced to $3\times10^5$ cells/mL. However, in the low serum concentration steps (1%, 0.5%), it is recommended to always use $6\times10^5$ cells/mL as the seeding density.

### 1. Materials

- Cell line: BHK21C13-2P (ECACC Cat. No.: 84111301, already adapted to suspension culture conditions), in late logarithmic growth phase, with normal morphology and cell viability > 95%.
- Serum-free cell culture medium: Cellvento™ BHK-200 medium (pH 7.2–7.4)
- Serum: Newborn Calf Serum (NCS) or Fetal Bovine Serum (FBS)
- Cryogenic vial: 2 mL
- Freezing media: 90% Cellvento™ BHK-200 medium +10% DMSO
- Cell culture vessel: 250 mL Erlenmeyer flasks (plastic with vent filter)
- Shaker incubator: temperature 36.6–37 °C, rotation speed 110 rpm, 80% humidity, 5% $CO_2$
- Centrifugation: rotation speed 200–300 g (1200–1500 rpm for a mid-size centrifuge)
- Cell counting and viability test: cell counting chamber and 0.4% (W/V) trypan blue staining or automatic cell counters.

## 2. Conservative cell adaptation procedure (works with every BHK21 clone)

**1st adaptation step from initial medium (usually equivalent to GMEM, 10% serum, 5% TPB) to Cellvento™ BHK-200 medium + 10% serum**

- Start with cells in mid-logarithmic phase, cultured in suspension in GMEM or an equivalent medium supplemented with 10% (V/V) serum and 5% TPB in shaker flasks, with > 95% viability and normal morphology.
- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium + 10% serum, with a culture volume of 100 mL in a 250 mL shake bottle/Erlenmeyer in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When the cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages in the medium
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ viable cells/mL, continue with the step 2.

**2nd adaptation step from Cellvento™ BHK-200 medium + 10% serum to Cellvento™ BHK-200 medium + 7% serum**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium + 7% serum, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages.
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ cells/mL
  - Freeze some cells (WCB at 7%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 7% serum + 10% DMSO
  - Continue with step 3.

**3rd adaptation step from Cellvento™ BHK-200 medium + 7% serum to Cellvento™ BHK-200 medium + 5% serum**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium + 5% serum, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages.
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ cells/mL
  - Freeze some cells (WCB 5%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 5% serum + 10% DMSO
  - Continue with step 4.

**4th adaptation step from Cellvento™ BHK-200 medium + 5% serum to Cellvento™ BHK-200 medium + 3% serum**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium + 3% serum, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages.
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ cells/mL
  - Freeze some cells (WCB 3%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 3% serum + 10% DMSO
  - Continue with step 5.

**5th adaptation step from Cellvento™ BHK-200 medium + 3% serum to Cellvento™ BHK-200 medium + 1% serum**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium + 1% serum, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages.
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ cells/mL
  - Freeze some cells (WCB 1%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 1% serum + 10% DMSO
  - Continue with step 6.

**6th adaptation step from Cellvento™ BHK-200 medium + 1% serum to Cellvento™ BHK-200 medium + 0.5% serum**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium + 0.5% serum, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages.
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ viable viable cells/mL
  – Freeze some cells (WCB 0.5%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 0.5% serum + 10% DMSO
  – Continue with step 7.

**7th adaptation step from Cellvento™ BHK-200 medium + 0.5% serum to Cellvento™ BHK-200 medium**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages.
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ cells/mL
  – Freeze some cells (WCB 0%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 10% DMSO
  – Continue with step 8.

**8th adaptation of high density culture in Cellvento™ BHK-200 medium**

- Subculture the cells at a seeding density of $6\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture cells for 2 additional passages
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ viable cells/mL
  – Freeze some cells (WCB 0%), with $1\times10^7$ viable cells/mL in Cellvento™ BHK-200 medium + 10% DMSO
  – Continue with step 9.

**9th adaptation of low density culture in Cellvento™ BHK-200 medium**

- Subculture the cells at a seeding density of $3\times10^5$ viable cells/mL in Cellvento™ BHK-200 medium, with a culture volume of 100 mL, in an incubator at 37 °C with a rotation speed of 110 rpm, 80% humidity and 5% $CO_2$
- When cell density reaches $1.5\times10^6$ viable cells/mL, subculture the cells for additional passages until stable doubling times are obtained (see Figure 1).
- When the culture is stable with a viability of > 95% and a cell density above $1.5\times10^6$ viable cells/mL
  – Prepare a MCB (min. 30 tubes in Cellvento™ BHK-200 medium + 10% DMSO, $1\times10^7$ viable cells/mL)
  – Prepare a WCB (min. 100 tubes in Cellvento™ BHK-200 medium + 10% DMSO, $1\times10^7$ viable cells/mL)

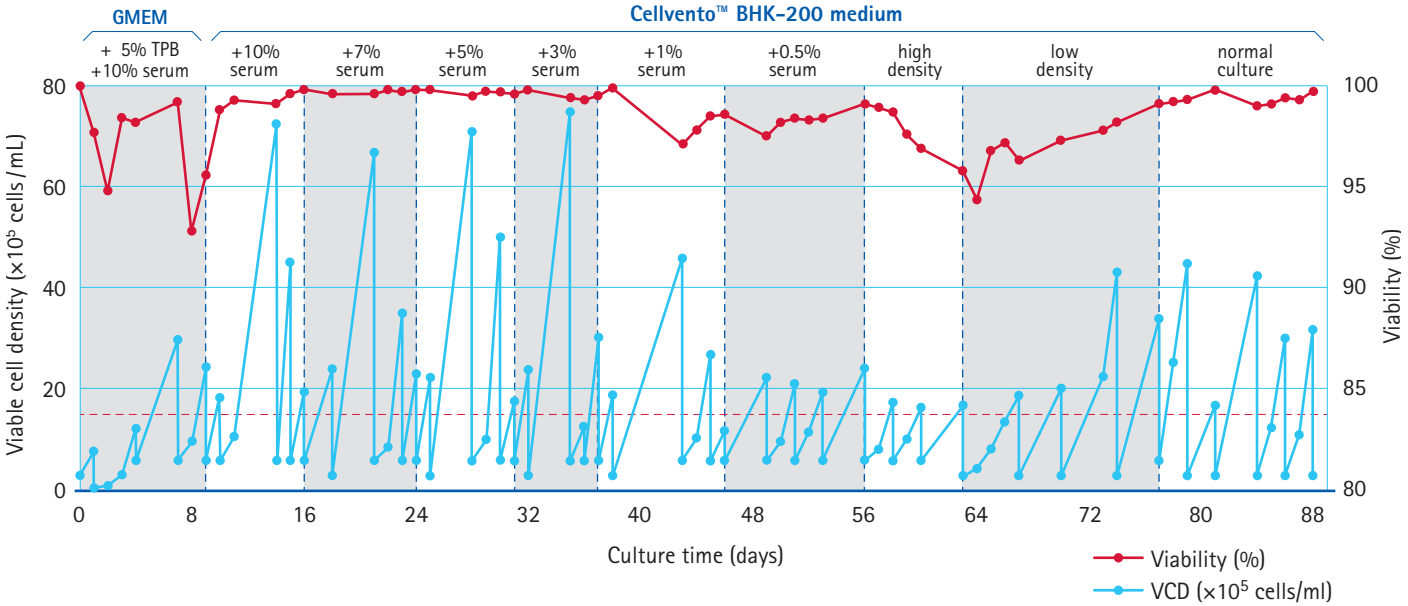


**Figure 1: Example of cell densities and cell viabilities observed during the conservative adaptation process.**

BHK21 cells were adapted by reducing the serum concentration progressively. In the first step with 0% serum, cells were cultivated with high seeding densities. In a second step with 0% serum, the seeding density was reduced to provide more stringent conditions leading to a full adaptation with stable doubling times.

## Diagram Protocol

BHK21 suspension cells at late logarithmic growth phase, cell viability > 95%
Medium: **GMEM + 10% serum + 5% TPB**
Inoculation density: 6×10$^5$ viable cells/mL, 1 passage

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Medium: **Cellvento™ BHK-200 medium + 10% serum**
Inoculation density: 6×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium + 7% serum**
Inoculation density: 6×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium + 5% serum**
Inoculation density: 6×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium + 3% serum**
Inoculation density: 6 ×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium + 1% serum**
Inoculation density: 6×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium + 0.5% serum**
Inoculation density: 6×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium**
Inoculation density (high): 6×10$^5$ viable cells/mL, 3 passages

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Medium: **Cellvento™ BHK-200 medium**
Inoculation density (low): 3×10$^5$ viable cells/mL, 3 passages

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**Cell banking MCB & WCB**
Cell density: 1×10$^7$ viable cells/mL

## 3. Short cell adaptation procedure (example with BHK21C13-2P cells)

With several BHK21 cell lines, it is possible to reduce the adaptation period. However, in all cases, cells need time to adapt fully to the new medium without serum. In this guide, a cell line is considered adapted when the doubling time remains constant.

In Example 1, BHK21C13-2P cells were thawed either in GMEM + 10% serum + 5% TPB or in Cellvento™ BHK-200 medium supplemented with 10% serum. Once the cells reached the minimum of $1.5 \times 10^6$ viable cells/mL, the cells in GMEM were passaged once in Cellvento™ BHK-200 medium supplemented with 0.5% serum and once in serum-free Cellvento™ BHK-200 medium. Cells thawed in Cellvento™ BHK-200 medium supplemented with 10% serum were directly passaged in serum-free Cellvento™ BH-200 medium and cell growth was monitored over several weeks. All adapted cells showed stable growth after 2 months in serum-free medium.

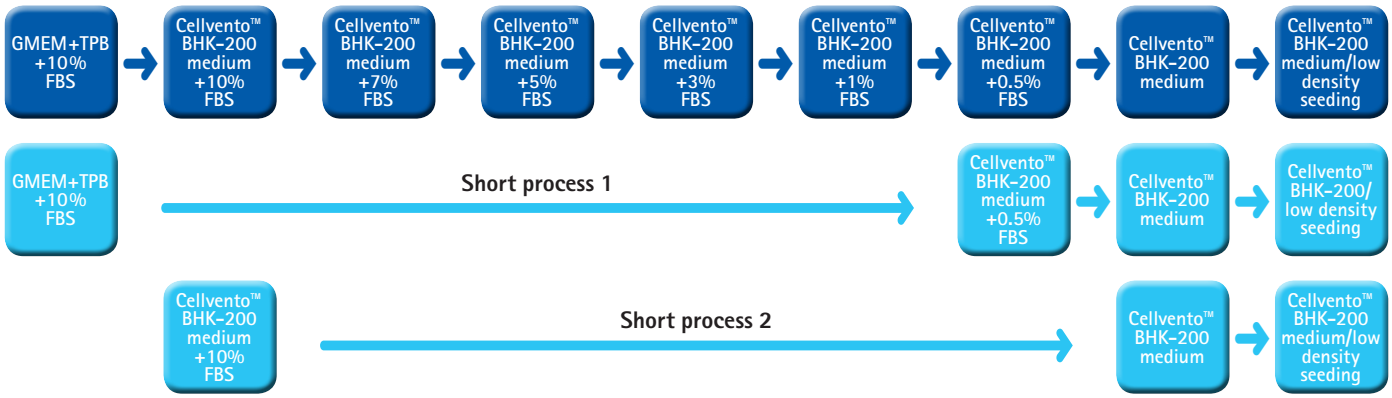


**Figure 2: Schematic illustration of two shorter adaptation processes when cells are thawed in GMEM + TPB + 10% serum or Cellvento™ BHK-200 medium + 10% serum.**

The cells stay in each condition for one passage only. Once in Cellvento™ BHK-200 medium, the first passages are performed at high seeding densities while final adaptation is performed with low seeding densities.

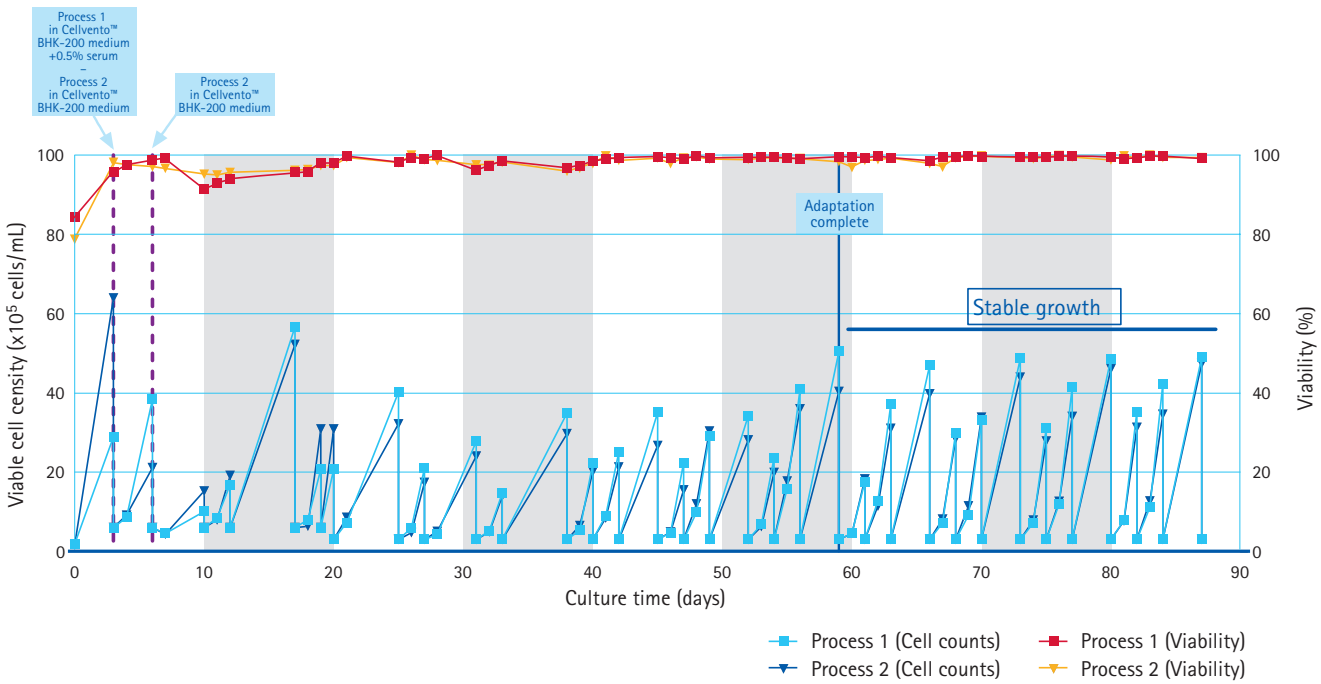


**Figure 3: Example of viable cell densities and viabilities during two short adaptation processes.**

In Process 1, cells are thawed in GMEM + TPB + 10% serum, then transferred to Cellvento™ BHK-200 medium + 0.5% serum after 3 days and in Cellvento™ BHK-200 medium after another 3 days. Growth is monitored with high seeding densities for 14 days and then with low seeding densities for 40 days. On day 60, the adaptation is considered complete as the doubling time is constant. In Process 2, cells are thawed directly in Cellvento™ BHK-200 medium + 10% serum (very rapid growth) and switched to serum-free Cellvento™ BHK-200 medium after 3 days. Growth is monitored as in Process 1.

In Example 2, BHK21C13-2P cells were thawed either in Cellvento™ BHK-200 medium supplemented with 1% serum or directly in serum-free Cellvento™ BHK-200 medium. Once the cells reached the minimum of 1.5 x $10^6$ viable cells/mL, the serum concentration was reduced from 1% to 0.5% then to 0% (one passage each). For the direct thaw in serum-free conditions, growth was monitored over several weeks and compared to the other conditions. All adapted cells showed stable growth after 2 months in serum-free medium.

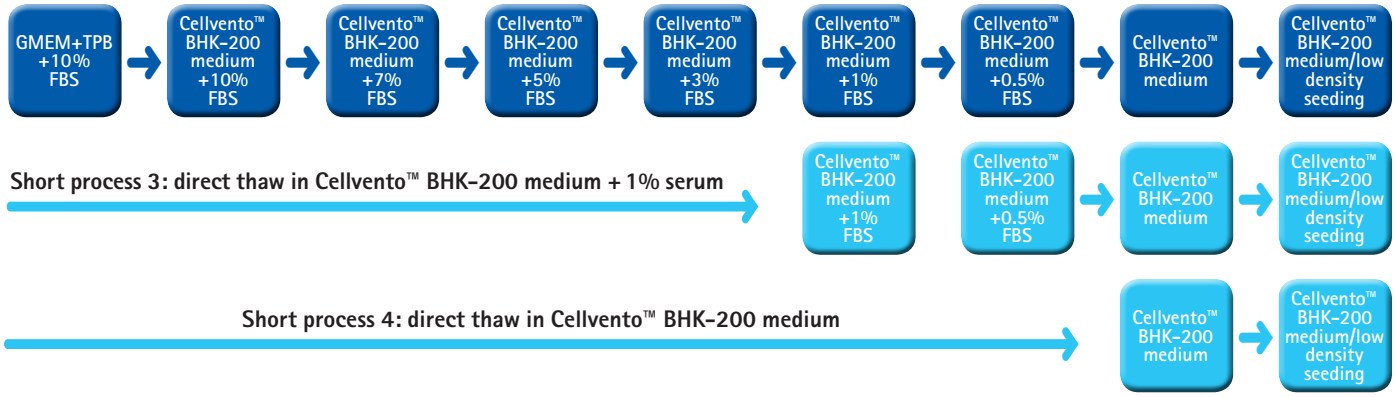


**Figure 4: Schematic illustration of two shorter adaptation processes when cells are thawed directly in Cellvento™ BHK-200 medium + 1% serum or serum-free Cellvento™ BHK-200 medium.**

First passages are performed at high seeding densities to allow recovery (increase in viability). Once the viability rises above 95%, the seeding density is reduced to provide more stringent conditions leading to a full adaptation with stable doubling times.

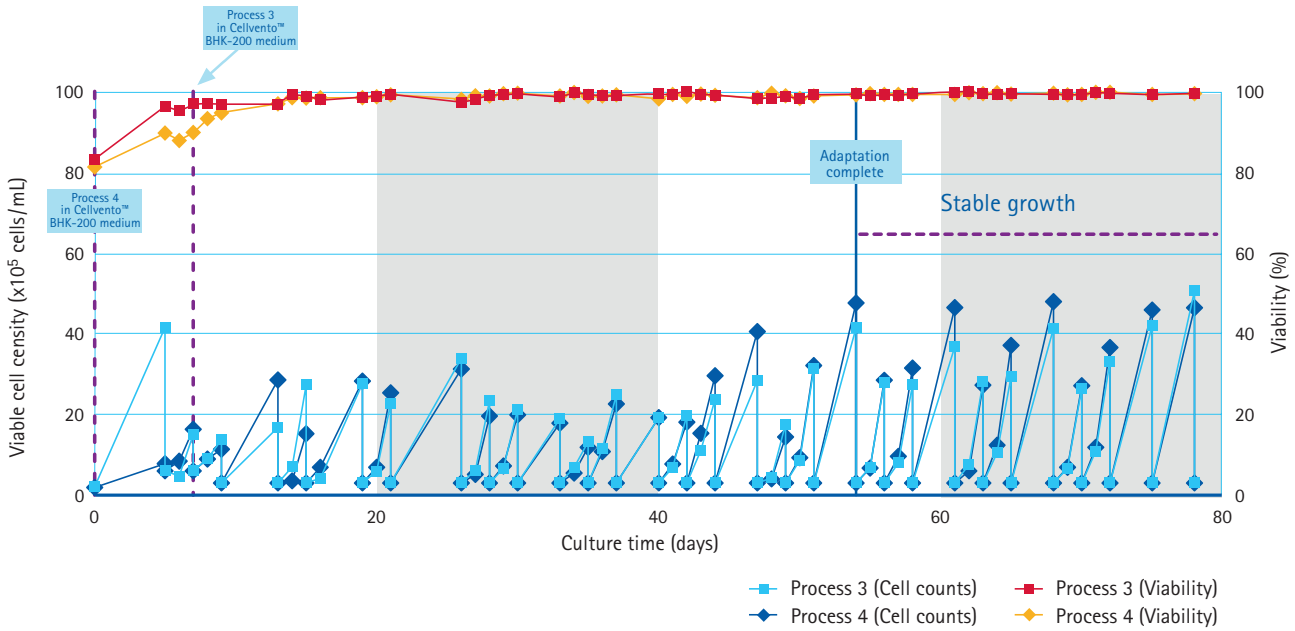


**Figure 5: Adaptation process when cells are thawed directly in Cellvento™ BHK-200 medium + 1% serum or serum-free Cellvento™ BHK-200 medium.**

In Process 3, cells are thawed in 1% serum, then transferred to Cellvento™ BHK-200 medium + 0.5% serum after 5 days, then Cellvento™ BHK-200 medium after another 2 days. In Process 4, cells are directly thawed in Cellvento™ BHK-200 medium leading to very slow cell growth. After several days in culture, the cells start to grow and the viability increases. Once the threshold of 1.5x$10^6$ viable cells/mL is reached, cells are passaged and allowed to recover slowly. Cells are considered fully adapted after 54 days (stable doubling times).

## Annex 1: Freezing procedure for BHK21 cells in serum-free medium

**1. Materials**

- Clean bench or laminar flow hood
- Centrifuge
- 2–8 °C, –20 °C and –80 °C refrigerator or automatic freezing system
- Liquid nitrogen tank
- Freezing medium: Cellvento™ BHK-200 medium + 10% DMSO
- Cryovials
- Freezing container
- Sterile pipette
- Sterile centrifuge tube

**2. Cell freezing operation procedure**

- Cell freezing medium preparation: Mix sterile DMSO and Cellvento™ BHK-200 medium with a 1:9 volume ratio under the clean bench or laminar flow hood. As DMSO dilution will release heat during preparation, the freezing medium should be prepared in advance and stored at 2–8 °C.
- Select cells in mid-logarithmic phase and with normal shape, cell density should be > $1.5\times10^6$ cells/mL and viability > 95%. Count the cells.
- Centrifugation: Transfer the cells into a centrifuge tube, centrifuge at 1200–1500 rpm for 5 mins.
- Discard the supernatant, resuspend cells in freezing medium, adjust the cell density to $1\times10^7$ cells/mL, and transfer the cell suspension to freezing vial with 1 mL in each vial.
- Freezing procedure with a freezing container containing isopropanol: Place the cryo vials into the cryobox, and freeze the cells following the sequential procedure with decreasing temperatures:
  - 30 min at 4 °C
  - 2–4 hr at –20 °C
  - overnight at –80 °C
  - Transfer and store the vials in the liquid nitrogen tank.

**Note:** *The freezing procedure can be standardized using an automatic cooling instrument. In this case, the cooling speed is controlled and the cell suspension is frozen 4 °C to usually –150 °C (normally) in 1 hour.*

## Annex 2: Thawing procedure of BHK21 cells in serum-free medium

**1. Materials**

- Clean bench or laminar flow hood
- Centrifuge
- Constant-temperature water bath
- $CO_2$ incubator
- Cellvento™ BHK-200 medium, pH 7.2-7.4
- 25 mL Erlenmeyer flask
- Sterile pipette
- 50 mL centrifuge tube

**2. Cell thawing and recovery procedure**

1. Prepare a water bath at 37 °C for cell thawing.
2. Prepare one 50 mL centrifuge tube, 10 mL culture medium under the clean bench or the laminar flow hood.
3. Transfer the cryovial of BHK21 cells from liquid nitrogen to the 37 °C water bath.
4. Take out the vial when ice particles detach from the side of the vial (DMSO may have a toxic effect at higher temperature).
5. Transfer the BHK21 cell suspension from the cryovial to the centrifuge tube, centrifuge at 1200–1500 rpm for 5 mins.
6. Discard the supernatant, resuspend the cells in fresh culture medium (Cellvento™ BHK-200 medium) and transfer to a 125 mL Erlenmeyer flask for cultivation. Culture the cells in a 37 °C $CO_2$ incubator with 5% $CO_2$, 80% humidity and a rotation speed of 110 rpm.

## Annex 3: Media preparation instructions for 10 L Cellvento™ BHK-200 medium

Powdered media are hygroscopic and should be protected from moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form.

### Media preparation instructions for 10 L

For the media preparation, sodium bicarbonate needs to be added (Art. No. 1.37013) following the instructions below.

1. Measure out 9.5 L of cell culture grade (Milli-Q®) water, by weight, into a 10 L carboy or equivalent.
2. Slowly add 0.217 kg of Cellvento™ BHK-200 medium powder to the water.
3. Allow to dissolve by gentle stirring (300 rpm) for 15 minutes.
4. Add sodium bicarbonate (20 g/10 L) and stir until dissolved (~15 minutes).
5. Measure the pH (should be approximately 6.85) and adjust the medium using 2 M sodium hydroxide (approx. 14–16 mL /10 L) to a pH of 7.1–7.2
6. Fill up to 10 L final volume using Milli-Q® water.
7. Sterilize by membrane filtration with Millipore Express® PLUS membrane (0.22 µm, polyethersulfone).
8. Measure the final pH and osmolality (pH = 7.2-7.4; osmolality = 362 +/-15 mOsmol/kg).
9. Store at 2–8 °C. Protect from light.

## Troubleshooting

| Issues | Recommendations |
|---|---|
| During adaptation, after reducing the serum concentration, the cells do not grow or grow very slowly. | This is normal. Leave the cells in the medium and wait for several days until the concentration reaches $1.5 \times 10^6$ viable cells/mL. |
| During adaptation, after reducing the serum concentration, the viability is reduced. | This is normal – the cells are adapting to the new conditions. If the viable cell density reaches $1.5 \times 10^6$ viable cells/mL, perform several passages in this serum concentration until the viability rises above 95% again. |
| During the adaptation, cells are dying because of reduced pH of the medium. | Be sure to use a controlled $CO_2$ incubator at 37 °C, 5% $CO_2$, 80% humidity, agitation rate 110 rpm. Be sure to use Erlenmeyers with vented caps (allowing gas exchange). $CO_2$ is used together with sodium bicarbonate as a buffering agent. |
| Some cell clumps appears during adaptation. | Continue with the adaptation, cell clumps will disappear or be reduced after several passages in serum-free medium. You can also gently triturate the clumps to break them up when passaging cells. The use of 50 mL vented spin tubes has proven helpful in reducing clumps. If possible, shake in spin tubes at 320 rpm in a controlled $CO_2$ incubator. |

The typical technical data above serve to generally characterize the cell culture media in industry-relevant expression systems.
The product information is available separately from the website: **www.emdmillipore.com**

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