Process Guidance

Cellvento® 4CHO Fed-batch Medium

Chemically defined cell culture medium for fed-batch applications

Mammalian cell expression systems are the dominant tool today for the production of complex biotherapeutic proteins. Different CHO hosts are used routinely to develop such biologics and are combined with several expression system like the dhfr, the UCOE or the glutamine synthetase (GS) system.

The platform described in this Process Guidance was developed to grow a broad range of CHO cells. The production medium supports initial cell growth and production while the highly concentrated, neutral pH feed is added to replenish depleted nutrients required for cellular function and to maintain and extend the production phase in fed-batch mode. This single feed is concentrated to more than 130 g/L allowing a reduction of the volume of feed added to the medium thereby increasing the volumetric productivity. It also contains cysteine and tyrosine derivatives which have been shown to release free cysteine and free tyrosine slowly throughout the culture. This time dependent release avoids tyrosine depletion which can lead to sequence variants. This process also results in free cysteine release, while maintaining a reduced redox environment. This is often related to higher cell growth and productivity.

As the performance of production media and their companion feed(s) are typically interdependent, optimizing a feeding strategy is a crucial step to achieve high cellular growth while maintaining high specific productivity. This document provides the basis for initiating feed optimization activities, but fine-tuning an effective feeding strategy should be considered.

The fed-batch media system

Cellvento® 4CHO medium and its companion Cellvento® 4Feed supplement are chemically-defined, non-animal origin products designed for use with CHO cell-based mammalian cell culture. The medium and its feeds are effective at achieving high-density cell growth and increased productivity with CHO suspension cell types. As with all fed-batch processes, optimization of feeding volumes and feed frequency are recommended.

Production medium and feed

- 1.03795.0010 Cellvento® 4CHO COMP
- 1.03796.0005 Cellvento® 4Feed COMP

Additives

- 1.02415.0400 Glucose for cell culture media
- 1.00286.1000 L-Glutamine EMPROVE® exp
- 1.37013.2500 Sodium hydrogen carbonate EMPROVE® bio
- 1.37020.5000 Sodium hydroxide pellets EMPROVE® bio
- On request HT supplement (50x)

Filtration

- GPWP02500 - Millipore Express® PLUS Membrane, 0.22 µm, 25 mm
- GVWP02500 – Durapore® Membrane 0.22 µm, 25 mm

All components are available individually.

Applications

- Cellvento® 4CHO medium and its companion feed have been designed for use with recombinant CHO suspension cells but may be suitable for other cell lines.
- The medium does not contain hypoxanthine and thymidine to allow a broad utilization, also in dhfr-transfected cells. The feed does not contain glucose to allow a fine tuning of the glucose concentration during fed-batch processes, thus allowing to minimize lactate production. The feed contains sources of both cysteine and tyrosine and should not be supplemented with any additional alkaline feed.
- Cellvento® 4CHO medium should be used as an amplification medium and a production medium in fed-batch applications.

Using Cellvento® 4CHO medium in fed-batch mode

- Add 4-8 mM L-Glutamine to Cellvento® 4CHO medium prior to use with non-GS CHO cells lines.
- Add 1x HT prior to use with non-dhfr systems
- Cellvento® 4Feed does not require any additional supplementation with L-glutamine, L-cysteine or L-tyrosine for use in fed-batch culture.
- Optimal volumes and timing of Cellvento® 4Feed should be determined experimentally (see guidance)
- Glucose should be monitored daily and added separately during feeding to maintain appropriate levels throughout the fed-batch culture.
- Cell selection agents should be added as required during the seed train expansion. In general, we recommend removing the selective pressure during the fed-batch production step and culture.

Options for Cellvento® 4CHO media system evaluation

1. Direct media adaptation

Cell lines may be adapted directly into Cellvento® 4CHO medium. Cells should be seeded at $3x10^5$ – $5x10^5$ cells/mL, then sub- cultured when densities reach $1x10^6$ – $3x10^6$ cells/mL and ≥ 80% viability. Adaptation is complete when cells attain a stable doubling time (20-30 hours) and VCD ≥ 90% over at least 2-3 passages.

Cells that are initially adapted to and cultured in any of the first generation Cellvento™ CHO product can be directly thawed or cultured in Cellvento® 4CHO.

2. Sequential media adaptation

The adaptation guidance provided below relies on regular sub-culturing of cells to maintain cultures in a logarithmic growth phase. This typically means that cells should be passaged every 3 to 4 days. At least two passages at each adaptation step are recommended to ensure that cells appropriately adjust to their new media environments.

Ratio of current media vs. Cellvento® 4CHO medium (in %)	Seeding density (x10^5 cells/mL)	Evaluation of cell growth	Acceptance criteria for next step
75:25	3.0	Cell density, viability in mid-log growth phase	Normal cell doubling time; Viability>80% over at least 2 passages
50:50	3.0	Cell density, viability in mid-log growth phase	Normal cell doubling time; Viability>80% over at least 2 passages
25:75	3.0	Cell density, viability in mid-log growth phase	Normal cell doubling time; Viability>80% over at least 2 passages
10:90	3.0	Cell density, viability in mid-log growth phase	Normal cell doubling time; Viability>80% over at least 2 passages
0:100	3.0	Cell density, viability in mid-log growth phase	Adaptation complete when cells maintain normal doubling time; Viability≥90% over at least 2 passages

3. Cryopreservation

Viable cell banks may be created by freezing cells in 90% Cellvento® 4CHO medium and cell culture grade 10% dimethyl sulfoxide (DMSO).

Cell freezing operation procedure:

- Mix sterile DMSO and Cellvento® 4CHO 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 prior to use.
- Select cells in mid-logarithmic phase and with normal shape, cell density should be >1.5x10^6 cells/mL and viability >95%.
- Centrifuge at 1200-1500 rpm for 5 min (200-300g).
- Discard the supernatant and re-suspend cells in cold freezing medium at 1x10^7 - 2x10^7 viable cells/mL, and transfer the cell suspension into sterile cryovials with 1mL each vial.
- Freezing procedure with a freezing container containing isopropanol: Place the cryovials into the cryobox, and freeze the cells following the sequential procedure with decreasing temperatures:
  - 30 min at 4°C
  - 2-4 hrs at -20°C
  - overnight at -80°C
  - Transfer and store the vials in the liquid nitrogen tank for long term storage.

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 in 1 hour.

Cell thawing and recovery procedure:

- Prepare a water bath at 37°C for cell thawing.
- In a 50 mL centrifuge tube, prepare 10 mL culture medium under the clean bench or the laminar flow hood.
- Transfer the cryovial of CHO cells from liquid nitrogen to the 37°C water bath.
- Take out the vial when ice particles detach from the side of the vial (DMSO may have a toxic effect at higher temperature).
- Transfer the CHO cell suspension from the cryovial to the centrifuge tube, centrifuge at 1200-1500 rpm for 5 min.
- Discard the supernatant, re-suspend the cells in fresh culture medium (Cellvento® 4CHO medium) in order to achieve a seeding density of 3x10^5 – 5x10^5 cells/mL, 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 100 rpm until densities reach ≥1x10^6 cells/mL. Thereafter, sub-culture following standard protocols.

Reconstitution of powder to prepare liquid medium

Reconstitution method to prepare 10L Cellvento® 4CHO medium

- Slowly add 237 grams of powder to 8.0 L of Milli-Q® or similar cell culture grade water in an appropriately sized container.
- Rinse medium container as necessary to remove remaining powder.
- Allow to dissolve with vigorous mixing for 30 minutes (solution will still be slightly turbid).
- Add 2 g/L sodium bicarbonate and stir until dissolved (~10 minutes).
- Add cell culture grade water to reach a final volume of 10L.
- Confirm a final pH of 7.0 ± 0.3.
- Measure the osmolality of the solution. Final osmolality should be at 310 ± 30 mOsmol/kg.
- Sterilize by membrane filtration using a 0.22 µm Millipore Express® PLUS or Durapore®, bottle cap or capsule filter.
- Store at 2-8°C protected from light.
  - Reconstituted Cellvento® 4CHO liquid medium is stable for at least 90 days.
  - When supplements are added, the liquid media is stable for max 4 weeks.

Note: This medium does NOT contain L-Glutamine or hypoxanthine and thymidine. Aseptically supplement as required prior to use.

Reconstitution method to generate 5L Cellvento® 4Feed

- Slowly add 651.7 grams of powder to 4.5L of Milli-Q® or similar cell culture grade room temperature water in an appropriately sized container.
- Rinse feed container as necessary to remove remaining powder.
- Vigorously mix for 45 min until fully dissolved.
- Slowly add 3.7 g/L of NaOH pellets.
- Add cell culture grade water to reach a final volume of 5L.
- Confirm final pH of 7.0 ± 0.3.
- Measure the osmolality of the solution. Final osmolality should be 1220 ± 50 mOsmol/kg.
- Sterilize by membrane filtration using a 0.22 µm Millipore Express® PLUS or Durapore®, bottle cap or capsule filter.
- Store at 2-8°C protected from light.
  - Reconstituted Cellvento® 4Feed liquid feed is stable for 60 days.
  - When a bottle is opened, liquid media is stable for max 3 weeks.

Recommended feeding strategy

Cellvento® 4CHO medium and companion feed have been developed to complement each other and enhance the performance of CHO cells in protein production. As with most upstream bioprocesses, optimization of feed volumes and timing of feed administration should be empirically determined on a process- and cell-line specific basis to maximize performance. The table below provides recommended ranges for evaluation, of both feed volumes and frequency of feeding, to optimize each parameter within the context of an overall feeding scheme.

Parameter	Recommended range for evaluation
Cellvento® 4Feed	1% – 6% (v/v)
Glucose	4 – 6 g/L (monitor daily and maintain at 4 g/L)
Frequency	48-72 hour feed intervals

Recommended process guidance for initial fed-batch medium and feed evaluation in shaker flasks:

Experimental condition	Operating Parameter
Culture type	Spin tubes with vented caps
Initial working volume	30 mL
Inoculation density	2-3 x10^5 cells/mL
Agitation rate	320 rpm
Production media	Cellvento® 4CHO Chemically defined cell culture medium
Feed media	Cellvento® 4Feed Chemically defined cell culture feed
Temperature	37.0 ± 0.5 °C
Incubator pCO_2	5%
Media pH	7.0+/-0.3
Harvest criterion	End culture when viability < 50-70%
Sampling points	Study days 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14
Cellvento® 4Feed volume	See table above
Feeding schedule	See tables below
Glucose feed addition	Daily addition, maintaining concentration above 1g/L and levels post-feeding at 4- 6 g/L

Although we recommend sampling the culture on day 0 to confirm the seeding density, the first proposed post-inoculation sampling time point is study day 3, followed by daily sampling.

Minimal sampling volume (i.e. < 800 µL) is recommended.

Measuring parameters at sampling days:

- Viable cell density
- Viability
- Glucose, glutamine (as appropriate)
- Recombinant protein product

Suggested initial feeding evaluation

Recommended Feeding

Initiate the feeding only when viable cell density is ≥ $2x10^6$ cells/ml and no earlier than day 3 (to avoid over-feeding).

Maintain supplementation with feed supplements and glucose until culture viability is less than 80%. Terminate and harvest cultures when viability drops below 50-70%.

Culture Day	Addition Order	0	1	2	3	4	5	6	7	8	9 or 10	11	12	13	14
Cellvento® 4Feed (%v/v)	1				3		3		6		3		3		
Glucose	2				Monitor daily and maintain at 4-6g/L										

Fed-batch performance in spin tubes

Cell culture performance tests have been evaluated.

Cell growth and protein production profiles were virtually indistinguishable in the 3 production lots tested, ensuring that Cellvento® 4CHO medium can be used confidently with minimal risk of process variability attributable to cell culture media raw materials.

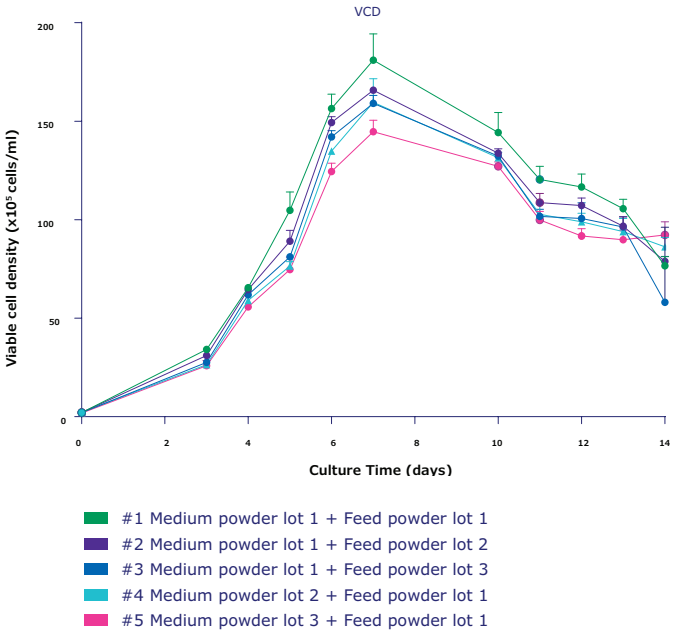

Figure 1: Growth and viability profiles in fed-batch culture. Cells from a CHOK1 GS were grown in Cellvento® 4CHO medium supplemented with complementary Cellvento® 4Feed product.

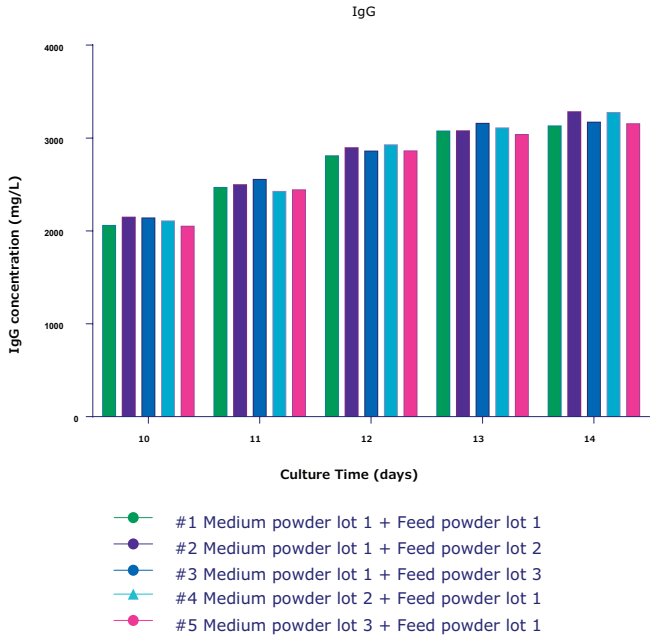

Figure 2: Consistent IgG titers were achieved using three different lots of Cellvento® 4CHO medium against one lot of Cellvento® 4Feed over a 14-day fed-batch culture with cells from CHO-K1 clone 1.

Troubleshooting Guide

No.	Question / Problem	Reason / Solution
1	The liquid media is still cloudy or hazy after mixing for 30 minutes during the first reconstitution step.	Depending on the agitation rate or mixing process and water temperature, there may still be some haziness following the first mixing step. This haziness will dissipate when adding sodium bicarbonate.
2	Can I add L-Glutamine prior to the sterile filtration step in order to prepare a complete medium?	Yes. You may add powder or liquid L-Glutamine during the first mixing step, and prior to the initial pH adjustment. Complete media supplemented with L-Glutamine should be used within 60 days to minimize impact on stability and ammonia accumulation.
3	Should I add HT to the medium and the feed?	If the cell line used requires, the 2 components have to be added to the medium. Addition in the feed is not required.
4	Do I need to supplement the medium Pluronic F68 prior to use?	No. Cellvento® 4CHO medium contains 2.0 g/L poloxamer 188, which is sufficient to protect suspension cultures from shear stress. Adding additional poloxamer may adversely impact cell growth and cause problems in down-stream processing and purification steps.
5	Can I use Nylon based filters for the media filtration?	No. We recommend the use of 0.22 µm Millipore Express® PLUS or Durapore® membrane filters. Nylon or cellulose acetate based filter membranes may non-specifically bind critical media components and adversely impact performance.
6	Can I use Cellvento® 4CHO medium with cells in 8-10% CO_2?	Cellvento® 4CHO is for use with 5% CO_2 incubation. You may need to increase the sodium bicarbonate concentration to offset and minimize the impact of the higher carbonic acid levels and decreased media pH on the cultures.
7	The osmolality of the complete Cellvento® 4CHO medium media prior to filtration, is >355 mOsmol/kg.	We recommend preparing fresh media as we typically observe (with multiple batches and media lots) final media osmolalities of 310 -315 mOsmol/kg in both our R&D and QC labs. An out of specification media osmolality is typically the result of a misformulation or multiple acid/base titrations during the pH adjustment steps.
8	Why do I need to store the complete liquid media protected from light?	Cellvento® products contain light sensitive components, including HEPES and vitamins, which are rapidly oxidized upon fluorescent light exposure, resulting in decreased stability and cellular performance.
9	There is a precipitate in the media or feed supplement following extended storage at 2-8°C.	Prepare fresh media or feed supplements. Cellvento® products contain components at high concentrations that are required to support high density batch and fed-batch cell culture applications. Components may come out of solution with time and/or following multiple uses and warming/cooling steps. Use Cellvento® 4CHO medium and Cellvento® 4Feed supplement within 90 days and 60 days of preparation, respectively.
10	We observe rapid cell growth, however low protein expression or antibody titers in fed-batch cultures using Cellvento® 4CHO medium.	Fed-batch cultures should reach 3-5 fold higher protein levels or antibody titers vs. batch culture concentrations. This is either the result of a low-expressing cell line or a nutrient rate limitation during the production phase of the fed-batch culture. Measure and maintain glucose concentrations at 4-6 g/L and optimize the feed addition timing and volume additions for the Cellvento® 4Feed.

To find out more about Cellvento® products and the Cellvento® CHO media platform visit our website:
www.emdmillipore.com/cellvento

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