Process Guidance

Cellvento™ CHO-210
Chemically defined cell culture medium for fed-batch applications

CHO-derived DG44 cells are characterized by a deletion of both DHFR (dihydrofolate reductase) alleles and consequently require hypoxanthine and thymidine for growth. The stable transfection of this cell line with an exogenous DHFR gene combined with a gene of interest (i. e., monoclonal antibody, recombinant protein) allows a selection of transfected clones in HT-deficient medium. To further increase the selection pressure and amplify clones with higher copies of the DHFR gene, methotrexate, an analog of folate, is commonly used. This molecule binds to the DHFR and inhibits the production of tetrahydrofolate, thus allowing the selection of clones with high DHFR expression and high product yield.

The media and feed system described in this Process Guidance was selectively developed to grow DHFR-deficient CHO cells. The production medium supports initial cell growth and production while the feed or supplement(s) are added to replenish depleted nutrients required for cellular function and to maintain and extend the production phase of the culture in fed-batch mode.

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.

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The fed-batch media system

Cellvento™ CHO-210 medium and its companion Cellvento™ Feed-210 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 and supporting high-density cell growth and competitive productivity with DHFR-negative CHO suspension cell types and expression systems, especially recombinant CHO-DG44 DHFR cell lines, but may also be appropriate for use with other CHO cell lines. As with all fed-batch processes, however, optimization of feeding volumes and feed frequency is recommended.

Production medium and main feed

1.02485.0010 Cellvento™ CHO-210 medium
1.02488.0005 Cellvento™ Feed-210

Additional feed supplements

1.02735.0100 Cysteine HCl EMPROVE® exp
1.02413.0100 Tyrosine di-sodium salt dihydrate

Additives

1.02415.0400 Glucose for cell culture media
1.00286.1000 L-Glutamine EMPROVE® exp
1.37013.1000 Sodium hydrogen carbonate
On request HT Supplement (50 ×)

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™ CHO-210 medium and its companion feeds have been designed for use with DHFR-negative expression systems in cell suspension culture, but may also be suitable for other CHO cell lines.
- Cellvento™ CHO-210 medium should be used as a production medium starting at the final N-1 expansion step in fed-batch applications, and then together with its companion feed product Cellvento™ Feed-210 during the final production culture.
- We recommend Cellvento™ CHO-110 medium for seed train expansion up to the N-1 step, as it is a richer formulation than Cellvento™ CHO-210 medium and does not require additional supplementation.
- Cellvento™ products are designed to allow and provide for flexibility in feed and feed supplement optimization of fed-batch processes.

Using Cellvento™ CHO-210 medium in fed-batch mode

- Supplement Cellvento™ CHO-210 medium with 100 µM hypoxanthine and 16 µM thymidine for parental dihydrofolate reductase deficient cell lines (DHFR-) and for all non-dihydrofolate reductase amplified cell lines. This can be accomplished by adding 20 mL/L HT (50 ×) supplement.
- Add 4 – 8 mM L-glutamine to Cellvento™ CHO-210 medium prior to use with non-GS CHO cells lines.
- Cellvento™ Feed-210 does not require any additional supplementation with L-glutamine for use in fed-batch culture.
- Optimal volumes and timing of Cellvento™ Feed-210 and Cys/Tyr feed administration should be determined experimentally (see point 5 in this Process 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. In general, we recommend removing the selective pressure during the fed-batch production step and culture.

Options for Cellvento™ CHO-210 media system evaluation

1. Direct media adaptation

Cell lines may be adapted directly into Cellvento™ CHO-210 medium. Cells should be seeded at $3 \times 10^5 – 5 \times 10^5$ cells/mL, then sub-cultured when densities reach $1 \times 10^6 – 3 \times 10^6$ 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 Cellvento™ CHO-110 growth medium can be sub-cultured directly into Cellvento™ CHO-210 medium. Cells banked in Cellvento™ CHO-110 medium should be thawed and maintained in Cellvento™ CHO-110 growth medium for at least 2 passages prior to sub-culturing in Cellvento™ CHO-210 medium.

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 2 passages at each adaptation step are recommended to ensure that cells appropriately adjust to their new media environments.

Ratio of current media vs. Cellvento™ CHO-210 medium (in %)	Seeding density ($\times 10^5$ 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™ CHO-210 medium and cell culture grade 10 % dimethyl sulfoxide (DMSO).

Cell freezing operation procedure:

- Mix sterile DMSO and Cellvento™ CHO-210 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 > 1.5×10^6 cells/mL and viability > 95 %.
- Centrifuge at 1,200 – 1,500 rpm for 5 minutes (200 – 300 g).
- Discard the supernatant and re-suspend cells in cold freezing medium at $1 \times 10^7 – 2 \times 10^7$ viable cells/mL, and transfer the cell suspension into sterile cryovials, 1 mL per vial.
- Freezing procedure with a freezing container filled with isopropanol. Place the cryovials in the cryobox and freeze the cells with a sequential decrease in temperature:
  – 30 minutes at 4 °C
  – 2 – 4 hours 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 from 4 °C down 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 1,200 – 1,500 rpm for 5 minutes.
- Discard the supernatant, re-suspend the cells in fresh culture medium (Cellvento™ CHO-210 medium) in order to achieve a seeding density of $3 \times 10^5 – 5 \times 10^5$ cells/mL, and transfer to a 125 mL vented cap 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 ≥ 1×10^6 cells/mL. Thereafter, sub-culture following standard protocols.

4. Transformation from powder to liquid medium

Reconstitution method to generate 10 L Cellvento™ CHO-210 medium

1. Slowly add 231 grams of powder to 8 L of Milli-Q® or similar cell culture grade water in an appropriately sized container. Rinse medium container as necessary to remove remaining powder.
2. Allow to dissolve with vigorous mixing for 30 minutes (solution will still be slightly turbid). Adjust pH to 6.2 +/– 0.1 using 5 M sodium hydroxide (typically requires ~1 mL/L to reach target pH).
3. Add 2 g/L sodium bicarbonate and stir until dissolved (~10 minutes).
4. Adjust the pH to 7.0 +/– 0.2 using 5 M sodium hydroxide or 1 M hydrochloric acid, if needed.
5. Add cell culture grade water to reach a final volume of 10 L. Confirm a final pH of 7.0 +/– 0.2.
6. Measure the osmolality of the solution. Final osmolality should be at 315 +/– 40 mOsmol/kg.
7. Sterilize by membrane filtration using a 0.22 µm Millipore Express® PLUS or Durapore® membrane filter (bottle cap or capsule filter).
8. Store at 2 – 8 °C protected from light. Reconstituted Cellvento™ CHO-210 liquid medium is stable for at least 90 days. When supplements are added, the liquid medium is stable for max. 4 weeks.

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

Reconstitution method to generate 5 L Cellvento™ Feed-210

1. Slowly add 408 grams of powder to 4 L of Milli-Q® or similar cell culture grade water in an appropriately sized container. Rinse feed container as necessary to remove remaining powder.
2. Vigorously mix for 45 – 60 minutes until fully dissolved.
3. Add cell culture grade water to reach a final volume of 5 L. Confirm final pH of 5.2 – 5.8.
4. Measure the osmolality of the solution. Final osmolality should be 658 +/– 30 mOsmol/kg. Sterilize by membrane filtration using a 0.22 µm Millipore Express® PLUS or Durapore® membrane filter (bottle cap or capsule filter).
5. Store at 2 – 8 °C protected from light. Reconstituted liquid Cellvento™ Feed-210 is stable for 90 days. When a bottle is opened, liquid feed is stable for max. 2 weeks.

Preparation of cysteine/tyrosine stock solution – 150 mL

Component	CAS#	FW	g/L	mM
L-Cysteine hydrochloride monohydrate	7048-04-6	175.63	52.67	299.90
L-Tyrosine disodium salt dihydrate	122666-87-9	261.19	149.64	573.0

1. Measure 0.1 L of Milli-Q® or similar cell culture grade water into an appropriate container and adjust the pH ≥ 13 using 5 M sodium hydroxide.
2. Slowly add 7.9 g of L-cysteine and 22.45 g of L-tyrosine.
3. Adjust the pH to 11.3 +/– 0.1 using 5 M sodium hydroxide or 1 M hydrochloric acid and mix for 10 – 30 minutes to dissolve all components.
4. Add cell culture grade water to reach a final volume of 0.15 L. Confirm final pH of 11.3 +/– 0.1.
5. Measure the osmolality of the solution. Final osmolality should be 3,100 +/– 100 mOsmol/kg.
6. Sterilize by membrane filtration using a 0.22 µm Millipore Express® PLUS or Durapore® membrane filter (bottle cap filter).
7. Store at 2 – 8 °C protected from light. Reconstituted stock solution is stable for 7 – 14 days.

The stock solution yields concentrations of cysteine and tyrosine of 300 mM and 573 mM respectively, which are subsequently diluted during feeding.

To find out more about Cellvento™ CHO media platform products, visit www.merckmillipore.com/cellvento

5. Recommended feeding strategy

Cellvento™ CHO-210 medium and companion feeds have been developed to complement each other and enhance the performance of CHO-DG44 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 on the right 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™ Feed-210	2 – 8 % (v/v)
Glucose	4 – 6 g/L (monitor daily and maintain at 4 g/L)
Cys/Tyr feed	0.1 – 0.2 % (v/v) of recommended stock solution
Frequency	48 – 72 hour feed intervals

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

Parameter	Parameter
Culture type	125 mL vented cap shaker flask, non-baffled
Initial working volume	45 mL
Inoculation density	2 – 5 ×10^5 cells/mL
Agitation rate	150 rpm (25 mm orbital)
Production medium	Cellvento™ CHO-210 chemically defined cell culture medium
Main feed	Cellvento™ Feed-210 chemically defined cell culture feed
Feed supplement	Cys/Tyr stock solution
Temperature	37.0 +/– 0.5 °C
Incubator pCO_2	5 %
Media pH	7.0
Harvest criterion	End culture when viability < 50 – 70 %
Sampling points	Study days 0, 3, 4, 5, 6, 7, 8, 9, 10, 11,12, 13, 14
Cellvento™ Feed-210 volume	See table above
Cys/Tyr stock solution	See table above
Feeding schedule	See tables for options 1 & 2 below
Glucose feed addition	Daily addition, maintaining concentration above 1 g/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.

Measurement parameters on sampling days:

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

Suggested initial feeding evaluation

Feeding option 1

Initiate the feeding only when viable cell density is ≥ 2 ×10^6 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	10	11	12	13	14
Cellvento™ Feed-210 (% v/v)	1				6		6		6		6		6		6	
Glucose	2	Monitor daily and maintain at 4 – 6 g/L														
Cys/Tyr stock solution (% v/v)*	3				0.2		0.2		0.2		0.2		0.2		0.2	

* Add to culture slowly

Feeding option 2 (slow growing cell lines)

Initiate the feeding only when viable cell density is ≥ 2 × 10^6 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	10	11	12	13	14
Cellvento™ Feed-210 (% v/v)	1				3		3		6		6		6		6	
Glucose	2	Monitor daily and maintain at 4 g/L														
Cys/Tyr stock solution (% v/v)*	3				0.10		0.15		0.2		0.2		0.2		0.2	

* Add to culture slowly

It is recommended that customers optimize the feeding process in order to meet the specific metabolic requirements of their cell lines.

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™ CHO-210 medium can be used confidently with minimal risk of process variability attributable to cell culture media raw materials. To confirm media performance and consistency over different lots of Cellvento™ CHO-210 production medium, performance trials were run in spin tubes on a recombinant CHO-DG44 cell line.

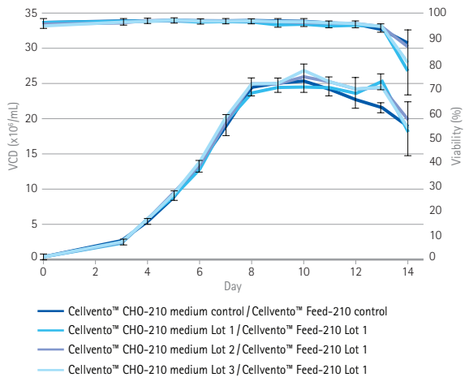

Figure 1:

Growth and viability profiles in fed-batch culture. Cells were grown in Cellvento™ CHO-210 medium supplemented with complementary Cellvento™ Feed products.

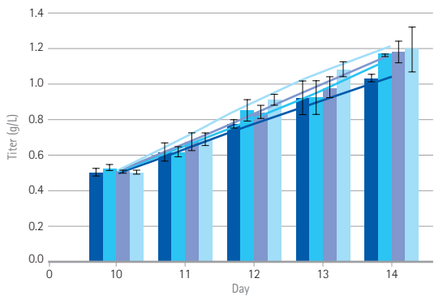

Figure 2:

Consistent IgG titers were achieved using three different lots of Cellvento™ CHO-210 medium against one lot of Cellvento™ Feed-210 over a 14-day fed-batch culture.

Troubleshooting

No.	Question/Problem	Reason/Solution
1	The liquid medium 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 the pH is adjusted to 6.20 (prior to addition of the 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	Do I need to supplement the medium with poloxamer prior to use?	No. Cellvento™ CHO-110 and Cellvento™ CHO-210 media contain 2.0 g/L poloxamer, which is sufficient to protect suspension cultures from sheer stress. Adding additional poloxamer may adversely impact cell growth and cause problems in down-stream processing and purification steps.
4	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.
5	Can I use Cellvento™ CHO-110 or Cellvento™ CHO-210 medium with cells in 8 – 10 % CO_2 incubators?	Cellvento™ CHO media have been optimized 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.
6	The osmolality of the complete Cellvento™ CHO-110 or Cellvento™ CHO-210 medium 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.
7	Why do I need to store the complete liquid media protected from light?	Cellvento™ CHO media and feed supplements contain light-sensitive components, including HEPES and vitamins, which are rapidly oxidized upon fluorescent light exposure, resulting in decreased stability and cellular performance.
8	There is a precipitate in the medium or feed supplement following extended storage at 2 – 8 °C.	Prepare fresh medium or feed supplements. Cellvento™ CHO media and feed supplements 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™ CHO-110 or Cellvento™ CHO-210 medium and Cellvento™ Feed-210 supplement within 90 days of preparation, respectively.
9	Viability of batch cultures in Cellvento™ CHO-110 medium drops rapidly after 6 days in culture.	This is likely a nutrient rate limitation. The glucose concentration in terminal batch cultures (used for the production of recombinant proteins) can be monitored daily and maintained at >1 g/L to avoid depletion.
10	We observe rapid cell growth but low protein expression or antibody titers in fed-batch cultures using Cellvento™ CHO-210 medium.	Fed-batch cultures should reach 3- to 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 Cellvento™ Feed-210 and the cysteine/tyrosine feed supplements.

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.merckmillipore.com**

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