



BIOSTAT® CultiBag RM Culturing Convenience



#2

Application
Note

#3

Protocol for fed
batch, serum free
cultivation of CHO
XM 111 suspension
cells in the BIOSTAT
CultiBag RM 20

#4

#5

#6

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Introduction

In this application note, we describe a protocol for the propagation of the model protein secreted alkaline phosphatase expressing *CHO XM 111* suspension cells (obtained from Prof. Dr. Martin Fussenegger, Swiss Federal Institute of Technology, Zurich) in selective, chemically defined, protein and peptide-free ChoMaster media (HP-1 and HP-5) using the disposable bioreactor BIOSTAT CultiBag RM 20 basic.

Generally, the inoculum for the bioreactor is prepared by pooling T-flasks. The pre-culture is routinely realized in 75 cm² and 175 cm² culture flasks containing CHOMaster FMX-8 growth medium, which was used for maintenance of the culture. In order to ensure optimum growth in T-flasks, the CHO suspension cells are incubated at 37°C in a humidified atmosphere of 10% CO₂ in air. Cells are seeded at a minimal density of 2–3 × 10⁵ viable



Fig. 1: BIOSTAT® CultiBag RM20 basic.

cells/mL and subcultured or inoculated in the larger scale when cell densities have reached values around 1*10⁶ viable cells/mL.

In our experience, this method described for *CHO XM 111* suspension cells can also be successfully applied to other animal cell lines such as non-transfected CHO suspension cells, *Sf-9/Sf-21* suspension cells (DSMZ), and engineered *HEK-293 EBNA* suspension cells (Cytos Biotechnology AG, Switzerland). Modifications mainly concern the culture medium.

1. Equipment and Material

- BIOSTAT® CultiBag RM 20 basic (Sartorius Stedim Biotech GmbH)
- CultiBag RM 2L (with Sartofluor 300 Capsule, 0.2 µm; Sartorius Stedim Biotech GmbH)
- CHOMaster media HP-1/HP-5 (Cell Culture Technologies GmbH)
- Cedex Cell Counter (Innovatis AG) or Cedex HiRes (Innovatis AG) or NucleoCounter (ChemoMetec A/S)
- Bioprofile Analyzer 100 or BioProfile Analyzer 100+ (Nova Biomedical)
- T-flasks (T-75, T-175)
- CO₂ incubator
- Water bath
- Magnetic stirrer
- Pipetboy (Integra Biosciences AG)
- Peristaltic-pump (e.g. Dose-it 803, Vitaris AG)
- Sterile syringes (10mL, 50 mL)
- Serological pipettes
- Reaction tubes and sample vials (1.5 mL)
- Sterile bottles
- Sterile aluminium foil
- Safety cabinet class II
- Laminar flow module
- Roll-Boy with tripod

2. Methods

a. Schedule

Day 1:	Establishment of preculture I in T-75 flask with rapidly growing, healthy <i>CHO XM 111</i> suspension cells characterized by logarithmic growth and doubling times ≤ 24 hours
Day 3:	Feeding of preculture I with ChoMaster FMX-8 growth medium
Day 5:	Establishment of preculture II (T-175 flask) from preculture I Passage cells into T-175 (minimal seeding density of $2\text{--}3 \times 10^5$ viable cells/mL), if cell density has reached 1×10^6 viable cells/mL
Day 7:	Pooling of preculture II, inoculation and starting-up BIOSTAT CultiBag RM 20 with the disposable bioreactor bag CultiBag RM 2L operating with 100 mL cell suspension (1×10^6 viable cells/mL) and 100 mL ChoMaster HP-1 growth medium (see section 2d, 2e, 2f and 2g)
Day 7:	Fermentor/Bioreactor and medium preparation (see section 2b and 2c)
Day 8, 9, 10, 11:	Sampling, successive feeding of ChoMaster growth medium (up to cell densities of 1.2×10^6 viable cells/mL HP-1, subsequent feeding of HP-5 growth medium), increase of rocking rate and IPC (see section 3 and 5). The feeding procedure should be also done in such a mode that glucose levels below 1.0 g/L are avoided.
Day 12 or 13:	Partial or complete harvest of cells (see section 4). Cell densities between 2 and 4×10^6 viable cells/mL may be achievable. Aim at viabilities above 95%.

b. Fermentor|Bioreactor preparation

100 mL of ChoMaster HP-1 growth medium containing 0.2 % Pluronic are filled in the CultiBag RM 2L in the safety cabinet (clamped air filters)

Keep in mind that there is no need to use Pluronic in media containing serum.

c. Media

Selective medium for T-flasks: filter-sterilized, conditioned (37 °C, pH 7.3) ChoMaster FMX-8 growth medium (Cell Culture Technologies).

Additional supplements for FMX-8 medium:

Used antibiotics to keep cells under selection pressure, support cell growth and prevent SEAP expression: 100 mg mL⁻¹ G418 sulphate, 5 mg mL⁻¹ puromycin dihydrochloride, 2.5 mg mL⁻¹ tetracycline hydrochloride.

Medium for BIOSTAT CultiBag RM 20: filter-sterilized, conditioned (37 °C, pH 7.3) ChoMaster HP-1- and HP-5 growth medium (Cell Culture Technologies)

Additional supplements for HP-1 and HP-5 medium:

2.5 mg mL⁻¹ tetracycline hydrochloride, supports cell growth and prevents SEAP expression and 0.2 % Pluronic F68 solution (Sigma) protects cells against shear (*only necessary in serum-free media!*)

d) Preculture and Inoculum for CultiBag RM 2L

For establishing the preculture II (T-175) representing the subsequent inoculum after pooling procedure approximately 4 days are required. In case of use of cryopreserved vials we recommend a previous T-flask cultivation of 14 days. In other words, the use of cryopreserved vials instead of T-75 will prolong the precultivation time.

In order to obtain the desired seeding cell density of about 5*10⁵ viable cells/mL for the CultiBag RM 2L, harvest of 5*10⁷ viable cells from T-flasks, pooling of the cell pellets and resuspension in 100 mL fresh ChoMaster HP-1 growth medium have to be carried out.

Consequently, the cells were transferred from T-175 into a sterile beaker (pipetting) covered with a sterile aluminum foil and

incubated (CO₂ incubator) for 3 hours in order to allow the cells to settle. Alternatively for other cell lines, the cells can be centrifuged at maximum 200 g.

The consumed growth medium (FMX-8) was then aspirated and replaced with fresh HP-1 growth medium (pH 7.3, 37°C) in the safety cabinet. After cell density check the cell suspension in the sterile beaker was ready for its use in CultiBag RM 2L.

e. Corrective agent

Acid:	0-10% CO ₂
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f. Culture conditions

Starting culture volume:	200 mL
Final culture volume:	1000 mL
Rocking rate:	14-30 rpm
Rocking angle:	6°
pH:	7.3-6.9
Temperature:	37 °C
Aeration rate:	0.2 vvm
Start cell density:	5 x 10 ⁵ viable cells/mL
Final cell density:	3-4 x 10 ⁶ viable cells/mL
Cultivation time:	6-7 days

g. Inoculation

- By inserting a syringe into the CultiBag's luer lock inoculation port, 100 mL of the prepared cell suspension (see section 2d) were added in the safety cabinet (exhaust air filter was clamped off).
- The filled CultiBag (100 mL HP-1 growth medium, see section 2b, and 100 mL cell suspension, see section 2d) was put back on the tray (clamped air filters), fixed and installed.
- The filter heater was installed and switched on.
- Air filter lines were opened and aeration (0.2 vvm), rocking (14 rpm, 6°) and heating (37 °C) were switched on.

4. Complete cell harvest or Scale-up

For harvesting the cells/product, one of the attached ports can be used. For the scale-up into a larger volume the following procedure can be used:

- The BIOSTAT CultiBag RM basic station was switched off.
- The air filters were closed.
- The CultiBag RM 2L was removed from the tray and transferred to a laminar flow module.
- The CultiBag was hung on a tripod standing on a Roll-Boy in the laminar flow module.
- The exhaust filter of the CultiBag was opened whereas inlet filter was closed.

3. Start-up and operation of BIOSTAT CultiBag RM 20

½ hours:

Sample 0: Analytics (section 5)

24 hours:

Sample 1: Analytics (section 5)

48 hours:

Sample 2: Analytics (section 5), feeding with 200 mL HP-1 growth medium and rocking rate increase (16 rpm)

72 hours:

Sample 3: Analytics (section 5), feeding with 200 mL HP-5 and rocking rate increase (18 rpm)

96 hours:

Sample 4: Analytics (section 5), feeding with 200 mL HP-5 and rocking rate increase 25 rpm

120 hours:

Sample 5: Analytics (section 5), feeding with 200 mL HP-5 and rocking rate increase 30 rpm

6 or 7 days:

Sample 6 and 7: Analytics (section 5), partial or complete cell harvest (section 4).

In case of partial cell suspension harvest, the adequate amount of fresh HP-5 growth medium is fed.

- 200 mL of HP-5 growth medium were fed.

- For about 3 hours the cells were allowed to settle on the bottom of the CultiBag RM 2L.

- The medium was removed from the CultiBag using the tube of the fill/harvest port.

5. Analytics

Daily one 2 mL sample is taken in order to determine:

Cell growth and viability (1 mL sample) by use of Cedex or NucleoCounter instead of traditional, time-consuming, manual cell counting (hemocytometer, Trypan Blue) Glucose, lactate, glutamine, glutamate, pH (1 mL sample) by use of Nova BioProfile Analyzer 100 or its successor. Alternatively, other automatized analyzers (e.g. YSI 2700 Bio-chemistry Analyzer, YSI Incorporated, and Eppendorf Ebio plus) or also test kits (for example, from Roche Diagnostics) are available.

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