Cultivation of hybridoma cell line CF-10H5 (DSMZ ACC477)

University of Applied Science Aachen, Applied Science and technology, Th. Schnitzler

Introduction

The cells of the immune system from humans and animals are able to produce antibodies for the recognition of pathogens. There are an enormous number of different antibodies, which react specifi cally in each case to certain exciters. Antibodies are required in many ranges of research, medicine and the diagnostic investigation. They are manufactured with hybridoma cells in different bioreactors in large quantities.

A hybridoma cell is a fusion cell from two different cells. On the one hand it is a myelom cell and on the other hand a plasma cell. This hybridoma cell unites the most important characteristics of the two cells, it lives "eternally" and procedures the desired monoclonal antibody.

1. Equipment and Material Used

- BIOSTAT® Aplus 2 L CC-Assembly
- 7 × 500 mL Schott flasks
- 1 × 100 mL Schott flask
- 1 × 50 mL Schott flask
- 2 × 25 mL Schott flasks
- 2 × 2 L Schott flasks with stainless steel head plate
- 1 × 5 L Schott flask with stainless steel head plate
- 25 × 25 mL Schott flasks
- T-Flasks (T25, T75)
- Spinner flask
- 1.5 mL – Reaction tubes
- 15 mL – Centrifuge tubes
- Glucose-Analyzer or Glucose Kit
- Glutaminanalyzer or Glutamin Kit
- Osmometer
- Centrifuge
- Microscop with Haemocytometer
- Erythrosin B
- Cell Counter (e. g. CASY, Schärfe)
- Balance (e. g. Sartorius CP serie)
- CO_2 incubator
- Clean bench
- Sterile pipettes
- High purity water
- 96 Well plates
- Well plate reader (wave length = 690 nm + 410 nm)
- Sterile filter (Midisart® 2000, Sartobran® P, Sartobran® 300)
- Sterile connector

2. Overview of setting up procedures

a) Timetable

Day 1: Sterilization storage bottles
Day 1: Preparation and sterile fi ltration of specific culture solutions
Day 1: Preparation and inoculation preculture I (T25)
Day 3: Preparation and inoculation preculture II (T75)
... Passage cells into new T75-Flasks, if cell density have reached 1× 10^6 cells/mL
... Preparation and inoculation Spinner Flask if cell density have reached 1× 10^6 cells/mL
Day 14: Preparation, cleaning and sterilization of the culture vessel
Day 14: Sterility test
Day 16: Inoculation bioreactor | fermenter

b) Bioreactor | Fermenter

- Cleaning of the culture vessel by incubation with 1% (w | v) NaOH
- Calibration and installation of the pH-electrode
- Installation of the pO_2 probe
- Washing with high purity water
- After this the vessel should be fi lled with high purity water to a level covering both probe membranes
- Connection of peripheral equipment (tubes and filter for air inlet & exhaust, sampling device, sterile connector for medium transfer etc.)
- Sterilization of the culture vessel by autoclaving according the manual

c) Media

Trace element solution
Salts were dissolved in 150 mL 1M HCl (individually) and fi lled up in a measuring flask with high-purity water to 1000 mL. The solution must have to be light yellow and completely clear. Store at +4°C.

Fe(III)-Citrat	1126.00 mg/L
$CuSO_4.5\ H_2O$	3.75 mg/L
$ZnCl_2$	518.00 mg/L

Vitamin solution
Dissolve ingredients in 1000 mL high purity water, store at -20°C for max. 6 month.

Choline chloride	100.00 mg/L
D-Ca-Panthogtenic acid	100.00 mg/L
Folic acid	100.00 mg/L
i-Inositol	200.00 mg/L
Nicotine amide	100.00 mg/L
Pyridoxal.HCl	100.00 mg/L
Ribofl avin	10.00 mg/L
Thiamine. HCl	100.00 mg/L

Fatty acid solution
At fi rst stock solutions of fatty acids have to be prepared: Dissolve each 100 mglipoic acid and 100 mg linolenic acid in10 mL ethyl alcohol (store at -20°C for 12month). Afterwards mix 760 µl lipoic acid - Stock solution, 320 µl linolenic acid – Stock solution as well as 8.9 mL ethyl alcohol and store until use at -20°C (stable for 6 month).

Ethanolamine solution
Dissolve 3 mL ethanolamine in 48 mL high purity water (stable for 3 month).

Sodium selenite solution
Dissolve 3.46 mg sodium selenite solution in 100 mL high purity water (stable for 6 month).

Insulin solution
Dissolve 100 mg Insulin in 25 mL high purity water (store at -20°C, stable for 6 month) until use.

Transferrin solution
Dissolve 500 mg transferrin in 25 mL high purity water (store at -20°C, stable for 12 month).

Culture medium
The powdered components are dissolved in approximately 4 liters high-purity water on a magnetic stirrer. Afterwards the liquid components are added an the solution is mixed for several minutes. The pH value is determined and adjusted to 7.1 if necessary (HCl | sodium hydroxide). It is fi lled up with high-purity water to 5 litres. The osmolality is determined and adjusted to 320–330 mosmol/kg if necessary (high purity water | sodium chloride).

DMEM (Gibco 52100)	
dependent on batch	33.30 g
HAM F-12 (Gibco 21700)	
dependent on batch	17.70 g
HEPES	4.37 g
$NaHCO_3$	12.00 g
BSA	0.50 g
Glutamin	1.27 g
Glucose*	16.00 g
Insulin	500.00 mL
Transferin	100.00 mL
Selenit	25.00 mL
Fettsäuren	305.00 mL
Aminoethanol	500.00 mL
Vitamins	25.00 mL
Oligo elements	4.30 mL
FcS	50.00 mL

*3,2 g/L - Please note DMEM specification!

The medium for preculture is filtered into sterile 500 mL bottles. The medium for the bioreactor | fermenter is filtered into a sterile 2 liter Schott bottle.

d) Preculture
For setting up the preculture approximately 14 days are required. The cells from a cryo vial are transferred into a T25-Flask. After 2 days, the cells are further cultivated into a T75-Flask. After a cell number of 1×10^6 cells/mL is reached, the cells are diluted to 2×10^5 cells/mL and are further cultivated. When a number of 1×10^6 cells/mL is reached and if the viability of the culture is > 70% the cells are transferred into a Spinner flask starting cell number 2×10^5 cells/mL. The Spinner flask is cultivated up to a suffi cient quantity of cells. The cell density in the spinner should not exceed 2×10^6 cells/mL. The starting cell density in the fermenter | bioreactor should be 1.8×10^5 cells/mL. To start with a culture volume of 1000 mL 1.8×10^8 cells are necessary, correspond to 120 mL of a Spinner culture with 1.5×10^6 cells/mL. The viability of the cells should be greater than 80%.

e) Corrective agent
Acid: CO_2

f) Culture conditions

Culture volume:	1000 mL
Stirrer:	150 rpm
DO:	60%; controlled
pH:	7.1; controlled
Temperature:	37°C
Start cell density:	1.8×10^5 cells/mL
Fermentation time:	100 h

g) Sterile test
- Connect the waste bottle to the vessel (via sterile connector)
- Pump the water from the vessel into the waste bottle
- Connect the bottle with the culture media to the vessel (via sterile connector)
- Fill 1000 mL of culture medium into the vessel
- Warm up the vessel to 37°C, aerate with air
- After 48h check the sterility of the culture medium, if it is still sterile go ahead
- Calibrate the pO_2-electrode (2-point calibration)
- Pump the medium from the vessel into the waste bottle
- Fill the vessel with 880 mL culture medium (if the volume of the starter culture is equal to 120 mL)

h) Inoculation
- Switch-on the temperature control, pO_2- and pH-control, wait until all set points are reached
- Connect the SuperSpinner to the culture vessel via STT connector
- Pump the culture into the vessel
- Take the first sample and determinate the start conditions (glutamine, glucose, number of cells, etc.)

3. Analytical procedure
Daily two samples are taken. The cell number, viability and the concentration of glutamine, glucose and osmolality should be determinate.

Determination of cell viability
For determination of cell number and viability mix 100 µl cell suspension with 100 µl Erythrosin B solution. If it is necessary dilute the mixture with PBS. The number of cells in one large square of a hemocytometer should be between 50 and 150 cells. Red colored cells are dead, not colored cells are viable.

Erythrosin B solution:

Erythrosin B (Sigma E9259)	2.00 g
NaCl	4.05 g
K_2HPO_4	0.30 g

Dissolve the components in approximately 400 mL high purity water, stir the solution until all components are completely dissolved (heating is necessary). After this adjust the pH to 7.2 – 7.3 by adding 1 M sodium hydroxide.

Fill up to 500 mL with high purity water. Dilute the solution 1:10 with PBS before using (store at 2-8°C, stable for 12 month).

$$\text{cells (viable)} = \frac{\text{viable cells in big squares}}{\text{number of squares}} \times 10^4 \times \text{dilution (cells/mL)}$$

$$\text{Viabilty (\%)} = \frac{\text{number of cells (viable)}}{\text{number of cells (dead and viable)}} \times 100$$

Determination of glucose, glutamine & osmolality
2 mL of the sample is centrifuged at 15,000 × g for 4 min, the concentration of glutamin, glucose and the osmolality are determinate in the supernatant.

For this analysis the following analyzers are suitable:
– YSI 2700 for glucose | glutamate | glutamine
– Eppendorf Ebio for glucose
– Gonotec Osmomat 030 for osmolality

Alternatively it is possible to use test kits (e. g. Roche Diagnostic) according the manufacturer protocol.

The remaining supernatant is frozen (-20°C) to measure the antibody concentration by ELISA test in 96-well plates after the fermentation is fi nished.

ELISA (enzyme linked immune assay)
Antibody (AB) assay should be performed using a sandwich ELISA (standard procedure).

Capture-AB:	Anti-Mouse IgG (Sigma M8642)
Blocking agent:	BSA (Sigma A9647)
Detection-AB:	Anti-Mouse IgG Alkaline Phosphatase conjugate (Sigma N-2765)
Standard:	IgG (Sigma M5284) Standard concentration 0.02 mg/mL-3 µg/mL

Detailed information regarding ELISA test are available from the author (schnitzler@fh-aachen.de).

Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen, Germany

Phone +49.551.308.0
Fax +49.551.308.3289
www.sartorius-stedim.com

USA Toll-Free +1.800.368.7178
UK +44.1372.737159
France +33.442.845600
Italy +39.055.63.40.41
Spain +34.91.3586102
Japan +81.3.3740.5407

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