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Bioreactors
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**CELLine**Attachment Dependent
Cell Cultivation on
MicroCarriers

Attachment Dependent Cell Cultivation on MicroCarriers: Secreted Protein Production

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Description:

Microcarriers provide surface area for cell attachment within the cell compartment of the CELLLine. Culture of attachment dependent cells on microcarriers within the CELLLine cell compartment provides the benefits of increased product concentration, reduced serum consumption, reduced handling and elimination of trypsin in maintenance of a long term culture of attachment dependent cells. This protocol is intended as a starting point for the scientist in successful establishment of an attachment dependent culture in the CELLLine.

Certain cell lines can be cultivated following suspension adaptation without the need for microcarriers as an attachment substrate. It should be determined by the researcher if suspension adaptation is possible for their cell line and if expression levels are influenced under these conditions. Additional information regarding these issues is available from Sartorius AG.

1. Cell and MicroCarrier Inoculum Preparation:

Microcarriers are provided sterile to simplify establishment of attachment dependent cultures. These microcarriers are polystyrene and provide a surface similar to that of commonly used polystyrene culture plates, flasks and roller bottles. Alternative chemistry and surface properties are available for those applications which may benefit from more rapid attachment or porous carriers for three dimensional cultures.

Suspend cells at $2.0 - 3.0 \times 10^6$ cells per ml in complete culture medium. Cells should be taken from a culture which is in growth phase. If trypsin has been used to harvest cells it must be neutralized. Cells taken from confluent cultures may not grow as rapidly as cells taken from non confluent cultures.

Microcarrier addition:

Slowly pour dry microcarriers directly into cell suspension. The microcarriers will accumulate on top of the medium and slowly sink. Add the microcarriers in several steps to aid in suspension. Swirling of the tube will aid in getting the microcarriers in suspension. Do not shake as this will cause foaming. Add sufficient volume of microcarriers to achieve the total volume listed above for the CELLLine to be used. For example, for a CL 350 add microcarriers to 5 ml of cell suspension by slow addition and mixing to achieve a total volume of 7 ml. The total volume of cells and microcarriers is now ready for inoculation into the cell compartment.

Cell inoculum:

Prepare appropriate volume of cell suspension for the CELLLine to be used.

CELLine	Cell Suspension	Microcarrier Volume	Total Volume
CL350	5 ml	2 ml	7 ml
CL1000	14 ml	6 ml	20 ml



2. Cell Seeding and Culture Initiation:

It is important to allow cells to attach to the microcarriers to initiate culture. This must be done under culture conditions, i.e. 37° C, 5% CO₂ and in complete culture medium.

Inoculate:

Draw complete volume of microcarriers and cells into the pipette and transfer into pre-wetted cell compartment of the CELLLine. Pre-wet upper membrane of cell compartment by placing small volume of culture medium into upper nutrient compartment and on top of semi permeable membrane. Remove any air bubbles by aspirating from cell compartment with pipette.

Cell Attachment:

Tighten white cell compartment cap securely. Place CELLLine in incubator standing on back end with green cap pointed upwards (Fig 1.). This allows cells and microcarriers to settle within the cell compartment and initiates cell attachment to the microcarriers. Every 15-20 minutes gently mix cell compartment volume by rocking the CELLLine to resuspend cells and microcarriers in the cell compartment (do not shake). Replace CELLLine on end into incubator and allow cell compartment contents to settle again. Cell attachment will be complete after approx. 100 minutes. To confirm cell attachment a sample of the cell compartment volume can be taken and free cells counted. A 80-90% decrease in the number of free cells indicates cell attachment has taken place (Fig. 2).

3. Culture:

After cell attachment onto the microcarriers is complete, the nutrient reservoir should be filled with nutrient medium to support cell proliferation. Gently rock the CELLLine to achieve an even distribution of microcarriers within the cell compartment. Place the CELLLine in normal operating position in incubator and begin culture.

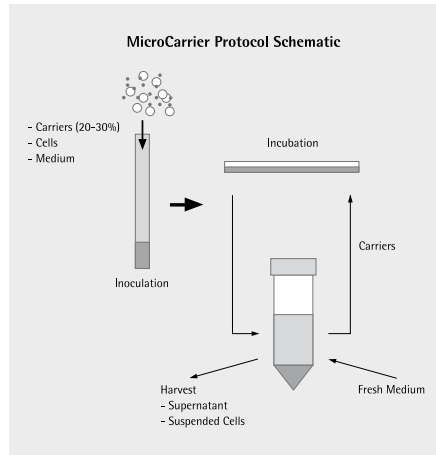


Figure 1: Schematic for microcarrier culture in CELLLine.

Cells and microcarriers are inoculated together into cell compartment. Standing CELLLine on end causes cells and microcarriers to settle and contact leading to cell attachment. Following cell attachment, the CELLLine is operated in normal horizontal position. Harvest is accomplished by removal of cell compartment contents and following settling of microcarriers, removal of supernatant. Addition of fresh medium to the microcarriers and return to cell compartment completes harvest cycle. Stripping cells off of microcarriers by pipetting will allow for reexposure of additional growth surface if needed.

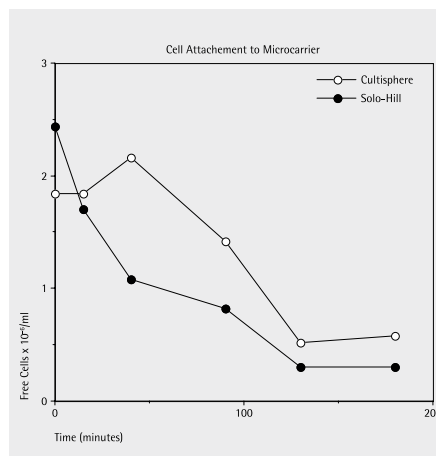


Figure 2: Cell attachment to microcarriers is shown by the depletion of free cells from suspension.

At start, 2.0×10^6 /ml CHO cells (harvested from a trypsinized monolayer culture) were mixed with microcarriers (35% v/v) and allowed to settle in cell compartment. At the indicated times the cell compartment was gently mixed and a small volume sampled and free cells were counted. The depletion in free cell numbers was associated with cell attachment to microcarriers as seen by microscopic examination. Two different microcarriers were evaluated; Cultisphere and SoloHill.

4. Harvest:

Remove entire cell compartment volume by gently mixing cell compartment volume by pipette and gentle rocking of CELLline. Place the removed volume in a sterile 50 cc tube and allow microcarriers to settle or gently centrifuge to pellet microcarriers. Remove supernatant, this is the harvest volume. Add fresh complete cell compartment medium to the remaining microcarrier pellet to achieve the initial culture inoculum starting volume (osmotic flux will influence cell compartment volume during culture). Suspend microcarriers/cells and return volume into the cell compartment. Exchange nutrient medium.

The frequency of harvest and nutrient medium exchange should follow the protocol provided for hybridoma cultivation in the CELLline. A harvest and nutrient medium exchange every three days after initial 6 day growth period should be satisfactory. More frequent nutrient medium exchange may be beneficial to support maximum numbers of cells within the cell compartment.

5. Results:

Secreted product (>10,000 dalton) will be recovered in the small cell compartment volume at elevated concentrations when compared to normal static culture supernatant. A prolonged culture can be maintained over extended periods (>30 days) by routine harvest and feeding. For production of chimeric molecules from BHK cells a nearly 28 fold increase in product concentration was obtained in the pooled cell compartment supernatant collected over a 30 day culture period (Fig 3.). Culture of CHO cells indicated at least 11 fold increase in product concentration was obtained in a short culture period (Fig 4.). These cultures were maintained without trypsinization or additional microcarriers. Differences in microcarrier types used did not appear to be significant.

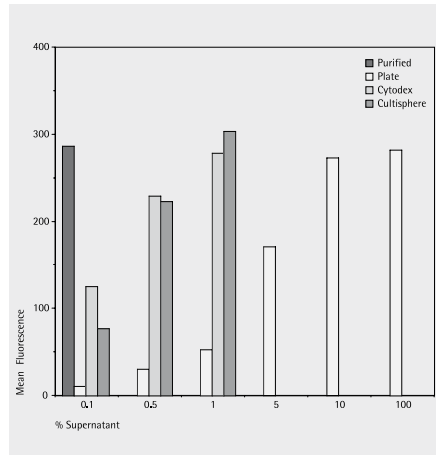


Figure 3: Comparison of biological activity of pooled supernatants taken from microcarrier cultures in CL 350 and control culture plates.

Purified CTLA4-Ig (0.7 mg/ml) was also tested as control. Culture supernatants were collected over a 30 day period from repetitive harvests (3-5 days) and pooled. Activity of CTLA4-Ig in supernatant was determined by staining B7C1 cells with supernatants and detecting bound CTLA4-Ig with goat antimouse Ig labeled with PE. Mean fluorescence determined by flow cytometry is shown for the various percentages of supernatant used in staining. Maximum fluorescence was achieved at 0.1% with the purified preparation (0.7 mg/ml). Subsequent purification of culture supernatants by Protein A chromatography yielded 25 X CTLA4-Ig when compared to equal volume of control supernatant. Two different cultures using either Cytodex or Cultisphere microcarriers gave similar results.

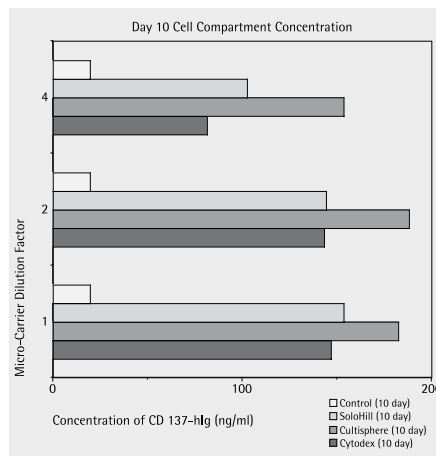


Figure 4: Cell compartment concentration of recombinant CD137-hlg from a culture of CHO cells engineered to express the recombinant molecule cultured in a single CL 6 WELL cell compartment.

Three different microcarriers were evaluated at three different concentrations. The microcarriers tested were as follows: Cytodex (DEAE Sepharose), Cultisphere (microporous collagen), SoloHill (Plastic). Microcarriers were used at a dilution of 1, 2 and 4 from a stock suspension of settled carriers. Comparison in product concentration to a control culture seeded onto a 75 cm² culture flask is shown. The microcarrier cultures produced significant increase in product concentration when compared to the control culture at 10 days.

Notes:

Cell growth on the microcarriers will proceed until surface of microcarriers is exhausted. In many cases cell growth will continue with cell bridging between microcarriers. Some sloughing of cells will take place off of the microcarriers when cell growth becomes dense. When cells slough off of the microcarrier they usually are seen as viable clusters of cells.

The handling of the microcarriers during harvest will influence culture dynamics. Vigorous mechanical pipetting will strip cells off of the microcarriers, re-exposing additional surface for renewed cell proliferation. To split back cell numbers during harvest, pipette microcarriers to remove cells and remove cells along with supernatant. To maintain cell numbers, gently pipette microcarriers and allow cells to settle with microcarriers prior to removal of supernatant.

A continuous culture can be maintained without addition of new microcarriers by partially stripping cells from microcarriers during harvest and returning microcarriers with fresh medium into the cell compartment.

6. Serum Strategies:

The same serum strategies used for hybridoma cells can be used with most attachment dependent cells. Serum supplementation of the cell compartment (15%) is generally sufficient. It may be beneficial to provide 5% serum to the nutrient medium during the initial phase of culture to aid in establishing the culture. Thereafter, serum can be removed from the nutrient medium completely or supplemented at 1% if it aids in expression levels.

Serum free medium formulations available for specific cell types (CHO, HEK 293) can also be used in place of serum. These media allow reduced protein concentrations in the cell compartment through the elimination of serum supplementation and can ease downstream processing demands.

Initially, the serum free medium should be provided in the cell compartment and the nutrient medium compartment. After culture establishment, the use of the basal medium for the serum free medium may be used in place of serum free medium in the nutrient compartment. Supplementation of the basal medium with serum (1-5%) may be beneficial in some cases. This strategy provides the benefits of serum free medium in the cell compartment without the associated expense of using serum free medium in the nutrient compartment. Any supplementation of serum into the nutrient medium will not contaminate the cell compartment with significant protein because the semipermeable membrane prevents entry of molecules greater than 10,000 daltons.



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