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Application Note

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Expansion of MRC-5 Cells on SoloHill® Microcarriers via Serial Passage in Stirred Vessels

1. Introduction

Common production platforms employed for manufacture of vaccines, biologics, and cell therapeutics include 2-dimensional culture systems such as roller bottles or cell cubes/factories. These systems are typically used for expansion of cells to seed large bioreactors. Although well-established, these formats occupy a large footprint, are labor intensive and are susceptible to frequent contamination problems due to numerous open handling steps.

Impeller-driven bioreactors provide logical alternatives to 2-dimensional culture systems. Advantages of bioreactors include the ability to precisely control and optimize cell growth conditions, ease of use and avoidance of contamination due "closed" nature of the system.

Microcarriers offer a large surface area for growth of anchorage-dependent cell types. Because specific cell types have different requirements for attachment and growth, the optimal microcarrier should be selected experimentally. This can be accomplished in small-scale studies with multiple microcarrier types to identify the best microcarrier for a particular application. In order to facilitate and streamline manufacturing processes, consideration should be given to the physical characteristics of the microcarriers. For example, durable and rigid microcarriers facilitate efficient harvest of cells from bioreactors. Additionally, some microcarrier types require minimal preparation steps prior to use.

MRC-5 cells are a human-derived lung fibroblast diploid cell line, commonly used for production of vaccines such as rubella, hepatitis A, varicella, rabies, and smallpox. Preliminary studies performed using SoloHill® Microcarriers and other microcarrier brands indicated that MRC-5 cells exhibit excellent growth characteristics on Collagen (SoloHill® Microcarriers C-221), FACT III (SoloHill® F-221), and Plastic Plus (SoloHill® Microcarriers PP-221) microcarriers with potential growth on Hillex® II microcarriers (SoloHill® Microcarriers H-170). In this application note we characterize MRC-5 cell growth on multiple SoloHill® and Cytodex 1 (GE Healthcare) microcarriers in stirred vessels and present data demonstrating feasibility of serial passage and scale-up into bioreactor expansion of MRC-5 cells (ATCC CCL17).

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2. Materials and Methods

2.1 Cells and Materials

MRC-5 cells purchased from ATCC (CCL-171; population doubling level 22) were expanded to create working cell banks. Cells from the working cell bank were used for these experiments. Corning* brand 250 mL spinner vessels (Fisher Scientific 10-203B) containing 200 mL of Minimal Essential Medium (MEM: ATCC 30-2003, Cellgro 25-025-CL, or Sigma M3024) supplemented with 5% fetal bovine serum (HyClone* SH30071.03) and pen/strep (ATCC 30-2300) were used for MRC-5 cell propagation. Microcarrier concentrations equivalent to a total surface area of 1030 cm^2 per vessel were employed and all microcarriers were prepared according to manufacturer's instructions.

Cytodex 1 microcarriers were prepared by hydrating in Dulbecco's Phosphate Buffered Saline (DPBS; HyClone SH30028.03) for 3 hours, followed by two PBS washes and autoclaving at 121 °C in 15 mL of PBS for 30 minutes. All SoloHill® Microcarriers were prepared by autoclaving at 121 °C for 30 minutes in 15 mL of deionized water.

2.2 First Passage on Microcarriers

For the first passage (P1), MRC-5 cells were harvested from roller bottles (Fisher 09-761-113) using 1X trypsin EDTA (Sigma T3924) after 2 washes with DPBS. Cell counts were performed using standard assays to assess cell numbers and viability. Spinners were seeded (Ni) at either 1.5×10^4 cells/cm^2 or 2.0×10^4 cells/cm^2 (approximately 18 to 19 cells per microcarrier). Hillex® II spinners were maintained at 60 rpms, whereas, all other microcarrier spinners were kept at 40 rpms for the first 48 hrs and increased to 60 rpms for remainder of the culture. Incubation conditions were 37 °C ± 0.5 in a cell culture incubator. Medium exchanges of 100 mL (50%) were performed on Days 3 and 5 of the culture.

2.3 Subsequent Passages on Microcarriers

For subsequent passages (P2 and P3), cells were harvested from microcarriers and passaged to new spinners. To harvest from Hillex® II microcarriers, spinners were transferred to a biological safety cabinet and microcarriers were allowed to settle to the bottom of the spinner. The higher relative density of Hillex® II microcarriers (1.1) compared to other microcarrier types, decreases settling time and expedites process steps. The supernatant was carefully decanted through the side arm of the vessel to a waste container and microcarriers were washed for 10 minutes in 100 mL dPBS with constant stirring at 40 rpm. The spinner was removed from the stir plate, microcarriers were allowed to settle, DPBS was decanted from the vessel and 20 mL of 1X trypsin EDTA was added. The spinner was then incubated for 30 minutes at room temperature with occasional swirling of the spinner by hand. Following trypsinization, the microcarrier/cell/trypsin slurry was pipetted to aid removal of cells from microcarriers. To further increase cell yield during harvest, the microcarrier/medium slurry was filtered into a sterile 50 mL conical tube through a 100 µM cell strainer (removing microcarriers from the flow through). The microcarriers were washed with equivalent volumes of medium (20 mL). Cells were used to seed spinner vessels containing surface area equivalents (1030 cm^2) of microcarriers.

The trypsinization of the lower specific gravity microcarriers (all carriers except Hillex® II microcarriers) followed a similar procedure. The only variation from the above protocol was replacement of one spinner arm cap with a 100 µM mesh filter. Due to the lower settling velocity and easily suspended characteristic of these carriers, media removal and washes were done by pipetting off as much volume as possible and subsequently decanting the remaining volume through the mesh filter.

During culture, samples were retrieved daily for nuclei counts using the citric acid/crystal violet method. Nuclei were counted using a Nexcelom counter and with associated software and the number of nuclei per cm^2 surface area was calculated for each sample.

2.4 Bioreactor Growth

To seed the bioreactor (5 L New Brunswick* Celligen* 310 with a working volume of 3.75 L), MRC-5 passage 5 cells were trypsinized off of roller bottles and 5150 cm^2/L Collagen microcarriers (14 g/L; 2.5 L total) were seeded at 1.5×10^4 cells/cm^2 in MEM (Sigma M3024; supplemented with sodium pyruvate). Due to previous spinners being grown in MEM (ATCC and Cellgro), two spinner controls were seeded. Spinner #1 contained cells/microcarriers in MEM from Cellgro, and Spinner #2 contained cells/microcarriers in MEM from Sigma. An additional spinner control (Spinner #3) was created by taking 200 mL from the bioreactor two hours after seeding.

*New Brunswick and Celligen are trademarks of Eppendorf AG, Germany; ATCC is a trademark of ATCC (American Type Culture Collection, Inc.); ProNectin is a trademark of Sanyo Chemical Industries, Ltd.; Cytodex is a trademark of General Electric Company and HyClone is a trademark of Hyclone Laboratories, Inc.

3. Results

3.1 Experimental Design

The goal of these studies was to demonstrate feasibility of performing serial passages of MRC-5 cells on SoloHill® Microcarriers and to demonstrate the development parameters for efficient cell growth in bioreactors. The study was designed to establish a reproducible method for expansion of these anchorage-dependent cells in stirred tank reactors. To this end, we performed independent experiments in spinner flasks where MRC-5 cells were passaged on SoloHill® Microcarriers or GE Cytodex 1 for three passages. The design for these experiments was chosen to provide feasibility data for a scenario in which 1 to 10 scale-up ratio between bioreactors would culminate in the seeding of a 1000 L bioreactor. Using this scenario, the train would proceed from a 10 L reactor, to a 100 L vessel, to a final seeding of a 1000 L production bioreactor seeded with 2×10^4 cells/cm² surface area of microcarriers (Figure 1).

Bioreactor Size [L]	10	100	1000
Hillex® II [g]	100	1,000	10,000
Surface Area [cm²]	51,500	515,000	5,150,000
Roller Bottles (850 cm²)	60.6	606	6,060

Figure 1
Overview

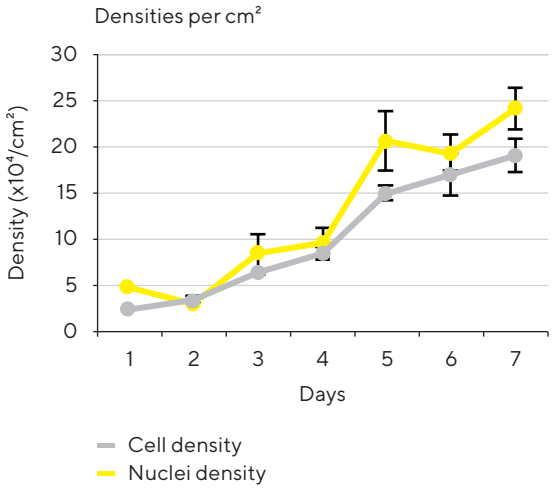
Diagrammatic representation of a microcarrier-based scale up manufacturing and roller bottle surface area equivalents.

The significance of this proposed bioreactor process is highlighted by the fact that a 10 L bioreactor is equivalent to approximately 61 × 850 cm² roller bottles and an 100 L bioreactor replaces 605 × 850 cm² roller bottles given equivalent confluent cell densities are reached. Outside of direct material cost savings for large scale production, each bottle manipulation represents an "open" step with a potential for introduction of contaminants and the need for extensive labor.

3.2 Baseline Growth on Flatware

To successfully implement a production scheme as proposed it is important to obtain sufficient data to warrant transition into larger bioreactor formats. The logical and most cost-effective path toward this goal is to obtain data in small-scale static and spinner-based feasibility studies prior to transitioning into costly studies in large bioreactors. In order to establish a baseline for cell growth and to establish a gauge to evaluate the performance of our stirred tank system, we first characterized growth of the MRC-5 cell line in static culture. MRC-5 cells were seeded into T25 flasks at 2×10^4 cells/cm² and a growth curve was generated (Figure 2).

Figure 2
Static culture



Growth curve of MRC-5 cells in static culture establishes baseline for comparison of growth in stirred tank vessels. Data is presented as means ± SEM (n = 3).

These cells grew with a population doubling time (PDT) of ~48 hours throughout the 7 day growth experiment. The average cell density was $19.1 \pm 1.8 \times 10^4$ cells/cm² whereas the nuclei counts were $24.1 \pm 2.2 \times 10^4$ nuclei/cm². The slight differences in counts between methods may reflect protocol differences or it is possible that a subset of the cell population exhibits synchronized growth and may be multinucleated at the time of harvest for counting.

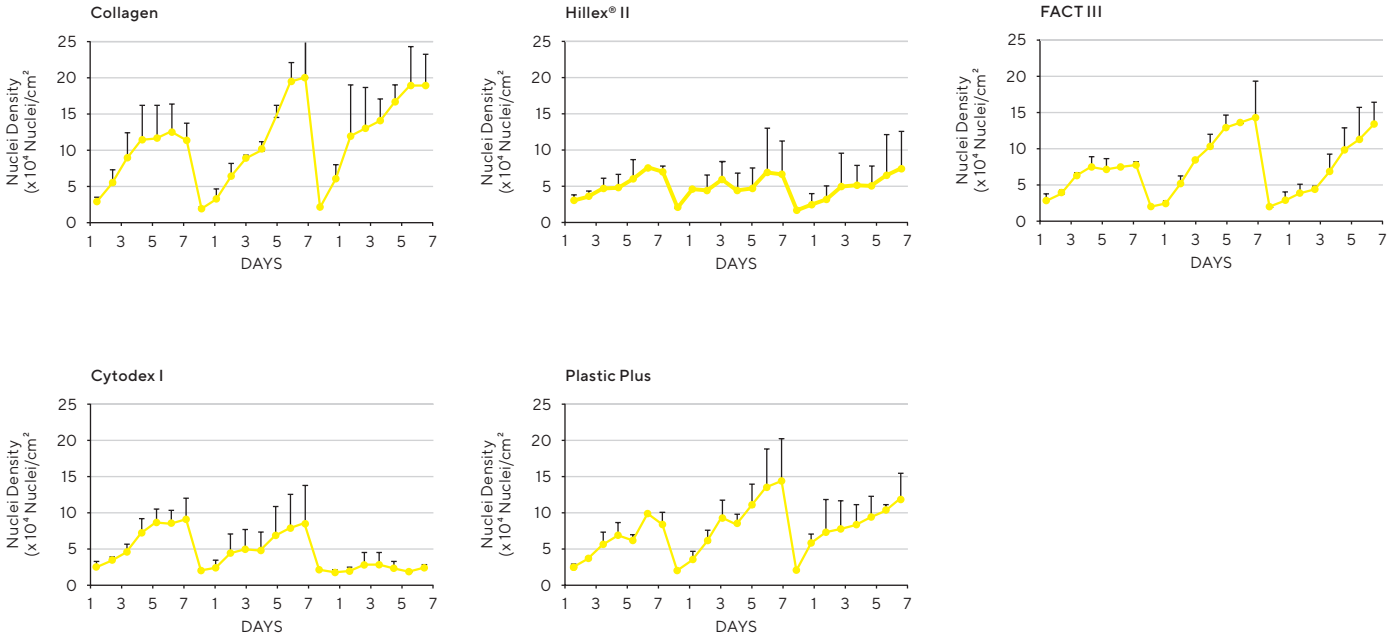
3.3 Growth Comparison

Figure 3 demonstrates the ability to expand MRC-5 cells on SoloHill® Microcarriers in stirred vessels for three passages with satisfactory growth on Collagen, FACT III, and Plastic Plus microcarriers. Under the conditions tested in this study, suboptimal growth was observed on Hillex® II and Cytodex 1 microcarriers. Both of these microcarrier types are animal product free and contain a positively charged surface. Whereas, growth on Hillex® II could be maintained over the passage regimen, cell numbers on Cytodex 1 steadily decreased over the three passages suggesting that scale-up on Cytodex 1 would not be feasible without further optimization.

Cells reached a maximum confluent cell density of 20.0 ± 5.5 x 104 nuclei/cm^2 in Collagen, 14.3 ± 4.2 × 10^4 nuclei/cm^2 in FACT III, 14.4 ± 5.8 × 10^4 nuclei/cm^2 in Plastic Plus spinner vessels containing MEM medium supplemented with 5 % FBS (n = 3). These nuclei counts were slightly lower than those obtained in static T flask culture which yielded 24.1 ± 2.2 × 10^4 nuclei/cm^2 on day 7 of culture (n = 3; cell density of 19.1 ± 1.8 × 10^4 cell/cm^2).

Figure 3

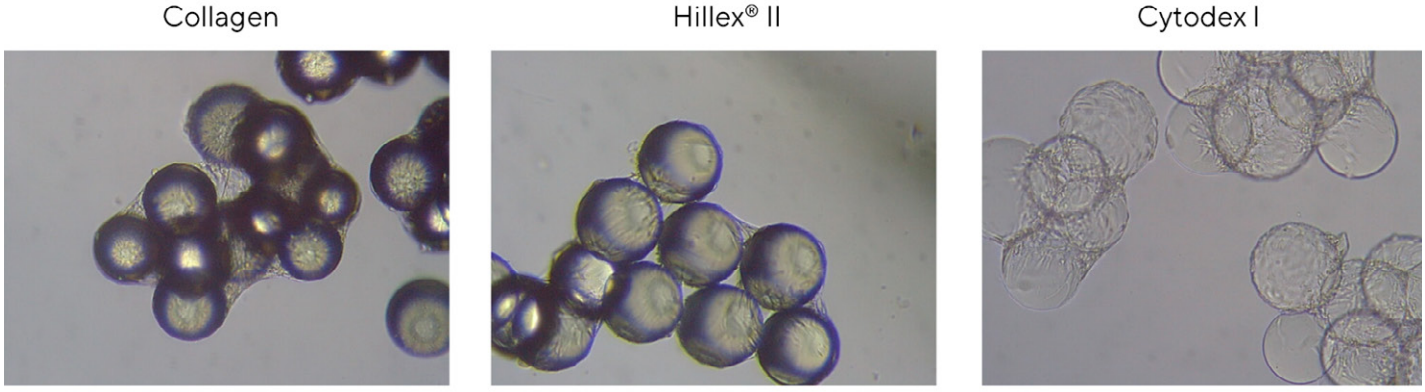
Serial passages



MRC-5 serial passages on SoloHill® Microcarriers and Cytodex 1 suggest potential for scale-up on Collagen, Fact III, and Plastic Plus and possibly with Hillex® II after further optimization. Data represents means ± SD (n = 3).

All SoloHill® Microcarriers outperformed Cytodex 1 under the conditions used in these studies. Cytodex 1 reached a maximum confluent cell density of $9.1 \pm 2.9 \times 10^4$ nuclei/cm^2 in the first pass on microcarriers. Not only is scale-up on Cytodex 1 not feasible due to a decrease in growth over several passages, but the lower maximum confluent cell density translates into a 37-55% lower cell yield per spinner when compared with Collagen, FACT III, Plastic Plus microcarriers.

Figure 4
Day 7 Expansion



Sample pictures of Day 7 expansion on SoloHill® or Cytodex 1 microcarriers. Note presence of clumping on confluent microcarriers as a function of cell density, i.e. more clumping as cells grow to higher densities.

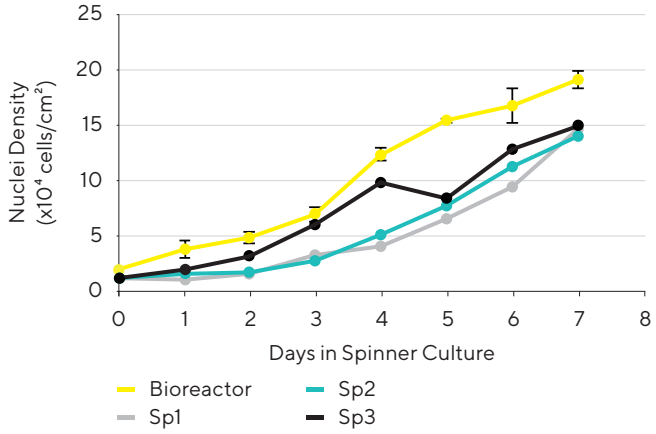
Visual observations of cells on microcarriers were consistent with these data as well as published data on MRC-5 (Figure 4). Cells and microcarriers began to clump as early as day 3 in spinner cultures. Clumping was more noticeable in spinner cultures with higher cell densities but did not appear to affect cell growth.

3.4 Scaling Up

To determine feasibility of scale-up production with the MRC-5 cell line, MRC-5 cells were expanded in roller bottles and used to seed Collagen microcarriers in a 2.3 L bioreactor. Figure 5 shows that MRC-5 cells grew to a density of 19.1 ± 0.78 × 10^4 nuclei/cm^2 which is similar to the densities obtained from multiple Collagen spinner experiments (shown in Figure 3).

Figure 5

Bioreactor Study

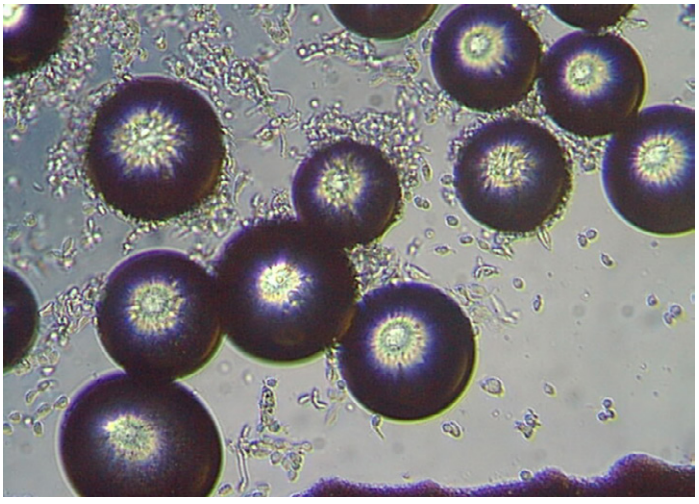


MRC-5 grown on Collagen microcarriers in a 2.3L bioreactor.

A critical feature of SoloHill® Microcarriers is the ability to easily and efficiently harvest cells from microcarriers. Standard enzymatic techniques can be used to readily harvest MRC-5 cells in a single-cell suspension. Figure 6 shows MRC-5 cells after a 15 minute trypsinization incubation. Once these cells and microcarriers are pipetted, a single cell suspension is achieved that can be used to seed new spinners.

Figure 6

Trypsinization



MRC-5 cells after a 15 minute trypsinization step. Pipetting creates a single cell suspension that can be used to see new spinners.

The rigid core of all SoloHill®Microcarriers not only imparts stability and durability but promotes cell removal by preventing fouling of screens or filters used to separate cells from microcarriers. Another benefit of these microcarriers is that they do not require lengthy microcarrier preparation steps. In this regard, microcarriers suspended evenly in deionized water can be autoclaved and used immediately after sterilization. SoloHill®Microcarriers can also be gamma irradiated without adverse effects on performance. Cytodex microcarriers require multiple long processing steps and if bead preparation is not performed properly and consistently, difficulties with cell release from microcarriers and subsequent detrimental effects on cell yield are encountered.

4. Conclusions

Results presented here demonstrate the feasibility of using several SoloHill® Microcarriers for expansion of MRC-5 cells via microcarrier-based serial passage in stirred vessels. The data indicate that growth of MRC-5 cells on microcarriers at a bioreactor levels is also achievable. In these studies, minimal effort was expended to optimize growth of MRC-5 cells in the spinner and bioreactor formats so it is likely that higher confluent cell densities can be achieved with further experimentation. Although the counts from microcarrier spinner cultures were slightly lower than static cultures, the number of 850 cm^2 roller bottles required to produce equivalent cell yields is still significant in that 45 or 450 roller bottles would be required to achieve the same cell yield as a single 10 L or 100 L bioreactor, respectively. Using SoloHill® Microcarriers, the cell numbers were sufficient for serial passage, and microcarriers supported serial passaging between spinners with no decrease in confluent cell densities. Cytodex 1 serial passages demonstrated a continual decrease in maximal cell densities over multiple passages.

These feasibility studies demonstrate that SoloHill®'s Microcarriers provide an ideal substrate for expansion of MRC-5 cells in a closed stirred vessel system and lay the groundwork for subsequent developmental studies in larger bioreactor formats.

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