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# Expansion of Porcine SK-RST Cells on Hillex<sup>®</sup> II Microcarriers via Serial Passage in Stirred Vessels

## 1. Introduction

Manufacturing processes used for production of vaccines, biologics and cell therapeutics routinely employ two-dimensional (2-D) culture systems such as roller bottles or cell cubes/factories for expansion of cells to seed large bioreactors. These formats occupy a large footprint, are labor intensive, and are susceptible to frequent contamination due to many open handling steps. Closed-impeller bioreactors provide logical alternatives to 2-D culture systems. Advantages of bioreactors include the ability to precisely control and optimize cell growth conditions, ease of use, and avoidance of contamination due to the “closed” nature of the system. Additionally, the recent emergence of disposable bioreactor technology eliminates the need for cleaning validation.

Microcarriers provide a large surface area for growth of adherent cell types and thus facilitate the use of bioreactors for attachment-dependent cell-based manufacturing. Because each cell type has its own requirements for attachment and growth, the optimal microcarrier and media conditions should be selected experimentally. The best approach is to perform small-scale studies with multiple microcarrier types and medias to identify the best microcarrier and media for the intended application. These feasibility studies should be performed in a manner that takes into consideration final bioreactor configurations and performance. This approach allows researchers to examine many parameters concurrently and limit costs of developmental studies before transitioning to large bioreactor formats.

In order to streamline manufacturing processes, consideration should also 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.

Porcine SK-RST kidney epithelial cells are commonly used for diagnostic testing of swine diseases and for production of veterinary and human pharmaceuticals. They offer the advantage of being free of endogenous porcine viruses and exhibit a cytopathic effect when infected with multiple viruses. Preliminary studies performed using SoloHill<sup>®</sup> and other commercially available microcarriers revealed that SK-RST cells exhibit excellent growth characteristics on Hillex<sup>®</sup> II (SoloHill<sup>®</sup> H-170) and FACT III (SoloHill<sup>®</sup> F-221) microcarriers. In this application note we present data demonstrating the feasibility of serial passaging porcine SK-RST cells on Hillex<sup>®</sup> II microcarriers in stirred vessels.

## 2. Materials and Methods

### 2.1 Material Preparation

Porcine SK-RST cells purchased from ATCC (CRL-2842; passage 64) were expanded to create master (P66) and working cell banks (P72). 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 OptiMEM\* media (Life Technologies 11058-021) supplemented with 5% fetal bovine serum (Thermo Scientific HyClone\* SH3007103) and antibiotic/antimycotic (Mediatech FBS 30 004-C1) were used for SK-RST cell propagation. Microcarrier concentrations equivalent to a total surface area of 900 cm<sup>2</sup> per vessel were employed and all microcarriers were prepared according to manufacturer's instructions by autoclaving at 121 °C in deionized water. Spinner cultures were essentially performed as described in SoloHill® microcarrier technical briefs.

### 2.2 First Passage Harvesting

For first passage (P1), SK-RST cells were harvested from vented T150 tissue culture flasks (Corning 430825) using TrypLE\* Express (Life Technologies 12604) after one wash with Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Scientific HyClone SH30028.03) and one incubation with cell culture-tested 0.02% EDTA solution (Sigma E8008). Cell counts were performed using standard assays to assess cell numbers and viability. Spinners were seeded at  $2.0 \times 10^4$  cells/cm<sup>2</sup> or 18 to 19 cells per microcarrier. Incubation conditions were 37 °C with 5% CO<sub>2</sub>. Media exchanges of 150 mL were performed every other day for the duration of the culture starting at Day 2 and 0.2 mL of a 10 mg/mL stock solution of folic acid was added daily to Hillex® II spinner cultures to promote growth during expansion and after passage.

### 2.3 Second and Third Passage Harvesting

For subsequent passages (P2 and P3), cells were harvested from microcarriers and passaged to either Hillex® II, FACT III or Cytodex\* 3 microcarriers (Sigma C3275-10g). 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 vessel. Supernatant was carefully decanted by pouring spent media through the side arm of the vessel into a waste container prior to washing microcarriers twice. The first wash was performed in 100 mL DPBS and the second wash with 100 mL of 0.02% EDTA; each wash was incubated at room temperature for 15 to 20 minutes at 40 RPM. The spinner was removed from the stir plate, microcarriers were allowed to settle by gravity, wash solution was decanted and any residual solution was removed by pipetting.

Ten mL of TrypLE was added and the vessel was swirled by hand to assure even distribution of the enzyme solution among the microcarriers. Microcarriers were incubated for 15 minutes with occasional swirling by hand. Cells were collected with four sequential 40 mL volumes of FBS-containing medium and were pooled in a sterile container. To increase cell yield during harvest, the microcarrier/media slurry was transferred to a sterile 50 mL conical tube, triturated and supernatant was transferred to a sterile 250 mL bottle through a 100 µm cell strainer. Each subsequent wash was performed in the same manner, progressing from spinner, to 50 mL conical tube, to 250 mL bottle.

At the third passage, cells were used to seed spinner vessels containing surface area equivalents (900 cm<sup>2</sup>) of Hillex® II, FACT III and Cytodex 3. Hillex® II and Cytodex 3 spinner cultures were stirred at 55 RPM continuously from culture initiation; FACT III cultures were stirred at 40 RPM continuously for the first two hours followed by an increase to 55 rpm continuous for the remainder of the culture. During culture, samples were retrieved daily and processed for nuclei counts using the citric acid/crystal violet method.

### 2.4 Counting Nuclei and Fluorescent Imaging

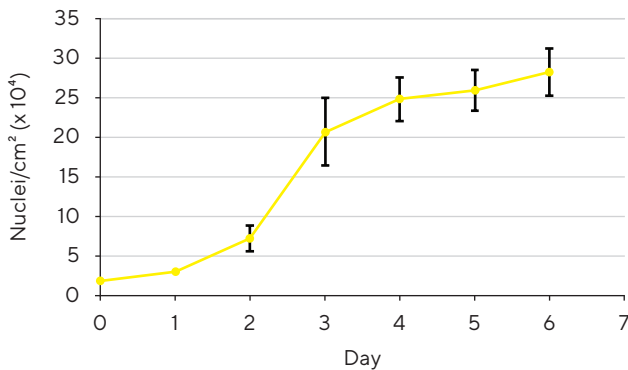
Nuclei were counted using a hemocytometer and the number of nuclei per cm<sup>2</sup> surface area was calculated for each sample. For fluorescence imaging, microcarriers were gently transferred to 1.5 mL Eppendorf Tubes\* (Fisher Scientific 02-681-284) and microcarriers were allowed to settle via gravity. The supernatant was then removed, and the cell-laden microcarriers were gently washed twice (5 minutes each wash) with DPBS at room temperature and then fixed with 10% neutral buffered formalin solution (Sigma HT5011-1CS) for 10 minutes at room temperature. Microcarriers were suspended in DPBS containing 300 nM 4',6-diamidino-2-phenylindole (DAPI). Phase contrast and fluorescence images were captured on a Nikon Eclipse Ti microscope equipped with a CoolSnap HQ2\* CCD camera and Nikon NIS Elements Imaging software.

\* ATCC is a trademark of ATCC (American Type Culture Collection, Inc.); Corning is a trademark of Corning Corporation; OptiMEM and TrypLE are trademarks of Life Technologies Corporation; HyClone is a trademark of HyClone Laboratories, Inc.; Cytodex is a trademark of General Electric Company and Eppendorf Tubes is a trademark of Eppendorf AG.

### 3. Results

In order to establish a baseline for cell growth as a gauge to evaluate the performance of our stirred tank system, we first characterized growth of the SK-RST cell line in static culture. SK-RST cells were seeded into T25 flasks at  $2.0 \times 10^4$  cells/cm<sup>2</sup> and a growth curve was generated (Figure 1). These cells experienced a growth lag phase of 1 day immediately after plating but grew with a population doubling time (PDT) of ~25 hours from days 2 to 4. On days 5 through 7, the cell PDT increased to an average value of 37 hours suggesting strong contact inhibition. During this time, the SK-RST cell population routinely formed “dome-like” structures which are indicative of confluence and renal epithelial cell polarization.

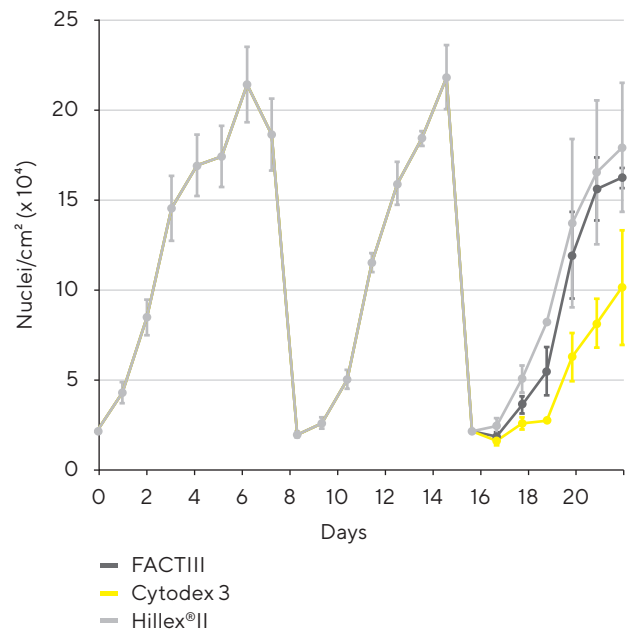
**Figure 1**  
Baseline Growth Curve



Growth curve of SK-RST cells in static culture establishes baseline for comparison of growth in stirred tank vessels. Data is presented as means  $\pm$  SEM (n = 5).

Figure 2 demonstrates the ability to expand porcine SK-RST cells on Hillex<sup>®</sup> II microcarriers in stirred vessels for two passages followed by a third pass to three different types of microcarriers.

**Figure 2**  
Serial Passages

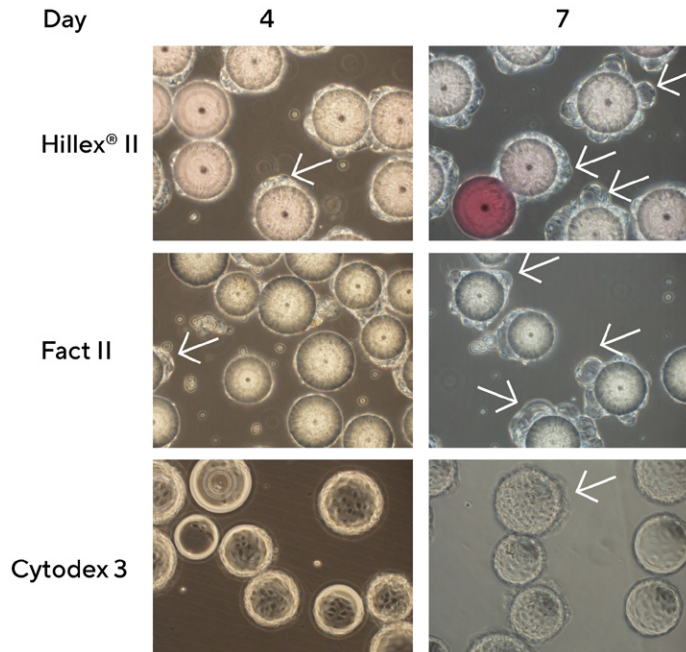


SK-RST serial passaged on Hillex<sup>®</sup> II microcarriers with final passage to either Hillex<sup>®</sup> II (light grey line), FACT III (dark grey line) or Cytodex 3 (yellow line) microcarriers. Data represents means  $\pm$  SEM (n = 3).

Cells reached a peak density on day 5 of culture, and a maximum confluent cell density of  $\sim 21.7 \pm 4.0 \times 10^4$  nuclei/cm<sup>2</sup> in spinner vessels containing OptiMEM media supplemented with 5% FBS and Hillex<sup>®</sup> II or FACT III microcarriers (n = 3). These nuclei counts were slightly lower than those obtained in static T flask culture which yielded  $\sim 29.2 \pm 3.0 \times 10^4$  nuclei/cm<sup>2</sup> on day 6 of culture (n = 5). Hillex<sup>®</sup> II and FACT III microcarriers outperformed Cytodex 3 under the conditions used in these studies. Cytodex 3 reached a maximum confluent cell density of  $10.0 \pm 3.2 \times 10^4$  nuclei/cm<sup>2</sup> which translates into a 46% lower cell yield per spinner when compared with Hillex<sup>®</sup> II or FACT III microcarriers.

Visual observations of cells on microcarriers were consistent with these data (Figure 3).

**Figure 3**

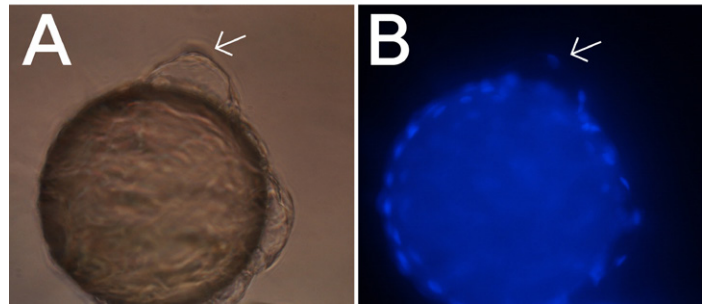


Day 4 and 7 images of passage 3 cells transferred to Hillex® II, FACT III or Cytodex 3 microcarriers. Note presence of doming on confluent microcarriers (white arrows) which appear earlier (day 4) and more frequently on SoloHill® Microcarriers (day 7).

Domes began to form on Hillex® II and FACT III microcarriers on day 4 and many structures were evident on day 7, whereas, the number formed on Cytodex 3 were minimal on day 4 and were fewer than those observed on Hillex® II or FACT III at later time points. Evidence of doming on microcarriers was confirmed by fluorescent imaging of nuclei on microcarriers.

Confluent layers of cells with intact nuclei raised from the microcarrier surface are clearly identified by corresponding bright field and fluorescent images as indicated by the arrows in Figure 4.

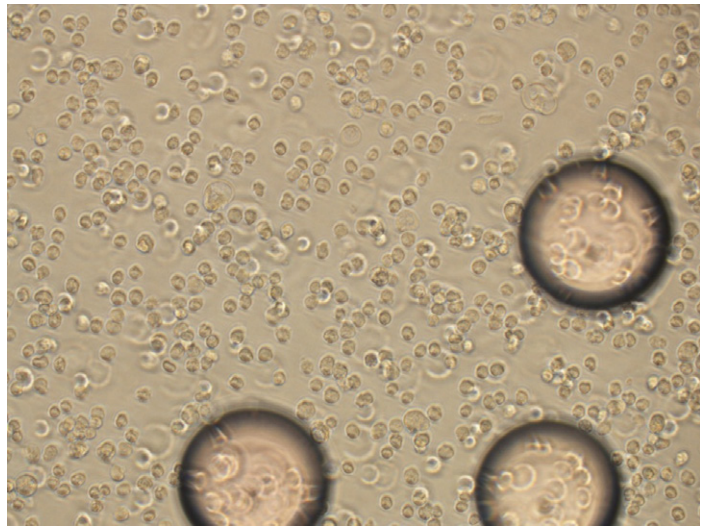
**Figure 4**



A.) Phase contrast image of passage 3 SK-RST cells on a Hillex® II microcarrier and;  
B.) Corresponding DAPI image of same microcarrier. White arrow highlights nucleus elevated from the surface of the microcarrier in area of doming.

A critical feature of SoloHill® Microcarriers is the ability to easily and efficiently harvest cells. Standard enzymatic techniques can be used to readily harvest SK-RST cells in a single-cell suspension (Figure 5).

**Figure 5**



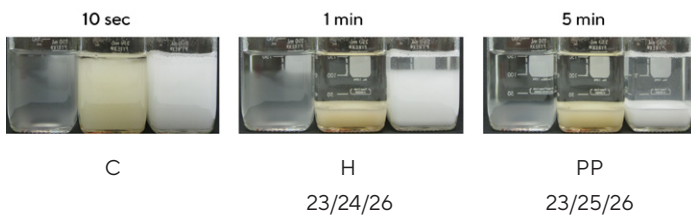
Porcine SK-RST cells separated from Hillex® II microcarriers. Single cell suspension obtained during harvest of SK-RST cells from Hillex® II microcarriers with TrypLE Express.

## 4. Conclusions

The rigid core of all SoloHill® Microcarriers not only imparts stability and durability during process runs, but also promotes cell removal by preventing fouling of screens or filters used to separate cells from microcarriers.

The higher relative density of Hillex® II microcarriers at 1.1 provides an added advantage in that Hillex® II microcarriers settle rapidly compared with other microcarrier types thereby quickly separating microcarriers from the cells in the media (Figure 6).

**Figure 6**



SoloHill Hillex® II microcarriers exhibit rapid settling which expedites process times. Time course of Cytodex 1 (C), Hillex® II (H) and SoloHill® Plastic Plus (PP) Microcarrier settling in DPBS.

Separation of Hillex® II microcarriers is seen as early as 10 seconds after termination of mixing. After 1 minute, they are completely settled. The opaqueness of Hillex® II also allows for easy visualization of microcarrier settling and facilitates cleaner process steps because microcarriers can be avoided during solution removal and transfer.

Results presented here demonstrate the feasibility of using Hillex® II microcarriers for expansion of porcine SK-RST cells via microcarrier-based serial passage in stirred vessels. The data indicated that subsequent passage to different microcarrier types after serial passage is also achievable. In these studies, minimal effort was expended to optimize growth of SK-RST cells in the spinner format so it is likely that higher confluent cell densities can be achieved with further experimentation. Regardless, in these experiments Hillex® II and FACT III microcarriers produced a cell yield that was ~74% of that achieved with static cultures. The Cytodex 3 reached 30%. Estimates using cell yields obtained for Hillex® II and FACT III spinner cultures in this study indicate that a 10 L or 100 L bioreactor could, at a minimum, produce cell yields equivalent to 45 or 450 roller bottles (850 cm<sup>2</sup>), respectively.

The goal of this study was to demonstrate the feasibility of performing serial passage of SK-RST cells on the Hillex® II microcarriers followed by transfer to several different types of microcarriers. The experiments in this study were designed to establish a reproducible method for expansion of these anchorage-dependent cells in stirred tank reactors.

To this end, we performed three independent experiments in spinner flasks where we passaged porcine SK-RST cells on Hillex® II microcarriers twice and completed a third passage to Hillex® II, FACT III, and Cytodex 3 microcarriers.

The design for these experiments was chosen to provide feasibility data for a scenario in which a 1 : 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 bioreactor, to a 100 L bioreactor, to a final transfer to a 1000 L production bioreactor seeded with  $2.0 \times 10^4$  cells per cm<sup>2</sup> surface area of microcarriers (Figure 7).

**Figure 7**

Diagram of a microcarrier-based scale up manufacturing



**Table 1**

Hillex® II and roller bottle-microcarrier surface area equivalents

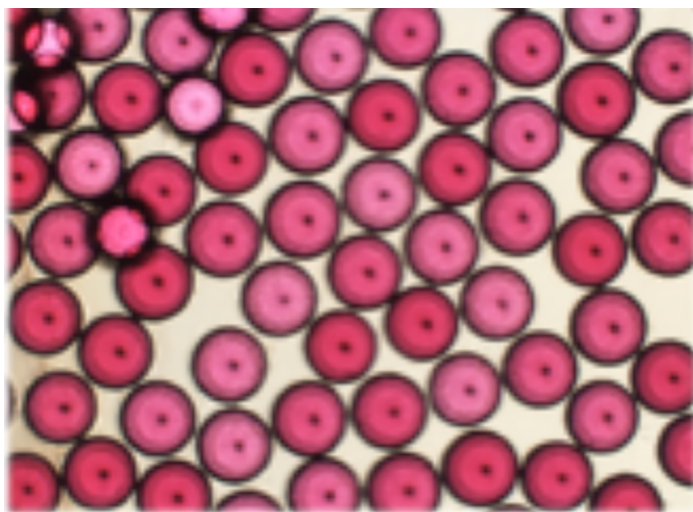
Bioreactor Size [L]	10	100	1000
Hillex® II [g]	100	1,000	10,000
Surface Area [cm <sup>2</sup> ]	51,500	515,000	5,150,000
Roller Bottles (850 cm <sup>2</sup> )	60.6	606	6,060

The significance of this proposed bioreactor process is highlighted by the fact that a 10 L bioreactor containing 10g/L of Hillex® II microcarriers is equivalent to approximately 61 × 850 cm<sup>2</sup> roller bottles (Table 1). One 100 L bioreactor replaces 606 × 850 cm<sup>2</sup> roller bottles, given that 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.

To successfully implement this proposed production method, it is important to obtain sufficient data to justify the use of larger bioreactor formats. The logical and most cost-effective approach is to obtain data in small-scale static and spinner-based feasibility studies prior to transitioning into more expensive studies in large bioreactors.

For SoloHill® Microcarriers, the cell numbers were sufficient for serial passage and harvest efficiency approached 95%. The lower cell yield on Cytodex 3 microcarriers in these studies was not attributable to serial passage on Hillex® II microcarriers because similar yields were obtained when cells were directly passaged to Cytodex 3 from static cultures. It is possible that these low cell numbers obtained on Cytodex 3 microcarriers could also benefit from additional optimization, for example, increased microcarrier concentration, seeding density, media component, etc.

These feasibility studies demonstrate that Hillex® II microcarriers provide an ideal substrate for expansion of Porcine SK-RST cells in closed stirred vessels and lay the groundwork for subsequent developmental studies in larger bioreactor formats.




Cell growth on Hillex® II microcarriers

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