GLOBAL CELL
SOLUTIONS

Small-Scale Microcarrier Screening Studies Using 24 Well Plates and Snap-Top Tubes

Introduction

Static cell culture experiments in multi-well plates and snap-top tubes are useful starting points for microcarrier users to assess microcarrier compatibility with cells. The protocols below provide a framework to begin the microcarrier screening process. Protocol 1 and the associated supporting techniques will familiarize investigators with microcarrier handling and the visualization of cells on microcarriers. Protocols 2 and 3 provide semi-quantitative measures of cell attachment and growth. These protocols are precursors to experiments aimed at optimization of conditions in small-scale stir vessels and subsequent transfer of developed protocols into larger bioreactor formats.

Protocol 1: *Cell attachment and spreading on microcarriers in non-tissue culture-treated multi-well plates under static conditions*

1. Standard multi-well cell culture plates can be used for one- to three-day studies (ULA 24-well plate catalog#: GAA-2003) If cells will be harvested after three or more days, plates should be modified with a hydrophobic coating such as poly(2-hydroxyethyl,methylacrylate; pHEMA, SIGMA ALDRICH P3932) or its equivalent from other commercial suppliers.

2. Prepare a stock of sterile microcarriers in water by autoclaving for 15 to 45 minutes at 121°C. For a rapid one day assessment of attachment and spreading, prepare a microcarrier stock of 25 mg per mL. Depending on goals, a more concentrated or dilute stock might be preferred. The stock can be stored and used for several months if handled aseptically.

3. Suspend the microcarrier stock solution via agitation or titration aseptically and then dispense a 2 mL aliquot of the microcarrier slurry (50 mg) directly into each well. Allow the microcarriers to settle to the bottom of the wells. Aspirate the autoclave liquid with a sterile pipette tip by lightly pushing a one-mL tip against the bottom of the dish and carefully drawing off the liquid.

   TIP: The amount of microcarriers dispensed into each well should be roughly equivalent. If uneven distribution is visually discernible the surface area in each well will not be equivalent and calculations of cell numbers attached to microcarriers will be inaccurate. If this occurs, remove microcarriers from wells and discard, rinse wells with sterile phosphate buffered saline (PBS) or water and re-aliquot microcarriers into the freshly cleaned wells. For the Collagen, FACT III, Plastic, Plastic Plus and ProNectin®F products, a 2mL aliquot of evenly mixed microcarrier slurry provides approximately 18 cm^2 of bead surface area and 23,000 beads per well.

4. Add 2 mL of your chosen media to each well and redistribute the microcarriers by gentle agitation or rocking the six-well plate.

5. Acclimate the microcarriers and media (see Global Cell Solutions cell culture media line) in a 37°C cell culture incubator for at least 20 minutes.

6. Seed each well with an appropriate quantity of viable cells. *For example 2 to 5 x 10^4 cells per cm^2.*

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The cells must be prepared in a manner to achieve a uniform, single-cell suspension. The total volume of medium in each well should be 2 to 2.5 mL depending on the seed stock volume and how thoroughly the well was aspirated to remove the autoclave liquid.

TIP: When better control of the final volume is desired, one can centrifuge the microcarriers, decant and rinse with the media, and then create a bead stock directly in the media.

7. Distribute the cells in each well by carefully agitating the multiwall plate 10 to 15 seconds with a side-to-side and a back-and-forth motion. This will insure a thorough mixing of the beads and cells without causing the media to splash onto the plate cover or between wells. Incubate at 37°C. For the first hour agitate the plate every 15 minutes.

8. Cell attachment and spreading can be observed at 100x magnification on an inverted microscope at various time intervals such as 2, 4, 18 or 24 hours and qualitative assessment of the attachment and spreading can be performed. Cells can be visualized at the edges or circumference of the microcarriers as rounded (initial attachment phase), "gumdrop-shaped" (early spreading) or flattened (completely spread).

   TIP: Ability to visualize spread cells on microcarriers is dependent upon cell characteristics. For example, fibroblast-like cells tend to be flatter than epithelial cells when they are spread on surfaces and are more difficult to visualize without using special techniques to aid in visualization (See Step 9 below.)

9. Several techniques can be used to enhance visualization cells on microcarriers if needed:
   - Fluorescence techniques (preloading of cells with fluorescent dyes prior to binding)
   - DAPI staining method
   - Acridine orange
   - Direct visualization by phase microscopy
   - Cell tracker staining for Hillex®II products

Protocol 2: *Semi-Quantitative cell attachment and growth in plates (Method #1)*

1. Prepare multiple samples as in Protocol #1. This allows one to harvest duplicate or triplicate wells and count the cells at the various time points up to four to five days. If comparing one microcarrier type to another, adjust the amount of microcarriers to assure that surface area among the several bead types is equivalent. See Table 2 below for examples. If significant cell growth is expected, lower the seeding density to 1 or 2 x 10^4 cells per cm^2. Replace media as needed.

Table 2. Amount of microcarriers to dispense into each well of a six-well plate to obtain equivalent surface area in each well

Microcarrier	Relative Density Range	Diameter (in µm)	Nominal Area per gram (cm^2)	Volume microcarrier slurry per well (mL)	Microcarrier quantity (mg)	Surface Area (cm^2)
Hillex®II	1.090-1.150	160-200	515	2.0	50.00	25.75
Collagen, FACT III, Plastic, Plastic Plus, ProNectin®F	1.022-1.030	125-212	360	2.9	71.53	25.75
* Denotes all microcarriers types contained in the standard microcarrier starter kit, including Plastic, Plastic Plus, Collagen-Coated, FACT III, ProNectin®F- Coated. Equivalent microcarrier surface areas are achieved by adding various quantities of microcarriers to each well						

2. Several methods can be used to successfully harvest cells. The simplest technique is to aspirate the media, wash with phosphate buffered saline (PBS, catalog# GSM-0100-501) and aspirate being careful not to remove cell-laden microcarriers from wells, add Accutase (catalog#: GSP-0101-100) directly to the well and collect all the microcarriers and free cells. Cell numbers can be counted with a hemocytometer. The microcarriers generally do not get under the cover slip so they do not interfere with the count.

3. For nuclei counts: Allow microcarriers to settle to the bottom of the dish or tube, remove as much media without removing microcarriers. If you are using a tissue culture plate, lightly push a pipet tip against the bottom of the dish and aspirate the liquid. Carefully remove medium and note the volume (X mL) that has been removed. Add X mL of crystal violet solution to the bead pack for a total equivalent to the sample volume (mLs). Incubate samples for one or more hours at 37°C. NOTE: Crystal Violet solution is 1 mg/ml crystal violet in 0.1M citric acid.

4. After the incubation period, vortex the sample vigorously for at least one minute to complete the nuclei release process. If using plates, vortex carefully so as to not cause liquid to splash and distribute between wells. The sample is now ready to count using a hemocytometer. Make sure to record the final volume of solution remaining in the wells because the amount will impact cell numbers. It is advisable to use techniques that minimize evaporation from wells if incubation proceeds for an extended period of time.

Protocol 3: *Semi-Quantitative cell attachment and growth assay (Method #2)*

1. Prepare microcarriers as in Protocol #1.

2. Dispense appropriate volume(s) of microcarriers to 17x100mm Tubes (Falcon; Catalog # 352059 or equivalent).

3. For each microcarrier, use 1mL media per tube.

4. Prepare one to two tubes for each time point, i.e. 1h, 2h, 4h, 24h.

5. Add appropriate amount of microcarriers to each tube to obtain equivalent surface areas.

6. Add 200,000 cells per tube.

7. Mix gently and incubate at 37°C.

8. Gently mix microcarriers every 15 minutes for the first hour.

9. At each time point, collect contents of each tube into conical tube.

10. Carefully separate supernatant from beads.

11. Perform cell count and viability on supernatant.

12. Examine cells on beads – take photomicrographs.

13. Perform nuclei release method or Accutase (GSP-0101-100) protocol on beads to count number of cells attached to beads as follows:

    a. Pellet remaining microcarriers and resuspend in either1 mL 0.1M citric acid containing 0.1% w/v crystal violet or enzyme dissociation mix.

    b. For nuclei release, vortex for 1 minute to mix well then incubate for 1 hr at 37°C in a humidified incubator. After incubation, vortex and count stained nuclei with a hemocytometer. Samples can be stored for up to one week at 4°C.

    c. For enzymatic dissociation, gently mix microcarriers to expose all microcarriers to enzyme. Incubate at room temperature for 15 minutes. Triturate microcarrier/cell slurry to dislodge cells from microcarriers and carefully remove supernatant to a fresh tube. Wash microcarriers with PBS and add to 1st aliquot of cells. Transfer microcarriers to a multi-well plate and visualize with microscope to verify that cells have been dissociated from microcarriers.

14. Perform cell counts on a Hemocytometer or other suitable cell counting device.

Table 3: Calculations for each microcarrier type for snap-top tube method of quantifying cell attachment

	Hillex II	Collagen, FACT III, Plastic, Plastic Plus, ProNectin®F
cm^2/g	515	360
#beads/g	120,000	460,000
mg/mL	14	20
cm^2/mL	7.2	7.2
Cells/mL	200,000	200,000
$Cells/cm^2$	27,800	27,800
#beads/mL	1,680	9,200
#cells/bead	119	22

Technical support is available at any time by contacting info@globalcellsolutions.com