

Protocol for Small-Scale Microcarrier Culture

Hillex[®] II Microcarriers

This document provides a general protocol for initiation of small-scale mammalian cell spinner cultures. This procedure is based upon a 200 mL Hillex[®] II microcarrier spinner culture. For preliminary studies, 10 grams (g) of Hillex[®] II (Cat. No. PGH-030-0010) per liter (L) or less is recommended. For a 200 ml culture, 2.0 g of this microcarrier type or less would be used. Hillex[®] II microcarriers have been successfully used in oxygen controlled bioreactors at concentrations up to 50 g/L. Hillex[®] II is an excellent substrate for extensive serial passage scale-up.

Table of Contents

1.0	Physical Characteristics of Hillex [®] II	2
2.0	Basic Equipment and Reagent List.....	2
3.0	Siliconizing Glassware	3
4.0	Autoclaving	3
5.0	Cell Dissociation Methods	4
6.0	Serum-Containing Cultures	5
7.0	Animal Protein-Free Adapted Cell Cultures.....	6
8.0	Harvesting Cells.....	7
9.0	Sampling Techniques	8
10.0	Traditional Nuclei Release Assay Method	9

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

1.0 Physical Characteristics of Hillex[®] II

Relative density range	Size (microns)	Surface Area (cm ² /g)	Microcarriers/g
1.090-1.150	160-180	515	5.5 x 10 ⁵

- Hillex[®] II absorbs phenol red from media; therefore we recommend using phenol red-free medium. However, extensive testing using multiple cell lines has demonstrated that Hillex[®] II-absorbed phenol red in culture is not harmful to cell viability or growth.
- Due to the rigidity of its core, Hillex[®] II is an excellent substrate for serial passage scale-up, i.e. microcarrier-to-microcarrier passage.

Please contact Global Cell Solutions for additional information at info@globalcellsolutions.com.

2.0 Basic Equipment and Reagent List

2.1 Equipment

- Two 4 to 5 place spinner plates
- Four 250 mL spinner vessels with impeller
- Hemocytometer or other preferred cell/nuclei counting device
- Vortex mixer

2.2 Reagents

- Accutase (GCS cat. no. GSP-0101-100) or Trypsin.
- Dulbecco's Phosphate Buffered Saline DPBS (GCS cat. no. GSP-0302-501)
- Crystal violet/citric acid solution for nuclei release assay; Prepare a 0.1 M citric acid solution in DI water, for the hypotonic/cell lysis effect and add 0.1% w/v crystal violet for nuclear staining.
- Sigmacote[®] (catalog no. SL-2, available from Sigma Chemical Co., tel: 800-325-5832)

2.3 Plasticware

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

- Twelve-well tissue culture plates
- Fifteen (15) and 50 mL conical tubes for sample collection
- Tissue culture flasks or roller bottles

3.0 Siliconizing Glassware

Treat glass surfaces with a silicon solution to avoid cell attachment to glassware. We recommend using Sigmacote[®]. Follow the manufacturer's instructions summarized below:

- 3.1 Start with clean glassware.
- 3.2 Apply the Sigmacote[®] product (approximately 20 mL for a 250 mL container), and swirl to thoroughly coat the surface of all glassware to be exposed to microcarriers.
- 3.3 Drain excess Sigmacote[®] from the glassware and allow to air dry (18 to 24 hours). Optional: Bake in glassware oven at 95°C for ½ hour. Only bake glass portion of spinners.
- 3.4 Wash with mild detergent, rinse the glassware, and perform a final thorough rinsing with DI water.
- 3.5 Clean and maintain vessels with tissue culture-approved solutions. Routinely monitor vessels to assure maintenance of coating. Aqueous solutions should form beads that are repelled from coated surfaces.

4.0 Autoclaving

- 4.1 Transfer 2.0 g of 160-180 micron microcarriers (1,030 cm² for a 200 mL culture) into a spinner flask and add in 25-30 mL of DI water before autoclaving at 121°C for at least 30 minutes.
- 4.2 After microcarriers have settled to the bottom of the spinner flask, carefully decant the autoclaved liquid by pouring through the sidearm of the flask, taking care not to dispense too many microcarriers. Alternatively, liquid can be aspirated from the bead pack with a pipette aid equipped with a 25 or 50 mL pipette. It is reasonable to expect that a very small amount of

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

microcarriers may be lost during this process; however, this should not adversely affect experimental results.

5.0 Cell Dissociation Methods

In order to obtain a uniform distribution of cells among microcarriers, it is essential to generate a robust, single-cell suspension that is free of aggregates and clumps. After preparation, the suspension should appear smooth and consistent.

To achieve satisfactory cell inoculums, a gentle, not necessarily fast process is recommended; therefore, the Accutase or trypsinization process must be optimized for each cell type. Matrix studies for Accutase or trypsin optimization can be performed in T-flasks or tissue culture plates. We recommend rinsing cell cultures up to two times with a solution like Ca/Mg-free DPBS, thus eliminating extraneous protein from the culture before starting the enzyme treatment. We typically use Ca/Mg-free DPBS as a rinse solution for our experiments, but other cell culture-approved solutions may be used for rinses. Titrate the concentration of your chosen dissociation solution for each cell type at various temperatures. Monitor progress of cell dissociation microscopically and note time required for complete dissociation. Some cells respond readily to low concentrations of enzyme at room temperature (for example, Vero cells). Other cell types, including MDCK cells, respond at 35-37°C over a longer time period. For these more tenacious cell types, several 30 minute (or longer) rinses with DPBS prior to a prolonged enzyme treatment may facilitate cell removal and promote viability. In such cases, solution of EDTA can be used to accelerate the process. The lowest temperature and concentration of dissociation solution identified in matrix studies should be used for cell removal.

Typically, minor cell agglutination occurring during processing can be disbursed by triturating with a pipette or an 18-gauge needle assembly.

Prior to microcarrier culture initiation, the cell suspension may be held at ambient temperature for up to 1 hour.

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

6.0 Serum-Containing Cultures

6.1 Place a stir plate in the biological hood with stirring speed set at 55-60 rpm. The impeller must be set at a rate that suspends all microcarriers throughout the vessel without forming a visible gradient.

6.2 The recommended cell inoculum range for preliminary studies is 2.0×10^4 cells/cm² to 3.0×10^4 cells/cm² depending on the culture cycle. At least 10 cells per microcarrier is the minimum cell seeding density.

Two grams of Hillex[®] II = 1,030 cm² per 200 mL; therefore if 2.5×10^4 cells/cm² are the desired rate of inoculum, 2.6×10^7 cells are required per culture.

6.3 In the biological hood, with spinners on the stir plate and the impeller mode set at 55-60 rpm, add a volume of cell suspension that contains the desired number of cells to pre-warmed spinners containing microcarriers in media. Finally, add warmed media to bring final spinner volume to 200 mL. Immediately transfer flasks to incubators equipped with a stir plate also set at 55-60 rpm.

Typically, the cell attachment rate to Hillex[®] II is faster than other PolyGEM[™] microcarriers; sometimes within 1 hour. The cell attachment rate can vary based on the enzyme brand used for cell dissociation.

Avoid cell aggregation: Single, healthy unattached cells are likely to attach to microcarriers, while cells that form clumps in culture before attachment are unsatisfactory and unlikely to attach or, at best, attach in clumps. Uniform cell distribution among microcarriers cannot be achieved with cells that are aggregated. If microcarrier aggregation occurs, it will skew cell counts making results difficult to interpret.

6.4 Stir the spinner flask at 55-60 rpm throughout the incubation period; particularly throughout the cell attachment period. The proper speed will

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

keep the microcarriers in suspension without forming a gradient or without microcarriers collecting at the bottom of the vessel.

- 6.5 Expect to observe cells attaching and spreading on microcarriers 18 to 24 hours after culture initiation with few, if any, unattached cells remaining in solution.
- 6.6 For microscopic observations and analysis over time, transfer 1 mL of homogeneous samples (microcarriers and media) to a 24-well tissue culture plate (cat. no. GAA-2003). These representative samples can be incubated and observed throughout the culture period while collecting data on the status of cells on the microcarriers; cells that have died, cells that did not attach to microcarriers or cells that have detached from microcarriers over time. These data are invaluable for trouble-shooting culture problems and designing optimization strategies.

NOTE: Media exchange strategies are based on the requirements of each cell line and culture conditions.

7.0 Animal Protein-Free Adapted Cell Cultures

- 7.1 To initiate animal protein-free adapted cell cultures or cell lines with low plating efficiency, a single-cell suspension is added to the Hillex[®]II culture. To achieve initial even cell attachment among microcarriers, constant stirring for 1-4 hours at 50-60 rpm is recommended. Starting the culture in the intermittent stirring mode before cell attachment may result in uneven cell distribution among Hillex II. During this period of attachment, cells must not aggregate in suspension. Cell attachment rate can vary based on the enzyme brand used for cell dissociation.
- 7.2 After cell attachment, typically within 1 to 4 hours on Hillex[®]II microcarriers, cells will spread using an intermittent stirring cycle for a period of 6 to 12 additional hours. For example 3 minutes on and 30 minutes off. After the cells have spread, resume constant stirring at 50-60 rpm.

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

- 7.3 For preliminary culture studies, maintain cells in static tissue culture based on their growth characteristics.

8.0 Harvesting Cells

- 8.1 Before harvesting cells from experimental cultures, test the Accutase or trypsinization process in 6-well or 12-well plates by taking homogeneous 1 mL samples from the spinner and transferring to wells. Conduct temperature, rinsing, and enzyme concentration studies to achieve smooth single cell suspensions required for microcarrier culture initiation.
- 8.2 Transfer the 200 mL culture from the incubator to a biological safety hood allowing the microcarriers to settle. Because Hillex[®] II microcarriers have a relatively high specific gravity (1.11-1.12) they tend to settle quickly. Avoid prolonged oxygen deprivation of the cells.
- 8.3 Carefully remove the media from the settled microcarrier pack by pouring the media through a side-arm of the vessel. Alternatively, aspirate the media from the vessel.
- 8.4 Thoroughly rinse the cell-laden microcarriers by adding 50-100 mL of Ca/Mg-free DPBS (cat. no. GSP-0302-501) to the vessel being careful not to dispense the liquid directly onto the microcarriers thereby dislodging cells. Stir the culture at 40 rpm, at the appropriate temperature, for about 10-15 minutes.
- 8.5 Repeat the rinse cycle, if required, according to the testing results carried out in the plate studies.
- 8.6 After removing the final rinse of DPBS, add enough dissociation solution at the optimal concentration to cover the microcarrier pack. For example, 30 mL of dissociation solution can be used in a 250 mL spinner flask containing 2.0 g of microcarriers. Allow the culture to incubate for 15 minutes or more at the appropriate temperature. Track the cell dissociation using samples transferred to wells in a plate configuration. Time for complete dissociation

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

may vary depending on the cell density and cell type. If the dissociation time is too short, cells will be clumped; alternatively, too long and the cells will be clumped with non-viable cells.

- 8.7 After cells are rounded and on the verge of dislodging, indicating complete dissociation, pipette the microcarrier-cell suspension gently up and down to dislodge the cells into a single cell suspension.

9.0 Sampling Techniques

- 9.1 Transfer the culture vessel from the incubator to the biological safety hood placing the vessel on a stir plate set at 60-70 rpm.
- 9.2 With the culture in the stir mode, remove the cap from one side-arm of the vessel. Lower the pipette tip mid-point into the culture vessel toward the side and free of the impeller assembly while quickly taking a known representative volume consisting of microcarriers and media. **Do not adjust the volume amount in the pipette; aspirate in one stroke.** Transfer the sample to a 15 mL conical tube and note final volume.

NOTE: The bead pack is part of the sample volume removed from the culture; the ratio of microcarriers to media volume should be representative of microcarriers in suspension at 60-70 rpm with no visible gradient. After taking samples, return the cultures to the incubator with the set point at 50-60 rpm.

- 9.3 Allow the Hillex[®] II microcarriers to settle in the sample tube before removing the media from the microcarrier pack. For precision and volume reference, transfer the media taken from the sample to a graduated cylinder documenting the volume. (For example, 9.5 mL of media was taken from the 10 mL sample leaving 0.5 mL of microcarriers.)
- 9.4 Rinse the microcarrier pack once or twice by adding 5 mL of DPBS to the tube, swirling several times before removing the supernatant. For this step, conducted at room temperature, volume accuracy is unimportant.

GLOBAL CELL SOLUTIONS

Protocol for Hillex[®] II Microcarriers

9.5 To remove the cells from the Hillex[®] II microcarriers, add the correct known volume (see reference volume Section 9.3 above) of dissociation solution such as Accutase or trypsin at 0.05 % to 0.25 % to the tube containing the rinsed cells-microcarriers and allow to set for 10 minutes or longer at the desired temperature. Gently pull the sample into the pipette and dispense, repeating the in-out motion several times thereby creating a single cell suspension.

9.6 The sample is now ready to count and/or process further.

NOTE: As stated earlier in Section 6.3, if microcarrier aggregation occurs, it will skew cell counts making samples difficult to measure.

10.0 Traditional Nuclei Release Assay Method

Transfer a 1–10 mL homogenous microcarrier culture sample and allow beads to settle before discarding the supernatant. Note the volume of the sample removed from the vessel prior to further processing. This volume will be used to calculate the total number of nuclei in the sample. Suspend the pelleted microcarriers in a volume of citric acid solution equal to the volume of supernatant removed from the tube. Incubate the contents of the tube for 1 hour or longer at 37°C. Avoid evaporation of the contents of the tube by using either a humidified incubator or by sealing the tube with plastic film or cap. After incubation, mix the contents of the tube using a vibromixer before counting the released stained nuclei with a hemocytometer. The microcarriers in the sample do not interfere with counting. The samples may be stored for up to one week at 4°C. This method of determining the number of cells in the culture is most accurate *when cultures are evenly suspended and when aggregation of microcarriers and cells in the culture has been avoided.*

NOTE: Inaccurate sampling may result to inaccurate counts.