

Application Note

Growth Kinetics of Primary CEF Cells on Hillex Microcarriers in Sigma's TiterHigh™ CEF Basal Medium with 2% Bovine Calf Serum.

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Introduction

Efficient propagation of cells in culture is essential for commercial production of virus vaccines. While many cell lines grow in suspension, many other cell lines (most primary cell lines) are anchorage-dependent and require substrates such as plastic culture flasks or roller bottles. Substituting a microcarrier substrate in a sophisticated, computer-controlled bioreactor — in place of numerous roller bottles in an incubator — offers major advantages to the vaccine industry. These advantages include ease of operations at large scale and the ability to precisely control cell growth conditions ultimately yielding increased virus titers. One major vaccine sector that has not utilized microcarrier technology is the production of viral vaccines using primary avian cell lines. In particular, primary chick embryo fibroblast (CEF) cells are commonly grown in roller bottles to produce avian viral vaccines using Dulbecco's Modified Eagles' Medium (DMEM) with 4% fetal bovine serum (FBS). Serum is an important nutritional component in medium and facilitates cell spreading after cells attach to a substrate, but FBS is costly. Typically, CEF cells require 4% or higher serum for growth and survival. However, lower serum levels are desirable in vaccine manufacturing for many reasons including favorable production costs.

The purpose of these studies was to determine the growth kinetics of primary CEF cells on a novel, new microcarrier substrate developed by SoloHill in a new medium developed for CEF cells by Sigma Chemical. The microcarrier (called Hillex¹), is a co-polymer trimethylamine bead designed to support attachment and growth of cells in advanced, low-serum or no-serum formulations such as Sigma's TiterHigh™ CEF Basal Medium. TiterHigh™ CEF Basal Medium was specifically designed for monolayer growth of CEF cells using low levels of iron-supplemented calf serum. Results of CEF cell growth using the Hillex microcarrier in a static culture system were compared between two media groups: DMEM supplemented with 4% FBS and TiterHigh™ CEF Basal Medium with 2% iron-supplemented calf serum. In these experiments, no attempt was made to optimize the culture system.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Tissue Culture Media

TiterHigh™ CEF Basal Medium (Product Code: C 3978) with 2% iron-supplemented calf serum (Product Code: C 8056) or DMEM (Product Code: D 5671) with 4% FBS (Product Code: F 2442) were used to support cell growth. MEM nonessential amino acids (Product Code: M 7145), L-glutamine (Product Code: G 7513) and a penicillin/streptomycin /Amphotericin B cocktail (Product Code: A 5955) were added to both media in the standard concentrations just prior to use.

Culture Substrates

Growth experiments were conducted on Hillex microcarriers (SoloHill Engineering Inc. Ann Arbor, MI) using techniques previously described.²⁻⁵ Hillex microcarriers are a copolymer of styrene and divinylbenzene derivatized with trimethylamine (CP-TMA).⁶ Fifty milligrams of Hillex microcarriers were used. This represents a surface area of 17.5 cm². Hillex microcarriers require no prehydration. The microcarriers were sterilized by autoclaving in PBS for 20 minutes at 121 °C. Prior to use the PBS was discarded and replaced with the selected media. Since the targeted substrate was the Hillex microcarriers, the surface of the wells of a six-well plate (non-tissue culture) was treated with a polyhydroxymethacrylate solution to further inhibit cell attachment.

CEF Cell Culture

Cells from ten-day-old specific pathogen free (SPF) embryonated eggs (SPAFAS Inc., Storrs, CT.) were prepared using standard dissociation procedures with trypsin.⁷ Cells were plated in six-well plates containing 2 ml of medium per well. Four seeding densities for each of the two media types were used. They were 1.0 x 10⁵, 3.0 x 10⁵, 10.0 x 10⁵ and 30.0 x 10⁵ per well. Cultures were incubated at 37 °C with 5% CO₂. Media were exchanged with fresh media at two and four days post-culture seeding. Experiments 1 and 2 were conducted on separate days using different primary CEF cell preparations.

Total Cell Counts

Cells were counted using a Coulter Multisizer II particle counter (Coulter Corp. Hialeah, FL). Primary cells were counted on the day cultures were seeded. On days two, four and six of post-culture seeding, secondary cells were removed from the microcarrier substrates with trypsin, resuspended in media, and counted. Cells from microcarriers in one well constituted a sample. Two samples per group were prepared for assay. Cell numbers were measured from each sample in duplicate and results of the two samples were averaged. Viable cells were not measured in this study.

Data Analysis

The trends of the CEF growth curves were determined with StatView® (Abacus Concepts Inc., Berkeley, CA). Using the formula for a straight line ($y = mx + b$), cell numbers were plotted along a linear trendline that best fit the values in the experimental data set and describes the cell growth per day (x-value) during the log phase of the growth curve. The variable 'm' represents the slope of the line and 'b' represents the y-intercept. R² (coefficient of determination) compares estimated and actual y-values and ranges in value from 0 to 1. Cell population-doubling levels were calculated using the formula $n = 3.32 (\log N_{H_i} - \log N_0)$.⁸

Results and Discussion

In two separate experiments, the efficiency of CEF cell growth on Hillex microcarriers in the two media groups was investigated. Cell numbers comparing DMEM and TiterHigh™ CEF Basal Medium were recorded on the day the culture was initiated (day 0) and on days 2, 4, and 6 as shown in Table 1. Comparing total cell numbers between media groups on the sixth and final day in Experiments 1 and 2, shows that CEF cells grown in the TiterHigh™ medium with only 2% iron-supplemented calf serum were consistently higher. In Experiment 2, however, the lag phase of the growth curve, between days 0 and 2, was shorter than the lag phase in Experiment 1. This may indicate a difference in the culture conditions between Experiments 1 and 2. Considering the consistency of the growth trends in all cell density groups and between the media groups on days 0 and 2, it is likely that the condition of the cells, not the media, resulted in an extended lag phase in Experiment 1. A more precise time when the lag phase ended and the log phase started cannot be determined since cells were not measured on day 1.

Table 1: Chick Embryo Fibroblast (CEF) Cell Growth in DMEM with 4% FBS or Sigma's TiterHigh™ CEF Basal Medium with 2% Iron-Supplemented Calf Serum on Hillex Microcarrier Substrate

Experiment 1				
Day (Post Seeding)	0	2	4	6
Media Group	<i>(total cells x 10⁵)</i>			
DMEM	1.0	0.57	0.54	0.44
	3.0	1.6	1.2	1.2
	10.0	5.5	5.6	15.0
	30.0	17.0	23.0	29.0
TiterHigh	1.0	0.72	0.73	0.85
	3.0	1.8	2.4	6.4
	10.0	7.7	14.0	16.0
	30.0	23.0	34.0	42.0
Experiment 2				
Day (Post Seeding)	0	2	4	6
Media Group	<i>(total cells x 10⁵)</i>			
DMEM	1.0	1.0	1.2	0.92
	3.0	3.1	3.7	2.2
	10.0	13.0	16.0	20.0
	30.0	48.0	55.0	40.0
TiterHigh	1.0	1.2	1.4	1.3
	3.0	3.8	5.4	13.0
	10.0	15.0	28.0	34.0
	30.0	40.0	60.0	63.0

Two additional measures of cell growth in culture systems are cell population-doubling level and a change in cell numbers each day in the log phase as determined by the trendline or slope (Tables 2 and 3). Again cells grown in the TiterHigh™ medium with 2% iron-supplemented calf serum outperformed cells grown in the DMEM with 4% serum irrespective of the initial seeding density. In the TiterHigh cultures, seeding densities of 3.0×10^5 and 10.0×10^5 produced the highest doubling levels. Line graphs in

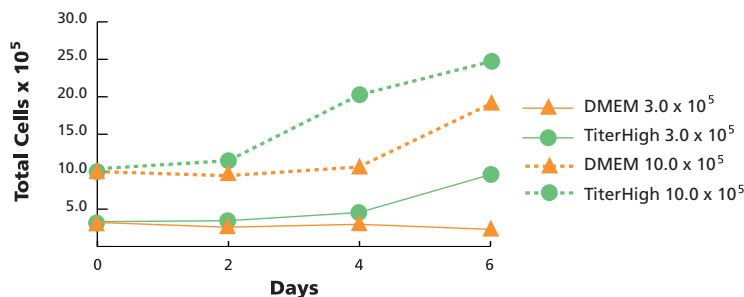


Figure 1: CEF Cell Growth Profile in DMEM or TiterHigh Media on Hillex Microcarriers. Averaged results of Experiments 1 and 2 for each media group and seeding densities 3.0×10^5 and 10.0×10^5 .

Figure 1 are based on data averaged from Experiments 1 and 2 and shows TiterHigh and DMEM with starting cell densities of 3.0×10^5 and 10.0×10^5 .

For comparison purposes experiments were also conducted with Hillex, Cytodex™ 1 and 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, N.J.). These data are not shown, but cell harvest was accomplished more easily on the Hillex microcarriers, consistent with earlier published results.^{2,6}

Table 2: Population-Doubling Levels of Chick Embryo Fibroblast (CEF) Cells Grown in DMEM with 4% FBS or Sigma's TiterHigh™ CEF Basal Medium with 2% Iron-Supplemented Calf Serum on Hillex Microcarrier Substrate.

Seeding Densities (1.0 x 10 ⁵)	Experiment 1			Experiment 2		
	TiterHigh	DMEM	(S-D)*	TiterHigh	DMEM	(S-D)
1.0	0	0	0	0.38	0	0.38
3.0	1.09	0	1.09	2.1	0	2.1
10.0	0.68	0.58	0.10	2.79	1.0	1.79
30.0	0.49	0	0.49	1.07	0.41	0.66

*S-D=difference in population-doubling levels in TiterHigh and DMEM media

Table 3: Change in Cell Numbers per Day During the Log Phase of Chick Embryo Fibroblast (CEF) Cells Grown in DMEM with 4% FBS or Sigma's TiterHigh™ CEF Basal Medium with 2% Calf Serum on Hillex Microcarrier Substrate.

Seeding Densities (1.0 x 10 ⁵)	Experiment 1		Experiment 2	
	TiterHigh	DMEM	TiterHigh	DMEM
1.0	0.018	-0.030	0.008	-0.010
3.0	0.137	-0.030	0.133	-0.037
10.0	0.077	0.110	0.088	0.048
30.0	0.065	0.058	0.050	0.020

*Refer to Table 4 at our Website for complete linear regression data

Conclusions

We describe results of CEF cell growth experiments on SoloHill's Hillex microcarriers using Sigma's TiterHigh™ CEF Basal Medium with 2% iron-supplemented calf serum or DMEM with 4% fetal bovine serum (FBS). Starting cell densities ranged from 1.0×10^5 to 30.0×10^5 per culture. Cell population-doubling levels and cell growth per day during the log phase were used to evaluate the media. These encouraging results show conclusively that:

- Hillex microcarriers provide a favorable substrate for the cultivation of CEF cells.
- In this culture system, TiterHigh™ CEF Basal Medium with only 2% iron-supplemented calf serum enables higher growth in the log phase compared to DMEM with 4% fetal bovine serum.

Future experiments will be conducted to explore the use of primary and secondary CEF cells in spinner flasks and controlled-bioreactor culture systems using Hillex microcarriers and TiterHigh™ CEF Basal Medium with low

serum. It is reasonable to expect that avian viral vaccines can be produced effectively and efficiently using these advanced culture systems, and that remains to be demonstrated.

References and Note

1. U. S. Patent Pending
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7. Ham, R.G. and McKeegan, W.L., *Methods in Enzymology*, Jakoby, W.B. and Pasta, I.H., (Eds.). Vol. **58**, p. 50 (Academic Press, New York, 1979).
8. McAtier, J.A. and Daview, J., *Basic Cell Culture. A Practical Approach.*, Davies, J.M. (Ed) p. 125 (Oxford University Press Inc, New York, 1998).

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ORDERING INFORMATION

Product Code	Product Name	Unit	1-11	12-59	60+
C 3978	TiterHigh™ Chick Embryo Fibroblast Basal Medium	1 liter	\$39.95	\$36.00	\$32.00
G 7513	L-Glutamine Solution (200 mM)		1-11	12+	
		20 ml	\$5.40	\$4.90	
		100 ml	\$16.80	\$12.60	
C 8056	Iron-supplemented Calf Serum		1-11	12+	
		100 ml	\$9.50	\$8.60	
		500 ml	\$36.60	\$33.00	
M 4060	Hillex Microcarrier Beads (75-150 microns; density=1.10 g/cm ³)	20g	\$102.00		

SUPPORTING LITERATURE

Media for Biotechnology/Products for Formulation brochure (DOJ)
Life Science Catalog 2000-2001, pages 354(C 3978), 427(G 7513), 445(C 8056)