

# Understanding Metabolic Needs of EB<sub>14</sub> Cells in Culture

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## Abstract

EB<sub>14</sub> is a genetically stable diploid cell line derived from chicken embryonic stem cells. EB<sub>14</sub> cells demonstrate great potential in virus and recombinant protein production for prophylactic and therapeutic purposes. The present study aims at identifying metabolic needs of the EB<sub>14</sub> cells so that specific nutrients can be provided adequately and in a timely manner. EB<sub>14</sub> cells exhibit unique metabolic patterns in various serum-containing and serum-free media and are highly sensitive to nutritional supply and waste accumulation in culture. Typically, EB<sub>14</sub> cells require frequent replenishment of culture media to stay viable. The ratio of aerobic to anaerobic metabolism was calculated based on carbon and nitrogen energy source consumption rates in EB<sub>14</sub> cells. As a result, optimal media formulations and culture processes were developed to support high viable cell growth supporting recombinant and attenuated viral vaccine production. Chemical and cellular analysis of EB<sub>14</sub> cell cultures with specific viable cell density (VCD), culture longevity and viral productivity (TCID<sub>50</sub>) will be discussed.

## Introduction

Current flu vaccine manufacturing process issues have caught the world's attention and revealed concerns about the ability to provide an adequate supply of vaccines. Currently the flu vaccine is produced primarily in chicken embryonic fibroblasts (CEFs). Processes based on CEFs are highly dependent on the tedious process of isolating and culturing CEFs from chicken eggs. EB<sub>14</sub> cells provide a brand-new cell culture platform to the vaccine industry with immediate benefits. This avian stem cell line was first successfully isolated in long-term culture by Vivalis (Nantes FRANCE). The application of such a revolutionary cell platform represents great potential for viral production and recombinant protein expression for research and biomanufacturing purposes. JRH Biosciences, Inc. (Lenexa, Kansas USA) and Vivalis scientists are the first team to develop a serum-free media and process to support high cell density and viral production in EB<sub>14</sub> cells. More interestingly, JRH and Vivalis scientists investigated EB<sub>14</sub> cells' energy expenditure and metabolic pathway control through media nutrient optimization and a fed-batch process development.

## Materials and Methods

### Cells

EB<sub>14</sub> is an avian stem cell line isolated from chicken embryos and was adapted to grow in suspension by Vivalis. Stock cultures were maintained in a 37 °C, 7.5% CO<sub>2</sub> humidified incubator. EB<sub>14</sub> cells were cultured in 125 mL flasks (25 mL culture volumes) at 90 rpm. The Influenza A/New Caledonia 2009 (A/NC/09) was obtained from the Centers for Disease Control and Prevention (CDC). The Madin Darby Canine Kidney (MDCK) cell line was from American Type Culture Collection (ATCC Number CCL-34).

### Cell Culture Media

Cell culture media was prepared by the imMEDIATE ADVANTAGE™ program (JRH Biosciences). Stock cultures were maintained in SFM-60947, SFM-65318, SFM-65319, SFM-65320 and SFM-65126 (all from JRH) and competitor Medium X. The SFM formulations are serum- and animal-component free media. They contain low levels of recombinant insulin and LONG<sup>®</sup>RIGF-1 (GroPep Limited, Adelaide AUSTRALIA). All formulations except for SFM-65318 were supplemented with 2.5 mM L-glutamine at time of use.

### Process Development

Bioreactor runs were conducted in 3 L stirred tank bioreactors (Applikon, Inc., Schiedam, Holland THE NETHERLANDS). Bioreactors were seeded at 0.26e6 cells/mL in SFM-60947 and Medium X. Dissolved oxygen (DO) and pH were monitored online and controlled using air, O<sub>2</sub>, N<sub>2</sub>, CO<sub>2</sub> and 7.5% NaHCO<sub>3</sub>, respectively. Temperature was maintained at 37 °C using a heating blanket. Samples (5 mL) were collected daily to monitor cell density, viability and metabolic consumption/production (data not shown).

### Viral Infection

Twenty-four hours prior to infection, EB<sub>14</sub> cells were seeded at 0.4e6 cells/mL in 125mL flasks. Cells were inoculated with a multiplicity of infection (MOI) of 0.01 of Influenza A/New Caledonia and allowed to adsorb for one hour at 34 °C. After one hour, additional media was added and the cultures were incubated at 34 °C for five to six days. Trypsin was added at 3 µg/mL each day while sampling cultures.

### Western Blot Analysis

10 µL of supernatant from infected cells were separated on NuPAGE® Novex 4-12% Bis-Tris Gels (1.5 mm) (Invitrogen Corporation, Carlsbad, California USA) and then transferred to PVDF membranes. The membranes were blocked for one hour at room temperature with WesternBreeze® Chromogenic Immunodetection Kit (Invitrogen). Influenza A Virus Hemagglutinin Monoclonal Antibody (HA) (QED Bioscience Inc., San Diego, California USA) was added to the membrane at 4 °C overnight. The blots were washed at room temperature three times for five minutes per wash and then incubated with Alkaline Phosphatase labeled secondary antibody for 30 minutes to one hour at room temperature. The blots were washed an additional time and HA-specific bands were detected using chromogenic reagents. HA-specific bands were detected within 5 - 10 minutes of incubation.

### Analytical Methods

Cell density and viability was determined by Cedex (Innovatis, Bielefeld GERMANY) and by trypan blue exclusion method with hemacytometer. Metabolic consumption of glucose and L-glutamine was monitored offline with the BioProfile® 100 (Nova Biomedical Corporation, Waltham, Massachusetts USA). Metabolic production of lactate, ammonium and glutamate was also monitored offline using the BioProfile® 100.

## Results and Discussion

**Figure 1: Growth Curve of EB<sub>14</sub> cells in SFM-60947 and Medium X in flasks**  
 EB<sub>14</sub> cells were passaged several times in SFM-60947 and Medium X to allow for adaptation. After passaging, EB<sub>14</sub> cells were seeded in 125 mL vented cap flasks at a concentration of 0.25e6 cells/mL as determined by trypan blue/hemocytometer counting. Cell viability and density measurements were taken daily over a four-day growth curve. EB<sub>14</sub> cells grew significantly higher in SFM-60947 and demonstrated greater longevity as compared to Medium X. EB<sub>14</sub> cells in SFM-60947 reached a maximum cell density of approximately 3e6 cells/mL on Day 2 and maintained this density through Day 4. EB<sub>14</sub> cells in Medium X reached maximum cell density of 2e6 cells/mL on Day 2 and then rapidly declined. Data represents the mean viable cell density for duplicate flasks.

**Figure 2: Growth and Viability of EB<sub>14</sub> cells in SFM-60947 and Medium X in Stirred Tank Bioreactors**  
 EB<sub>14</sub> cells were passaged several times in SFM-60947 and Medium X to allow for adaptation. After passaging, EB<sub>14</sub> cells were seeded into 3 L stirred tank bioreactors at a cell density of 0.26e6 cells/mL. Bioreactors were sampled daily for 10 days. Cell viability and density were measured by trypan blue/hemocytometer. EB<sub>14</sub> cells grew significantly higher in SFM-60947 and demonstrated greater longevity compared to Medium X. EB<sub>14</sub> cells in SFM-60947 reached a maximum cell density of approximately 5e6 cells/mL on Day 8 and then slowly declined for the remaining two days. EB<sub>14</sub> cells in Medium X reached maximum cell density of 2.75e6 cells/mL on Day 6 and then rapidly declined.

**Figure 3: Metabolite Consumption and Production Rate of EB<sub>14</sub> cells in SFM-60947 and Medium X**  
 EB<sub>14</sub> cells were passaged in SFM-60947, SFM-60947 with 1x nucleotides, SFM-60947 with 1x nucleotides and recombinant transferrin and Medium X. A growth curve experiment comparing all formulations was completed. During this experiment, cell culture supernatant samples were taken and metabolites were measured. The rate of metabolite consumption and production for Day 2 growth was calculated relative to Day 1 measurements. The data is plotted per metabolite. The rate of lactate and ammonia production was much higher for Medium X than all SFM-60947 formulations. The rate of glucose and glutamine consumption was similar among all formulations tested.

**Figure 4: L-Glutamine Consumption of EB<sub>14</sub> cells in SFM-60947, SFM-65318 and SFM-65319**  
 EB<sub>14</sub> cells were passaged in SFM-60947, SFM-65318 and SFM-65319. A growth-curve experiment comparing all formulations was completed. During the growth curve, cell culture supernatant samples were taken and L-glutamine was measured using a NOVA 100 bioanalyzer. The absolute amount of L-glutamine consumption was plotted over the seven-day growth curve. The original concentration of L-glutamine in SFM-60947 and SFM-65319 was 2.5 mM. The SFM-65318 formulation was supplemented with L-alanyl-L-glutamine. Glutamine is consumed at a constant rate until Day 4. After Day 4, much less glutamine is being utilized by EB<sub>14</sub> cells. SFM-65318 accumulates glutamine after Day 1. This is most probably due to the breakdown of the L-alanyl-L-glutamine bond during culture.

**Figure 5: Ammonia Production of EB<sub>14</sub> cells in SFM-60947, SFM-65318 and SFM-65319**  
 During the growth curve of EB<sub>14</sub> in SFM-60947, SFM-65318, and SFM-65319, cell culture supernatant samples were taken and ammonia was measured using the NOVA 100 bioanalyzer. The absolute amount of ammonia production was plotted over the seven-day growth curve. Ammonia was shown to be produced at a constant rate in SFM-65318 and SFM-65319 up to a maximum level of 3.5 mM on Day 5. SFM-65318 produced much less ammonia than the other formulations tested and reached a maximum of approximately 2.4 mM.

**Figure 6: Hemagglutinin Production in EB<sub>14</sub> Cells grown in SFM-60947, SFM-65126, SFM-65318, SFM-65319 and SFM-65320**  
 EB<sub>14</sub> cells adapted in SFM-60947, SFM-65126, SFM-65318, SFM-65319 and SFM-65320 were infected with Influenza A/New Caledonia. Cultures were treated with trypsin when samples were collected for six days following infection. Western blot analysis indicates that SFM-60947 supports the production of Influenza A/New Caledonia, as demonstrated by the presence of HA<sub>0</sub> and HA<sub>2</sub> (Lane 9 and 10); however, modifications to the formulation increased the amount of hemagglutinin produced following infection with Influenza (Lanes 1-8). These results suggest that modified formulations, such as SFM-65319 support increased Influenza virus production in comparison to SFM-60947.

## Conclusion

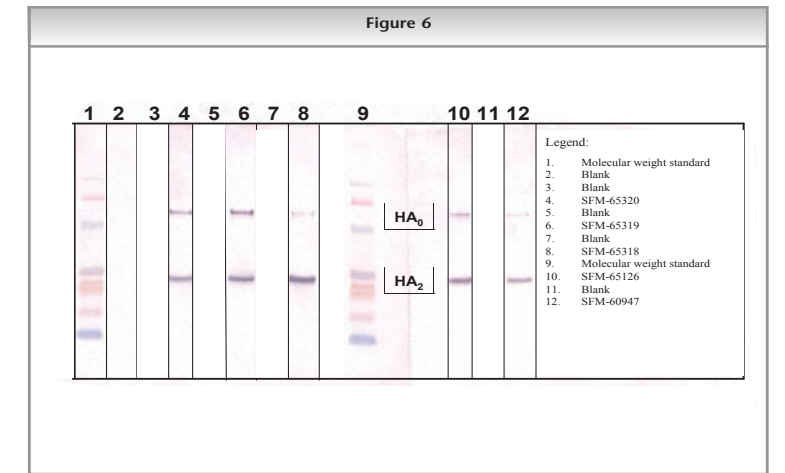
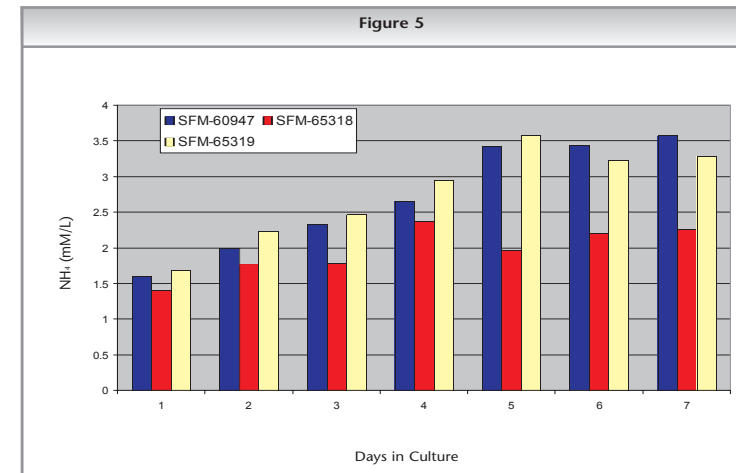
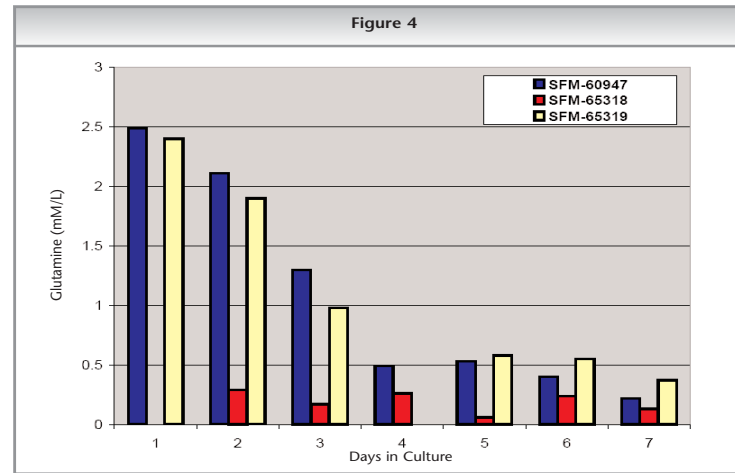
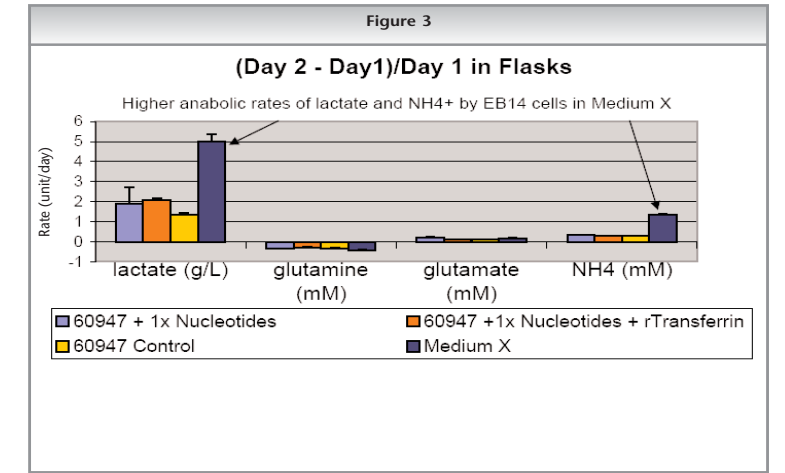
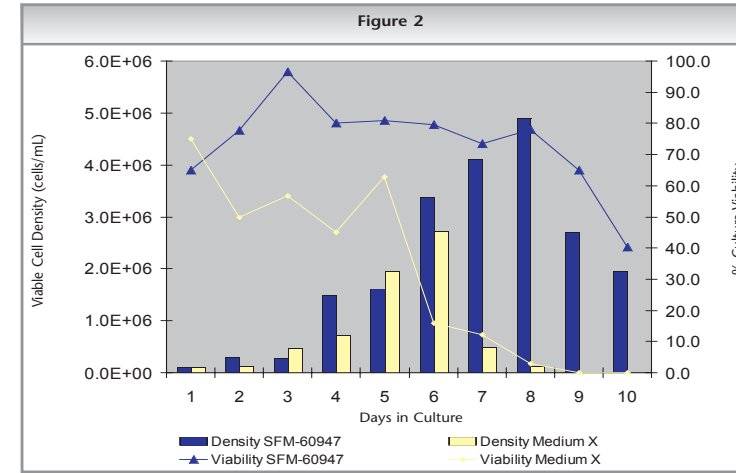
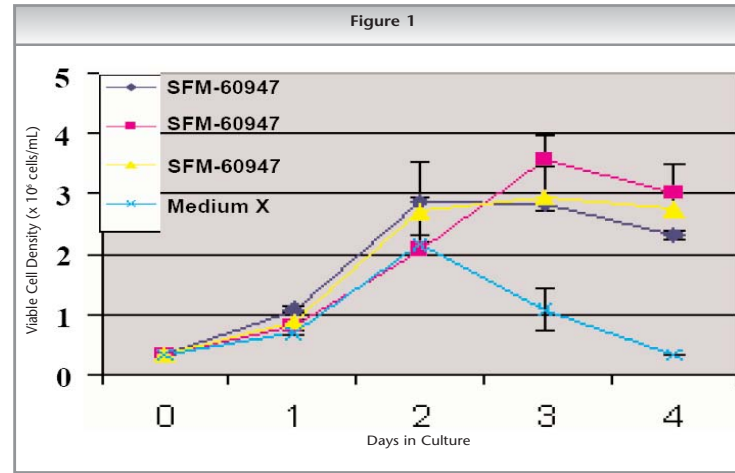
- SFM-60947 is a serum-free, animal-component free medium developed for EB<sub>14</sub> cells in suspension culture.
- SFM-60947 supports higher cell densities, improved viabilities and greater culture longevity in comparison with a competitor medium.
- SFM-60947 supports Influenza virus production.
- Optimization of key components and metabolic analysis has facilitated the development of additional media formulations; these media exhibit improved viral productivity.
- Research into the mechanisms of viral infection in EB<sub>14</sub> cells is ongoing; further medium optimization is in progress.

## References

<sup>1</sup>Reed, L. J., and Muench, H.A., A Simple Method of Estimating Fifty Per Cent Endpoints, Am. J. Hyg. 1938; 27:493-7.

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The authors would greatly like to thank Vivalis and the JRH R&D teams in Lenexa, for without their dedicated efforts and expertise, this project would not have been possible.



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