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Application Note

May 21, 2021

Keywords or phrases
4Cell® MDCK CD medium, Influenza, Suspension, PoC, Growth Profile

Evaluation of Influenza Production in 4Cell® MDCK CD Medium

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Abstract

Madin-Darby canine kidney (MDCK) cells are shown to be one of the foremost cell lines for supporting high titer influenza virus replication. Other advantages of using MDCK cells for virus production include: the versatility to produce some influenza B vaccines and the suitability for large-scale productions, and MDCK cells are non-allergenic. Influenza viruses also replicate more rapidly in MDCK cells than any other cell lines, adapting to produce high titers in MDCK cells in as few as three passages. 4Cell® MDCK CD Medium is a chemically defined, serum-free, protein-free, animal component-free, hydrolysate-free medium designed as a singular solution for the growth and infection of MDCK cells in suspension. 4Cell® MDCK CD Medium has also been optimized for the direct scalability of MDCK cells from bench to manufacturing processes. Initial screenings to infectivity efficiency of MDCK cells with two different influenza virus strains, A/Puerto Rico/8/34/ (H1N1), and A/Hong Kong/8/68/ (H3N2), were evaluated in 4Cell® MDCK CD to produce 5.56×10^7 $TCID_{50}$/mL (48 h) and 8.32×10^5 $TCID_{50}$/mL (72 h), respectively.

Introduction

During the 2019–2020 flu season, the demand for an influenza vaccine was estimated to be 165.5 million doses for the United States alone. Market demand has been estimated to increase approximately 5% each year. However, the current predominant manufacturing technology for influenza vaccines has been in use since the 1940s, a nearly 100-year-old process reliant upon eggs and first discovered in the 1930s. The process yield from these egg-based vaccines can require up to two eggs per dose, thus placing an added burden on the raw material supply chain. Using embryonated hen eggs carries a multitude of other disadvantages such as allergens, adventitious viruses and viral compatibility. The individuality of embryonated egg cultures also induces variability resulting in hemagglutinin (HA) mutations, causing antigenic mutations and affecting the efficacy of vaccines.

The challenges facing egg-based vaccine production have driven the recent rise of alternative methods to generate vaccines via cell-based technology without the inherent risk of allergens or adventitious viruses. Furthermore, cell-based flu vaccines offer several advantages over the traditional method because cell lines are fully characterized and highly regulated with strict adherence to guidelines (1), and the raw materials for production may be defined and can be quickly produced (2). From cell-based cultures, three primary cell lines stand out as alternatives for influenza vaccines: African green monkey kidney-derived (Vero) cells, Insect cells (Sf9), and Madin-Darby canine kidney (MDCK) cells. Compared to other cell lines, the MDCK cells present several advantages for influenza vaccine production. MDCK cells are safe cell substrates and equivalent or better than embryonated chicken eggs or Vero cells for producing vaccines (3). Viral strains such as influenza H3N2 grow better in MDCK cells, while in eggs over 90% of the human isolates are unable to be recovered (4). Additionally, MDCK cells are the most suitable for large-scale production of the influenza virus. A singular 30 L batch can supply equivalent yield of live-attenuated influenza virus doses compared to thousands or millions of embryonated eggs in some instances (5, 6). Finally, MDCK cells are refractory to human and mouse prions, and *in vitro* data suggest that MDCK-derived components are not allergenic (7). To reduce manufacturing costs and improve health safety, it is crucial to move towards the use of chemically defined media and compatible cell line system for vaccine manufacturing. MDCK cells can be easily adapted to be grown in serum-free media in suspension and maintained under various bioreactor conditions. The performance of 4Cell® MDCK CD medium to support influenza virus production was evaluated with A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/8/68 (H3N2) influenza strains.

Materials

Frozen suspension MDCK cells (Cat. No. NBL-2) were previously adapted from adherent and kindly provided by Max Planck Institute. The cells were cultured in a reconstituted 4Cell® MDCK CD medium titrated to pH 7.0–7.2 and sterile filtered. The suspension cells were subcultured every two to three days within duplicate 125 mL shake flasks with a working volume of 25–30 mL. The cultures were cultivated in an incubator with a 25 mm throw orbital shaker at a speed of 110–125 rpm with temperature set at 37 °C and CO_2 of 5%. A Vi-CELL cell counter was used to determine the cell concentration and viability approximately every 24 hours until the total cell count reached maximum and viability declined below 90%.

Influenza Virus Infection

McGill University provided two influenza virus strains for use in this study, A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/8/68 (H3N2), with infectious titer of 5.9×10^7 and 1.5×10^6 $TCID_{50}$/mL, respectively. Infection occurred in 125 mL shake flasks with a working volume of 30 mL. Using 1×10^6 cells/mL of viable MDCK suspension cells, viral strains were inoculated with variations in the multiplicity of infection (MOI) ranging from 10^{-1} and 10^{-2}. Trypsin-TPCK was also supplemented into the media at the time of infection: 1 µg/mL for iInfluenza A/Puerto Rico/8/34 (H1N1), and 2 µg/mL for influenza A/Hong Kong/8/68 (H3N2).

Viable cell density and viability were determined every 24 hours throughout virus production, while samples for viral quantification were taken at 48 hours post-infection (hpi) and at the time of harvest, approximately 67–72 hpi. Viral samples were centrifuged at 4,000 g for 15 minutes to remove cells and cell debris. Sterile supernatant was stored at –80 °C in a 10% DMSO solution before measurements were taken.

Influenza Virus Quantification

$TCID_{50}$ assays were used to quantify virus particles, and adherent MDCK cells were seeded in 96-well plates 24 hours before infection. Cells were then infected with a 1:5 serial dilution of virus stock sample previously stored at –80 °C. Infected MDCK cells were cultivated at 37 °C and 5% CO_2. On the third to sixth day post-infection, positive and negative wells for each plate were counted using a microscope. The infectious virus titer was calculated from seven replicates according to the Spearman-Kärber method. Biorad-QX200 was used for droplet digital PCR (ddPCR) to determine the total viral genome.

Results

Cell Growth Analysis

The adapted suspension MDCK cells achieved a maximum viable cell concentration of $9.91 \times 10^6 \pm 0.46$ cells/mL in 4Cell® MDCK CD medium after five days in culture with doubling time of approximately 26 hours (Figure 1). In both cultures, viability was consistently maintained throughout the cell growth phase between 94–98% after declining in the stationary phase to 89% on Day 7 (Figure 1).

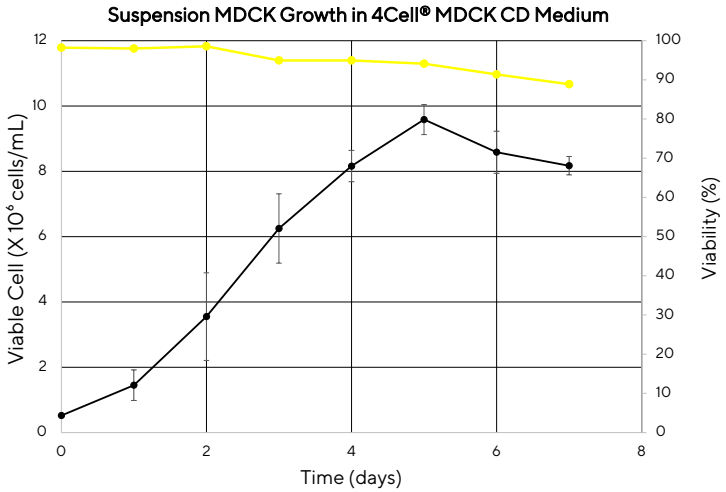

Figure 1: Suspension MDCK growth in 4Cell® MDCK CD medium. MDCK cells were cultivated in duplicate in 125 mL shake flasks at 37 °C, 5% CO_2, 125 rpm. Standard deviations were calculated from individual measurements of viable cell density performed in duplicate.

Assessment for Media Performance to Support Influenza A Virus Production

Influenza A production capability was determined in shake flask cultures (30 mL w.v.) of MDCK cells at a density of 1×10^6 cells/mL for up to 72 hpi. At MOI 10^{-3}, H1N1 virus production after supplementation with 1 µg/mL of Trypsin-TPCK was determined to have an infectious titer of 1.33×10^7 $TCID_{50}$/mL (Table 1). Infectious titer was validated with ddPCR of 2.57×10^7 vp/mL (data not shown). MOI 10^{-1} produced the maximum virus titer within 48 hpi with a viral titer of 5.56×10^7 $TCID_{50}$/mL, while the same MOI measured 2.28×10^7 $TCID_{50}$/mL after 67 hpi. H3N2 at a MOI 10^{-2} after 72 hpi was measured to produce an infectious titer of 8.32×10^5 $TCID_{50}$/mL.

Influenza Virus Strain	A/PR/8/34 (H1N1)			A/HK/8/68/ (H3N2)
MOI	0.1		0.001	0.01
Time Post-infection	48 h	67 h	72 h	72 h
Infectious Titer ($TCID_{50}$/mL)	5.56×10^7	2.28×10^7	1.33×10^7	8.32×10^5

Table 1: Influenza A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/8/68/ (H3N2) virus production by $TCID_{50}$ assay.
1×10^6 cells/mL of suspension MDCK cells infected with various MOI and time of infection (TOI) conditions.

Conclusion

This study serves as a proof of concept that 4Cell® MDCK media supports the growth of MDCK cells in suspension along with viral production and infection of both A/PR/8/34 (H1N1) and A/HK/8/68 (H3N2) influenza A strains. Among the test conditions evaluated, MOI 10^{-1} produced the largest concentration of H1N1 with an infectious titer of 5.56×10^7 $TCID_{50}$/mL after 48 hours of inoculation. The infectious titer of A/HK/8/68 (H3N2) (8.32×10^5 $TCID_{50}$/mL at MOI 10^{-2}) was significantly lower than H1N1. However, within embryonated eggs, H3N2 viruses commonly experience a similar low replication efficiency in addition to difficulties in recovery and hurdles induced by critical antigenic modification with adaptation (8). After adaptation to the cell line, influenza viruses have been noted to produce more rapidly, with higher titers in MDCK cells than other cell lines (2). The adaptation of viruses to MDCK cells can occur within as few as 3–10 passages. These production benefits imposed using MDCK cells have the potential to significantly reduce lead times for vaccine production and alleviate constraints on raw material supply in comparison to embryonated egg processes.

To further improve viral titer and production efficiency, additional studies will be required to evaluate performance in bioreactor systems where more control can be allotted to performance.

Metabolic uptake and production along with pH have been found to impact the yield of influenza production in MDCK. At ammonium chloride concentrations above 20 mM, the yield of influenza viruses was significantly impacted (9). To further define the performance from this study, further optimization would be needed to adapt the viral stock, and ensure stability, along with defining ideal MOI, TOI, Trypsin concentration, and temperature for infectivity. Although more development is required to fully define the production capacity of 4Cell® MDCK, a serum-free media capable of producing influenza A, can significantly improve the process workflow without the need for serum removal prior to infection. Additionally, suspension MDCK cells have also been observed to have the least amount of amino acid substitutions from virus isolate mutations, reducing changes to hemagglutinin, and retaining genetic and antigenic properties during propagation when compared to adherent MDCK (10).

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Status: 08 | 13 | 2021