

Research Report

Evaluation of MDCK Cell Growth and Virus Production in EX-CELL[®] MDCK

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Abstract

EX-CELL MDCK is a serum-free, animal-protein free medium designed and optimized to support high-density culture of Madin Darby Canine Kidney (MDCK) cells. The MDCK cell line can be easily adapted to EX-CELL MDCK using a direct adaptation method or a sequential adaptation method.

EX-CELL MDCK supports MDCK cell density up to 5×10^5 cells/cm² with doubling time as short as 30 hours.

MDCK cells in EX-CELL MDCK were also infected with Canine Adenovirus (CAV) and produced $10^{7.5}$ TCID₅₀/mL at a multiplicity of infection (MOI) of 0.1 and $10^{7.4}$ TCID₅₀/mL at a MOI of 0.01. It was concluded that EX-CELL MDCK supports rapid MDCK cell growth and produces high adenovirus production.

Introduction

The MDCK cell line is used in applications such as development of viral vaccines, anticancer agents and the production of recombinant adenoviral vectors.

Traditionally, MDCK cells are grown in serum-supplemented basal medium such as Minimum Essential Medium (MEM). EX-CELL MDCK is a serum-free, animal-protein free medium specially formulated to support large-scale, high-density MDCK culture and adenovirus production. The medium contains very low levels of recombinant protein (approximately 1.1 mg/L), facilitating downstream processing of expressed products and eliminating regulatory concerns associated with serum and animal proteins. Additionally, the liquid formulation of EX-CELL MDCK is formulated without L-glutamine, which avoids problems associated with L-glutamine degradation, therefore improving product shelf-life. Our experiments show that EX-CELL MDCK supports high-density, serum-free MDCK cell growth and the production of high yields of adenovirus.

Materials

Cells

- Madin Darby Canine Kidney cell line, American Type Culture Collection, Catalog No. CCL-34

Virus

- Canine Adenovirus (CAV) Strain: Toronto A 26/61, American Type Culture Collection, Catalog No. VR-800, Lot Number 217526

Serum-Free Media

- EX-CELL MDCK, SAFC, Catalog No. 14580C

Other Media and Supplements

- Minimum Essential Medium, Alpha Modification (α -MEM), SAFC, Catalog No. 51451C
- Fetal Bovine Serum (FBS) Gamma Irradiated, SAFC, Catalog No. 12107C
- L-glutamine 200mM, SAFC, Catalog No. 59202C
- Trypsin 0.25%, 0.1% EDTA, Gamma Irradiated, SAFC, Catalog No. 59429C
- Dulbecco's Phosphate Buffered Saline (DPBS), without Calcium and Magnesium, SAFC, Catalog No. 59321C
- Soybean Trypsin Inhibitor (STI), Sigma-Aldrich, Catalog No. T6522C
- Trypan Blue 0.4%, Sigma-Aldrich, Catalog No. T8154C
- Dimethyl sulfoxide (DMSO), Sigma-Aldrich, Catalog No. D2650C

Methods

Media and Supplement Preparation and Storage

EX-CELL MDCK was prepared by adding L-glutamine at a final concentration of 6 mM at time of use. Unless otherwise noted, all EX-CELL MDCK was supplemented before use. 10% FBS was added to α -MEM at time of use (referred to as α -MEM-10%). Soybean Trypsin Inhibitor (STI) was prepared as a concentrated (10 mg/mL) solution in DPBS and filter sterilized (0.2 μ m). Working stock solutions of STI were diluted to 1 mg/mL with sterile DPBS as needed.

All media were stored at 2 to 8 C protected from light. Other supplements were stored at their recommended temperatures.

Basic Culture Techniques

Cells were routinely subcultured every 3 days (72 hours \pm 6 hours) at a seeding density of 1×10^5 cells/cm² (in EX-CELL MDCK) or 5×10^4 cells/cm² (in α -MEM-10%) in 75 cm² vent-cap T-flasks (Corning). The total volume of media was 20 mL per flask. Cultures were maintained at 37 C \pm 1 C in a humidified

incubator with 5% CO₂. All experiments were performed in triplicate flasks, except where noted. Cell counts were determined by standard counting technique using a hemocytometer (i.e. typically a 1:10 dilution in 0.04% Trypan Blue, with 5 squares of the hemocytometer counted). All cultures were maintained using aseptic technique and without the use of antibiotics or fungicides.

Trypsinization

MDCK Cultures in α -MEM-10%

Spent medium was aspirated and the flasks rinsed with 5 mL DPBS. The DPBS was aspirated and 3 mL trypsin was added to the flasks. The flasks were incubated at 37 C for 13 - 15 minutes until the cells dissociated. 10 mL of α -MEM-10% was then added to the flasks, the cells gently resuspended and a sample taken for counting. Cells were subsequently diluted in the appropriate quantity of medium and incubated as above.

MDCK Cultures in EX-CELL® MDCK

Spent medium was aspirated and the flasks rinsed with 5 mL DPBS. The DPBS was aspirated and 3 mL trypsin was added to the flasks. The flasks were incubated at 37 C for 13 - 15 minutes until the cells dissociated. 7 mL of STI was added and the cells gently resuspended and transferred to sterile 15 mL conical tubes. The tubes were centrifuged at 1000 rpm (228 g) for 5 minutes to pellet the cells. The supernatant was removed, discarded and the pellet resuspended in 10 mL of prepared medium. A small aliquot was taken for counting and the cells were subsequently diluted in the appropriate quantity of medium and incubated as above.

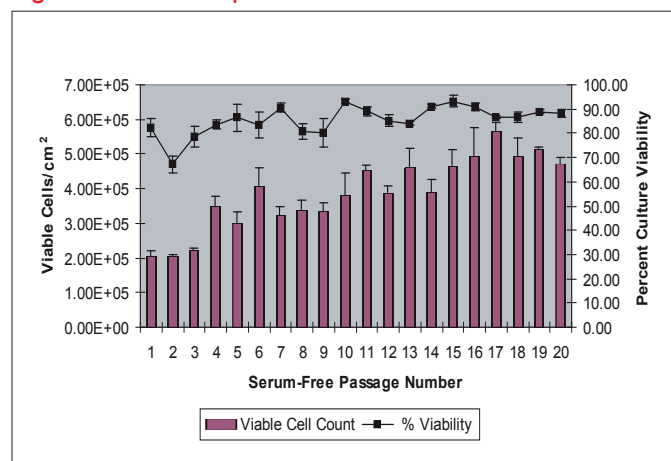
Notes

1. MDCK cells in EX-CELL MDCK medium appeared sensitive to mechanical force. It was found that rapping the flask to dislodge the cells resulted in decreased viability.
2. Over-trypsinizing the cells may occur quickly. It is important to inactivate the trypsin within 15 minutes (less time in smaller flasks). Over-trypsinized cells do not recover.
3. The use of STI is not absolutely required. After trypsinization, cells may be resuspended in a small quantity of EX-CELL MDCK and then centrifuged to remove the trypsin. The use of STI may, however, ease the adaptation to EX-CELL MDCK.

Direct Adaptation

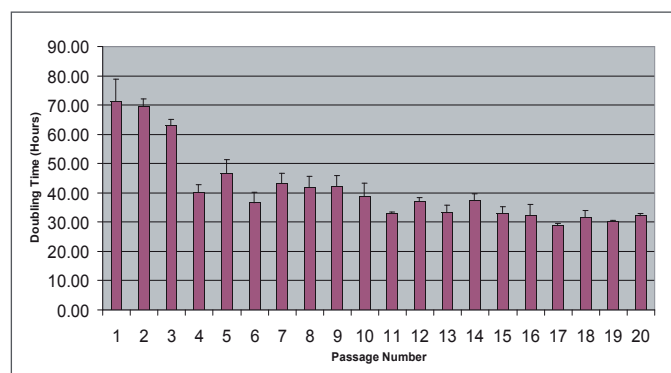
MDCK cells were started from frozen cells in α -MEM-10% in 25 cm² T-flasks, expanded and maintained in 75 cm² T-flasks. MDCK cells were subcultured directly into EX-CELL MDCK at a seeding density 1 x 10⁵ cells/cm², the cells reached 100% confluency within 3 days and displayed normal doubling times.

Figure 1. Direct Adaptation of MDCK Cells to EX-CELL MDCK



MDCK cells were maintained in EX-CELL MDCK for 20 passes after direct adaptation. Cell counts during the first 3 - 4 passes in EX-CELL MDCK mimicked those seen in α -MEM-10%, then cell counts gradually increased during the course of the study.

Figure 2. Doubling Times (Hours) of MDCK Cells in EX-CELL MDCK

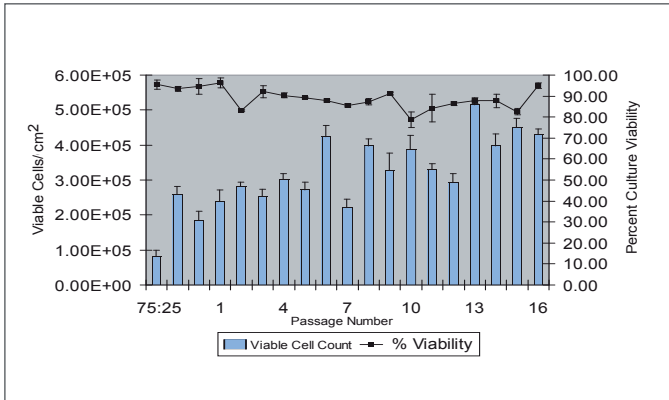


As MDCK cell densities increased, the cell doubling times decreased. In addition, as the cell counts increased, it was noted the morphology of the cells appeared more normal. Average MDCK cell density in EX-CELL MDCK was 3.9 x 10⁵ cells/cm² and average doubling time was 30 - 40 hours. In comparison, the average cell density in α -MEM-10% was approximately 2 x 10⁵ cells/cm² with doubling times of about 60 hours.

Gradual (Sequential) Adaptation

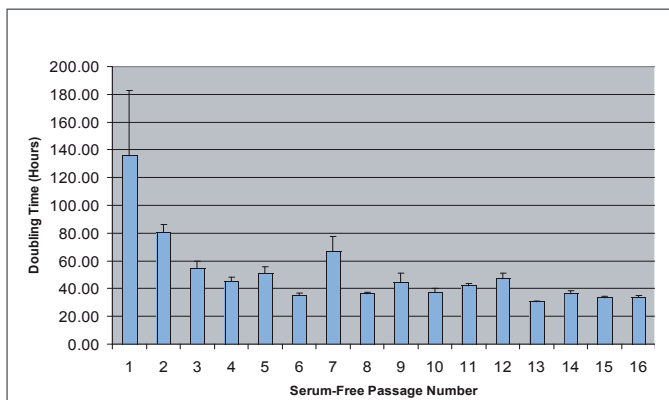
MDCK cells were adapted over 4 passages whereby the concentration of serum-containing medium was reduced by 25% at each pass. Cell densities and doubling times were similar to those seen during the direct adaptation.

Figure 3. Sequential Adaptation of MDCK Cells to EX-CELL® MDCK



MDCK cells were adapted to EX-CELL MDCK in a sequential manner with decreasing concentration of serum-containing medium.

Figure 4. Doubling Times (Hours) During Sequential Adaptation to EX-CELL MDCK



As MDCK cell densities increased, the cell doubling times decreased (Figure 2). In addition, as the cell counts increased, it was noted the morphology of the cells appeared more normal.

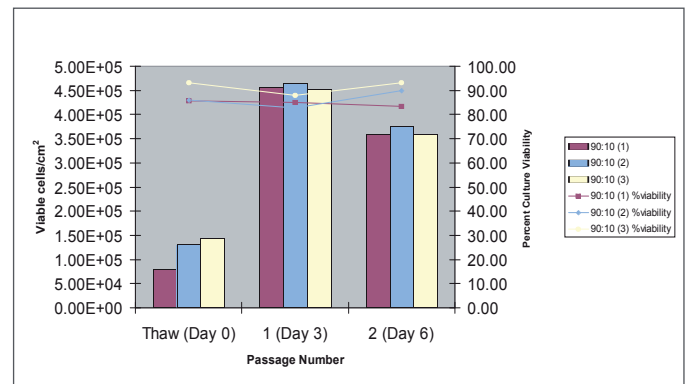
Cryopreservation

MDCK cells were frozen using a cryopreservation medium consisting of 90% fresh EX-CELL MDCK with 10% DMSO (referred to as “90:10”). Three 175 cm² T-flasks were seeded with 1 x 10⁵ cells/cm² in EX-CELL MDCK. After 3 days, the cells were harvested and resuspended in the cryopreservation media as follows: flasks were rinsed with 10 mL DPBS and trypsinized with 6 mL of trypsin per flask (37 C for 15 minutes). 10 mL of STI was added to each flask and the cells were gently resuspended and pooled together in one flask. A sample was then taken to assess viability (>90%). The cells were then transferred to conical tubes and centrifuged at 1000 rpm (228 g) for 5 minutes. The cells were resuspended in cold “90:10” medium to a final concentration of approximately 1 x 10⁷ cells/mL. The cell

mixture was dispensed in 1 mL aliquots into sterile cryovials. The tubes were placed in a Styrofoam container and placed at -20 C for 4 hours, then transferred to -70 C overnight. The next day, the tubes were placed in liquid nitrogen vapor.

MDCK cells were recovered from liquid nitrogen after 3 days. Cells were quickly thawed and resuspended in 10 mL fresh medium in 15 mL conical tubes. A sample was taken to determine viability and then to remove residual DMSO, the cells were centrifuged at 1000 rpm (228 g) for 5 minutes. The medium was discarded and the cells resuspended in 20 mL fresh medium and plated into 75 cm² T-flasks.

Figure 5. MDCK Cells Frozen in 90% Fresh EX-CELL MDCK with 10% DMSO



MDCK cells recovered from cryopreservation with initial thaw viabilities > 85%. Cells grew to confluency within 3 days and displayed no apparent lag in growth. Cells were monitored for 2 additional passages and grew to normal densities in both passages.

Canine Adenovirus Production

To determine the capability of EX-CELL MDCK to support the production of virus, MDCK cells were infected with CAV. Prior to testing, the CAV was amplified twice on MDCK cells in α -MEM-10% to a final titer of 2.54 x 10⁶ TCID₅₀/mL. CAV production was assessed at two different MOIs, 0.01 and 0.1, over 7 days in EX-CELL MDCK.

MDCK cells were seeded in EX-CELL MDCK in 25 cm² T-flasks at 1 x 10⁵ cells/cm² in a total volume of 5 mL medium per flask. Flasks were incubated overnight at 37 C with 5% CO₂. The next day, one flask was sacrificed and the total cell count was determined. The cells were then infected with the appropriate amount of virus to yield a MOI of 0.01 or 0.1. Flasks were incubated as above and a single flask from each trial was removed daily over the next 7 days and frozen at -70 C. During the infections the cells were monitored and displayed cytopathic effect (CPE). The cells became rounded and many, although not all, of the cells detached from the bottom of the flask.

Prior to titration, each flask was subjected to 3 rounds of thawing (37 C) and freezing (-70 C) to lyse the cells. Any remaining attached cells were sloughed off the flask by rapping the flasks against the palm of the hand and by pipetting the lysate forcefully against the bottom of the flask. The cell lysates were transferred to 15 mL conical tubes and centrifuged at 3000 rpm (~2000 g) for 10 minutes to pellet the cell debris. The supernatants were transferred to new tubes and stored at -70 C until titration.

TCID₅₀/mL Titrations

CAV production in EX-CELL® MDCK was determined by TCID₅₀/mL. The titration procedure was devised by modifying an existing procedure from Qbiogene (AdenoVator™ Adenoviral Vector System, Applications Manual, Version 1.1).

MDCK cells growing in α-MEM-10% were harvested by trypsinization, counted and diluted to a final concentration of 1 x 10⁵ cells/mL in α-MEM supplemented with 5% FBS (α-MEM-5%). Using a 12-channel pipettor, 100 mL of cells were dispensed into 96-well microtiter plates (1 x 10⁴ cells/well) and allowed to attach at 37 C.

Duplicate serial dilutions of each lysate were made in α-MEM-0% in sterile snap-cap disposable tubes (1:10 dilutions, 10⁻³ to 10⁻¹⁰ plated). **Note:** α-MEM used for dilutions was not supplemented with FBS, therefore, the final concentration of FBS in the wells was 2.5%. The dilutions were poured into sterile reservoirs and 100 mL of each dilution was dispensed in wells 1 - 10 (wells 11 and 12 in all rows served as controls). The rows were dispensed with the highest (10⁻¹⁰) dilution in the bottom row, the lowest dilution (10⁻³) in the top row. The plates were incubated at 37 C, 5% CO₂ for 7 days and then observed on an inverted microscope for CPE. Wells were considered positive even if only a small area of the well showed CPE. The titer (T) was determined as follows:

$$T = 10^{1+d(S-0.5)}$$

Where d = Log₁₀ of the dilution and S = Sum of the ratios

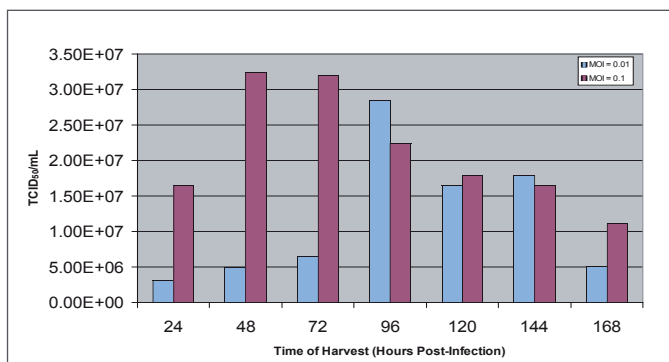
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Figure 6. Canine Adenovirus Production in EX-CELL MDCK



EX-CELL MDCK supported the production of CAV in MDCK cells adapted to the medium. Cells infected at a MOI of 0.1 produced slightly better yields than those infected at a MOI of 0.01. Peak production (TCID₅₀/mL=10^{7.5}) occurred between days 2 - 3 at a MOI of 0.1, and a day later at the MOI of 0.01 (TCID₅₀/mL=10^{7.4}). At both MOI's production declined after day 4, presumably due to unfavorable conditions for CAV such as cell lysis, pH shift and accumulation of toxins.

Conclusions

MDCK cells were adapted from α-MEM-10% to EX-CELL MDCK in static cultures. During adaptation a seeding density of 1 x 10⁵ cells/cm² was used to obtain cultures that reached confluency within 3 days. MDCK cells took 3 - 4 passages to adapt to EX-CELL MDCK. However, the cells appeared to be continually adapting to the medium, with increasing cell densities and shorter doubling times as the culture time in EX-CELL MDCK increased. Cell densities in EX-CELL MDCK were approximately twice those seen in α-MEM-10%.

Cryopreservation and recovery of adapted cells in EX-CELL MDCK was easily accomplished with only the addition of 10% DMSO. It was not found to be necessary to add conditioned medium or supplements (e.g. Bovine Serum Albumin).

EX-CELL MDCK also supported CAV production in MDCK cells. The cells exhibited classic CPE in culture and produced CAV titers in the range of 10^{7.5} TCID₅₀/mL. This range is comparable with those seen in serum-supplemented cultures.

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