

Chapter 8

Propagation of Chikungunya Virus Using Mosquito Cells

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Abstract

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that transmits in between a mosquito host vector to a primate host and then back to the mosquito host vector to complete its life cycle. Hence, CHIKV must be able to replicate in both host cellular systems that are genetically and biochemically distinct. The ability to grow and propagate the virus in high titers in the laboratory is fundamentally crucial in order to understand virus replication in different host cellular systems and many other CHIKV research areas. Here, we describe a method on CHIKV propagation using C6/36, a mosquito cell line derived from *Aedes albopictus* in both serum-containing and serum-free media.

Key words CHIKV, Virus propagation, Mosquito cells, C6/36, Serum-free

1 Introduction

Chikungunya virus (CHIKV) is an alphavirus of the *Togaviridae* family. Its genome comprises a single positive-stranded RNA of around 11.8 kb containing a 5'-methylguanylate cap, two open reading frames, and a 3'-polyadenylate tail [1, 2]. It encodes for four nonstructural proteins (nsP1–4), three structural proteins (C, E1, and E2), and two small peptides (E3 and 6K). CHIKV is maintained in two distinct transmission cycles: a sylvatic cycle that is mainly confined within African countries involving forest-dwelling *Aedes* mosquitoes and nonhuman primates on a smaller epidemic scale [3]; and a human–mosquito–human cycle involving urban mosquitoes *Aedes aegypti* and *Aedes albopictus* being the transmission vectors during large epidemics [4].

Chikungunya has re-emerged as a disease of global significance following major outbreaks with millions of infections reported since 2005 [5]. Despite causing public health threat globally, there is currently no vaccine or effective therapeutics toward CHIKV infections. Therefore, extensive research to understand CHIKV pathogenesis as well as CHIKV diagnosis and drug development is needed to better control CHIV epidemics. Generation of virus

infectious particles in the laboratory is fundamentally essential in many areas of CHIKV research. Since CHIKV is able to replicate both in vertebrate and invertebrate cellular systems that are biochemically and genetically divergent in nature, CHIKV infectivity in different established cell lines derived from mammalian and mosquito origins have been tested [2, 6]. Cells in general possess innate defense mechanisms, e.g. type-I interferon response in mammalian cells [7] and RNA interference (RNAi) response in insect cells [8, 9] to suppress virus replication. However, Vero cells (African green monkey kidney epithelia) lacks type-I interferon, making them highly permissive for CHIKV infection and therefore commonly used for CHIKV propagation [10]. Similarly, C6/36 (a genetically homogenous isolate of Singh's *Aedes albopictus* cells) lacks functional RNAi response [11] where Dcr2 fails to cleave double-stranded viral RNA molecules into small RNA effector molecules which in turn silence complimentary RNA molecules [12]. CHIKV infection in C6/36 only causes mild cytopathic effect (CPE) and apoptosis compared to that of Vero cells [13], making it a better cell line of choice for persistent CHIKV infection to produce higher titer of virus progeny.

In this chapter, we will describe protocols for culturing C6/36 cells, followed by CHIKV infection on the cells for virus propagation in a detailed step-by-step manner. To ensure high virus titer production, tips will be given on the steps that require special attention on the methods described.

2 Materials

2.1 Culture of C6/36 Cells

1. *Aedes albopictus* C6/36 cells.
2. Leibovitz (L15) media containing 10 % heat-inactivated fetal calf serum (FCS).
3. 1× Trypsin-EDTA solution: 0.05 % trypsin, 0.02 % EDTA, 0.1 % glucose, 0.8 % NaCl, 0.04 % KCl, 0.058 % NaHCO₃.
4. 1× Phosphate Buffered Saline (PBS).
5. T75 cm² flasks.

2.2 Virus Propagation

1. Chikungunya virus.
2. C6/36 cells at 80 % confluency.
3. L15 media containing 2 % FCS.
4. L15 media containing 0.2 % BSA.
5. 1× PBS.

3 Methods

3.1 Culture of C6/36 Cells

1. Thaw 2 vials (1 ml each) of cryo-preserved C6/36 stock culture (*see Note 1*).
2. Aliquot 10 ml of L15 media supplemented with 10 % heat-inactivated FCS into a sterile T75 cm² flask and add thawed cells into the flask (*see Note 2*).
3. Mix well by gently pipetting up and down several times to dissociate clumped cells while preventing the formation of excessive air bubbles in the media. Lay the flask flat and swirl gently to equally distribute the cells for adherence.
4. Alternatively, upon thawing, suspend cells in 10 ml of L15 media and centrifuge at 300×*g* for 10 min. Decant the media carefully and gently resuspend cell pellet in 10 ml of L15 media before adding the cell suspension into a T75 cm² flask (*see Note 3*).
5. Incubate the cells at 28 °C overnight in the absence of CO₂.
6. On the next day, check the cells under light microscope. Most of the cells will have attached and there may be some dead cells. Replace the media with fresh L15 supplemented with 10 % heat-inactivated FCS.
7. Incubate at 28 °C for 5–6 days until ~80 to 100 % confluency.
8. To expand cells, aspirate and decant the culture media from the flask, wash the cells once with 1× PBS followed by the addition of 2 ml trypsin-EDTA solution sufficient to cover the cell monolayer.
9. Swirl gently to allow trypsin to be in contact with cells for ~2 min (*see Note 4*).
10. Add 8 ml of L15 media supplemented with 10 % heat-inactivated FCS. If the cells have yet to dislodge after 5 min, replace with fresh trypsin and/or gently hit the flask against your palms.
11. Resuspend cells by pipetting up and down several times to avoid cell clumping (*see Note 5*).
12. Add 3 ml of cells to 6 ml of L15 media (1:3 dilution) per flask and incubate at 28 °C for 3–5 days until ~80 % confluency (*see Note 6*).

3.2 Virus Propagation

1. Thaw 3 vials (1 ml each) of cryo-preserved CHIKV from –80 °C to room temperature or 37 °C (*see Note 7*).
2. Aspirate and decant culture media from T75 cm² flasks and wash C6/36 cell monolayer with 1× PBS.

3. Infect the cells with 1 ml of virus/flask at a multiplicity of infection (MOI) of at least 0.1 (*see Note 8*). Swirl gently to ensure equal distribution of virus to the cells.
4. Incubate the cells at 37 °C for 1.5 h, with gentle swirling at 15 min interval (*see Note 9*).
5. After 1.5 h, add 9 ml of L15 media supplemented with 2 % heat-inactivated FCS to the flask (*see Note 10*). Incubate CHIKV-infected cells at 28 °C for 3–5 days until the cells are observed to be unhealthy (*see Note 11*).
6. Alternatively, to have the virus cultured serum-free, incubate CHIKV-infected cells at 28 °C for 1 day in L15 media supplemented with 2 % heat-inactivated FCS. Then, remove media, wash twice with 1× PBS and then top up with 10 ml L15 media supplemented with 0.2 % BSA. Incubate for 3–4 days until the cells are observed to be unhealthy.
7. Collect viral supernatant from the flasks into a sterile centrifuge tube and centrifuge at 1000×*g* for 10 min at 4 °C (*see Note 12*).
8. Clarified supernatant containing virus can now be used directly for quantification by viral plaque assays. Alternatively, virus supernatant should be stored as 1 ml aliquots in cryo-preservation tubes at –80 °C (*see Note 13*).

4 Notes

1. C6/36 cells must be thawed rapidly and completely in a water bath set at the cell's normal growth temperature, i.e. 28 °C. This is to ensure maximal recovery of cell viability upon thawing in the presence of dimethyl sulfoxide (DMSO), which is a cryo-preservant [14]. During thawing, ensure cryovials are half-submerged in the water bath to prevent water from leaking in or contaminating screw caps of any loosen vials.
2. For adherent cell line like C6/36, it is optimal to have at least 3×10^4 cells/cm² per flask [14]. The routine sub-culturing ratio of C6/36 cells is 1:5. However for thawing purpose, this ratio can be increased to 1:10 where more media can dilute the toxic effect of DMSO. A higher dilution ratio can also initiate rapid cell growth as some cells might be lost due to damage from thawing.
3. This additional centrifuge step removes DMSO in the thawed media which helps in cell recovery.
4. Trypsin enables cells to detach from one another and from the substratum by digesting extracellular matrix protein and cell junction proteins, while EDTA disaggregates cells by chelating calcium ions. Excessive trypsinization is detrimental to cell

recovery and may undesirably lead to cell death. Therefore, it is important to use the smallest possible volume of trypsin solution at 0.025–0.05 w/v and the shortest exposure trypsinization time to detach the cells from the flask. Once the cells round up and begin to dislodge from the monolayer, trypsin activity must be inhibited immediately by adding fresh media containing 5–10 % FCS to the cell-trypsin solution [15].

5. After detachment from a monolayer culture, C6/36 cells have the tendency to form clusters if they are not well-dissociated by pipetting forces. Therefore, it is important to dissociate the clusters into single cells in order for the cells to have better growth and adaptation to the fresh culture environment [16].
6. Sufficient cells of at least 80 % confluency is optimal to achieve a high-titer CHIKV progeny since C6/36 cells are able to support persistent CHIKV infection [13, 17].
7. Thawing of frozen virus should be performed in water bath set at 37 °C so that the viral supernatant is equilibrated for optimal cell absorption upon adding to the cell monolayer.
8. With a MOI of 0.1, the volume of virus (volume from virus stock: L15 media dilution) to add per flask can be calculated by taking the expected cell density of C6/36 cells at the time of confluency divided by the virus titer. If possible, use a higher MOI to allow multiple rounds of CHIKV infection and achieve a culture of higher virus titer.
9. Regular swirling of the culture flask allows CHIKV to be well-distributed which helps in virus adsorption to the cell surface.
10. Heat-inactivated 2 % FCS is sufficient to maintain cell growth and metabolism so that CHIKV propagation can occur for the next few days.
11. Due to the presence of certain host factors in C6/36 cells, these mosquito cells do not exhibit apparent CPE and apoptosis after CHIKV infection [11, 13].
12. Low-speed centrifugation causes dead cells to pellet down and thus helps to purify the viral supernatant.
13. Repeated freeze–thawing should be minimized as this may decrease virus titer.

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