Chapter 27

Production of Chikungunya Virus-Like Particles and Subunit Vaccines in Insect Cells

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Abstract

Chikungunya virus is a reemerging human pathogen that causes debilitating arthritic disease in humans. Like dengue and Zika virus, CHIKV is transmitted by *Aedes* mosquitoes in an epidemic urban cycle, and is now rapidly spreading through the Americas since its introduction in the Caribbean in late 2013. There are no licensed vaccines or antiviral drugs available, and only a few vaccine candidates have passed Phase I human clinical trials. Using recombinant baculovirus expression technology, we have generated CHIKV glycoprotein subunit and virus-like particle (VLP) vaccines that are amenable to large scale production in insect cells. These vaccines, in particular the VLPs, have shown high immunogenicity and protection against CHIKV infection in different animal models of CHIKV-induced disease. Here, we describe the production, purification, and characterization of these potent CHIKV vaccine candidates.

Key words Chikungunya virus, Baculovirus, Insect cells, Secreted E1 and E2, Virus-like particles, Production and purification

1 Introduction

Chikungunya virus (CHIKV) is an arthropod-borne (arbo)virus, which is transmitted by *Aedes* mosquito species. After its re-emergence in 2004 in Kenya, CHIKV has caused large-scale epidemics in Africa, Indian Ocean, and (South-East) Asia [1]. More recently, CHIKV has found its way to Europe (Italy 2007 [2]; France 2010 [3]) and emerged in the Caribbean in late 2013 [4]. The virus is expected to continue spreading to Central and South America, where it will then co-circulate with the most important arbovirus, dengue [5]. CHIKV causes significant morbidity such as the sudden onset of fever, myalgia, rash and in some cases severe, chronic arthralgia [6, 7]. CHIKV (genus *Alphavirus*; family *Togaviridae*) has a positive sense, single-stranded RNA genome of ~11 kb long and encodes two open reading frames (ORFs), the non-structural ORF and the structural ORF. The viral genome is encapsidated in a nucleocapsid which is tightly surrounded by a host-derived lipid

Justin Jang Hann Chu and Swee Kim Ang (eds.), *Chikungunya Virus: Methods and Protocols*, Methods in Molecular Biology, vol. 1426,DOI 10.1007/978-1-4939-3618-2_27, © Springer Science+Business Media New York 2016

envelope displaying trimeric E1/E2 glycoprotein spikes that mediate cell binding and entry [8].

The global threat of CHIKV is affecting millions and the unprecedented rapid spread of the virus demands effective countermeasures, including an efficacious vaccine. In the past decade, many studies have focused on generating CHIKV vaccine candidates, using a large variety of vaccine platforms, e.g., live-attenuated viruses, DNA vaccines, chimeric vector vaccines, glycoprotein subunits, and virus-like particle (VLP) vaccines [9–16]. Different expression platforms, i.e., mammalian cell transfection and baculovirus expression in insect cells, have been used for the production of CHIKV-E1 and E2 subunits and VLPs [14, 15]. The baculovirus insect cell expression technology is a well-established eukaryotic protein production platform with applications in veterinary and human vaccinology [17] and is well suited to produce essentially authentic arbovirus proteins [18, 19]. Indeed, the system has been used for the expression of secreted (s)E1 and secreted (s)E2 glycoprotein subunits [20] and CHIKV-VLPs [15]. Both modalities were immunogenic in animal models and conferred protection against CHIKV-induced disease with VLPs being better immunogens than subunits [15, 16]. In this chapter, we describe the production, purification, and analysis of the highly immunogenic CHIKV-VLPs [15, 16] and the glycoprotein subunits sE1 and sE1 [20], which were expressed in insect cells using recombinant baculovirus vectors. The generation of vector constructs and recombinant baculoviruses expressing the CHIKV-subunits and VLPs is explained in detail. Next, we detail the procedures of the production and purification of the subunits and VLPs, which are based on affinity chromatography and sucrose density gradient purification, respectively. Finally, a step-by-step description of subunit and VLP characterization based on glycosylation, furin-cleavage, and morphology is presented.

2 Materials

2.1 Cell Culture

1. *Spodoptera frugiperda* (*Sf*)21 insect cells (Invitrogen).
2. *Sf*9-easy titration (ET) insect cells [21].
3. Grace's insect-cell medium (Invitrogen) supplemented with 10 % fetal bovine serum (FBS).
4. *Sf*900II insect-cell medium (Invitrogen) supplemented with 5 % FBS and 200 μg/ml geneticin.
5. 75 cm^2 culture flasks.
6. 6-well culture plates.
7. Cell scrapers.

2.2 Generation of Recombinant Baculovirus Ac-sE1, Ac-sE2, and Ac-S27

1. Synthetically generated CHIKV S27 structural polyprotein DNA (GeneArt®).
2. Phusion High Fidelity DNA polymerase.
3. 10 mM dNTPs mix.
4. Forward primers (*see* **Note 1**):
   - sE1-Fw (ggggacaagtttgtacaaaaaagcaggcttaggatccaccatggccacataccaagaggctgc).
   - sE2-Fw (ggggacaagtttgtacaaaaaagcaggcttaggatccaccatgagtcttgccatcccagttatg).
5. Reverse primers (*see* **Note 1**):
   - sE1-Rv (ggggaccactttgtacaagaaagctgggtaaagcttctaatgatgatgatgatgatgcatccatgacatcgccgtagcgg).
   - sE2-Rv (ggggaccactttgtacaagaaagctgggtaaagcttctaatgatgatgatgatgatgctgcagcagctataataatacagaa).
6. MilliQ water.
7. Silica Bead DNA Gel Extraction Kit (Thermo Scientific).
8. pDONR207 donor plasmid (Invitrogen).
9. pDEST8 expression plasmid (Invitrogen).
10. BP Clonase™ II enzyme mix (Invitrogen).
11. LR Clonase™ II enzyme mix (Invitrogen).
12. Proteinase K solution (100 μg/ml).
13. Electrocompetent DH5α-*E. coli* bacteria.
14. Luria broth (LB) liquid culture medium.
15. LB-agar plates containing 7 μg/ml gentamycin.
16. LB-agar plates containing 7 μg/ml gentamycin and 100 μg/ml ampicillin.
17. Tris–EDTA (TE) buffer: 10 mM EDTA, 25 mM Tris–HCl pH 8.0.
18. DH10Bac electrocompetent *E. coli* bacteria (*see* **Note 2**).
19. LB-agar plates containing 7 μg/ml gentamycin, 50 μg/ml kanamycin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG.
20. Primers for analyzing recombined bacmids:
    - M13-Fw (taaagcacggccag).
    - M13-Rv (caggaaacagatatgac).
    - Genta-Rv (agccacctactcccaacatc).
21. *Sf*21 insect-cells.
22. Serum-free Grace's insect-cell medium.

23. Grace's insect-cell medium supplemented with 10 % FBS and 50 μg/ml gentamycin.
24. 6-well cell culture plate.
25. FectoFly™ I (Polyplus transfection).
26. Rocking plateau.
27. 75 cm^2 culture flasks.
28. Cell scrapers.
29. *Sf*9-ET insect-cells.
30. *Sf*900II insect-cell medium supplemented with 5 % FBS and 200 μg/ml geneticin (G418).
31. 60-well microtiter plate.
32. Fluorescent microscope.

2.3 Production of CHIKV-sE1 and -sE2 Subunits and VLPs

1. *Sf*21 insect-cells.
2. Serum-free Grace's insect-cell medium.
3. Phosphate buffered saline (PBS).
4. Rocking plateau.

2.4 Purification of sE1 and sE2 Subunits

1. Talon® spin columns 0.5 ml (Clontech).
2. Talon washing buffer (TWB): 20 mM Tris–HCl, 100 mM NaCl, pH 7.9.
3. Talon elution buffer (TEB): 20 mM Tris–HCl, 100 mM NaCl, 300 mM imidazole, pH 7.9.
4. Roller plateau.

2.5 Purification of CHIKV-VLPs

1. 7 % (w/v) polyethylene glycol (PEG)-6000.
2. 0.5 M NaCl.
3. Glycine-tris-sodium chloride-EDTA (GTNE) buffer: 200 mM Glycine, 50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.3.
4. Discontinuous 70 % (w/v), 40 % (w/v) sucrose in GTNE gradient at 4 °C.
5. SW55 soft ultracentrifuge tubes 5 ml (Beckman).
6. SW55 rotor (Beckman).
7. Pasteur pipettes.
8. Roller plateau.

2.6 CHIKV Protein Analysis

1. Glycoprotein denaturing buffer (New England Biolabs).
2. G7 reaction buffer (New England Biolabs).
3. 10 % NP40 buffer (New England Biolabs).
4. 500 units/μl PNGase F (New England Biolabs).

5. MilliQ water.
6. SDS-PAGE and Western blot equipment.
7. Rabbit-polyclonal anti-E1 and anti-E2 [20].
8. *Sf*21 insect-cells.
9. Grace's insect-cell medium (Invitrogen) serum free.
10. 50 μM furin inhibitor I (Calbiochem).
11. 6-well culture plate.
12. PBS.
13. Rocking plateau.
14. PBS–Tween 0.05 %.
15. *Sf*9-ET insect-cells.
16. *Sf*900II insect-cell medium supplemented with 5 % FBS and 200 μg/ml geneticin.
17. *Sf*900II insect-cell medium supplemented with 0.2 mg/ml cholesterol.
18. HCl-acidified *Sf*900II medium with pH 5.8, 5.5, and 5.0.
19. Fluorescent microscope.
20. Copper 400 square mesh grids (Veco).
21. Argon gas discharger.
22. Filter paper.
23. 2 % uranyl acetate.
24. Transmission electron microscope.

3 Methods

3.1 Cell Culture

1. Maintain adherent *Sf*21-cells as monolayer cell cultures in a closed 75 cm^2 culture flask at 27 °C (without additional CO_2) in Grace's insect cell medium, supplemented with 10 % FBS. Passage cells when the culture reaches ~80 % confluency, by dislodging them from the bottom of the flask using a Pasteur pipette and resuspending them in the culture medium by pipetting up and down. Split the cells 1:5 in fresh supplemented medium for culture maintenance. Leave the cells for 1 h at 27 °C for attachment to the bottom of the culture flask or plate.
2. Maintain *Sf*9-easy titration (ET) cells as a monolayer cell culture in *Sf*900II insect cell medium supplemented with 5 % FBS and 200 μg/ml geneticin at 27 °C. Passage cells when the culture reaches ~80 % confluency, by scraping them from the flask bottom using a cell scraper and resuspending them in the culture medium by pipetting up and down. Split the cells 1:5 in fresh supplemented medium for culture maintenance.

3.2 Generation of Recombinant Baculovirus *Ac*-sE1, *Ac*-sE2, and *Ac*-S27

Generation of recombinant baculovirus expressing the sE1 and sE2 subunits and the complete CHIKV S27 structural cassette is based on the Bac-to-Bac baculovirus expression system, using an adapted *Autographa californica* nucleopolyhedrovirus (*Ac*MNPV) backbone [22]. The synthetically generated S27 structural polyprotein cloning fragment (Genbank accession # AF369024) containing AttB1/2 recombination sites that enable Gateway ® cloning will be used as a template for generation of the sE1 and sE2 cloning fragments.

1. To PCR amplify the sE1 and sE2 coding fragments, set up a 20 μl PCR mix containing:
   - 20 ng synthetic S27 DNA.
   - 4 μl 5× Phusion HF buffer.
   - 0.25 μl 10 mM dNTPs.
   - 0.5 μl 10 mM Fw-primer (*see* **Note 1**).
   - 0.5 μl 10 mM Rv-primer (*see* **Note 1**).
   - 0.5 μl Phusion DNA polymerase.
   - Fill to 20 μl with MilliQ water.
2. To amplify the target sequences, start with initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 3 min. Finish with a final elongation at 72 °C of 7 min. Purify the amplicons from agarose gel by using Silica Bead DNA Gel Extraction Kit following the manufacturer's protocol.
3. Add 150 ng of synthetic S27 DNA or sE1 and sE2 amplicons to 150 ng of pDONR207 donor plasmid in 8 μl TE-buffer.
4. Add 2 μl BP Clonase™ II enzyme mix and incubate for 1 h at room temperature (RT).
5. Stop the reaction by adding 1 μl Proteinase K and incubate for 10 min at 37 °C.
6. Transform 1 μl of the BP-reaction mix into electrocompetent DH5α *E. coli* bacteria. After transformation, recover the bacteria for 1 h in 1 ml LB-medium.
7. Plate 100 μl of transformed bacteria on LB-agar plates supplemented with 7 μg/ml gentamycin and incubate overnight at 37 °C.
8. Verify correctly recombined pDONR-sE1, pDONR-sE2, and pDONR-S27 colonies by sequencing.
9. Add 150 ng of pDONR-sE1, pDONR-sE2, or pDONR-S27 to 150 ng of the pFastBacI (pFB) analog pDEST8 destination vector in 8 μl TE-buffer.
10. Add 2 μl LR Clonase™ II enzyme mix and incubate for 1 h at RT.

11. Stop the reaction by adding 1 μl Proteinase K and incubate for 10 min at 37 °C.
12. Transform 1 μl of the LR-reaction mix into electrocompetent DH5α *E. coli* bacteria. After transformation, recover the bacteria for 1 h in 1 ml LB-medium.
13. Plate 100 μl of transformed bacteria on LB-agar plates supplemented with 7 μg/ml gentamycin and 100 μg/ml ampicillin and incubate overnight at 37 °C.
14. Verify correctly recombined pFB-sE1, pFB-sE2, and pFB-S27 by PCR using any available internal primers and restriction/digestion analysis.
15. Transform 1 μl of pFB-sE1, pFB-sE2, and pFB-S27 into electrocompetent DH10Bac *E. coli* bacteria and recover for 4 h in 1 ml LB medium at 37 °C.
16. Plate 100 μl of 10^{-1} and 10^{-2} dilutions of the transformed DH10Bac cells on LB-agar plates supplemented with 7 μg/ml gentamycin, 50 μg/ml kanamycin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG. Incubate overnight at 37 °C.
17. White colonies indicate correct recombination of sE1, sE2 or the S27 structural cassette into the bacmid. Analyze correctly recombined bacmids by PCR using GentaFw, M13Fw, and M13Rv primers (*see* **Notes 3** and **4**).
18. Store the bacmids at 4 °C.
19. Seed 8×10^5 *Sf*21-cells per well in a 6-well plate in 2 ml of Grace's medium without antibiotics and let cells attach to the bottom of the well (*see* **Notes 5** and **6**).
20. Add 10 μl bacmid DNA to 90 μl serum-free Grace's medium.
21. Add 5 μl FectoFly I to 95 μl serum-free Grace's medium.
22. Mix the bacmid DNA and the FectoFly solution and incubate for 30 min at RT.
23. After incubation, add the 200 μl FectoFly I-DNA mix dropwise to the cells and incubate for 4 h at 27 °C on a rocking plateau.
24. Replace the medium with Grace's medium supplemented with 10 % FBS and 50 μg/ml gentamycin and incubate for ~4–5 days until clear baculovirus cytopathic effect (CPE) is visible (*see* **Notes 7** and **8**).
25. Harvest cells by pipetting up and down and separate the supernatant from the cell fraction by centrifugation of 5 min at $1500 \times g$.
26. Seed 8×10^6 *Sf*21-cells in a 75 cm^2 culture flask in 12 ml of Grace's medium without antibiotics and let cells attach to the bottom of the flask.

27. Remove the medium from the cells and add the 2 ml of *Ac*-sE1, *Ac*-sE2, or *Ac*-S27 containing supernatant retrieved after bacmid transfection.
28. Add an additional 2 ml of SFM to the cells and incubate for 4 h on a rocking plateau at 27 °C.
29. Remove medium from the cells and add 12 ml of Grace's medium supplemented with 10 % FBS and 50 μg/ml gentamycin and incubate at 27 °C for ~2–3 days until clear baculovirus CPE is visible.
30. Harvest the cells by scraping them from the bottom of the flask and resuspend them in the supernatant. Spin down the supernatant for 5 min at $1500 \times g$ and store the medium at 4 °C.
31. Prepare a 90 μl dilution series of 10^{-1} to 10^{-9} of the baculovirus (BV) suspension in 1.5 ml Eppendorf tubes (*see* **Note 9**).
32. Dilute *Sf*9-ET cells to a final concentration of 1.5×10^6 cells/ml in Sf900II medium supplemented with 5 % FBS and 200 μg/ml geneticin.
33. Add 90 μl of the *Sf*9-ET cell suspension to each virus dilution and mix well.
34. Add 10 μl of cell/virus suspension to each well of a 60-well microtiter plate. Fill six wells per dilution and start with six wells of uninfected *Sf*9-ET cells at the bottom of the plate. Fill the remaining rows by the different virus dilution, starting with the lowest dilution.
35. Incubate cells for ~5 days at 27 °C.
36. Observe infected wells using an inverted fluorescence microscope.
37. Read out by using GFP expression as a sign of infection.
38. Accumulate the number of infected and uninfected wells per dilution, starting with the lowest concentration for infected wells and with the highest concentration for uninfected cells.
39. Calculate the percentage of accumulated infected wells (AIW) per dilution and calculate the virus titer using the following formula (*see* **Note 10**):

    $TCID_{50}/\text{ml} = 10^{(a+x)} \times 200/\text{ml}$.

    $a = -(\log n)$.

    n = the highest dilution of which the percentage of AIW is higher than 50 %.

    b = percentage of AIW of dilution n.

    c = percentage of AIW of a ten times dilution of dilution n.

    x = relative percentage of AIW $= (b - 50)/(b - c)$.

3.3 Production of CHIKV-sE1 and -sE2 subunits and VLPs

1. Seed 8×10^6 *Sf*21-cells in a 75 cm^2 culture flask in 12 ml of Grace's medium without antibiotics and let cells attach to the bottom of the flask.
2. Infect the cells with a multiplicity of infection (MOI) = 10. Based on the titer of *Ac*-sE1, *Ac*-sE2, and *Ac*-S27, calculate the correct amount of virus needed and add this to 4 ml of serum-free Grace's medium.
3. Replace the medium by the virus solution and incubate for 4 h at 27 °C on a rocking plateau.
4. Replace the medium for 10 ml SFM and incubate for ~72 h until clear baculovirus CPE is visible.
5. Harvest the subunit or VLP-containing medium fraction by separating it from the cell fraction by centrifugation. Wash the cell fraction with PBS and finally store it at −20 °C for subsequent protein expression analysis.

3.4 Purification of sE1 and sE2 Subunits

The CHIKV-sE1 and -sE2 subunits contain a C-terminal poly-histidine tail to enable efficient purification using Talon® spin columns.

1. Equilibrate the Talon® spin columns with 8 ml TWB.
2. Load the subunit-containing supernatant on the column and collect the flowthrough.
3. Reload the flowthrough onto the same column.
4. When all medium has passed the column, wash three times with 5 ml TWB.
5. To elute the bound subunits from the Talon-resin, add 0.2 ml TEB to the column and vortex vigorously (*see* **Notes 11** and **12**). Leave the column on a roller bench for 10 min at 4 °C.
6. Centrifuge the column at $100 \times g$ for 2 min at 4 °C and collect the eluted fraction. This contains the sE1 and sE2 subunits.
7. Store the subunits at −80 °C for further analysis and use.

3.5 Purification of CHIKV-VLPs

1. Precipitate the secreted VLPs and other proteins fractions from the medium by adding 7 % (w/v) polyethylene glycol (PEG)-6000 and 0.5 M NaCl to the medium fraction and incubate for 2 h on a roller bench at 4 °C.
2. Centrifuge the precipitates for 15 min at $4000 \times g$ at 4 °C and dissolve the pellet in 1 ml GTNE-buffer of 4 °C.
3. Prepare a discontinuous 70 % (w/v), 40 % (w/v) sucrose in GTNE gradient at 4 °C (*see* **Notes 13–15**).
4. Load the 1 ml VLP solution carefully on top of the 40 % sucrose fraction without disrupting the gradient.
5. Centrifuge the loaded gradient for 2 h at $70{,}000 \times g$ in an ultracentrifuge at 4 °C.

6. Carefully isolate the 70–40 % VLP-containing interphase band and resuspend it in 5 ml GTNE buffer.
7. Pellet the VLPs by centrifugation for 30 min at 85,000 × *g* in an ultracentrifuge at 4 °C and resuspend in 50 μl GTNE before storage at −80 °C for subsequent analysis and use.

3.6 CHIKV Protein Analysis

Characterization of the produced CHIKV subunits and VLPs are based on glycosylation states, furin-dependent cleavage maturity, fusogenic activity, and morphology.

1. Treat protein samples with 1 μl denaturing buffer in 9 μl MilliQ water for 10 min at 95 °C.
2. Add 2 μl G7 reaction buffer, 2 μl 10 % NP40 buffer, 0.5 μl PNGase F in 4.5 μl MilliQ water and incubate for 1 h at 37 °C.
3. Analyze both treated and non-treated protein samples by SDS-PAGE and Western blot (WB) using rabbit-polyclonal anti-E1 and anti-E2 [20], 1:15,000 and 1:20,000 diluted in PBS-Tween.
4. Seed 8×10^5 *Sf*21-cells per well in a 6-well plate in 2 ml of Grace's medium without antibiotics and let cells attach to the bottom of the well.
5. Infect the cells with an MOI of 10 with *Ac*-sE2 and *Ac*-S27 and add this to 1 ml of serum-free Grace's medium.
6. Replace the medium by the virus solution and incubate for 4 h at 27 °C on a rocking plateau.
7. Remove the 1 ml infection medium and add 2 ml Grace's medium containing 50 μM of furin inhibitor I and incubate for 72 h at 27 °C.
8. Harvest the cell and medium fraction by pipetting the cells loose. Separate the cells from the medium by centrifugation.
9. Wash the cells once with PBS and finally resuspend in 100 μl PBS.
10. Store the cell and medium fraction at −20 °C.
11. Analyze both treated and non-treated protein samples by SDS-PAGE and WB using rabbit-polyclonal anti-E1 and anti-E2 [20], 1:15,000 and 1:20,000 diluted in PBS-Tween.
12. Seed 8×10^5 *Sf*9-ET cells per well in a 6-well plate in 2 ml of *Sf*900II medium supplemented with 5 % FBS and 200 μg/ml geneticin and let cells attach to the bottom of the well.
13. Infect the cells with an MOI of 10 with *Ac*-GFP (*see* **Note 16**), *Ac*-sE1, *Ac*-sE2, and *Ac*-S27 and add this to 1 ml of *Sf*900II medium supplemented with 5 % FBS and 200 μg/ml geneticin. Incubate for 4 h at 27 °C on a rocking plateau.
14. Replace the infection medium with *Sf*900II medium supplemented with 0.2 mg/ml cholesterol and incubate for 72 h at 27 °C.

15. Subject infected cells for 2 min to acidified medium with pH 5.8, 5.5, and 5.0.
16. Score syncytia formation 4 h post induction with an inverted fluorescence microscope.
17. Treat copper 400 square mesh grids with argon gas discharge. Once discharged, use the grids within 1 h. After 1 h, discharge again.
18. Load 5 μl purified VLP sample to the grid and incubate for 2 min at RT.
19. Remove sample carefully using filter paper.
20. Wash the grid 5 × 2 min in MilliQ water by placing the grid upside down on 5 μl MilliQ water droplets placed on Parafilm.
21. Treat the grids with 2 % uranyl acetate for 15 s and remove the excess uranyl acetate carefully using filter paper.
22. Air-dry the grids and analyze the samples with a transmission electron microscope.

4 Notes

1. The primers used to amplify the sE1 and sE2 coding fragments are based on the CHIKV-S27 sequence (Genbank accession # AF369024). The primers contain AttB1/2 recombination sequences to enable Gateway® cloning (underlined sequences). In addition, sE1-Rv and sE2-Rv have a poly-His tail for efficient purification of the secreted glycoproteins.
2. The electrocompetent DH10Bac *E. coli* cells are stably transformed with a bacmid encoding the complete AcMNPV genome, a mini-F replicon to enable single-copy replication in bacteria and the attTn7 transposition site needed for insertion of de CHIKV coding fragments. In addition to the bacmid, the cells maintain at tetracycline-resistant helper plasmid encoding the enzymes required for transposition.
3. To verify successful Tn7 transposition of sE1, sE2, and S27 structural cassette into the bacmid, perform a PCR using M13-Fw and M13-Rv. The annealing sites of these primers flank the Tn7 transposition site (attTn7) and the size of the amplicon indicates successful incorporation of the insert. An additional PCR using M13-Fw and Genta-Rv is performed. The gentamycin resistance marker is co-recombined into the bacmid together with the CHIKV coding sequences. Thus, by using Genta-Rv as an internal primer, one can be sure that the recombination was successful.
4. PCR analysis of white colonies and thus correctly recombined bacmids can show a mixed phenotype of correct transposition

and so-called "empty bacmids." As long as amplicons indicate at least partial recombination, one can retransform the isolated bacmid into DH10β *E. coli* bacteria, which results in the loss of the empty bacmids.

5. During transfection of the bacmid DNA, it is important that cells are in their log-phase. If cell densities are too high or cells have reached their growth plateau, transfection efficiency declines rapidly.
6. The presence of FBS in the medium does not affect transfection efficiency.
7. Transfected cell will develop CPE caused by both the FectoFly I transfection reagent as well as baculovirus replication, which is characterized by enlarged nuclei and thus enlarged cells, cell fusion, and stalling of cell division.
8. When cells are transfected or infected with the S27 structural cassette, clear CPE caused by CHIKV capsid can be observed, which is a good indication of the level of infection. Dense nuclear bodies are formed due to the auto-assembly of nucleocapsids in the nucleus of infected cells.
9. During the preparation of the virus dilution series and the loading of the wells, it is important to refresh pipet tips at every dilution. Using the same tips results in false virus titers.
10. Average baculovirus titers range from of 5×10^7 to 5×10^8 $TCID_{50}$/ml. It is advised to redo the EPDA when viral titers are $>2 \times 10^9$.
11. When the subunits are eluted from the Talon-resin, it is important to completely resuspend the resin in the elution buffer to maximize elution efficiency.
12. To ensure protein stability, all purification steps should preferably be executed at 4 °C.
13. To ensure VLP stability, all purification steps should preferably be executed at 4 °C.
14. Before the soft SW55-tubes are used to prepare the sucrose gradient, leave them in water for 1 h at RT. This will decrease the risk of crack being formed during ultracentrifugation.
15. When preparing the 70 %, 40 % sucrose gradient, start with pipetting 3 ml of 40 % sucrose into the tube. Next, place a glass Pasteur pipette in the tube and pipet 1 ml 70 % sucrose into the Pasteur pipette. This will place the 70 % sucrose underneath the 3 ml 40 % sucrose by gravity and will ensure a clear and tight gradient boundary.
16. To ensure that the formation of syncytia is caused by CHIKV-E1 and not by the GP64 fusion protein of the baculovirus itself, one should include a GFP control (*Ac*-GFP). This control will only show syncytia formation when the pH of the medium drops to the range in which GP64 is fusogenically active ($pH < 5.0$).

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